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Hormonal Control of Meiosis by Cyclic GMP and the Guanylyl Cyclase NPR2 in the Mouse Ovarian Follicle

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Leia Cristina Shuhaibar, PhD

University of Connecticut, 2015

The meiotic cell cycle of mammalian oocytes starts during embryogenesis and then pauses until luteinizing hormone (LH) acts to restart the cycle. Within the ovary, granulosa cells surrounding each oocyte maintain meiotic arrest, by providing cGMP, which diffuses through gap junctions into the oocyte.

LH acts on receptors located on granulosa cells up to 10 cell layers away from the oocyte; thus it has been an open question how this signal that is initiated in a distant tissue is transmitted to the oocyte. In this dissertation, this question is addressed by developing a method for imaging cGMP in live ovarian follicles from mice. By expressing a FRET sensor for cGMP in all cells of the ovarian follicle, we visualized the spatial and temporal dynamics of cGMP before and after treatment with LH. After stimulation with LH, cGMP first decreases in the peripheral granulosa cells, where LH receptors are expressed, then in the cumulus cells, and lastly in the oocyte, as a consequence of diffusion through gap junctions. These findings show that diffusion of cGMP is not only responsible for maintaining meiotic arrest, but also for transmission of the signal that reinitiates meiosis from the follicle surface to the oocyte.

cGMP is produced in granulosa cells by the guanylyl cyclase NPR2 (natriuretic peptide receptor 2). We found that LH decreases NPR2 guanylyl cyclase activity by 20

minutes after treatment with the hormone, and that this LH-induced decrease in guanylyl cyclase activity is caused by dephosphorylation of seven regulatory serine and threonine sites in the juxtamembrane region of the NPR2 protein. We mutated these regulatory sites to glutamates, such that the receptor behaves as if it is constitutively phosphorylated and active, and produced knockin mice expressing this receptor. We found that these mutations inhibit the rapid LH-induced decrease in guanylyl cyclase activity in the follicle and delay meiotic resumption in follicle-enclosed oocytes. Mice with these mutations also had longer vertebrae. Our findings demonstrate that dephosphorylation of the seven serine and threonine regulatory sites of NPR2 is important for hormonal regulation of meiosis in oocytes and for the regulation of bone growth.

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Hormonal Control of Meiosis by Cyclic GMP and the Guanylyl Cyclase NPR2 in the Mouse Ovarian Follicle

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PREFACE

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Finally, I would like to thank my wonderful husband Gerardo for his love and unconditional support during these years pursuing my graduate studies, even though it meant I would be living in another country away from him.

I would like to dedicate this effort to my beloved country Venezuela and to a person who, without knowing me, with his example of fight and wise words has helped me stand up in though times “Strength and Faith” Mr. Leopoldo López.

TABLE OF CONTENTS

INTRODUCTION

INTRODUCTION

The meiotic cycle in mammalian oocytes

Meiosis is the cell cycle that occurs exclusively in germ cells and is responsible for reducing the number of each chromosome per cell from two to one, such that at fertilization chromosomes from the oocyte can combine with chromosomes from the sperm to make a new individual. In the female, oocytes are formed during embryonic development and are arrested at Prophase I until Luteinizing Hormone (LH), which is released from the pituitary gland beginning at puberty, restarts the cycle to continue oocyte maturation in preparation for fertilization. In women, this meiotic arrest can last up to 50 years.

Within the ovary (Fig. 1A), each oocyte is surrounded by somatic cells called granulosa cells, forming the ovarian follicle. Surrounding the follicle is a layer of theca cells; rich in blood vessels that deliver LH to the follicle. During development, follicle-stimulating hormone (FSH) induces follicle growth to its full size (~400-500μm in diameter) and expression of the LH receptors required for resumption of meiosis (Hunzicker-Dunn and Mayo, 2015). In a pre-ovulatory follicle (Fig. 1B), which is the stage responsive to LH, approximately 10 layers of granulosa cells surround each oocyte. The cells immediately surrounding the oocyte are called cumulus cells, whereas cells more distant to the oocyte are called mural granulosa cells. Between the cumulus and mural granulosa cells is a fluid-filled cavity called the antrum. Oocytes arrested at prophase I are recognized by the presence of the nuclear envelope and nucleolus; these structures disappear when meiosis resume.

Gap junctions connect mural and cumulus cells, as well as cumulus cells to the oocyte, forming a syncytium that allows equilibration of signaling molecules within the follicle cells (Gilula *et al*., 1978; Norris *et al*., 2008). Gap junction communication between

the oocyte and the granulosa cells is essential to maintain the arrest in oocytes in pre-ovulatory follicles. This was evident from early studies showing that rabbit oocytes are unable to maintain meiotic arrest if isolated from the follicle, raising the idea that an inhibitory signal from the granulosa cells must be controlling the arrest in the oocyte (Pincus and Enzmann, 1935). The requirement for cell contact to maintain arrest was established by experiments with hamster oocytes, showing that disconnecting the cumulus-oocyte complex from the surrounding mural granulosa cells, within the antral follicle, was sufficient to cause meiotic resumption (Racowsky and Baldwin, 1989). The requirement for gap junction communication was established by studies showing that inhibitors of gap junction permeability also caused meiotic resumption in follicle-enclosed oocytes (Edry et al., 2006, Norris et al., 2008).

By 2010 when I began my studies of meiotic regulation in mammalian oocytes, much had been learned about the signaling mechanisms that inhibit meiotic progression, but it remained uncertain how LH action on receptors 10 layers away from the oocyte causes meiosis to resume. This chapter will briefly summarize what was known about these questions when I began my thesis research and will outline the questions that my thesis addresses.

Inhibition of meiotic progression by cAMP in the oocyte

In the oocyte, meiotic arrest is maintained by high levels of cyclic adenosine monophosphate (cAMP), which is synthesized by adenylyl cyclase 3 (AC3) linked to the constitutively active G-protein coupled receptor, GPR3 (in mouse and humans) and GPR12 (in rats) (Mehlmann *et al*., 2002; Mehlmann *et al*., 2004; Hinckley *et al*., 2005; Ledent *et al*., 2005; DiLuigi *et al*., 2008). Constitutive activity of GPR3 and its linked $G\alpha_s$ G-protein and

AC3 are necessary to maintain high levels of cAMP in the oocyte, and the absence of any of these components in the oocyte causes meiosis to resume (Mehlmann *et al*., 2002; Horner *et al*., 2003; Mehlmann *et al*., 2004; Ledent *et al*., 2005; DiLuigi *et al*., 2008).

Synthesis of cAMP in the oocyte leads to activation of protein kinase A (PKA) (Bornslaeger *et al*., 1986; Kovo *et al*., 2006), which in turn inhibits the activity of the phosphatase CDC25B and stimulates the activity of the kinases WEE1B and MYT1 (Conti *et al*., 2012; Holt *et al*., 2013; Mehlmann, 2013). This results in inactivation of a protein complex composed of cyclin B and the kinase CDK1 (the meiosis promoting factor, MPF) responsible for inhibiting the prophase-to-metaphase transition (Jones, 2004; Mehlmann, 2005).

The level of cAMP in the oocyte is determined by a balance between its synthesis, by GPR3-G_S-AC signaling, and its degradation by phosphodiesterase activity. The main phosphodiesterase expressed in the oocyte is PDE3A, which is competitively inhibited by cyclic guanosine monophosphate (cGMP) (Norris *et al*., 2009; Vaccari *et al*., 2009).

Inhibition of meiotic progression by cGMP from granulosa cells.

As mentioned above, connection between granulosa cells and the oocytes is crucial for maintenance of meiotic arrest. It is now known that this is because the inhibitory molecule cGMP, which is produced in granulosa cells, diffuses through gap junctions into the oocyte to inhibit the phosphodiesterase activity of PDE3A and maintain high levels of cAMP (Norris *et al*., 2009; Vaccari *et al*., 2009). If cGMP in a follicle-enclosed oocyte is experimentally decreased, by injection of the catalytic domain of a cGMP-specific phosphodiesterase, cAMP is decreased, and as a consequence meiosis resumes (Norris *et al*., 2009).

Generation of the cGMP that maintains meiotic arrest requires the function of the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also known as guanylyl cyclase-B) and its extracellular agonist C-type natriuretic peptide (CNP, also known as natriuretic peptide C, NPPC) (Zhang *et al*., 2010). Deficiency in the *Npr2* or *Nppc* genes causes meiosis to resume precociously in the absence of LH signaling, supporting the fundamental role of the CNP-NPR2 signaling pathway in maintaining meiotic arrest in the oocyte (Zhang *et al*., 2010). In order for meiosis to resume, LH signaling must remove this inhibitory signal of cGMP from the oocyte to allow activation of PDE3A and degradation of cAMP in the oocyte.

Resumption of meiosis in response to luteinizing hormone

LH is an ~30 kDa protein secreted from the anterior pituitary; it is comprised of two subunits, alpha and beta (Hunzicker-Dunn and Mayo, 2015). LH is released into the bloodstream at low levels throughout the female reproductive cycle, and this release increases dramatically just preceeding ovulation, reaching levels of up to1-2 μg/ml (35-70 nM) (Kovacic and Parlow, 1972; Blake, 1976). The increase in LH secretion is caused by rising levels of estrogen, which acts on receptors on cells of the hypothalamus to stimulate the release of peptides including kisspeptin that in turn stimulate the release of gonadotropin releasing hormone that in turn causes the synthesis and release of LH (Dungan *et al*., 2006; Oakley *et al*., 2009).

This surge of LH is delivered to the pre-ovulatory follicle by blood vessels in the theca layer surrounding the follicle, and acts on receptors in the outer layers of the granulosa cells (Bortolussi *et al*., 1977; Wang and Greenwald, 1993). The LH receptor is a 7-transmembrane

G-protein-coupled receptor (Hunzicker-Dunn and Mayo, 2015). Activation of the LH receptor causes multiple responses (Hunzicker-Dunn and Mayo, 2015): meiotic resumption in the oocyte, 2) changes in the oocyte's ability to release calcium, allowing it to respond to fertilization by establishing a block to polyspermy and beginning embryonic development (Mehlmann and Kline, 1994), 3) ovulation of the oocyte from the follicle, and 4) transformation of the follicle into the corpus luteum. My research focussed on the first of these responses.

LH signaling reinitiates meiosis by decreasing cGMP in the oocyte thus releasing the inhibition of PDE3A (Norris *et al*., 2009; Vaccari *et al*., 2009), but at the time that I started my dissertation research, it was unknown how this signaling from the LH receptor in the outer granulosa cells to the oocyte 10 cell layers away was accomplished.

Dissertation aims

The question addressed by my dissertation research involved how LH signaling in the granulosa cells acts to lower cGMP in the oocyte. As will be described in the following chapters, the primary factor that leads to the cGMP decrease in the oocyte is a decrease in cGMP in the granulosa cells.

The first aim of this research was to test the hypothesis that the decrease in cGMP in the oocyte results from a decrease in cGMP in the large volume of the granulosa cells surrounding it, thus establishing a concentration gradient such that cGMP diffuses out of the oocyte down this gradient. To do this, we developed a method for visualizing the spatio-temporal dynamics of cGMP in live ovarian follicles, and found that luteinizing hormone lowers cGMP in the outer cells of the follicle, and then cGMP in the oocyte decreases as a

consequence of diffusion through gap junctions. Our findings for this aim are described in publication one (Shuhaibar *et al*., 2015a).

The second aim was to test whether and how LH signaling decreased the production of cGMP in the granulosa cells by regulating the NPR2 guanylyl cyclase. To do this we investigated the effect of LH treatment on NPR2 guanylyl cyclase activity in granulosa cells. We found that luteinizing hormone induces dephosphorylation and inactivation of NPR2 guanylyl cyclase activity as described in publications two and three (Robinson et al., 2012; Egbert et al., 2014).

The third aim was to test whether the LH-induced decrease in NPR2 phosphorylation and activity was a regulator of meiotic resumption in the oocyte. To do this we developed a knock-in mouse in which seven juxtamembrane serine and threonine residues are changed to glutamates, such that NPR2 behaves as if it is constitutively phosphorylated, and show that dephosphorylation of these sites is required for the rapid resumption of meiosis in response to LH. Results from these studies are described in publication four (Shuhaibar *et al*., 2015b, manuscript in preparation).

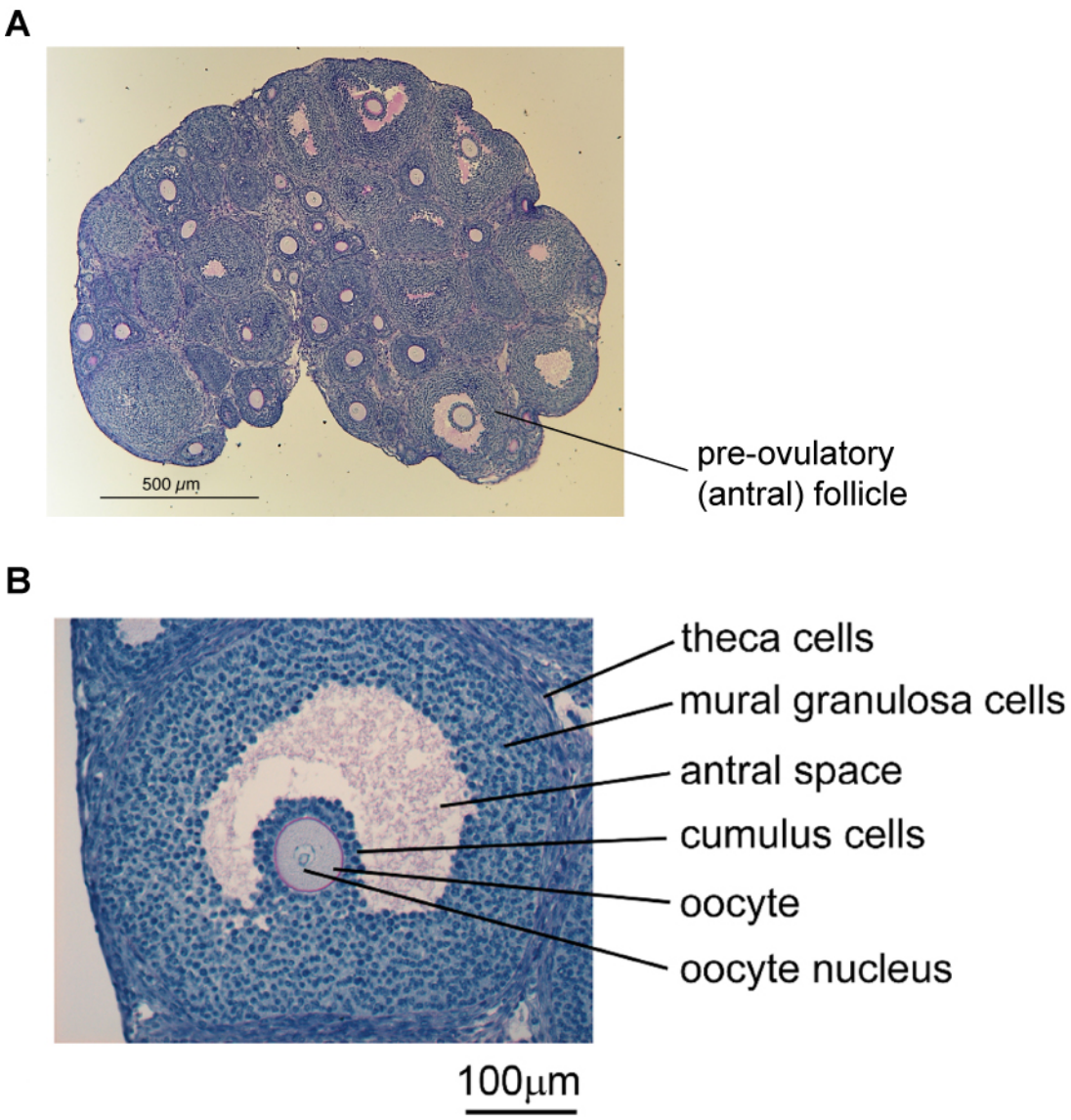


Figure 1. Structure of a mouse ovary and pre-ovulatory follicle. (A) Histological section of a mouse ovary showing follicles at different developmental stages. Each follicle contains one oocyte surrounded by layers of granulosa cells. During follicular growth, granulosa cells divide increasing the number of layers around the oocyte and becoming a pre-ovulatory (antral) follicle. (B) histological section showing an antral follicle, and indicating cell types and other structures in and around the follicle. A nuclear envelope and nucleolus within the oocyte is an indicator of meiotic arrest.

SUMMARY OF PUBLICATIONS

Publication 1: Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles.

Shuhaibar, L.C., Egbert, J.R., Norris, R.P., Lampe, P.D., Nikolaev, V.O., Thunemann, M., Wen, L., Feil, R., Jaffe, L.A. (2015). *Proc. Natl. Acad. Sci. U. S. A*. 12, 5527-5532.

Contribution: L.C.S. performed all the confocal microscopy experiments, and made all the figures. She also contributed importantly to the design of the experiments, and did some of the writing of the paper along with L.A.J. The other authors also contributed to the experiments, experimental design, figure preparation, and editing of the paper.

Meiosis in mammalian oocytes is paused until luteinizing hormone (LH) activates receptors in the mural granulosa cells of the ovarian follicle. Prior work has established the central role of cyclic GMP (cGMP) from the granulosa cells in maintaining meiotic arrest, but it is not clear how binding of LH to receptors that are located up to 10 cell layers away from the oocyte lowers oocyte cGMP and restarts meiosis. Here, by visualizing intercellular trafficking of cGMP in real-time, in live follicles from mice expressing a FRET sensor, we show that diffusion of cGMP through gap junctions is responsible not only for maintaining meiotic arrest, but also for rapid transmission of the signal that reinitiates meiosis from the follicle surface to the oocyte. Prior to LH exposure, the cGMP concentration throughout the follicle is at a uniformly high level of a few micromolar. Then within one minute of LH application, cGMP begins to decrease in the peripheral granulosa cells. As a consequence, cGMP from the oocyte diffuses into the sink provided by the large granulosa cell volume,

such that by 20 minutes, the cGMP concentration in the follicle is uniformly low, approximately 100 nanomolar (See Fig. 1). The decrease in cGMP in the oocyte relieves the inhibition of the meiotic cell cycle. This direct demonstration that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions could provide a general mechanism for diverse cellular processes.

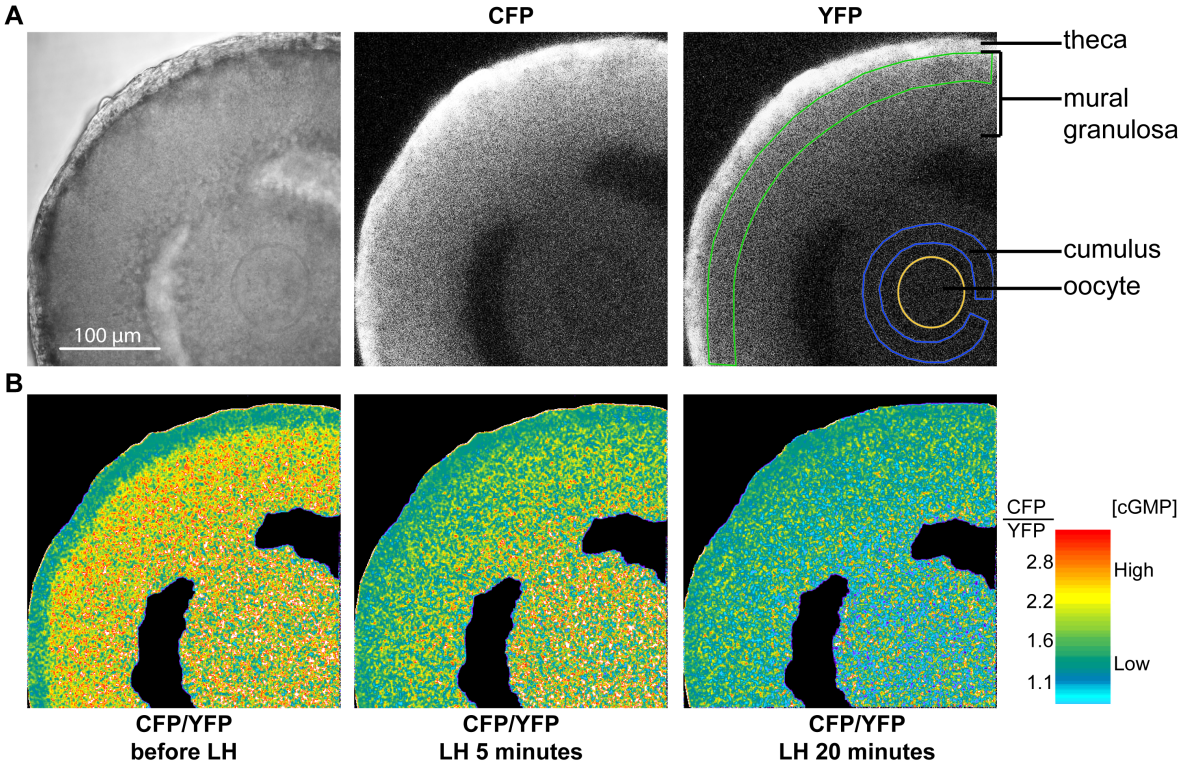


Figure 2. LH receptor activation initiates an inwardly propagating cGMP decrease in the mouse ovarian follicle. (A) Isolated follicle expressing the cGi500 sensor for cGMP, showing a scanning transmission image (left), CFP fluorescence (center), and YFP fluorescence with the regions of measurement indicated (right). (B) Images of the CFP/YFP ratio before LH perfusion, and at 5 and 20 minutes afterwards, for the follicle shown in A. Before LH application, cGMP is at a uniformly high level throughout the follicle. After LH application, cGMP decreases first in the mural granulosa cells, then in the cumulus cells and oocyte, reaching a plateau at the same value in all regions.

Publication 2: Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes.

Robinson, J. W., Zhang, M., **Shuhaibar, L. C.,** Norris, R. P., Geerts, A., Wunder, F., Eppig, J. J., Potter, L. R. and Jaffe, L. A**.** (2012). *Dev. Biol.* 366, 308-316.

Contribution: L.C.S. participated in preparing follicle, granulosa cell and ovary samples, and contributed to the preparation of the paper.

In preovulatory ovarian follicles of mice, meiotic prophase arrest in the oocyte is maintained by cyclic GMP from the surrounding granulosa cells that diffuses into the oocyte through gap junctions. The cGMP is synthesized in the granulosa cells by the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2) in response to the agonist C-type natriuretic peptide (CNP). In response to luteinizing hormone (LH), cGMP in the granulosa cells decreases, and as a consequence, oocyte cGMP decreases and meiosis resumes. Here we report that within 20 min, LH treatment results in decreased guanylyl cyclase activity of NPR2, as determined in the presence of a maximally activating concentration of CNP. This occurs by a process that does not reduce the amount of NPR2 protein. We also show that by a slower process, first detected at 2 h, LH decreases the amount of CNP available to bind to the receptor. Both of these LH actions contribute to decreasing cGMP in the follicle, thus signaling meiotic resumption in the oocyte.

Publication 3: Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes.

Egbert, J.R., **Shuhaibar. L.C**., Edmund, A.B., Van Helden, D.A., Robinson, J.W., Uliasz, T.F., Baena, V., Geerts, A., Wunder, F., Potter, L.R., Jaffe, L.A**.** (2014). *Development* 141, 3594-3604.

Contribution: L.C.S. participated in preparing follicle, granulosa cell and ovary samples, and contributed to the preparation of the paper.

In mammals, the meiotic cell cycle of oocytes starts during embryogenesis and then pauses. Much later, in preparation for fertilization, oocytes within preovulatory follicles resume meiosis in response to luteinizing hormone (LH). Before LH stimulation, the arrest is maintained by diffusion of cyclic GMP into the oocyte from the surrounding granulosa cells, where it is produced by the guanylyl cyclase natriuretic peptide receptor 2 (NPR2). LH rapidly reduces the production of cyclic GMP, but how this occurs has been unknown. Here, using rat follicles, we show that within 10 minutes, LH signaling causes dephosphorylation and inactivation of NPR2, by a process that requires PPP family protein phosphatase activity. The rapid dephosphorylation of NPR2 is accompanied by a rapid phosphorylation of the cGMP phosphodiesterase PDE5, an enzyme whose activity is increased by phosphorylation. Later, the NPR2 agonist, C-type natriuretic peptide, decreases in the follicle, and these sequential events contribute to the decrease in cGMP that causes meiosis to resume in the oocyte.

Publication 4: Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase regulates oocyte meiotic resumption and longitudinal bone growth.

Shuhaibar, L.C., Egbert, J.R., Edmund, A.B., Feil, R, Yee, S.P., Potter, L. R, Jaffe, L.A. (2015, *in preparation*)

Contribution: L.C.S. participated in making the Npr2-7E mice, conducted all the experiments except for the guanylyl cyclase assays, and made all of the figures. She contributed importantly to the design of the experiments and wrote the text of the paper with editing by L.A.J. The other authors also contributed to the experiments, experimental design, figure preparation, and editing of the paper.

In mammals, the meiotic cell cycle of oocytes starts during embryogenesis and then pauses until luteinizing hormone (LH) acts to restart the cycle. This meiotic arrest is maintained by cGMP, which is produced in the granulosa cells by the natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase B. LH signaling decreases cGMP in the granulosa cells and via equilibration through gap junctions, cGMP also decreases in the oocyte, thus releasing the meiotic arrest. Previous work has shown that one mechanism by which LH causes the cGMP decrease is by dephosphorylation and inactivation of NPR2, but it has not been directly tested whether NPR2 dephosphorylation is required for meiotic resumption. Studies of cells expressing point-mutated forms of NPR2 have identified seven

serine and threonine phosphorylation sites that regulate NPR2 activity, but this mechanism has not been tested in physiological settings. Here, by use of a knock-in mouse in which seven juxtamembrane serine and threonine residues are changed to glutamates (NPR2-7E), such that NPR2 behaves as if it is constitutively phosphorylated, we show that dephosphorylation of these sites is required for the rapid resumption of meiosis in response to LH (See Fig. 3). In the NPR2-7E follicles, LH failed to decrease guanylyl cyclase activity, and the cGMP decrease was attenuated, thus causing a delay in meiotic resumption. Additionally, the NPR2-7E mutation resulted in increased longitudinal bone growth. Our results demonstrate that dephosphorylation of these seven serine and threonine regulatory sites of NPR2 is important for hormonal regulation of meiosis in oocytes as well as for bone growth.

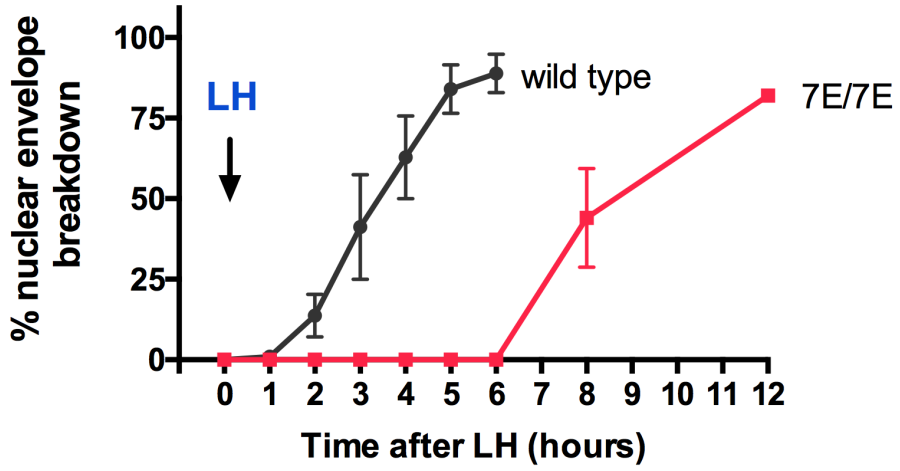


Figure 3. Delay of LH-induced meiotic resumption in NPR2-7E/7E follicle-enclosed oocytes. Time course of nuclear envelope breakdown in follicle-enclosed oocytes from NPR2-7E/7E and wild-type mice. LH was applied to the medium in the dish holding the Millicell membrane, and oocytes within follicles on the Millicell membrane were observed for the presence or absence of a nuclear envelope and nucleolus at 1-h intervals.

PERSPECTIVES

PERSPECTIVES

The research described in this thesis has contributed to our understanding of the question of how signaling by luteinizing hormone in the ovarian follicle reinitiates meiosis in mammalian oocytes. Before starting my studies in the Jaffe lab, many of the signaling pathway components in the oocyte and the granulosa cells had already been identified. It had been shown (by our lab and others) that cGMP is the inhibitory molecule from the granulosa cells responsible for maintaining the arrest in the oocyte, and that LH signaling restarts meiosis by reducing cGMP in the oocyte (Norris *et al*., 2009; Vaccari *et al*., 2009). It it had just been discovered that the guanylyl cyclase responsible for making cGMP in the follicle is NPR2 (Zhang *et al*., 2010).

The work presented in this dissertation contributed several new findings to our understanding of the mechanisms by which luteinizing hormone restarts meiosis: 1) LH signaling rapidly decreases cGMP in the mural granulosa cells and, as a result of diffusion through gap junctions, cGMP drains out of the oocyte into the larger compartment of granulosa cells. 2) LH signaling reduces the activity of NPR2 by dephosphorylation of serine and threonine sites in the juxtamembrane domain of the guanylyl cyclase. 3) LH signaling increases the phosphorylation of the cGMP phosphodiesterase PDE5, thus increasing hydrolysis of cGMP. The working model presented below (Fig. 4) summarizes these findings, and the timeline (Fig. 5) puts these events in the context of other knowledge about the regulation of oocyte meiosis by LH.

In addition to contributing to understanding of the regulation of meiosis in oocytes, the findings presented in this dissertation suggest that other physiological processes might be regulated by related mechanisms, particularly bone growth. The role of NPR2 in this system

is well established (Tamura *et al*., 2004), but our findings show for the first time that dephosphorylation of NPR2 is important in regulating bone growth. It is not known what hormonal signal could be regulating NPR2 activity in the chondrocytes of bone.

Many additional questions were generated by the studies presented in this dissertation. Among these, some future directions that I would like to pursue are described briefly below:

1. What phosphodiesterases contribute to lowering cGMP in the follicle, and are they activated by LH signaling? As shown in this dissertation PDE5 becomes phosphorylated by LH signaling, but it would be interesting to investigate the spatio-temporal dynamics of cGMP in cGi500 follicles treated with drugs that inhibit PDE5 activity, and determine the effect on the LH-induced decrease in cGMP in granulosa cells. Additionally, granulosa cell culture studies have suggested a role for the phosphodiesterase PDE1 in regulating cGMP in the follicle. The activity of PDE1 is regulated by calcium; thus an LH-induced increase in cytosolic calcium would increase PDE1 activity. This question could be investigated by using a transgenic mouse expressing a fluorescent sensor for calcium, and methods like those we used for the cGi500 follicles.

2. What hormonal signals might regulate the NPR2 guanylyl cyclase in growing bone, and does cGMP diffusion convey a signal in the growth plate as it does in the ovarian follicle? To investigate this question, histological studies of the growth plate would be used to first address the effect of the NPR2-7E mutations in the resting, proliferative, and hypertrophic chondrocyte layers. As mentioned in the discussion of publication #4 of this dissertation, we propose that FGFR3 signaling could potentially regulate NPR2 activity. It would be interesting to perform studies

using cultured long bones (e.g. tibia, femur) from young NPR2-7E mice and to evaluate their growth under treatment with FGF18 (an agonist for FGFR3). If dephosphorylation of NPR2 regulates the FGFR3-induced decrease in bone growth, then bones from mice with the 7E mutations would show increased growth compared to wild-type controls.

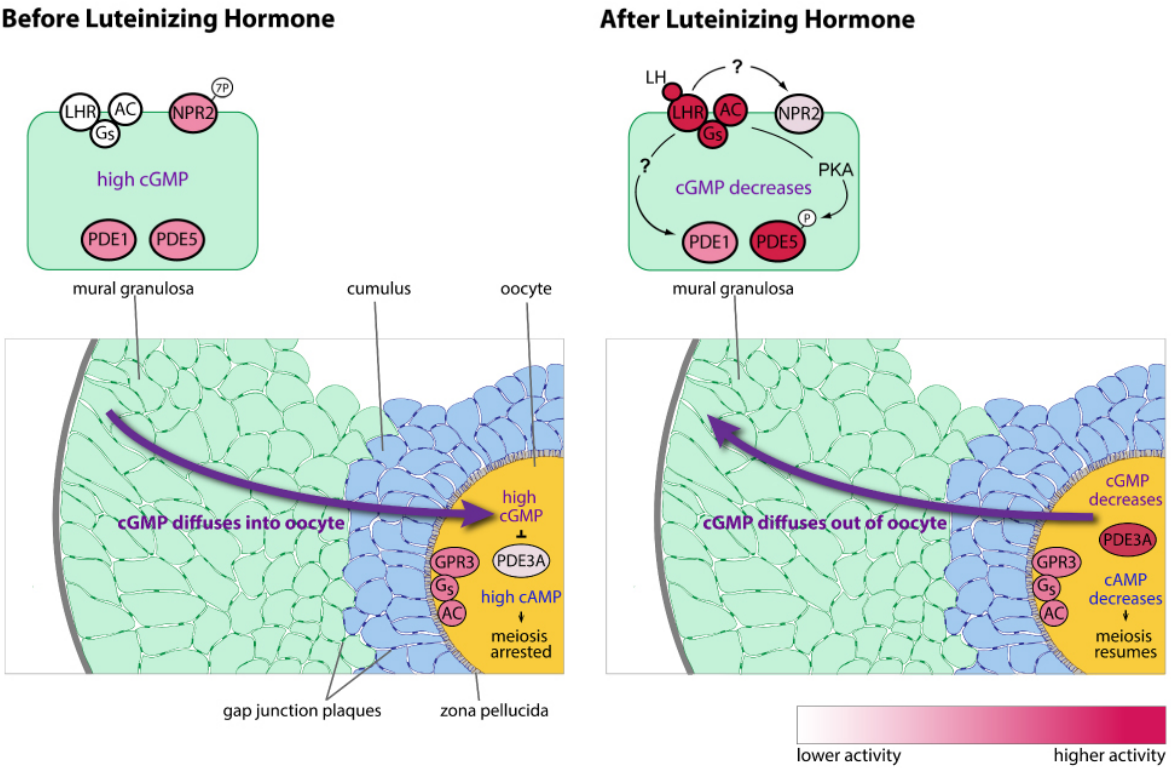


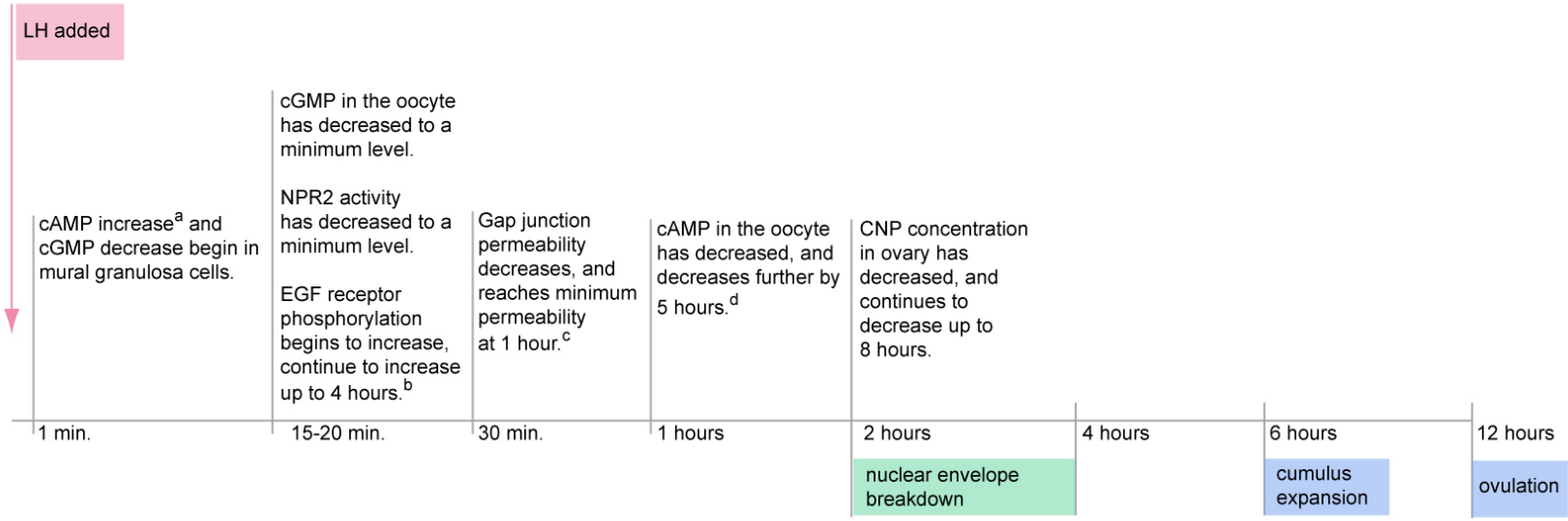
Figure 4. Working model of how LH signaling rapidly decreases cGMP in the mural granulosa cells, and then via cGMP diffusion through gap junctions, decreases cGMP in the oocyte, leading to meiotic resumption.

Before LH exposure (left), cGMP concentrations are elevated throughout the follicle, due to a high rate of production of cGMP by the NPR2 guanylyl cyclase in the mural granulosa and cumulus cells. cGMP phosphodiesterases, including PDE5 and PDE1, degrade cGMP at a rate equal to its production, thus keeping the cGMP concentration at a constant level. Through gap junctions that connect all cells of the follicle, cGMP diffuses into the oocyte, where it inhibits the activity of PDE3A, maintaining cAMP at a level that inhibits meiotic resumption. The cAMP in the oocyte is produced by adenylyl cyclase 3 in the oocyte, and AC3 is kept active by the constitutive activity of the Gs -coupled receptor GPR3.

When LH binds to its receptor in the mural granulosa cells (right), the activation of Gs and possibly other G-proteins results in dephosphorylation of NPR2, which decreases its rate of production of cGMP. Activation of the LH receptor also increases phosphorylation of PDE5, which increases its rate of degradation of cGMP. Due to reduced NPR2 activity and increased cGMP phosphodiesterase activity, the concentration of cGMP in the mural granulosa cells decreases. Through the series of gap

junctions that connects the oocyte to the large volume of the mural granulosa cells, cGMP in the oocyte diffuses down its concentration gradient, and the resulting decrease in oocyte cGMP relieves the inhibition of PDE3A in the oocyte, such that cAMP decreases. This model depicts only events occurring in the first 20 minutes after LH exposure. Subsequent events, including a decrease in gap junction permeability, an increase in EGF receptor ligands, and a decrease in C-type natriuretic peptide, also contribute to maintaining cGMP at the low level that triggers meiotic resumption. EGF receptor activation may also contribute to the early decrease in cGMP, although findings about this question are variable. References and further discussion of this model are included in the publications in the Appendix.

Timeline of the LH-induced events leading to meiotic resumption in mouse follicles



[a] Rachael Norris, Viacheslav Nikolaev, and Laurinda Jaffe, personal communication.

[b] Panigone et al., 2008; Liu et al., 2014.

[c] Norris et al., 2008.

[d] Norris et al., 2009.

Events regulated by NPR2

Events not regulated by NPR2

Figure 5. Timeline of the LH-induced events leading to meiotic resumption in mouse follicles. Key events, from previous work and this dissertation, are shown as a function of time after LH addition to isolated follicles. Footnotes indicate references to events investigated in previous studies.

REFERENCES

Blake, C.A. (1976). A detailed characterization of the proestrous luteinizing hormone surge. *Endocrinology*. **98**, 445-450.

Bornslaeger, E. A., Mattei, P. and Schultz, R. M. (1986). Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. *Dev. Biol.* **114**, 453-462**.**

Bortolussi, M., Marini, G. and Dal Lago, A. (1977). Autoradiographic study of the distribution of LH(HCG) receptors in the ovary of untreated and gonadotrophin-primed immature rats. *Cell Tissue Res.* **183**, 329-342.

Conti, M., Hsieh, M., Zamah, A. M. and Oh., J. S. (2012). Novel signaling mechanisms during oocyte maturation and ovulation. *Mol. Cell. Endocrinol*. **356**, 65-73.

DiLuigi, A., Weitzman, V.N., Pace, M.C., Siano, L.J., Maier, D., Mehlmann, L.M. (2008). Meiotic arrest in human oocytes is maintained by a Gs signaling pathway**.** *Biol. Reprod*. **78,** 667-672.

Dungan, H.M., Clifton, D.K., Steiner, R.A. (2006). Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. *Endocrinology* **147,** 1154-1158.

Edry, I., Sela-Abramovich, S., Dekel, N. (2006). Meiotic arrest of oocytes depends on cell-to-cell communication in the ovarian follicle. *Mol. Cell. Endocrinol.* **252,** 102-106.

Egbert, J.R., Shuhaibar. L.C., Edmund, A.B., Van Helden, D.A., Robinson, J.W., Uliasz, T.F., Baena, V., Geerts, A., Wunder, F., Potter, L.R., Jaffe, L.A. (2014) Dephosphorylation and inactivation of the NPR2 guanylyl cyclase in the granulosa cells contributes to the LH-induced cGMP decrease that causes resumption of meiosis in rat oocytes. *Development* **141,** 3594-3604.

Gilula, N.B., Epstein, M.L., and Beers, W.H. (1978). Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. *J. Cell Biol*. **78**, 58-75.

Hinckley, M., Vaccari, S., Horner, K., Chen, R., Conti, M. (2005). The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes. *Dev. Biol.* **287,** 249-261.

Holt, J. E., Lane, S. I. R. and Jones, K. T. (2013). The control of meiotic maturation in mammalian oocytes. *Curr. Top. Dev. Biol*. **102**, 207-226.

Horner, K., Livera, G., Hinckley, M., Trinh, K., Storm, D., Conti, M. (2003). Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Dev. Biol*. **258**(2): 385-96.

Hunzicker-Dunn M. and Mayo K. (2015). Gonadotropin signaling in the ovary. Knobil and Neill's Physiology of Reproduction, eds Plant TM, Zeleznik AJ (Academic, San Diego), 4th Ed, pp 895–945.

Jones, K.T. (2004). Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. *Mol. Hum. Reprod*. **10,** 1-5.

Kovacic, N., Parlow, A.F. (1972). Alterations in serum FSH-LH ratios in relation to the estrous cycle, pseudopregnancy, and gonadectomy in the mouse. *Endocrinology* **91**, 910-915.

Kovo, M., Kandli-Cohen, M., Ben-Haim, M., Galiani, D., Carr, D. W. and Dekel, N. (2006). An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes. *Reproduction* **132**, 33-43.

Ledent, C., Demeestere, I., Blum, D., Petermans, J., Hämäläinen, T., Smits, G., Vassart, G. (2005). Premature ovarian aging in mice deficient for Gpr3. **102,** 8922-8926.

Mehlmann, L. M. (2005). Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction.* **130,** 791-799.

Mehlmann, L. M. (2013). Signaling for meiotic resumption in granulosa cells, cumulus cells, and oocyte. In *Oogenesis* (ed. G. Coticchio, D. F. Albertini and L. De Santis), pp. 171-182. London: Springer-Verlag.

Mehlmann, L.M., Kline, D. (1994). Regulation of intracellular calcium in the mouse egg: calcium release in response to sperm or inositol trisphosphate is enhanced after meiotic maturation. *Biol. Reprod.* **51,** 1088-1098.

Mehlmann, L.M., Jones, T.L., Jaffe, L.A. (2002). Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science* **297,** 1343-1345.

Mehlmann, L.M., Saeki, Y., Tanaka, S., Brennan, T.J., Evsikov, A.V., Pendola, F.L., Knowles, B.B., Eppig, J.J., Jaffe, L.A. (2004). The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science*. **306,** 1947-1950.

Norris, R.P., Freudzon, M., Mehlmann, L.M., Cowan, A.E., Simon, A.M., Paul, D.L., Lampe, P.D., Jaffe, L.A. (2008). Luteinizing hormone causes MAPK-dependent phosphorylation and closure of Cx43 gap junctions in mouse ovarian. *Development* **135,** 3229-3238.

Norris, R. P., Ratzan, W. J., Freudzon, M., Mehlmann, L. M., Krall, J., Movsesian, M. A., Wang, H., Ke, H., Nikolaev, V. O. and Jaffe, L. A. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* **136**, 1869-1878.

Oakley, A.E., Clifton, D.K., Steiner, R.A. (2009). Kisspeptin signaling in the brain. *Endocr. Rev*. **30,** 713-743.

Pincus, G., Enzmann, E.V. (1935). The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs. *J. Exp. Med*. **62,** 665-675.

Racowsky, C., Baldwin, K.V. (1989). In vitro and in vivo studies reveal that hamster oocyte meiotic arrest is maintained only transiently by follicular fluid, but persistently by membrana/cumulus granulosa cell contact. *Dev. Biol*. **134**, 297-306.

Robinson, J. W., Zhang, M., Shuhaibar, L. C., Norris, R. P., Geerts, A., Wunder, F., Eppig, J. J., Potter, L. R. and Jaffe, L. A. (2012). Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. *Dev. Biol.* **366**, 308-316.

Shuhaibar, L.C., Egbert, J.R., Norris, R.P., Lampe, P.D., Nikolaev, V.O., Thunemann, M., Wen, L., Feil, R., Jaffe, L.A. (2015a). Intercellular signaling via cyclic GMP diffusion through gap junctions in the mouse ovarian follicle. *Proc. Natl. Acad. Sci. U. S. A*. **12**, 5527-5532.

Shuhaibar, L.C., Egbert, J.R., Edmund, A.B., Feil, R.,Yee, S.P., Potter, L. R., Jaffe, L.A. (2015b) Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase regulates oocyte meiotic resumption and longitudinal bone growth. *(in preparation, see appendix)*

Vaccari, S., Weeks, J. L., Hsieh, M., Menniti, F. S. and Conti, M. (2009). Cyclic GMP signaling is involved in the LH-dependent meiotic maturation of mouse oocytes. *Biol. Reprod.* **81**, 595-604.

Wang, X., Greenwald, G. S., (1993). Hypophysectomy of the cyclic mouse. II. Effects of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chroionic gonadotropin receptors, and steroidogenesis. *Biol Reprod*. **48,** 595-605.

Zhang, M., Su, Y.-Q., Sugiura, K., Xia, G., and Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* **330**, 366-369.

APPENDIX

Publication 1: Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles.

Shuhaibar, L.C., Egbert, J.R., Norris, R.P., Lampe, P.D., Nikolaev, V.O., Thunemann, M., Wen, L., Feil, R., Jaffe, L.A. (2015). *Proc. Natl. Acad. Sci. U. S. A*. 12, 5527-5532.

Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles

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Meiosis in mammalian oocytes is paused until luteinizing hormone (LH) activates receptors in the mural granulosa cells of the ovarian follicle. Prior work has established the central role of cyclic GMP (cGMP) from the granulosa cells in maintaining meiotic arrest, but it is not clear how binding of LH to receptors that are located up to 10 cell layers away from the oocyte lowers oocyte cGMP and restarts meiosis. Here, by visualizing intercellular trafficking of cGMP in real-time in live follicles from mice expressing a FRET sensor, we show that diffusion of cGMP through gap junctions is responsible not only for maintaining meiotic arrest, but also for rapid transmission of the signal that reinitiates meiosis from the follicle surface to the oocyte. Before LH exposure, the cGMP concentration throughout the follicle is at a uniformly high level of ∼2–4 μM. Then, within 1 min of LH application, cGMP begins to decrease in the peripheral granulosa cells. As a consequence, cGMP from the oocyte diffuses into the sink provided by the large granulosa cell volume, such that by 20 min the cGMP concentration in the follicle is uniformly low, ∼100 nM. The decrease in cGMP in the oocyte relieves the inhibition of the meiotic cell cycle. This direct demonstration that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions could provide a general mechanism for diverse cellular processes.

cyclic GMP | gap junctions | ovarian follicle | oocyte meiosis | luteinizing hormone

Meiosis in mammalian oocytes begins during embryonic development and then arrests in late prophase, for up to 50 y in women and for many months in mice. At the time of ovulation, luteinizing hormone (LH) acts on the granulosa cells of the follicle surrounding the oocyte to release the arrest and restart meiosis in preparation for fertilization (1–3). In the mouse preovulatory follicle, inhibition of meiotic progression is dependent upon the cyclic nucleotide cyclic GMP (cGMP), which diffuses from the granulosa cells into the oocyte through gap junctions that connect all cells of the follicle (4–6). The cGMP is produced by the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also known as guanylyl cyclase B), which is present in all of the granulosa cells, but not in the oocyte (7–11). In the oocyte, cGMP inhibits the degradation of another cyclic nucleotide, cAMP, which depends primarily on the phosphodiesterase PDE3A, an enzyme whose activity is antagonized by cGMP (4, 5). The resulting high level of cAMP, through a series of intermediate steps, inhibits meiotic progression (2, 3, 12).

LH signaling is initiated in the outer (mural) layers of granulosa cells; receptors for LH are absent in the oocyte and in the granulosa cells that directly surround it (the cumulus cells) (13–15). Ensuing events cause meiosis to resume by reducing cGMP in the oocyte (4, 5), but how LH receptor activation up to 10 cell layers away lowers oocyte cGMP is uncertain. LH signaling reduces gap junction permeability (16, 17), but meiosis can resume without the permeability decrease (17), arguing against gap junction closure as a primary mechanism for transmitting the signal. LH signaling also reduces cGMP in the follicle as a whole (4, 5, 18–20), suggesting that the decrease in oocyte cGMP is a consequence of the fall in cGMP in the large volume around the oocyte, to which it is connected by gap junctions (21).

Alternatively, recent work has suggested that the LH signal is transmitted to the oocyte by regulation of the release from the mural granulosa cells of peptides that diffuse through the extracellular space to the cumulus cells. In support of this concept, LH signaling decreases the ovarian content of the NPR2 agonist C-type natriuretic peptide, thus decreasing cGMP in the cumulus–oocyte complex (22); however, levels of this peptide decrease only after the decrease in oocyte cGMP (9, 20). LH signaling also increases the ovarian content of the EGF receptor ligands epiregulin and amphiregulin (23), and by a pathway that is not well understood, EGF receptor activation in isolated cumulus–oocyte complexes lowers their cGMP content (24). However, it is unknown if the increases in epiregulin and amphiregulin occur fast enough to cause the initial decrease in oocyte cGMP.

One missing link in understanding how LH lowers oocyte cGMP is precise information on the kinetics of the cGMP decreases in the oocyte and other regions of the follicle. Within 20 min after applying LH to isolated mouse follicles, the cGMP content of the whole follicle decreases from ∼2–4 μM to ∼100 nM

Significance

By imaging cyclic GMP (cGMP) in live ovarian follicles from mice, we show how luteinizing hormone signaling in the follicle periphery results in a rapid decrease in cGMP in the oocyte, thus reinitiating meiosis. Luteinizing hormone signaling lowers cGMP in the outer cells of the follicle, then cGMP in the oocyte decreases as a consequence of diffusion through gap junctions. These findings demonstrate directly that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions.

Author contributions: L.C.S., J.R.E., R.P.N., P.D.L., V.O.N., M.T., L.W., R.F., and L.A.J. designed research; L.C.S., J.R.E., R.P.N., and L.A.J. performed research; P.D.L., V.O.N., M.T., L.W., and R.F. contributed new reagents/analytic tools; L.C.S., J.R.E., R.P.N., V.O.N., and L.A.J. analyzed data; and L.C.S. and L.A.J. wrote the paper.

The authors declare no conflict of interest.

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(4, 19, 20) (Fig. S1*A*). This decrease occurs as a consequence of dephosphorylation and inactivation of the NPR2 guanylyl cyclase; activation of cGMP phosphodiesterases may also contribute (9, 11) (*Discussion*). By 1 h, cGMP in the oocyte decreases similarly, as measured with a fluorescent sensor of cGMP, cGi500, injected into the follicle-enclosed oocyte (4). However, the cGMP signals that occur in living follicles upon LH treatment have not been monitored except in the oocyte, and not before 1 h (4). Here we investigate how LH signaling causes the oocyte to resume meiosis by determining the spatiotemporal dynamics of the decrease in cGMP in the living follicle, and by examining whether gap junction permeability is needed for LH to lower cGMP in the cumulus–oocyte complex.

Results

The LH-Induced cGMP Decrease Occurs Sequentially in the Mural Granulosa Cells, Cumulus Cells, and Oocyte. Using antral follicles from mice that globally express the cGi500 sensor for cGMP (Fig. 1*A*) (25), we measured cGMP levels in mural granulosa, cumulus, and oocyte regions before and after addition of LH. Binding of cGMP to cGi500 decreases FRET between CFP and YFP, such that the CFP/YFP emission ratio measured after CFP excitation indicates cGMP concentration; the EC_{50} of cGi500 for cGMP is 500 nM (25–27), which is appropriate for detection of cGMP in the range of concentrations in mouse follicles before and after LH treatment. ELISA measurements of the cGMP content of follicles from cGi500-expressing mice showed an LH-induced decrease (Fig. S1*A*), and follicle-enclosed oocytes from these mice underwent nuclear envelope breakdown in response to LH with a normal time course (Fig. S1*B*). The cGi500-expressing follicles, which were spheres 320–400 μm in diameter when dissected, flattened to disks ∼200 μm in thickness after culture on an organotypic membrane. The follicles were imaged by confocal microscopy in a 200-μm-deep glass-bottomed chamber, with the focus at the oocyte equator, before and after perfusion of LH (Fig. 1 and Movie S1).

In the mural granulosa cells, cumulus cells, and oocyte, the CFP/YFP ratios before LH treatment were similar, indicating that the cGMP concentration was uniform in all parts of the follicle (Figs. 1*B*, *Left*, and 2 *A*–*D*). Similarly, at 20 min after LH treatment, the ratio values in the three compartments had decreased to the same plateau level (Figs. 1*B* and 2 *A* and *C*, and Fig. S2*A*), and remained at that level at 2 h (Fig. 2*D*). No change was seen with perfusion of control medium (Fig. 2*B* and Fig. S2*B*). However, the time for cGMP to decrease was greater in interior regions of the follicle (Figs. 1*B* and 2*A*, and Movie S1). In the mural granulosa cells, 10% of the decrease in CFP/YFP ratio had occurred at approximately 1 min after LH application, and 50% of the decrease had occurred at 2.8 ± 0.3 min (mean ± SEM, $n = 16$) (Fig. 2 *A*, *E*, and *F*). In the cumulus cells, 10% of the decrease had occurred at ∼5 min, and 50% of the decrease had occurred at 7.8 ± 0.4 min (Fig. 2 *A*, *E*, and *F*). In the oocyte, 10% of the decrease had occurred at ∼7 min, and 50% of the decrease had occurred at 9.9 ± 0.4 min (Fig. 2 *A*, *E*, and *F*). cGMP levels in the theca cells, located outside of the basal lamina of the follicle, remained at a constant low level before and after LH exposure (Fig. 1*B*).

The Rapid Decrease in cGMP in the Cumulus Cells Occurs via Outward Diffusion of cGMP Through Gap Junctions. To examine whether the LH-induced cGMP decrease in the cumulus cells occurs by diffusion through gap junctions, we preincubated follicles with carbenoxolone, which inhibits gap junction communication within the follicle (17). Carbenoxolone treatment itself had no effect on the cGMP level in the mural granulosa and cumulus cells (Fig. 3*B*). However, as previously reported (4), carbenoxolone treatment lowered cGMP in the oocyte, as a consequence of disconnecting it from the mural granulosa and cumulus cells where cGMP is produced (Fig. 3 *A* and *B*). Correspondingly, carbenoxolone treatment causes meiosis to resume (17).

With carbenoxolone present, LH caused cGMP to decrease in the mural granulosa cells, but not in the cumulus cells, during the initial 20 min (Fig. 3 *A* and *C*). Thus, only the cells in which LH

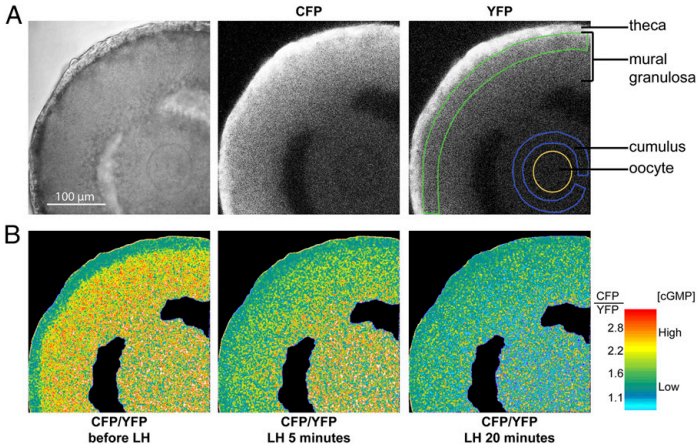

Fig. 1. LH receptor activation initiates an inwardly propagating cGMP decrease in the mouse ovarian follicle. (*A*) Isolated follicle expressing the cGi500 sensor for cGMP, showing a scanning transmission image (*Left*), CFP fluorescence (*Center*), and YFP fluorescence with the regions of measurement indicated (*Right*). (*B*) Images of the CFP/YFP ratio before LH perfusion, and at 5 and 20 min afterward, for the follicle shown in *A*. Before LH application, cGMP is at a uniformly high level throughout the follicle. cGMP in the surrounding theca cells is lower; the theca cells are not connected by gap junctions to the granulosa cells (17). After LH application, cGMP decreases first in the mural granulosa cells, then in the cumulus cells and oocyte, reaching a plateau at the same value in all regions. Movie S1 shows this time series. Based on ELISA measurements in wild-type follicles, the cGMP concentration before LH application is ∼4 μM, and the plateau value after LH is ∼100 nM (Fig. S1). No change in cGMP occurs in the theca cells.

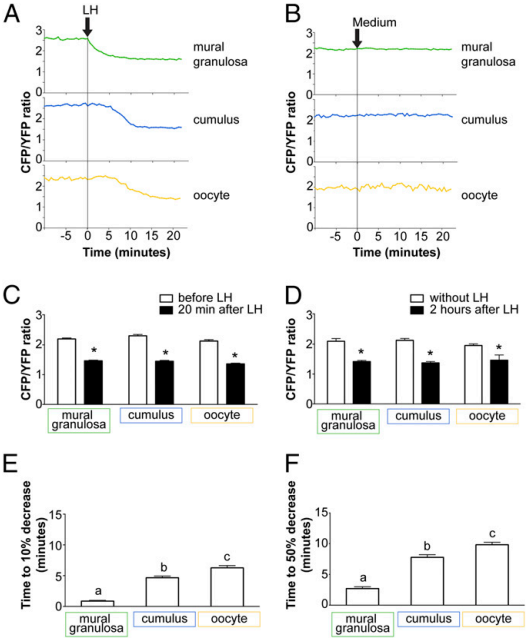

Fig. 2. Kinetics of the LH-induced cGMP decrease in mural granulosa, cumulus, and oocyte. (*A*) Time courses of the CFP/YFP ratios for the follicle shown in Fig. 1. For this and other graphs, ratios were calculated by dividing the mean CFP intensity in each region of interest, as shown in Fig. 1*A*, by the mean YFP intensity. (*B*) Recording from a follicle perfused with control medium without LH (representative of four experiments). (*C* and *D*) CFP/YFP ratios for mural granulosa cells, cumulus cells, and oocyte, before and at 20 min (*C*) or 2 h (*D*) after treatment with LH (16–20 follicles for each condition for *C*; 3–6 follicles for each condition for *D*). (*E* and *F*) Time to 10% or 50% of the decrease in CFP/YFP ratio in each region; results from 15 sets of measurements. Values that are indicated by an asterisk are significantly different from the control, and values not indicated by the same letter are significantly different from each other ($P < 0.05$); values indicate mean ± SEM.

receptors were present (the mural granulosa) showed a cGMP decrease. The lack of propagation of the cGMP decrease to the cumulus cells in the carbenoxolone-treated follicles indicates that after LH exposure, gap junction communication with the mural granulosa cells is needed for the rapid cGMP decrease to occur in the cumulus cells. However, by 2 h gap junction-independent signaling contributes to maintaining a low level of cGMP in the cumulus cells (Fig. 3*D*). This slower gap junction-independent cGMP decrease most likely results from EGF receptor ligands released from the mural granulosa cells acting on the cumulus cells (5, 19, 20), by an unknown pathway, and from a decrease in the NPR2 agonist C-type natriuretic peptide (9, 20, 22). Thus, different processes are responsible for the initial cGMP decrease in the cumulus cells, and for maintenance of low cGMP in the cumulus cells at later time points. Once cGMP in the mural granulosa and cumulus cells decreases, cGMP in the small volume of the oocyte (~0.2 nL) equilibrates with that in the large volume of the follicle (~20 nL), to which the oocyte is connected by gap junctions (17, 28).

Diffusion of cGMP Out of the Cumulus Cells Precedes the LH-Induced Decrease in Gap Junction Permeability. In response to LH, the permeability of the connexin-43 gap junctions between the mural granulosa cells and between the mural granulosa and cumulus cells decreases (17), raising the question of whether the permeability decrease occurs on a time scale that would interfere with the diffusion of cGMP from the cumulus–oocyte complex into the mural granulosa cells. The decrease in gap junction permeability occurs by 30–60 min after LH exposure, but earlier time points have not been examined (17). To investigate how rapidly the permeability decreases, we examined the rate of fluorescence redistribution after photobleaching of a small fluorescent tracer in the mural granulosa cells, as previously described (17). No decrease in redistribution rate was detected after a 10-min exposure to LH, indicating no decrease in permeability during this period (Fig. 4 *A* and *B*). Limitations of photobleaching deep in the follicle precluded similar measurements in the cumulus cells (17), but activation of LH receptors in the mural granulosa cells is unlikely to close gap junctions more rapidly in the cumulus cells than in the mural granulosa cells. Phosphorylation of key regulatory serines of connexin-43 that leads to the permeability decrease (29) was detectable by 10 min (Fig. 4*C*), but the resulting change in channel permeability did not occur until after 10 min (Fig. 4 *A* and *B*), after cGMP diffusion out of the cumulus cells is largely complete (Fig. 2*A*). Thus, cGMP diffusion out of the cumulus cells precedes the gap junction permeability decrease, such that diffusion would not be impeded.

Discussion

These results, obtained by imaging live ovarian follicles, show that diffusion of cGMP through gap junctions is responsible not only for maintaining meiotic arrest, but also for rapid transmission from the follicle surface to the oocyte of the hormonal signal that reinitiates meiosis. Before LH exposure, the cGMP

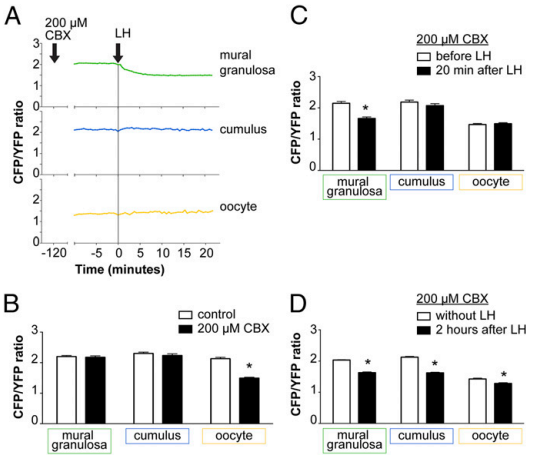

Fig. 3. The initial cGMP decrease in the cumulus cells requires gap junction communication. (*A*) A cGi500-expressing follicle was pretreated for 2 h with 200 μM carbenoxolone (CBX), and then perfused with LH. (*B*) CFP/YFP ratios for mural granulosa cells, cumulus, and oocyte, without LH exposure, for 10 follicles with CBX treatment; these are compared with the 20 control follicles from Fig. 2*C*. (*C* and *D*) CFP/YFP ratios for mural granulosa, cumulus cells, and oocytes, before and at 20 min (*C*) or 2 h (*D*) after treatment with LH, for follicles in the presence of CBX (7–15 follicles for each condition). Because CBX treatment lowers cGMP in the oocyte to approximately the same level attained after LH (*B*), LH treatment of follicles in the presence of CBX caused little further change in cGMP in the oocyte (*A*, *C*, and *D*). Values that are indicated by an asterisk are significantly different from the control ($P < 0.05$); values indicate mean ± SEM.

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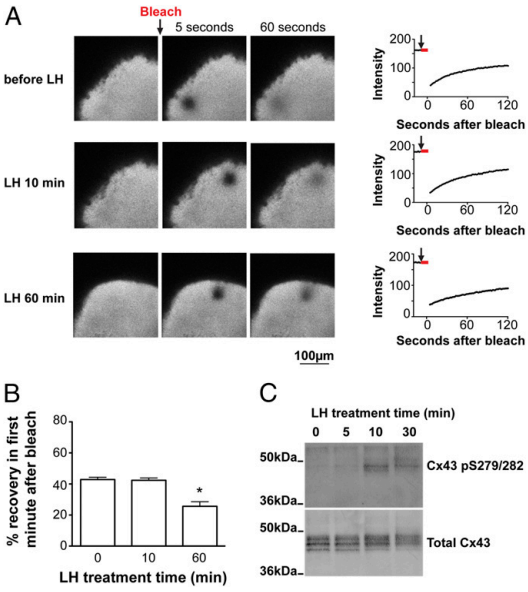

Fig. 4. Kinetics of the LH-induced decrease in gap junction permeability. (*A* and *B*) Fluorescence redistribution after photobleaching of Alexa-488 in the mural granulosa cells. (*A*) shows images of a follicle before photobleaching and at 5 and 60 s afterward, before (*Top*) or 10 min after LH perfusion (*Middle*). The bottom row shows a separate follicle that was photobleached 60 min after LH treatment. The graphs show the time courses of fluorescence recovery. (*B*) The percent recovery in the first minute after bleaching, for eight follicles before and after a 10-min LH treatment, and three follicles after a 60-min LH treatment. (*C*) Kinetics of Cx43 phosphorylation on serines 279 and 282 after applying LH to follicles. Similar results were obtained in another identical experiment. The value indicated by an asterisk is significantly different from the control ($P < 0.05$); values indicate mean ± SEM.

concentration throughout the follicle is at a uniformly high level of ∼2–4 μM. Then within 1 min of LH application, cGMP begins to decrease in the mural granulosa cells. As a consequence, cGMP from the oocyte diffuses into the sink provided by the large granulosa cell volume, such that by 20 min the cGMP concentration in the follicle is uniformly low, ∼100 nM. The decrease in cGMP in the oocyte relieves the inhibition of the meiotic cell cycle.

The signaling events leading to the rapid decrease in cGMP in the follicle begin with activation of G_s and other G proteins by the LH receptors in the mural granulosa cells (3) (Fig. 5). Through incompletely understood steps, this leads to dephosphorylation and inactivation of the NPR2 guanylyl cyclase, thus reducing the production of cGMP. Because cGMP phosphodiesterases are actively hydrolyzing cGMP in the granulosa cells (5), the decrease in the rate of cGMP production results in a lower equilibrium level of cGMP. In addition, there could be an increase in the activity of the cGMP phosphodiesterase PDE5, as indicated by evidence that PDE5 is phosphorylated (11) and evidence from studies of other cells that phosphorylation of PDE5 is associated with increased activity (30–32). G_s-mediated elevation of cAMP in the mural granulosa cells is very likely a step in this process, because in response to adenylyl cyclase activation by forskolin, cGMP in these cells decreases rapidly, reaching levels comparable to those seen with LH (Fig. S3). LH signaling also leads to activation of the EGF receptor (19, 20, 23), but our results do not indicate a role for EGF receptor activation in the initial decrease in cGMP (Fig. S4).

Previous studies have indicated that gap junction-mediated cyclic nucleotide diffusion can convey signals between pairs of cells (33–35), and the present findings are a direct demonstration that a physiological signal initiated by a stimulus in one region of an intact tissue can travel across many layers of cells via cyclic nucleotide diffusion through gap junctions. Another example of rapid communication through a complex of cells via diffusion of a cyclic nucleotide through gap junctions occurs in immune cell signaling, where cGMP-AMP diffuses from a cell infected by a virus to neighboring cells, causing them to increase synthesis of interferons (35). With the development of mice expressing cyclic nucleotide sensors (25, 36), it should now be possible to investigate whether signals could be transmitted similarly in other processes in gap junction-coupled tissues where cyclic nucleotides are essential regulators. For example, both cGMP (37) and connexins (38) can suppress tumor growth, suggesting that cGMP diffusion from adjacent cells connected by gap junctions could suppress cell division, as it does in the ovarian follicle. As occurs during hormonal signaling in the ovary, signals that increase or decrease cGMP in the cells surrounding a tumor could, via gap junctions, affect its growth.

Methods

Imaging of cGi500 Fluorescence in Mouse Preovulatory Follicles. Antral follicles were dissected from 23- to 26-d-old transgenic mice [R26-CAG-cGi500(L1)] in which the cGi500 sensor for cGMP was introduced by targeted integration into the *Rosa26* locus and expressed under the control of the CAG promoter (25). All measurements of cGi500 fluorescence were done with heterozygous mice expressing one copy of the cGi500 transgene. All animal protocols were approved by the University of Connecticut Health Center Animal Care Committee. Based on Western blot immunodensity, the concentration of the cGi500 sensor in the follicles of heterozygous mice was ∼20 μM (Fig. S5). Before use for imaging, the cGi500-expressing follicles were cultured for 24–30 h on organotypic membranes (Millipore; cat. no. PICMORG50), in the presence of follicle-stimulating hormone (17, 19). For some experiments, follicles were incubated with carbenoxolone (Tocris Bioscience).

Follicles were imaged while held between a plastic slide (ibidi; cat. no. 80161) and a glass coverslip; slides without adhesive were custom ordered from ibidi and assembled using silicon grease. The slide was constructed such that medium containing ovine LH (National Hormone and Peptide Program; 10 μg/mL) could be perfused through a 200-μm-deep channel holding the follicle. Temperature was maintained at 30–34 °C, by use of a warm air blower (Nevtek). Follicles were imaged using a Zeiss Pascal confocal system with a 40×/1.2 numerical aperture C-Apochromat objective with Immersol between the coverslip and objective (Carl Zeiss Microscopy). The excitation laser and emission filters were as previously described (4). The microscope was focused on the oocyte equator, with the confocal pinhole set for an ∼14-μm optical section. The laser attenuation was adjusted to avoid saturation. Images were collected using 1.6-s scans at 30-s intervals, for 10 min before LH addition, and for 20 min afterward. Values for ratios "2 hours after LH" were obtained by incubating follicles on an organotypic membrane for 2 h, then placing them in an ibidi slide for measurement. Files were saved as 12-bit images.

Measurements of CFP and YFP emission intensities were from regions as shown in Fig. 1*A*; the mural granulosa region included the 25-μm-wide band just inside the basal lamina, and the cumulus region included the 15-μm-wide zone just outside of the oocyte. Oocyte intensities were measured from a circular region slightly smaller than the oocyte diameter. Measurements were corrected for autofluorescence and for spectral bleed-through of CFP into the YFP channel (4). Values for ratios "before LH" are averages for the 10 measurements before LH addition; values for ratios "20 minutes after LH" are averages of the 10 measurements between 15 and 20 min after LH perfusion through the ibidi slide. Ratios were calculated by dividing the mean CFP intensity in each region of interest by the mean YFP intensity. Data analysis was done using ImageJ and Excel software.

Ratio images shown in Fig. 1*B* and Movie S1 were made using Metamorph software (Molecular Devices) and ImageJ, masking the antral space and the space outside of the follicle. Ratios were calculated by binning measurements of CFP and YFP intensities over 16 pixel regions (2.5 × 2.5 μm), and then dividing the binned values.

Evaluation of Gap Junction Permeability and Connexin-43 Phosphorylation. To evaluate gap junction permeability by fluorescence redistribution after

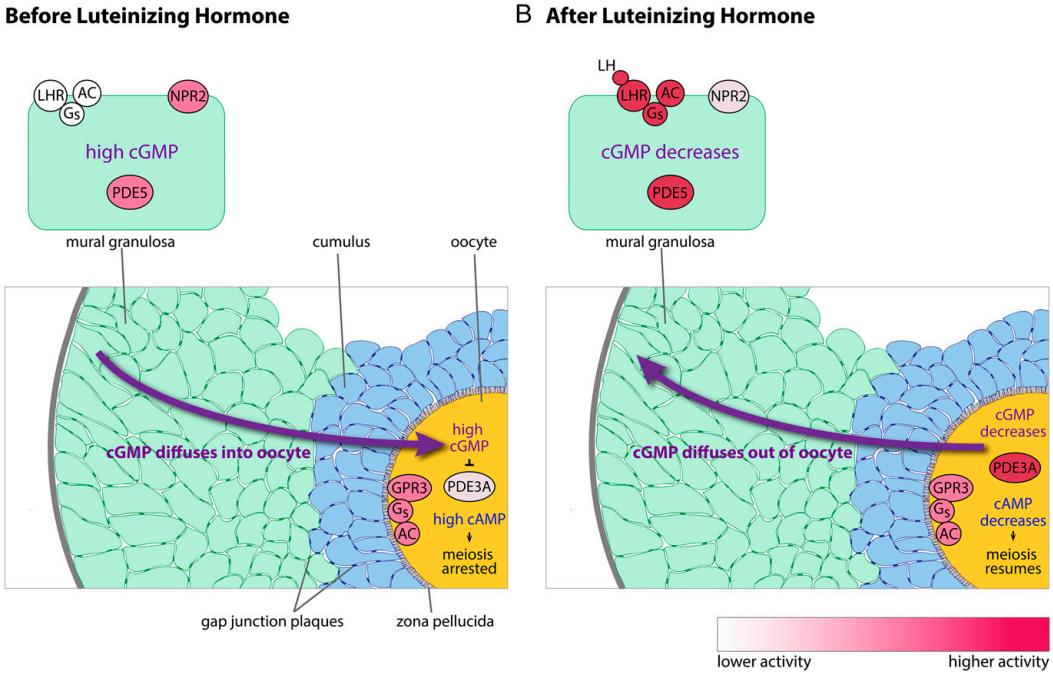

Fig. 5. Working model of how LH signaling rapidly decreases cGMP in the mural granulosa cells, and then via cGMP diffusion through gap junctions, decreases cGMP in the oocyte, leading to meiotic resumption. (*A*) Before LH exposure, cGMP concentrations are elevated throughout the follicle, because of a high rate of production of cGMP by the NPR2 guanylyl cyclase in the mural granulosa and cumulus cells. cGMP phosphodiesterases, including PDE5, degrade cGMP at a rate equal to its production, thus keeping the cGMP concentration at a constant level. Through gap junctions that connect all cells of the follicle, cGMP diffuses into the oocyte, where it inhibits the activity of PDE3A, maintaining cAMP at a level that inhibits meiotic resumption. The cAMP in the oocyte is produced by adenylyl cyclase 3 in the oocyte (39), and AC3 is kept active by the constitutive activity of the G_s-coupled receptor GPR3 (40). (*B*) When LH binds to its receptor in the mural granulosa cells, the activation of G_s and possibly other G proteins results in dephosphorylation of NPR2, which decreases its rate of production of cGMP. Activation of the LH receptor also increases phosphorylation of PDE5, and from studies of other cells, this should increase its rate of degradation of cGMP. Because of reduced NPR2 activity and increased cGMP phosphodiesterase activity, the concentration of cGMP in the mural granulosa cells decreases. Through the series of gap junctions that connects the oocyte to the large volume of the mural granulosa cells, cGMP in the oocyte diffuses down its concentration gradient, and the resulting decrease in oocyte cGMP relieves the inhibition of PDE3A in the oocyte, such that cAMP decreases. This model depicts only events occurring in the first 20 min after LH exposure. Subsequent events, including a decrease in gap junction permeability, an increase in EGF receptor ligands, and a decrease in C-type natriuretic peptide, also contribute to maintaining cGMP at the low level that triggers meiotic resumption. EGF receptor activation may also contribute to the early decrease in cGMP, although findings about this question are variable (Fig. S4). References and further discussion of this model are included in the text.

photobleaching, we loaded follicles with a fluorescent tracer by injecting follicle-enclosed oocytes with Alexa-488 (#A10436, Invitrogen; $M_r = 534$) and incubated the follicles on organotypic membranes for 3–4 h to allow the tracer to spread through gap junctions into the granulosa cells (17). Alexa-488 was used at a stock concentration of 2 mM, resulting in an initial concentration in the oocyte of 100 μM. Follicles were then placed in ibidi slides for FRAP analysis using a Zeiss Pascal confocal microscope, before and 10 min after perfusion of LH. For measurements at 60 min after LH exposure, follicles were exposed to LH before putting them in ibidi slides.

Using a 40×/1.2 NA objective and the 488 line of an Argon laser, we photobleached a 28 × 28-μm square in the mural granulosa cell layer, ∼20 μm below the follicle surface. The photobleaching was accomplished by using a zoom setting of 8. A 10-s laser exposure decreased the fluorescence intensity in the bleached region to ∼20% of the initial value. Postbleach images were collected with the same objective, but with the zoom setting reduced to 0.7 and the laser intensity reduced to 0.2% of that used for bleaching. The confocal pinhole was set for an ∼14-μm optical section, and a 505-nm long-pass filter was used to collect the emitted light; images were collected at 1.6-s intervals, and corrected for minor autofluorescence. These monitoring conditions did not significantly bleach the Alexa-488. To compare the time course of fluorescence redistribution with and without LH, we measured the change in Alexa-488 intensity in the bleached region during the first minute (between 5 and 60 s) after the end of the bleach.

To evaluate the time course of phosphorylation of connexin-43 after LH treatment, follicles were exposed to LH while positioned on an organotypic membrane, then washed in PBS and sonicated in Laemmli sample buffer containing protease and phosphatase inhibitors (17). For 5-min samples, the wash procedure was started at 3.5 min, and sample buffer was added at 5 min. For 10-min samples, the wash procedure was started at 8.5 min and sample buffer was added at 10 min. Western blots for phosphorylated and total connexin-43 were performed as previously described (17).

Statistics. Differences between multiple treatment conditions were analyzed by one- or two-way ANOVA followed by post hoc *t* tests with Bonferroni correction, using Prism software (GraphPad). Graph values that are indicated by an asterisk are significantly different from the control, and values not indicated by the same letter are significantly different from each other ($P < 0.05$); values indicate mean ± SEM.

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1. Clift D, Schuh M (2013) Restarting life: Fertilization and the transition from meiosis to mitosis. *Nat Rev Mol Cell Biol* 14(9):549–562.
2. Holt JE, Lane SIR, Jones KT (2013) The control of meiotic maturation in mammalian oocytes. *Curr Top Dev Biol* 102:207–226.
3. Hunzicker-Dunn M, Mayo K (2015) Gonadotropin signaling in the ovary. *Knobil and Neill's Physiology of Reproduction*, eds Plant TM, Zeleznik AJ (Academic, San Diego), 4th Ed, pp 895–945.
4. Norris RP, et al. (2009) Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* 136(11):1869–1878.
5. Vaccari S, Weeks JL, II, Hsieh M, Menniti FS, Conti M (2009) Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes. *Biol Reprod* 81(3):595–604.
6. Richard S, Baltz JM (2014) Prophase I arrest of mouse oocytes mediated by natriuretic peptide precursor C requires GJA1 (connexin-43) and GJA4 (connexin-37) gap junctions in the antral follicle and cumulus-oocyte complex. *Biol Reprod* 90(6):137, 1–10.
7. Jankowski M, et al. (1997) C-type natriuretic peptide and the guanylyl cyclase receptors in the rat ovary are modulated by the estrous cycle. *Biol Reprod* 56(1):59–66.
8. Zhang M, Su Y-Q, Sugiura K, Xia G, Eppig JJ (2010) Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* 330(6002):366–369.
9. Robinson JW, et al. (2012) Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. *Dev Biol* 366(2):308–316.
10. Geister KA, et al. (2013) A novel loss-of-function mutation in *Npr2* clarifies primary role in female reproduction and reveals a potential therapy for acromesomelic dysplasia, Maroteaux type. *Hum Mol Genet* 22(2):345–357.
11. Egbert JR, et al. (2014) Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes. *Development* 141(18):3594–3604.
12. Dupré A, Daldello EM, Nairn AC, Jessus C, Haccard O (2014) Phosphorylation of ARPP19 by protein kinase A prevents meiosis resumption in *Xenopus* oocytes. *Nat Commun* 5:3318.
13. Wang X-N, Greenwald GS (1993) Hypophysectomy of the cyclic mouse. II. Effects of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chorionic gonadotropin receptors, and steroidogenesis. *Biol Reprod* 48(3): 595–605.
14. Eppig JJ, Wigglesworth K, Pendola F, Hirao Y (1997) Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol Reprod* 56(4):976–984.
15. Eppig JJ, Wigglesworth K, Pendola FL (2002) The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc Natl Acad Sci USA* 99(5):2890–2894.
16. Sela-Abramovich S, Chorev E, Galiani D, Dekel N (2005) Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology* 146(3):1236–1244.
17. Norris RP, et al. (2008) 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. *Development* 135(19):3229–3238.
18. Hubbard CJ (1986) Cyclic AMP changes in the component cells of Graafian follicles: Possible influences on maturation in the follicle-enclosed oocytes of hamsters. *Dev Biol* 118(2):343–351.
19. Norris RP, Freudzon M, Nikolaev VO, Jaffe LA (2010) Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. *Reproduction* 140(5):655–662.
20. Liu X, Xie F, Zamah AM, Cao B, Conti M (2014) Multiple pathways mediate luteinizing hormone regulation of cGMP signaling in the mouse ovarian follicle. *Biol Reprod* 91(1):9, 1–11.
21. Törnell J, Billig H, Hillensjö T (1991) Regulation of oocyte maturation by changes in ovarian levels of cyclic nucleotides. *Hum Reprod* 6(3):411–422.
22. Kawamura K, et al. (2011) Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Hum Reprod* 26(11):3094–3101.
23. Park JY, et al. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 303(5658):682–684.
24. Wang Y, et al. (2013) Epidermal growth factor receptor signaling-dependent calcium elevation in cumulus cells is required for NPR2 inhibition and meiotic resumption in mouse oocytes. *Endocrinology* 154(9):3401–3409.
25. Thunemann M, et al. (2013) Transgenic mice for cGMP imaging. *Circ Res* 113(4): 365–371.
26. Russwurm M, et al. (2007) Design of fluorescence resonance energy transfer (FRET)-based cGMP indicators: a systematic approach. *Biochem J* 407(1):69–77.
27. Thunemann M, Fomin N, Krawutschke C, Russwurm M, Feil R (2013) Visualization of cGMP with cGi biosensors. *Methods Mol Biol* 1020:89–120.
28. Veitch GI, Gittens JEI, Shao Q, Laird DW, Kidder GM (2004) Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface. *J Cell Sci* 117(Pt 13):2699–2707.
29. Warn-Cramer BJ, Cottrell GT, Burt JM, Lau AF (1998) Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem* 273(15):9188–9196.
30. Corbin JD, Turko IV, Beasley A, Francis SH (2000) Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur J Biochem* 267(9):2760–2767.
31. Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, Beavo JA (2002) Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J Biol Chem* 277(5):3310–3317.
32. Jäger R, Schwede F, Genieser H-G, Koesling D, Russwurm M (2010) Activation of PDE2 and PDE5 by specific GAF ligands: Delayed activation of PDE5. *Br J Pharmacol* 161(7): 1645–1660.
33. Lawrence TS, Beers WH, Gilula NB (1978) Transmission of hormonal stimulation by cell-to-cell communication. *Nature* 272(5653):501–506.
34. Kanaporis G, et al. (2008) Gap junction channels exhibit connexin-specific permeability to cyclic nucleotides. *J Gen Physiol* 131(4):293–305.
35. Ablasser A, et al. (2013) Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. *Nature* 503(7477):530–534.
36. Calebiro D, et al. (2009) Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol* 7(8):e1000172.
37. Zhu H, et al. (2011) Restoring soluble guanylyl cyclase expression and function blocks the aggressive course of glioma. *Mol Pharmacol* 80(6):1076–1084.
38. Ableser MJ, Penuela S, Lee J, Shao Q, Laird DW (2014) Connexin43 reduces melanoma growth within a keratinocyte microenvironment and during tumorigenesis in vivo. *J Biol Chem* 289(3):1592–1603.
39. Horner K, et al. (2003) Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Dev Biol* 258(2):385–396.
40. Mehlmann LM, et al. (2004) The G_s-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science* 306(5703):1947–1950.

Supporting Information

Shuhaibar et al. 10.1073/pnas.1423598112

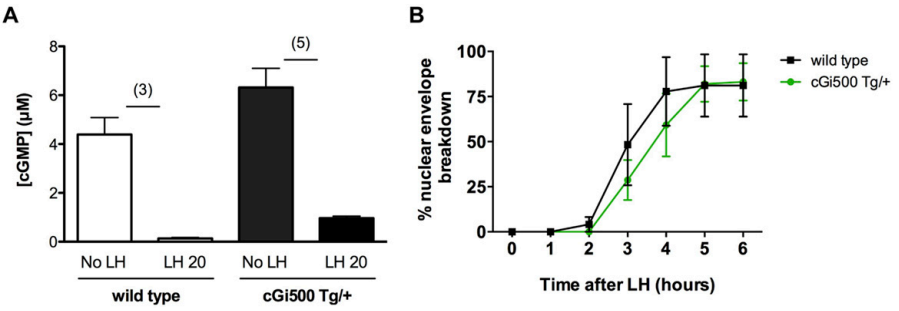


Fig. S1. ELISA measurements of the LH-induced cGMP decrease, and the time course of nuclear envelope breakdown, in cGi500-expressing and wild-type follicles. (*A*) cGMP ELISA measurements from follicles with or without a 20-min treatment with LH. Values indicate the mean ± SEM for three experiments with wild-type follicles, and for five experiments with Tg/+ follicles, both from the cGi500 transgenic mouse line. Measurements from follicles with a 20-min LH treatment were significantly different from controls without LH (paired *t* tests, for both genotypes $P < 0.01$). In the cGi500-expressing follicles, the cGMP level measured at 20 min after LH application was ∼15% of the basal level, compared with ∼3% in wild-type follicles, possibly because of some sequestration of cGMP by the sensor or to interference of the sensor with the ELISA. (*B*) Time course of nuclear envelope breakdown in follicle-enclosed oocytes from transgenic mice expressing the cGi500 sensor or from wild-type littermates. LH was applied to the medium in the dish holding the Millicell membrane, and oocytes within follicles on the Millicell membrane were observed for the presence or absence of a nucleus and nucleolus at 1-h intervals (1). Observations were made using a 20×/0.4 NA long-working distance objective. The graph shows the results from three experiments in which follicles from cGi500 and wild-type littermates were tested in parallel. Fifty-three follicles were scored for each genotype. Bars show mean ± SEM. Methods for the ELISA measurements were as previously described (1), except that the ELISA kit was from Enzo Life Sciences (#ADI-900-014), and the wash procedure was performed more rapidly. For measurements at "20 minutes" after LH, the follicles were removed from the incubator at 15 min after LH addition, washed in PBS at room temperature, transferred to a microfuge tube, and then solubilized in 0.1 M HCl at 20 min. In our previous study (1), the "20 minute" sample preparation involved removal of the follicles from the incubator at 10 min after LH, followed by a 10-min wash procedure, and HCl addition at 20 min. This technical difference may explain why the wild-type cGMP value reported here for the 20-min time point is somewhat lower (∼100 nM) compared with that reported previously (∼500 nM).

1. Norris RP, Freudzon M, Nikolaev VO, Jaffe LA (2010) Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. *Reproduction* 140(5):655–662.

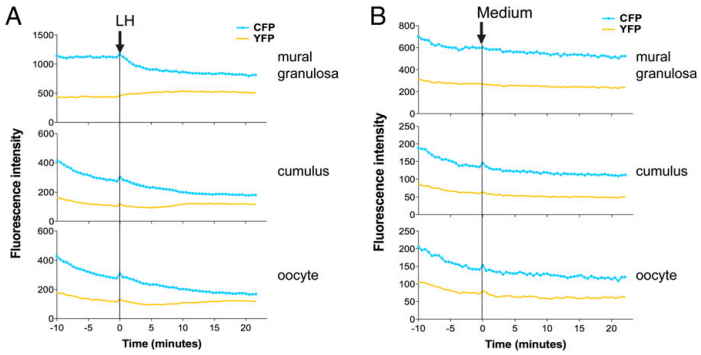


Fig. S2. Time courses of CFP and YFP emission intensities from follicles after perfusion of LH or medium. (*A*) Records from the same follicle for which CFP/YFP ratios are shown in Fig. 2*A* (perfusion of LH). (*B*) Records from the same follicle for which CFP/YFP ratios are shown in Fig. 2*B* (perfusion of medium). In the mural granulosa cells (*Top*), LH perfusion (*A*) caused a decrease in CFP emission and a corresponding increase in YFP emission, indicating a change in FRET efficiency. Perfusion of medium (*B*) caused no change in CFP or YFP intensity. Absolute intensities of CFP and YFP emission depended on the intensity of the excitation light, which varied somewhat in individual experiments, and on the clarity of individual follicles. In some examples, downward drift in emission intensities occurred, corresponding to darkening of the follicle as it was held in the imaging slide over the 30-min recording period. In the cumulus cells and oocyte (*Middle* and *Lower*), LH perfusion caused a clear increase in YFP intensity, but a decrease in CFP intensity was usually not detectable over the gradual downward drift because of time-dependent follicle darkening, which was also seen in the absence of LH. Darkening of the image was more pronounced in the follicle interior (cumulus and oocyte regions), and was also seen in follicles compressed in the imaging slide but not exposed to excitation light; therefore, it is unlikely to be caused by light-induced bleaching of the cGi500 sensor. Ratio imaging corrects for this artifactual change.

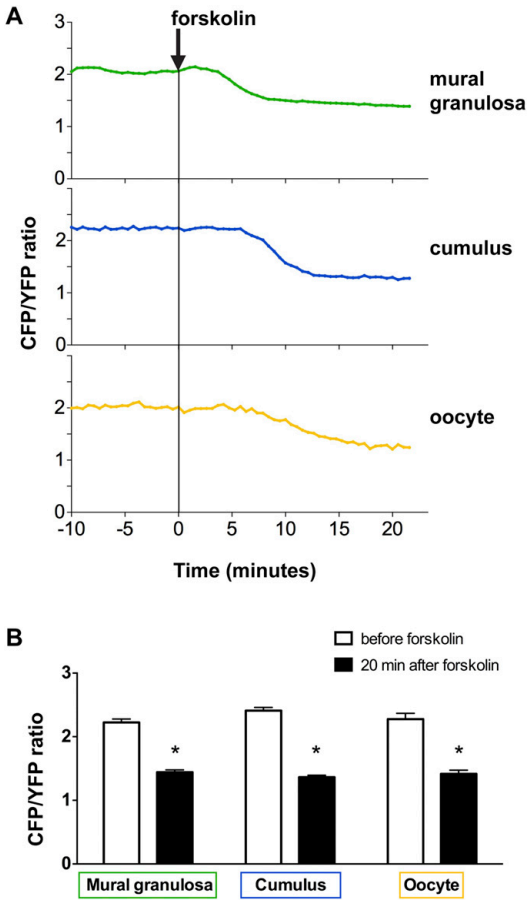


Fig. S3. cGMP in the follicle decreases rapidly in response to 10 μM forskolin, reaching a level similar to that seen in response to LH. (*A*) Representative records. (*B*) Results from seven follicles. Values that are indicated by an asterisk are significantly different from the control ($P < 0.05$); values indicate mean ± SEM. Unlike LH, for which receptors are present only in the mural granulosa cells, forskolin activates adenylyl cyclases in all cells of the mouse follicle (1, 2). Therefore, the forskolin-induced decreases in cGMP in the cumulus cells and oocyte could result in part from direct actions of forskolin on adenylyl cyclases in these cells, in addition to diffusion of cGMP into the mural granulosa cells. As a consequence of forskolin-stimulation of adenylyl cyclases in the oocyte, and the resulting cAMP elevation, continuous exposure of mouse follicles to forskolin does not cause nuclear envelope breakdown, although a transient pulse of forskolin does (3). Similarly, continuous exposure to forskolin inhibits LH-induced nuclear envelope breakdown in mouse follicles (3). Methods were the same as described for measurements of cGMP in response to LH. Forskolin was obtained from Sigma.

1. Schultz RM, Montgomery RR, Belanoff JR (1983) Regulation of mouse oocyte meiotic maturation: Implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. *Dev Biol* 97(2):264–273.
2. Bornslaeger EA, Schultz RM (1985) Regulation of mouse oocyte maturation: Effect of elevating cumulus cell cAMP on oocyte cAMP levels. *Biol Reprod* 33(3):698–704.
3. Hashimoto N, Kishimoto T, Nagahama Y (1985) Induction and inhibition of meiotic maturation in follicle-enclosed mouse oocytes by forskolin. *Dev Growth Differ* 27(6):709–716.

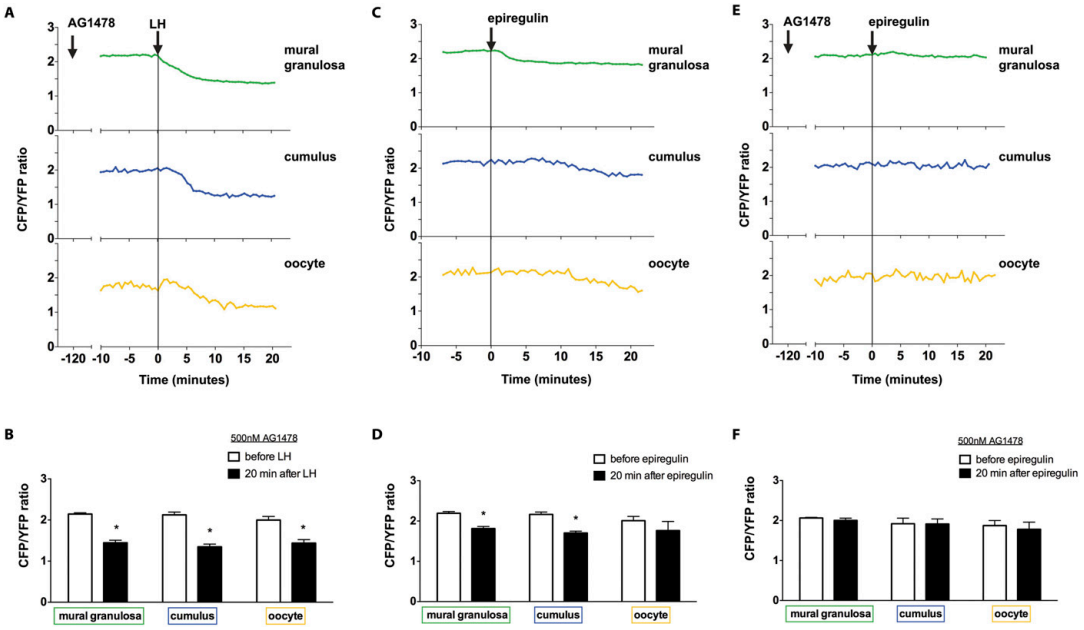


Fig. S4. Inhibition of EGF receptor kinase activity with 500 nM AG1478 does not inhibit the initial LH-induced cGMP decrease in the follicle. (*A*) Representative records. (*B*) Results from four follicles. (*C*–*F*) Control records showing that epiregulin causes a partial decrease in cGMP in the follicle (*C* and *D*; four follicles), and that this decrease is inhibited by 500 nM AG1478 (*E* and *F*; two follicles). Values that are indicated by an asterisk are significantly different from the control ($P < 0.05$); values indicate mean ± SEM. These control records confirm previous evidence that 500 nM AG1478 effectively inhibits EGF receptor kinase activity under these conditions (1). In contrast to our findings with AG1478, a previous study (2) found that 500 nM AG1478 inhibits most of the cGMP decrease measured by ELISA at 15 min after LH application. Several differences in experimental methods (strain of mice, gonadotropin injection of mice, follicle culture conditions, cGMP measurement method) could contribute to the difference in results. Our findings with epiregulin confirm previous reports that it causes cGMP to decrease in mouse follicles (1, 3). The cGMP decrease that we measured at 20 min was less than that seen with LH, but a previous study showed that by 2 h after epiregulin treatment, the cGMP level in the follicle is similar to that after LH treatment (3). Although the cGMP concentration in the oocyte has not been measured at 2 h after epiregulin treatment of the follicle, it is likely to be similar to that in the follicle as a whole, due to equilibration through gap junctions (see Fig. 2*D*). Correspondingly, follicles treated with epiregulin resume meiosis (1, 4). Methods were the same as described in the main text. AG1478 was obtained from EMD Chemicals. Epiregulin was obtained from R&D Systems.

1. Norris RP, Freudzon M, Nikolaev VO, Jaffe LA (2010) Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. *Reproduction* 140(5):655–662.
2. Liu X, Xie F, Zamah AM, Cao B, Conti M (2014) Multiple pathways mediate luteinizing hormone regulation of cGMP signaling in the mouse ovarian follicle. *Biol Reprod* 91(1):9, 1–11.
3. Vaccari S, Weeks JL, II, Hsieh M, Menniti FS, Conti M (2009) Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes. *Biol Reprod* 81(3):595–604.
4. Park JY, et al. (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 303(5658):682–684.

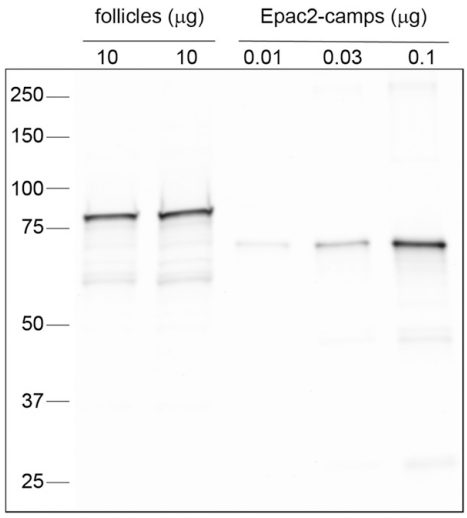
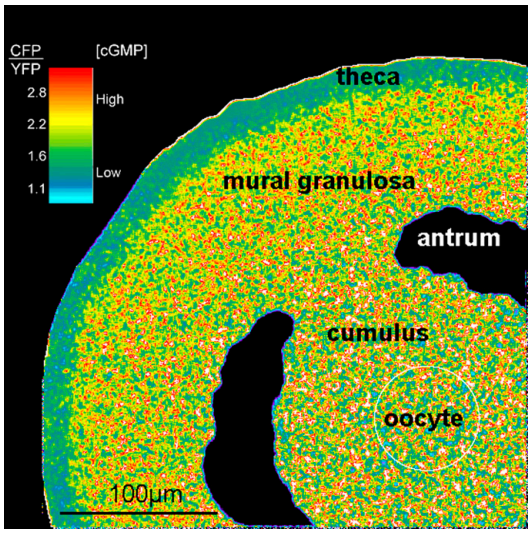


Fig. S5. Quantification of cGi500 protein in follicles from transgenic mice expressing one copy of the transgene. Ten-microgram samples of follicle protein from two separate preparations were separated by SDS/PAGE, in parallel with various amounts of purified Epac2-camps protein used as a standard (1). Epac2-camps has a structure similar to that of cGi500 (CFP and YFP separated by a linker); the protein was purified from insect cells and was >90% pure, based on Coomassie staining. Proteins were transferred to a nitrocellulose membrane and stained with an antibody that recognizes CFP and YFP (Cell Signaling Technology; cat. no. 2555) and a fluorescent secondary antibody for detection using a LI-COR Biosciences Odyssey infrared imaging system. Densitometry using ImageJ showed that 10 μg of follicle protein contained the same number of moles of cGi500 as the number of moles in 0.09 μg of Epac2-camps protein. Based on the relative molecular weights of cGi500 and Epac2-camps, an average protein content per mouse follicle of 3.5 μg, and an estimated cytoplasmic volume per mouse follicle of 20 nL (2), the concentration of cGi500 in the cytoplasm was estimated to be ~20 μM.

1. Nikolaev VO, Bünemann M, Hein L, Hannawacker A, Lohse MJ (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem* 279(36):37215–37218.
2. Norris RP, Freudzon M, Nikolaev VO, Jaffe LA (2010) Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. *Reproduction* 140(5):655–662.



Movie S1. Live imaging of the decrease in cGMP after applying LH to an ovarian follicle from a transgenic mouse expressing the cGi500 sensor. Images of the follicle were taken every 30 s for a 10-min period before LH perfusion, and for a 20-min period afterward. Frames from this movie are shown in Fig. 1*B*.

Movie S1

Publication 2: Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes.

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Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes

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ABSTRACT

In preovulatory ovarian follicles of mice, meiotic prophase arrest in the oocyte is maintained by cyclic GMP from the surrounding granulosa cells that diffuses into the oocyte through gap junctions. The cGMP is synthesized in the granulosa cells by the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2) in response to the agonist C-type natriuretic peptide (CNP). In response to luteinizing hormone (LH), cGMP in the granulosa cells decreases, and as a consequence, oocyte cGMP decreases and meiosis resumes. Here we report that within 20 min, LH treatment results in decreased guanylyl cyclase activity of NPR2, as determined in the presence of a maximally activating concentration of CNP. This occurs by a process that does not reduce the amount of NPR2 protein. We also show that by a slower process, first detected at 2 h, LH decreases the amount of CNP available to bind to the receptor. Both of these LH actions contribute to decreasing cGMP in the follicle, thus signaling meiotic resumption in the oocyte.

Introduction

Mammalian oocytes are maintained in meiotic prophase for prolonged periods. During prophase arrest, the oocyte is located in a follicle in which it is surrounded by granulosa cells (Fig. 1(A)). As the follicle grows to its full size (~400–500 μm in mice), the oocyte acquires the ability to resume meiosis, but due to inhibitory signals from the granulosa cells, the oocyte remains in prophase (Jaffe and Norris, 2010; Conti et al., 2012). Then during each reproductive cycle, luteinizing hormone (LH) from the pituitary acts on the granulosa cells of the fully grown follicle to cause the oocyte to mature into a fertilizable egg and be ovulated. This process begins with the transition from prophase to metaphase, marked by the breakdown of the nuclear envelope about 2 h after LH exposure. However, other events of the prophase-to-metaphase transition occur before nuclear envelope breakdown: microtubule organizing centers assemble (Schuh and Ellenberg, 2007), chromatin condenses (Racowsky and Baldwin, 1989), and cell cycle regulatory proteins undergo changes in activity and localization (Solc et al., 2010).

Recent studies of the mouse ovary have shown that a key inhibitory substance for maintaining prophase arrest is cGMP, which diffuses from the granulosa cells into the oocyte through gap junctions (Norris et al., 2009; Vaccari et al., 2009). In the oocyte, cGMP inhibits the cAMP phosphodiesterase PDE3A, and thus prevents the degradation of cAMP. Elevated cAMP activates protein kinase A, which acts through a complex of mechanisms to inhibit the activity of the CDK1-cyclin B kinase and thus to inhibit the prophase-to-metaphase transition (Solc et al., 2010; Conti et al., 2012). If cGMP in a follicle-enclosed oocyte is experimentally decreased, by injection of a cGMP-specific phosphodiesterase, cAMP is decreased, and as a consequence meiosis resumes (Norris et al., 2009).

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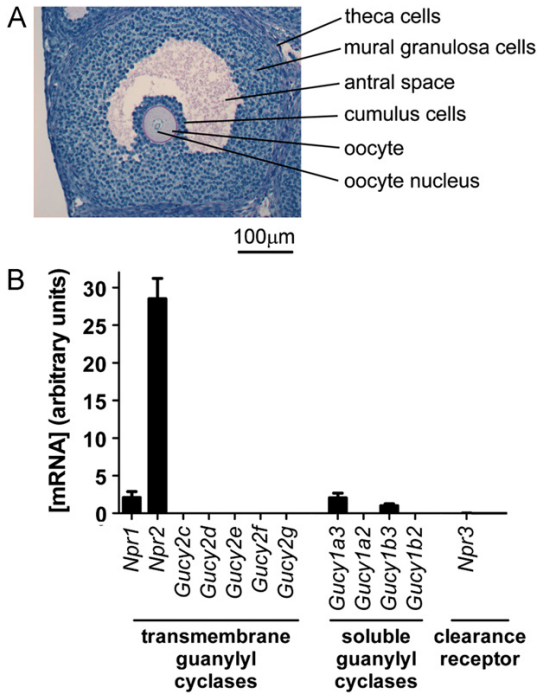

Fig. 1. In mural granulosa cells, *Npr2* mRNA is present at a higher concentration than mRNAs for other guanylyl cyclases. (**A**) Histological section of a mouse ovary, showing an antral follicle, and indicating the mural granulosa cells collected for analysis, as well as other cell types and structures in and around the follicle. (**B**) Relative concentrations of each guanylyl cyclase mRNA in isolated mural granulosa cells. Results for the natriuretic peptide clearance receptor, *Npr3*, are also shown. Where no bars are visible, concentrations of mRNAs were $<0.1\%$ of *Npr2*. The results show the mean $\pm$ s.e.m. for 3 RNA preparations.

The generation of the cGMP that maintains meiotic arrest requires the function in the granulosa cells of the transmembrane guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also known as guanylyl cyclase-B) and its extracellular agonist C-type natriuretic peptide (CNP, also known as natriuretic peptide C, NPPC) (Zhang et al., 2010). In ovaries of mice carrying mutations in *Npr2* or *Nppc* genes, meiosis resumes precociously (Zhang et al., 2010). Although there is also evidence for expression of other guanylyl cyclases in granulosa cells (Sriraman et al., 2006) and some evidence that these may contribute to the maintenance of meiotic arrest (Törnell et al., 1990; Sela-Abramovich et al., 2008; Vaccari et al., 2009) and the response of the follicle to LH (Sriraman et al., 2006), CNP-dependent activation of NPR2 is fundamental for generating the inhibitory levels of cGMP.

CNP is synthesized by the outer (mural) granulosa cells, and binds to NPR2 throughout the follicle to stimulate cGMP production (Jankowski et al., 1997; Zhang et al., 2010). The connection of the cumulus cells to the mural granulosa cells is essential for maintaining meiotic arrest, since when this connection is broken, leaving the cumulus-oocyte complex free in the antral space, meiosis resumes (Racowsky and Baldwin, 1989). This supports the concept that although *Npr2* mRNA is most concentrated in the cumulus cells (Zhang et al., 2010), cGMP generated by NPR2 in the mural layers also provides a critical part of the inhibitory cGMP to the oocyte.

Despite this knowledge of how CNP, NPR2, and cGMP function to maintain meiotic arrest, less is known about how signaling by LH reverses the arrest. LH acts on a G-protein-linked receptor (LHCGR) (Rajagopalan-Gupta et al., 1998), which in rats and mice, is located in the mural granulosa cells, mostly within the outer several layers of cells, and is absent in the cumulus cells (Amsterdam et al., 1975; Eppig et al., 1997). In response to LH, the permeability of the gap junctions between the granulosa cells throughout the follicle is reduced, such that intercellular diffusion within the follicle of molecules of the size of cGMP is slowed (Sela-Abramovich et al., 2005; Norris et al., 2008). In parallel, cGMP levels in the follicle decrease (Hubbard, 1986; Norris et al., 2009; Vaccari et al., 2009), from a basal level of $\sim$3 μM, to $\sim$0.5 μM at 20 min and $\sim$0.1 μM at 1 h after applying LH (Norris et al., 2010). CNP levels also decrease (Jankowski et al., 1997; Kawamura et al., 2011), but the earliest of these measurements were made at 4 h after LH application, while the cGMP decrease occurs by 20 min, so their functional significance has not been certain. As cGMP in the follicle decreases, cGMP in the interconnected oocyte falls correspondingly, to a few percent of the basal level at 1 h. As a consequence, the inhibition of PDE3A is relieved, cAMP decreases, and meiosis resumes (Norris et al., 2009; Vaccari et al., 2009).

The decrease in cGMP in the follicle could be caused by a decrease in cGMP synthesis, an increase in cGMP degradation, and/or an increase in cGMP efflux. Here we report that one mechanism by which LH signaling reduces cGMP is by reducing the activity of the guanylyl cyclase NPR2.

Materials and methods

Mice and hormones

Ovaries were obtained from prepubertal B6SJLF1 mice (23–25 day old) from The Jackson Laboratory (Bar Harbor, ME); procedures were approved by the animal care committees of the University of Connecticut Health Center, China Agricultural University, and The Jackson Laboratory. For granulosa cell collection, cumulus-oocyte complex collection, CNP ELISA assays, and histological analysis, the mice were injected with 5 I.U. equine chorionic gonadotropin (eCG) 40–48 h before use, to stimulate follicle growth and LH receptor expression. Mice for antral follicle isolation were not injected with eCG; instead the follicles were exposed to 10 ng/ml follicle stimulating hormone (FSH) in vitro.

Ovine LH, human LH, ovine FSH, and eCG, purified from biological sources, were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). Human recombinant LH was obtained from EMD Serono Research Institute, Inc. (Rockland, MA). Human chorionic gonadotropin (hCG) was purchased from Sigma-Aldrich (St. Louis, MO). Ovine LH was used for studies of isolated follicles (10 μg/ml). Because of their slower rate of degradation (Mock and Niswender, 1983), human LH or hCG was used for injection into mice (10 μg or 5 I.U., respectively).

Measurement of relative amounts of guanylyl cyclase mRNAs in granulosa cells

Mural granulosa cells were collected by puncturing antral follicles of isolated ovaries with 30 gauge needles. RNA was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). DNAse I digestion was performed to remove residual genomic DNA, and mRNAs were reverse transcribed using random hexamers.

Quantitative TaqMan analysis was performed using the Applied Biosystems PRISM 7900 sequence detection system, to determine the relative concentration of each guanylyl cyclase mRNA in granulosa cells. Primer sequences are listed in Table S1 in the supplementary material. Differences in primer efficiency

were determined by measuring the cycle threshold (C_t) values for each primer pair using 30 ng of genomic DNA. Only small differences were detected, and these were corrected for by use of the following formula:

C_t (corrected) = C_t (measured) + C_t (mean of all probes, genomic DNA) − C_t (genomic DNA)

Normalization was performed using the housekeeping gene *Rpl32* as a control. The resulting expression is given in arbitrary units.

2.3. Measurement of guanylyl cyclase activity in a crude membrane fraction of follicles

For each experiment, antral follicles from 4 mice were isolated and cultured for 24–30 h in the presence of FSH to stimulate follicle growth and LH receptor expression (Norris et al., 2008, 2010). The follicles were divided into 2 equal groups, and half were exposed to LH for the indicated time. The 40–50 follicles in each group were washed in PBS and lysed in phosphatase inhibitor buffer (Dickey et al., 2007) in a 100 μl glass homogenizer. To obtain a crude membrane fraction, the homogenate (200 μl volume) was centrifuged at 10,000 × *g* for 20 min; the pellet (~1 μl volume) was resuspended in 50 μl of phosphatase inhibitor buffer and sonicated briefly. Protein content was determined by solubilizing a 4 μl aliquot in 1% SDS and performing a BCA assay (Pierce, Thermo Fisher Scientific, Rockford, IL). The crude membrane fraction contained ~1 μg of protein per follicle. The samples were frozen in liquid N_2 and stored at −80 °C.

Guanylyl cyclase assays were conducted for each pair of follicle samples prepared as described above (one sample that had been treated with LH, one control sample without LH), using methods as previously described (Robinson and Potter, 2011). Assays were performed at 37 °C using 1–2 μg of follicle protein per assay tube, in the presence or absence of 1 μM CNP (or ANP), which are maximally activating concentrations for their respective receptors (Abbey-Hosch et al., 2005; Dickey et al., 2008). 0.5 mM isobutylmethylxanthine (IBMX) was included to inhibit cGMP phosphodiesterase activity. CNP (or ANP) dependent guanylyl cyclase activity refers to the activity measured in the presence of the natriuretic peptide minus the activity measured in the absence of the natriuretic peptide. Statistical significance of the data was tested using two-way repeated measures ANOVA with a Bonferroni post-test; control and LH-treated samples that had been prepared and assayed together were analyzed as pairs. The analysis was performed using Prism software (GraphPad Software, Inc., La Jolla, CA).

Measurement of cGMP in cumulus cells

Cumulus-oocyte complexes were isolated at various times after hCG injection, and cultured as previously described (Zhang et al., 2010), with or without 30 nM CNP for 1 h. Cumulus cells were then separated for measurement of cGMP using an ELISA method as previously described (Zhang et al., 2010). Statistical significance of the data was tested using one-way ANOVA with a Dunnett multiple comparisons post-test.

Measurement of CNP in ovaries

CNP in ovaries was assayed by an ELISA method (FEK-012-03, Phoenix Pharmaceuticals Inc., Burlingame, CA) with a primary antibody made against the 22 amino acid form of CNP. This antibody also recognizes the 53 amino acid form of CNP, and presumably the precursor forms, which include the same 22 amino acids at their C-termini (Wu et al., 2003; Potter et al., 2009).

Samples were prepared by a method modified from Jankowski et al. (1997). Two ovaries in 70 μl of 1.0 M acetic acid were heated at 95 °C for 10 min, then lysed with a probe sonicator. 350 μl of MeOH was added to solubilize lipids, and the tube was centrifuged at 30,000 × *g* at 4 °C for 15 min. The supernatant ("ovary extract") contained ~250 μg of protein. ~50% of the CNP was recovered in this extract (determined by adding a known amount of CNP to the ovaries before extraction). For each sample, 10, 5, and 2 μg of the extract protein were lyophilized and assayed, following the manufacturer's instructions. Data were analyzed using Prism software. Statistical significance of the data was tested using one-way ANOVA with a Dunnett multiple comparisons post-test. The concentration of CNP in the ovary, if CNP was uniformly distributed, was estimated based on a volume per ovary of ~4 μl (~4 mg wet weight).

Histological analysis of nuclear envelope breakdown kinetics

Serial sections of mouse ovaries were prepared as previously described (Mehlmann et al., 2004). Follicles with a diameter of ≥350 μm in at least one dimension, as measured in the section containing the nucleolus or chromosomes, were analyzed for the presence of an intact nucleus (see Fig. 1(A)).

Results and discussion

In mural granulosa cells, mRNA encoding NPR2 is present at a higher concentration than mRNAs encoding other guanylyl cyclases

Although NPR2 is known to be present in mural granulosa cells and functionally important for maintaining meiotic arrest, there is also evidence that NPR1 and soluble guanylyl cyclase subunits could contribute to the control of meiotic arrest and progression (see Introduction). Because previous studies did not determine the relative expression levels of mRNA for NPR2 and other guanylyl cyclases in mouse granulosa cells, and because not all of the guanylyl cyclases were investigated, we quantitatively compared the amounts of mRNA in mural granulosa cells for each of the mouse guanylyl cyclase genes (Fig. 1(B)).

The mouse genome contains 7 transmembrane and 4 soluble guanylyl cyclase genes (Potter, 2011a). We detected mRNA encoding two transmembrane guanylyl cyclases, NPR1 and NPR2, and two soluble guanylyl cyclase subunits, GUCY1A3 (soluble guanylyl cyclase alpha 1) and GUCY1B3 (soluble guanylyl cyclase beta 1). Among these, *Npr2* mRNA was expressed at a high level, ≥14 times higher than any of the other guanylyl cyclases. We also tested for mRNA encoding NPR3, which has sequence similarity to the extracellular domains of NPR1 and NPR2, but lacks the guanylyl cyclase domain and activity (Potter, 2011a). NPR3 is a clearance receptor for natriuretic peptides. Little or no *Npr3* mRNA was detected.

Although concentrations of mRNAs are not directly proportional to the amounts of the proteins they encode, these measurements further support the conclusion that NPR2 is the primary guanylyl cyclase that produces cGMP in the follicle.

LH signaling reduces NPR2 activity in the follicle

One way that LH activation of its receptors in the mural granulosa cells could decrease cGMP levels within the follicle is by reducing NPR2 activity. Two aspects of this question were considered: (1) whether LH signaling decreases NPR2 activity in the follicle as a whole, of which most of the volume is mural granulosa cells, and (2) whether LH signaling decreases NPR2 activity in the cumulus cells. A decrease in NPR2 activity in either

or both of these regions could contribute to the measured decrease in cGMP in the oocyte. This section describes our studies of a crude membrane fraction from whole follicles, and a subsequent section describes our studies of cumulus cells.

Guanylyl cyclase activity was measured using the particulate fraction obtained by centrifuging a homogenate of follicles. When this crude membrane fraction was incubated without CNP, guanylyl cyclase activity was too low to measure accurately, but addition of 1 μM CNP increased the activity to 0.21 ± 0.02 nmole cGMP/mg protein/minute ($n=15$ follicle preparations, see Fig. 2(A) and (B)). After a 20 min exposure of follicles to LH, CNP-dependent guanylyl cyclase activity fell to 50% of the activity measured in the membrane fraction from follicles without LH exposure, and remained depressed for 2 h after applying LH (Fig. 2 (A) and (B)).

The decrease in follicle cGMP that will result from a 50% decrease in NPR2 activity depends on the cGMP affinity of the phosphodiesterases present in the granulosa cells (Fig. S1). If the affinity is higher (lower K_m), the cGMP concentration will fall to a lower level. Much of the cGMP phosphodiesterase activity in the follicle is sensitive to sildenafil and tadalafil, indicating an important PDE5 component (Vaccari et al., 2009). Based on K_m values for PDE5, a 50% reduction in NPR2 activity could potentially account for the decrease in follicle cGMP from 3 μM before LH treatment to ~0.5 μM after 20 min (Norris et al., 2010) (Fig. S1).

Because a small amount of *Npr1* mRNA is also expressed in granulosa cells (Fig. 1(B)), we also evaluated the effect of LH on NPR1 activity, by measuring guanylyl cyclase activity in the presence of 1 μM atrial natriuretic peptide (ANP). Studies of human NPR1 and NPR2 have shown that 1 μM ANP activates NPR1, but has almost no effect on NPR2 (Dickey et al., 2008). In the crude membrane fraction from follicles, ANP-dependent guanylyl cyclase activity was 0.07 ± 0.03 nmole cGMP/mg protein/minute, or 33% of the CNP-dependent activity ($n=4$). However, the ANP-dependent activity was unchanged by LH (Fig. 2(C) and (D)). Some of the ANP-dependent guanylyl cyclase activity that we measured might be due to NPR1 expressed in membranes from theca cells and blood vessels that were not completely removed from the follicle by microdissection (Fig. 1(A)). The lack of effect of LH on ANP-dependent cGMP accumulation serves as a control to indicate that the LH-induced decrease in CNP-dependent cGMP accumulation is not due to an LH effect on phosphodiesterase activity that could have been present in the crude membrane fraction despite the presence of IBMX.

The LH-induced decrease in NPR2 activity in the follicle occurs without a corresponding decrease in NPR2 protein

Previous studies have shown that other biological factors that rapidly decrease NPR2 activity in cultured cells do so in a manner that is independent of NPR2 protein levels (Abbey and Potter, 2003; Abbey-Hosch et al., 2004). To test if LH decreased the amount of NPR2 protein in follicles, we first tried Western blotting, and immunoprecipitation followed by Western blotting. However, with the available antibodies, it was not possible to detect endogenous levels of the protein using these methods. So instead, we measured guanylyl cyclase activity in follicle membrane fractions after treatment with 1% Triton X-100 and 5 mM $MnCl_2$, a condition known to maximally activate NPR1 and NPR2 in the absence of natriuretic peptide and to be indicative of guanylyl cyclase protein levels (Potter and Hunter, 1999; Abbey and Potter, 2003). Guanylyl cyclase activity measured in the presence of Triton X-100 and $MnCl_2$ is independent of modification of the NPR2 protein by phosphorylation (Potter and Hunter, 1998).

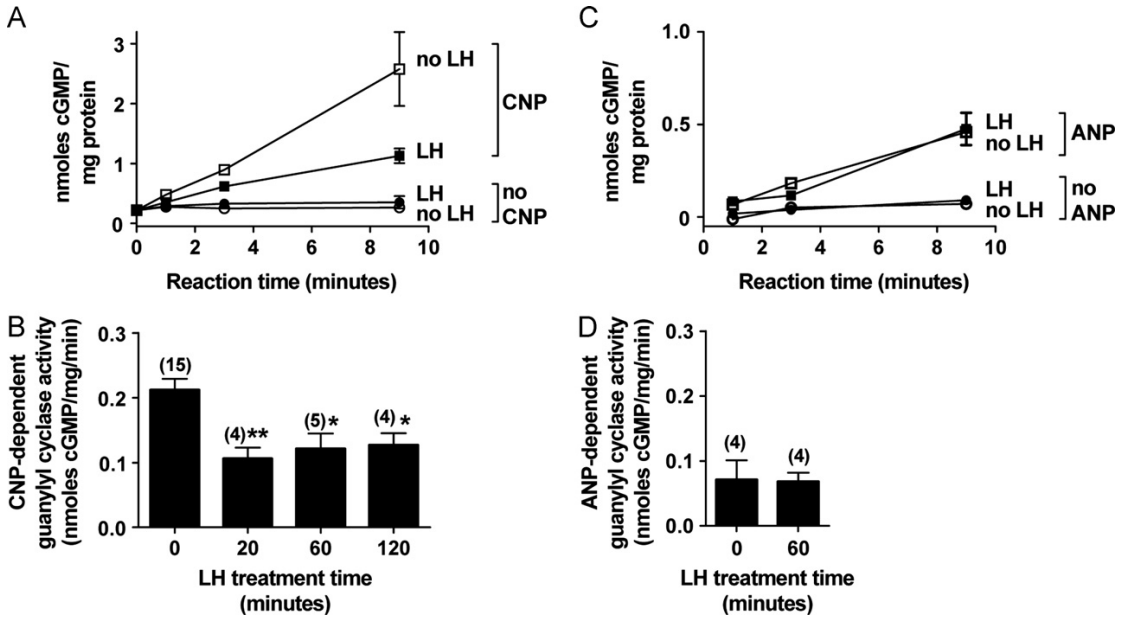

Fig. 2. LH signaling reduces NPR2 activity in the follicle. (A) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 20 min was measured with or without 1 μM CNP. Values indicate the mean ± range of duplicate measurements for each condition, using one follicle preparation made after LH treatment, and another preparation made in parallel but without LH treatment. Where not visible, the error bars are contained within the symbol. (B) Combined data from 15 experiments like that in A, showing CNP-dependent guanylyl cyclase activity of crude membrane fractions from follicles treated with LH for the indicated times. CNP-dependent activity values were determined by subtracting the basal values measured in the absence of CNP. Activities are expressed as the mean ± s.e.m. for n follicle preparations. Activities for follicles treated with LH for 20, 60, or 120 min differed significantly from the activity for follicles without LH treatment (*$p<0.05$; **, $p<0.01$). (C) and (D) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 60 min was measured with or without 1 μM ANP. Data are presented as described for A and B. LH did not decrease the ANP-dependent guanylyl cyclase activity.

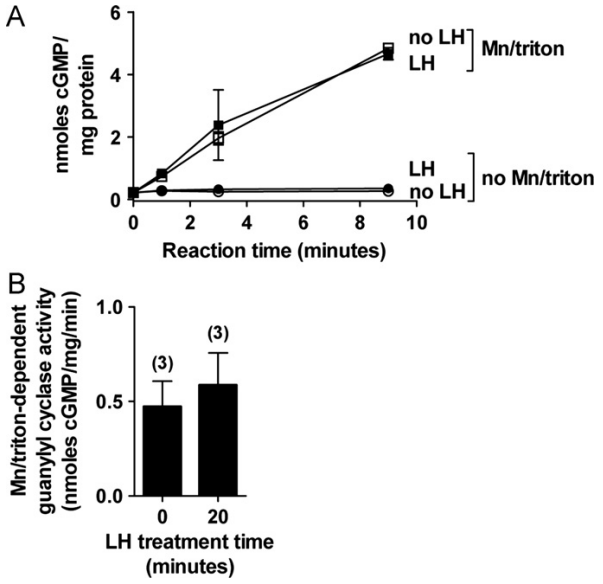

Fig. 3. The LH-induced decrease in NPR2 activity occurs without a corresponding decrease in NPR2 protein. (A) Guanylyl cyclase activity of crude membrane fractions prepared from follicles treated with or without LH for 20 min was measured with or without 1% Triton X-100 and 5 mM $MnCl_2$, to maximally activate guanylyl cyclase. Values indicate the mean ± range of duplicate measurements for each condition, using one follicle preparation made after LH treatment, and another preparation made in parallel but without LH treatment. (B) Combined data from 3 experiments like that in A, showing Mn/triton-dependent guanylyl cyclase activity of crude membrane fractions from follicles treated with or without LH for 20 min (mean ± s.e.m.). LH did not decrease the Mn/triton-dependent guanylyl cyclase activity.

Detergent-dependent guanylyl cyclase activity was the same in samples from follicles with or without LH treatment for 20 min (Fig. 3). Since NPR1 and NPR2 are the only detectable membrane guanylyl cyclases in granulosa cells, and since NPR1 is a relatively minor component, these detergent measurements indicate that at 20 min, LH does not decrease the amount of NPR2 protein. A possible cause of the rapid LH-induced decrease in NPR2 activity is dephosphorylation, which can result from elevation of intracellular Ca^{2+} and/or activation of protein kinase C (Abbey-Hosch et al., 2005; Potter, 2011b).

In the cumulus cells, CNP-dependent cGMP production decreases in response to LH receptor stimulation, but more slowly than in the mural granulosa cells

Because of the direct connection between the cumulus cells and the oocyte, and because of the higher level of *Npr2* mRNA in the cumulus cells compared with the mural cells (Zhang et al., 2010), it was of particular interest to investigate whether LH signals that are initiated in the mural granulosa cells regulate NPR2 activity in the cumulus cells. As described above, LH receptors are not present in the cumulus cells, so such regulation would have to involve signaling between different regions of the follicle.

Due to the small amount of protein that could be obtained, we could not analyze guanylyl cyclase activity in a cumulus cell membrane fraction as we did for the more abundant material from whole follicles. Instead, we isolated cumulus-oocyte complexes from ovaries at various times after injection of mice with human chorionic gonadotropin (hCG) to stimulate the LH receptor, incubated the complexes in the presence or absence of 30 nM CNP for an additional hour, then isolated the cumulus cells and measured their cGMP content. hCG is often used instead of LH, since both hormones act on the same receptor. 30 nM CNP was used because this is approximately the minimum concentration needed to prevent spontaneous meiotic resumption in isolated cumulus-oocyte complexes (Zhang et al., 2010). Under these experimental conditions, measurements of a change in cellular cGMP content in response to LH receptor stimulation could indicate a change in guanylyl cyclase activity, or a change in cGMP phosphodiesterase activity, or a change in cGMP efflux. However, by measuring the effect of LH receptor stimulation on cGMP content in the presence and absence of CNP, we were able to distinguish between these possibilities.

Without injection of the mice with hCG, addition of 30 nM CNP to cumulus-oocyte complexes elevated the cGMP content of the cumulus cells by 4.1 ± 0.9 times ($n=6$). When cumulus-oocyte complexes were isolated from mice at 1 h after hormone injection, and then incubated in the presence of CNP for an additional hour, the cGMP content of the cumulus cells was the same as that in cumulus cells from mice without hormone injection (Fig. 4). However, when the cumulus-oocyte complexes were isolated at 2 h after hormone injection, and incubated with CNP until 3 h, cGMP had decreased to 70% of values obtained without hormone treatment (Fig. 4). With isolation of the complexes at 3 h, followed by a CNP incubation and measurement at 4 h, cGMP had decreased to 42% (Fig. 4).

In the absence of CNP, the cGMP content of the cumulus cells was low, as expected for this in vitro condition in which NPR2 would not be activated. Under this condition, the cGMP content was not decreased by LH receptor stimulation (Fig. 4), indicating that LH receptor signaling does not increase cGMP phosphodiesterase activity in these cells, or cause an increase in cGMP efflux. Thus the LH receptor-induced decrease in cumulus cell cGMP seen in the presence of CNP can be attributed to a decrease in cGMP production. These findings indicate that LH signaling decreases NPR2 activity in the cumulus cells, but only after 2–3 h, versus 20 min in the mural cells. This delay is likely to be a consequence of the localization of the LH receptors in separate cells (the mural granulosa). As will be discussed below, this intercellular signaling is most likely mediated by the release of EGF-like growth factors from the mural granulosa cells.

The decrease in cGMP production in the cumulus cells could result from a decrease in the amount of NPR2 protein, or from a

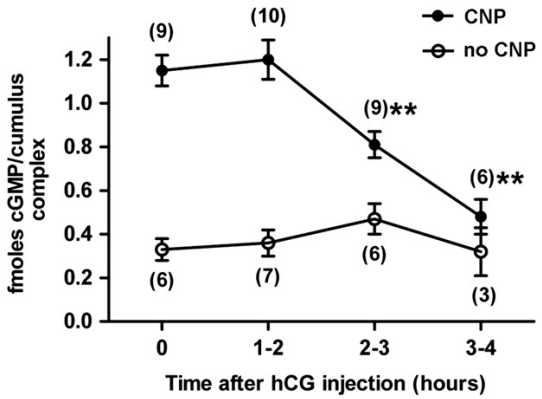

Fig. 4. In the cumulus cells, CNP-dependent cGMP production decreases in response to LH receptor stimulation, but more slowly than in the mural granulosa cells. Cumulus-oocyte complexes were collected at various times after hCG injection into mice and incubated for one hour with or without 30 nM CNP. The cumulus cells were separated and cGMP content was measured. Values indicate the mean ± s.e.m. for the number of experiments shown in parentheses. Values for 2–3 and 3–4 h post hCG were significantly different from the no hCG value ($^{**}p < 0.01$).

modification of the NPR2 protein such as dephosphorylation. In support of the first possibility, the amount of *Npr2* mRNA in the cumulus cells decreases to ~50% of the basal level at 3 h after LH receptor stimulation (Zhang et al., 2011). However, it is unknown how rapidly NPR2 protein would decrease as a consequence. Protein turnover rates for NPR2 have not been investigated, but turnover of the closely related protein NPR1 in cultured cells is a slow process, with a half-time of ≥ 8 h (Flora and Potter, 2010).

The amount of CNP in the ovary decreases in response to LH, preceding nuclear envelope breakdown

Another factor that could contribute to the LH-induced reduction in cGMP levels in the follicle is a decrease in CNP. CNP decreases have been reported previously for rat, mouse, and human (Jankowski et al., 1997; Kawamura et al., 2011), but the earliest of these measurements were made at 4 h after LH receptor stimulation, so it was unclear if the CNP decrease occurred early enough to contribute to causing nuclear envelope breakdown and the events that precede it, vs later events leading to ovulation. To investigate the time course of the decrease in CNP, we injected mice with LH, and at various times afterwards, collected their ovaries for analysis of CNP content using an ELISA based on an antibody that should recognize all forms of CNP and its precursor NPPC.

Without LH injection, there were ~150 fmoles of CNP per mg of ovary extract protein (Fig. 5(A)), corresponding to an overall concentration of ~10 nM. However, the immunoreactive material detected by the ELISA contains both extracellular peptide and intracellular precursor protein, and only the peptide that has been secreted into the extracellular space can activate NPR2. Thus the concentration of peptide that could function to regulate NPR2 is unknown.

No change from the pre-LH level of CNP was seen at 1 h after injection of LH, but by 2 h, the amount of CNP had decreased to 37% (Fig. 5(A)). The decrease in CNP at 2 h corresponds to the time at which nuclear envelope breakdown in the oocyte is beginning, as determined from histological sections of ovaries of similarly treated mice (Fig. 5(B)). Thus, the CNP decrease occurs well after cGMP decreases in the follicle (detected at 20 min after LH, Norris et al., 2010), but early enough to potentially contribute to stimulating nuclear envelope breakdown. After nuclear envelope breakdown, CNP continued to decrease, reaching 15% at 4 h after LH, and 7% at 8 h (Fig. 5(A)).

A likely cause of the CNP decrease is that *Nppc* mRNA decreases to about half of the basal level by 2 h after LH receptor stimulation (Kawamura et al., 2011). Thus LH signaling might reduce *Nppc* mRNA synthesis or increase its degradation. Because the turnover of CNP is very rapid, with a half-life of about 3 min in plasma (Hunt et al., 1994), a decrease in *Nppc* mRNA could rapidly decrease the amount of CNP. Other possible factors that could contribute to the decrease in CNP are an increase in the natriuretic peptide clearance receptor NPR3, and an increase in the activity of proteases that degrade CNP (Potter, 2011c).

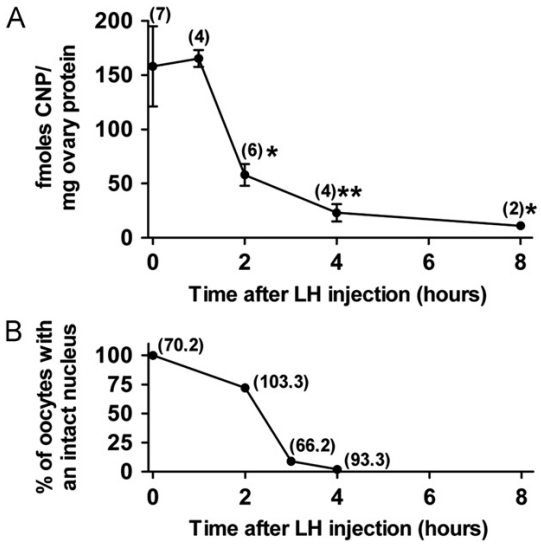

Fig. 5. The amount of CNP in the ovary decreases in response to LH, preceding nuclear envelope breakdown. (A) Time course of the decrease in the CNP content of ovary extracts, following LH injection into mice. Values indicate the mean ± s.e.m. for the number of mice shown in parentheses. Values for 2, 4, and 8 h LH treatments were significantly different from the no LH value (*$p < 0.05$; **$p < 0.01$). (B) Time course of nuclear envelope breakdown, following LH injection into mice. Values indicate the percentage of fully grown follicles (≥ 350 μm in diameter) in which the oocyte contained a prophase-arrested nucleus; the number of follicles and the number of mice counted are indicated.

The amount of CNP in the ovary increases as follicles develop to the preovulatory stage

We also examined the effect of equine chorionic gonadotropin (eCG, also called PMSG) on CNP levels. Unlike human chorionic gonadotropin (hCG), which binds to the LH receptor, eCG binds to the follicle stimulating hormone receptor, which stimulates antral follicle growth and LH receptor expression. eCG is often used experimentally to cause follicles to grow and to progress to the preovulatory stage; it was used for this purpose for the CNP measurements described above.

We found that eCG injection of the mice, 44 h before collecting the ovaries, increased their CNP content (Fig. 6). The increase in CNP in response to eCG is consistent with findings that mRNA encoding the CNP precursor (NPPC) increases in mouse ovaries in response to eCG (Kawamura et al., 2011), and that CNP and cGMP increase between the days of diestrus and proestrus, in rats and hamsters (Jankowski et al., 1997; Hubbard and Greenwald, 1982). Thus our findings add to the accumulating evidence that during follicle growth to the preovulatory stage, CNP and cGMP content of the ovary increases. At the preantral stage, cyclic nucleotide regulation is not needed to maintain meiotic arrest (Erickson and Sorensen, 1974). Then with follicle growth, as the oocyte accumulates more CDK1 and other factors that result in meiotic competence (Chesnel

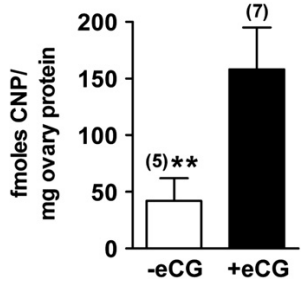

Fig. 6. The amount of CNP in the ovary increases in response to activation of follicle stimulating hormone receptors. The mice to be used for the CNP measurements shown in Fig. 5(A) were injected 44 h previously with eCG to stimulate follicle growth. In response to eCG, the amount of CNP per mg of ovary protein increased ~4 times. The +eCG data shown in Fig. 6 are the same as the no LH (0 h) data shown in Fig. 5(A), so the statistical significance of these data was tested together. Values for ovaries from mice with or without eCG treatment were significantly different.

and Eppig, 1995), CNP is synthesized in order to prevent premature meiotic progression.

Pathways by which LH signaling in the mural granulosa cells causes cGMP to decrease in the oocyte

The connections between LH-induced activation of G-proteins in the outer layers of the mural granulosa cells and the ensuing events in the follicle that lead to the decrease in cGMP and resumption of meiosis in the oocyte are only partially understood (Fig. 7) (Vaccari et al., 2009; Norris et al., 2010; Hsieh et al., 2011; Conti et al., 2012). These connections include not only the pathways leading to a decrease in granulosa cell guanylyl cyclase activity, but also pathways that reduce gap junction permeability through MAP kinase-dependent phosphorylation of connexin 43 (Sela-Abramovich et al., 2005; Norris et al., 2008), thus reducing diffusion of cGMP into the oocyte. Both the decrease in gap junction permeability and the decrease in granulosa cell guanylyl cyclase activity contribute to the decrease in oocyte cGMP.

At the level of the mural granulosa cells, LH receptor signaling activates G_s and adenylyl cyclase (Rajagopalan-Gupta et al., 1998), thus elevating cAMP. LH receptor signaling also activates G_i, G_q, and phospholipase Cβ (Rajagopalan-Gupta et al., 1998; Kühn and Gudermann, 1999), thus elevating calcium (via IP_3) in granulosa cells in culture (Davis et al., 1986; Flores et al., 1998). However, activation of protein kinase C by the diacylglycerol that is generated by phospholipase C has, to our knowledge, not been detected so far (Salvador et al., 2002). Further studies of these signaling events using intact follicles will be informative, because both calcium elevation and protein kinase C activation can lead to dephosphorylation and inactivation of NPR2 (Abbey-Hosch et al., 2005). Calcium elevation could also increase the activity of the PDE1 family of cGMP phosphodiesterases (Francis et al., 2011).

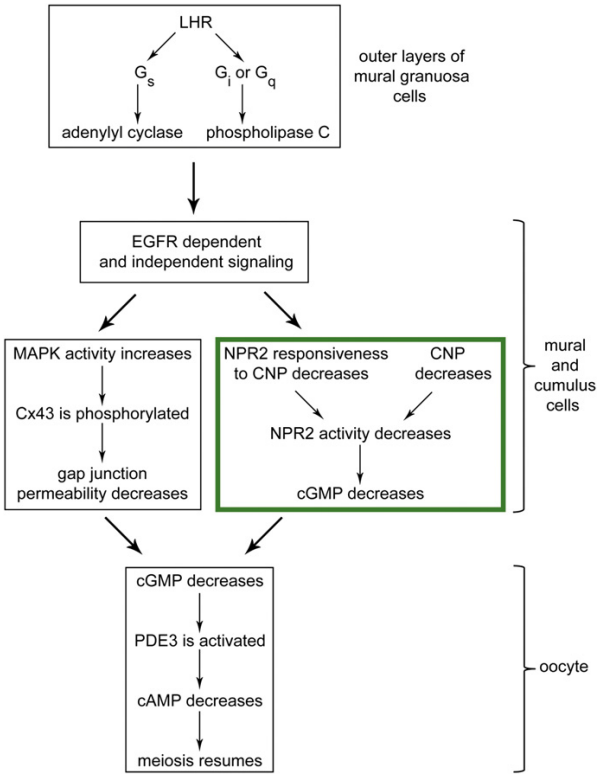

Fig. 7. Signaling pathways connecting LH binding to its receptors in the outer layers of the mural granulosa cells to resumption of meiosis in a mammalian oocyte. The green box indicates the findings of this study in the context of other aspects of the signaling network.

Since the mRNA encoding the precursor protein of CNP is expressed in the same cells as the LH receptor (Zhang et al., 2010), exposure to LH could result in a reduction in the amount of CNP by signaling within the same cells. Likewise, since the LH receptor is expressed in the outer several layers of the mural granulosa cells (Amsterdam et al., 1975), the LH-induced decrease in NPR2 activity in these cells could result from signaling within the same cell. However, the LH-induced decrease in NPR2 activity in the cumulus cells, or in the inner layers of the mural cell epithelium, both of which regions lack LH receptors (Amsterdam et al., 1975; Eppig et al., 1997), must involve signaling between cells.

Based on evidence that the cGMP decrease in the follicle is partially dependent on EGF receptor signaling (Vaccari et al., 2009; Norris et al., 2010; Hsieh et al., 2011; Conti et al., 2012), EGF-like growth factors released from the outer layers of mural granulosa cells in response to LH are mediators of the paracrine signals, and could also contribute to autocrine signaling in the outer layers of the mural granulosa cells. EGF receptor signaling is essential for LH-induced nuclear envelope breakdown (Park et al., 2004), and EGF receptor activation, as indicated by increased phosphorylation of the receptor protein, occurs as early as 30 min after LH treatment (Panigone et al., 2008). However, it remains unknown how LH receptor signaling triggers the synthesis and/or release of the EGF-like growth factors epiregulin and amphiregulin. RNA encoding precursors of these growth factors increases by 2 h after LH receptor stimulation (Park et al., 2004), but in addition, LH signaling might activate the proteases that release epiregulin and amphiregulin from pre-existing precursors (Blobel et al., 2009; Killock and Ivetic, 2010).

EGF receptor signaling is required for much of the increase in MAP kinase activity in response to LH (Panigone et al., 2008), and thus contributes to phosphorylation of connexin 43 and the resulting decrease in gap junction permeability (Norris et al., 2008; Conti et al., 2012). EGF receptor signaling also activates phospholipase Cγ (Chattopadhyay et al., 1999), and could thus elevate calcium and protein kinase C activity, amplifying the LH receptor signaling that may occur through through phospholipase Cβ. As discussed above, these signaling events could decrease NPR2 activity, and possibly increase PDE1 activity, thus lowering cGMP in the granulosa cells and oocyte.

Conclusions

By 20 min after applying LH to ovarian follicles, the guanylyl cyclase activity of NPR2 elicited by a saturating concentration of CNP is decreased by half. This correlates with a similarly rapid decrease in follicle cGMP. There is then a slower decrease in NPR2 responsiveness to CNP in the cumulus cells, first seen at 2–3 h. By 2 h, LH signaling also induces a decrease in the amount of CNP in the ovary. Together, these 3 factors that decrease guanylyl cyclase activity contribute to the decrease in cGMP in the follicle. Because the mural granulosa cells, cumulus cells, and oocyte are connected by gap junctions to form a syncitium with respect to cGMP, cGMP in the oocyte equilibrates with that in the surrounding somatic cell compartment, and the resulting decrease in oocyte cGMP promotes meiotic resumption.

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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.2012.04.019.

References

Abbey, S.E., Potter, L.R., 2003. Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. Endocrinology 144, 240–246.
Abbey-Hosch, S.E., Cody, A.N., Potter, L.R., 2004. Sphingosine-1-phosphate inhibits C-type natriuretic peptide activation of guanylyl cyclase B (GC-B/NPR-B). Hypertension 43, 1103–1109.
Abbey-Hosch, S.E., Smirnov, D., Potter, L.R., 2005. Differential regulation of NPR-B/GC-B by protein kinase C and calcium. Biochem. Pharmacol. 70, 686–694.
Amsterdam, A., Koch, Y., Lieberman, M.E., Lindner, H.R., 1975. Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. J. Cell. Biol. 67, 894–900.
Blobel, C.P., Carpenter, G., Freeman, M., 2009. The role of protease activity in ErbB biology. Exp. Cell. Res. 315, 671–682.
Chattopadhyay, A., Vecchi, M., Ji, Q.-S., Mernaugh, R., Carpenter, G., 1999. The role of individual SH2 domains in mediating association of phospholipase C-γ1 with the activated EGF receptor. J. Biol. Chem. 274, 26091–26097.
Chesnel, F., Eppig, J.J., 1995. Synthesis and accumulation of $p34^{cdc2}$ and cyclin B in mouse oocytes during acquisition of competence to resume meiosis. Mol. Reprod. Dev. 40, 503–508.
Conti, M., Hsieh, M., Zamah, A.M., Oh, J.S., 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. Mol. Cell. Endocrinol. 356, 65–73.
Davis, J.S., Weakland, L.L., West, L.A., Farese, R.V., 1986. Lutenizing hormone stimulates the formation of inositol trisphosphate and cyclic AMP in rat granulosa cells. Biochem. J. 238, 597–604.
Dickey, D.M., Flora, D.R., Bryan, P.M., Xu, X., Chen, Y., Potter, L.R., 2007. Differential regulation of membrane guanylyl cyclases in congestive heart failure: natriuretic peptide receptor (NPR)-B, not NPR-A, is the predominant natriuretic peptide receptor in the failing heart. Endocrinology 148, 3518–3522.
Dickey, D.M., Burnett, J.C., Potter, L.R., 2008. Novel bifunctional natriuretic peptides as potential therapeutics. J. Biol. Chem. 283, 35003–35009.
Eppig, J.J., Wigglesworth, K., Pendola, F., Hirao, Y., 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. Biol. Reprod. 56, 976–984.
Erickson, G.F., Sorensen, R.A., 1974. In vitro maturation of mouse oocytes isolated from late, middle, and pre-antral Graafian follicles. J. Exp. Zool. 190, 123–127.
Flora, D.R., Potter, L.R., 2010. Prolonged atrial natriuretic peptide exposure stimulates guanylyl cyclase-a degradation. Endocrinology 151, 2769–2776.
Flores, J.A., Aguirre, C., Sharma, O.P., Veldhuis, J.D., 1998. Luteinizing hormone (LH) stimulates both intracellular calcium ion ($[Ca^{2+}]_i$) mobilization and transmembrane cation influx in single ovarian (granulosa) cells: recruitment as a cellular mechanism of LH-$[Ca^{2+}]_i$ dose response. Endocrinology 139, 3606–3612.
Francis, S.H., Blount, M.A., Corbin, J.D., 2011. Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. Physiol. Rev. 91, 651–690.
Hsieh, M., Thao, K., Conti, M., 2011. Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation. PLoS ONE 6, e21574.
Hubbard, C.J., 1986. Cyclic AMP changes in the component cells of Graafian follicles: possible influences on maturation in the follicle-enclosed oocytes of hamsters. Dev. Biol. 118, 343–351.
Hubbard, C.J., Greenwald, G.S., 1982. Cyclic nucleotides, DNA, and steroid levels in ovarian follicles and corpora lutea of the cyclic hamster. Biol. Reprod. 26, 230–240.
Hunt, P.J., Richards, A.M., Espiner, E.A., Nicholls, M.G., Yandle, T.G., 1994. Bioactivity and metabolism of C-type natriuretic peptide in normal man. J. Clin. Endocrinol. Metab. 78, 1428–1435.
Jaffe, L.A., Norris, R.P., 2010. Initiation of the meiotic prophase-to-metaphase transition in mammalian oocytes. In: Verlhac, M.-H., Villeneuve, A. (Eds.), Oogenesis: The Universal Process. John Wiley & Sons Ltd., Chichester, UK, pp. 181–197.
Jankowski, M., Reis, A.M., Mukaddam-Daher, S., Dam, T.-V., Farookhi, R., Gutkowska, J., 1997. C-type natriuretic peptide and the guanylyl cyclase receptors in the rat ovary are modulated by the estrous cycle. Biol. Reprod. 56, 59–66.
Kawamura, K., Cheng, Y., Kawamura, N., Takae, S., Okada, A., Kawagoe, Y., Mulders, S., Terada, Y., Hsueh, A.J.W., 2011. Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. Hum. Reprod. 26, 3094–3101.
Killock, D.J., Ivetic, A., 2010. The cytoplasmic domains of TNFα-converting enzyme (TACE/ADAM17) and L-selectin are regulated differently by p38 MAPK and PKC to promote ectodomain shedding. Biochem. J. 428, 293–304.
Kühn, B., Gudermann, T., 1999. The luteinizing hormone receptor activates phospholipase C via preferential coupling to G_{i2}. Biochemistry 38, 12490–12498.
Mehlmann, L.M., Saeki, Y., Tanaka, S., Brennan, T.J., Evsikov, A.V., Pendola, F.L., Knowles, B.B., Eppig, J.J., Jaffe, L.A., 2004. The G_s-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. Science 306, 1947–1950.
Mock, E.J., Niswender, G.D., 1983. Differences in the rates of internalization of ^{125}I-labeled human chorionic gonadotropin, luteinizing hormone, and epidermal growth factor by ovine luteal cells. Endocrinology 113, 259–264.
Norris, R.P., Freudzon, M., Mehlmann, L.M., Cowan, A.E., Simon, A.M., Paul, D.L., Lampe, P.D., Jaffe, L.A., 2008. Luteinizing hormone causes MAPK-dependent phosphorylation and closure of Cx43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development 135, 3229–3238.
Norris, R.P., Ratzan, W.J., Freudzon, M., Mehlmann, L.M., Krall, J., Movsesian, M.A., Wang, H., Ke, H., Nikolaev, V.O., Jaffe, L.A., 2009. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development 136, 1869–1878.
Norris, R.P., Freudzon, M., Nikolaev, V.O., Jaffe, L.A., 2010. Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. Reproduction 140, 655–662.
Panigone, S., Hsieh, M., Fu, M., Persani, L., Conti, M., 2008. Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway. Mol. Endocrinol. 22, 924–936.
Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L., Conti, M., 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303, 682–684.
Potter, L.R., 2011a. Guanylyl cyclase structure, function and regulation. Cell. Signalling 23, 1921–1926.
Potter, L.R., 2011b. Regulation and therapeutic targeting of peptide-activated receptor guanylyl cyclases. Pharmacol. Ther. 130, 71–82.
Potter, L.R., 2011c. Natriuretic peptide metabolism, clearance and degradation. FEBS J. 278, 1808–1817.
Potter, L.R., Hunter, T., 1998. Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. J. Biol. Chem. 273, 15533–15539.
Potter, L.R., Hunter, T., 1999. A constitutively ''phosphorylated'' guanylyl cyclase-linked atrial natriuretic peptide receptor mutant is resistant to desensitization. Mol. Biol. Cell. 10, 1811–1820.
Potter, L.R., Yoder, A.R., Flora, D.R., Antos, L.K., Dickey, D.M., 2009. Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. Handb. Exp. Pharmacol. 191, 341–366.
Racowsky, C., Baldwin, K.V., 1989. In vitro and in vivo studies reveal that hamster oocyte meiotic arrest is maintained only transiently by follicular fluid, but persistently by membrana/cumulus granulosa cell contact. Dev. Biol. 13, 297–306.
Rajagopalan-Gupta, R.M., Lamm, M.L.G., Mukherjee, S., Rasenick, M.M., Hunzicker-Dunn, M., 1998. Luteinizing hormone/choriogonadotropin receptor-mediated activation of heterotrimeric guanine nucleotide binding proteins in ovarian follicular membranes. Endocrinology 139, 4547–4555.
Robinson, J.W., Potter, L.R., 2011. ATP potentiates competitive inhibition of guanylyl cyclase A and B by the staurosporine anoalog, Go6976: reciprocal regulation of ATP and GTP binding. J. Biol. Chem. 286, 33841–33844.
Salvador, L.M., Maizels, E., Hales, D.B., Miyamoto, E., Yamamoto, H., Hunzicker-Dunn, M., 2002. Acute signaling by the LH receptor is independent of protein kinase C activation. Endocrinology 143, 2986–2994.
Schuh, M., Ellenberg, J., 2007. Self-organization of MTOC's replaces centrosome function during acentrosomal spindle assembly in live mouse oocytes. Cell 130, 484–498.
Sela-Abramovich, S., Chorev, E., Galiani, D., Dekel, N., 2005. Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. Endocrinology 146, 1236–1244.
Sela-Abramovich, S., Galiani, D., Nevo, N., Dekel, N., 2008. Inhibition of rat oocyte maturation and ovulation by nitric oxide: mechanism of action. Biol. Reprod. 78, 1111–1118.
Solc, P., Schultz, R.M., Motlik, J., 2010. Prophase I arrest and progression to metaphase I in mouse oocytes: comparison of resumption of meiosis and recovery from G2-arrest in somatic cells. Mol. Human Reprod 16, 654–664.
Sriraman, V., Rudd, M.D., Lohmann, S.M., Mulders, S.M., Richards, J.S., 2006. Cyclic guanosine 5′-monophosphate-dependent protein kinase II is induced by luteinizing hormone and progesterone receptor-dependent mechanisms in

granulosa cells and cumulus oocyte complexes of ovulating follicles. Mol. Endocrinol. 20, 348–361.
Törnell, J., Carlsson, B., Billig, H., 1990. Atrial natriuretic peptide inhibits spontaneous rat oocyte maturation. Endocrinology 126, 1504–1508.
Vaccari, S., Weeks, J.L., Hsieh, M., Menniti, F.S., Conti, M., 2009. Cyclic GMP signaling is involved in the LH-dependent meiotic maturation of mouse oocytes. Biol. Reprod. 81, 595–604.
Wu, C., Wu, F., Pan, J., Morser, J., Wu, Q., 2003. Furin-mediated processing of pro-C-type natriuretic peptide. J. Biol. Chem. 278, 25847–25852.
Zhang, M., Su, Y.-Q., Sugiura, K., Xia, G., Eppig, J.J., 2010. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. Science 330, 366–369.
Zhang, M., Su, Y.-Q., Sugiura, K., Wigglesworth, K., Xia, G., Eppig, J.J., 2011. Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro. Endocrinology 152, 4377–4385.

SUPPLEMENTARY MATERIAL

Luteinizing Hormone Reduces the Activity of the NPR2 Guanylyl Cyclase in Mouse Ovarian Follicles, Contributing to the Cyclic GMP Decrease that Promotes Resumption of Meiosis in Oocytes. Jerid W. Robinson, Meijia Zhang, Leia C. Shuhaibar, Rachael P. Norris, Andreas Geerts, Frank Wunder, John J. Eppig, Lincoln R. Potter, and Laurinda A. Jaffe

TABLE S1. Primers and fluorescent probes used for qRT-PCR.

target	forward primer, 5'-3'	probe, 5'-3'	reverse primer, 5'-3'
Npr1	GCACTCGAGGCTGACCTACT	CCCTGCTATTCCTTGTCACCTCCCC	GTGTCATTGCTGGCACAGG
Npr2	GGAAAAGGAAAAATGCGAACT	ACTGGCTCTTGGGAGAGCAAAAGGG	TTTACAGGAGTCCGGGAGGT
Gucy2c	CCGCTGGAAGTTATGGATTG	AAGGAAACCCTAACCAGGATCCTGC	ATCAGCTTCCTGGCTGGAG
Gucy2d	CTGGCCCTGGACATCCTTAG	TATGCAGGCAACTTTCGGATGAGGC	CCTGACACGGATGGGTACAT
Gucy2e	GGAGATTCCCCCAGAGAGAC	CAGGCCAGGCCAGTTTACTGGGAAG	CTGTCCCCAGTAGAGCTTCA
Gucy2f	CTTGCAACCAGCAGAGATTG	TTCCAAAGGAGAAAAGCAGAGAGGC	GGCTTGTTTCGCACCAACT
Gucy2g	AAAGGTGATGGAAGGATTGTG	CCCTAGTGAGGGAGTGCTGGGATGA	GAAGGGAAGATGGGCCTTAG
Gucy1a3	GACTGTCCTGGCTTTGTGTTC	CCCCGAGATCAAGGGAGGAGCT	TCACTAGGGAAGTTTGGTGGA
Gucy1a2	CCAGACAACTTTCCGAAGGA	TTCCTGGGGTCTGCTATTTCCTGGA	GGCTTTGGGTCAGTCCTTAAC
Gucy1b3	CTCGGATCCACTGTTCCATT	AGAGGCCCAGTGTCTATGAAGGGCA	ACCAGACTTGCATTGGTTCC
Gucy1b2	CAAGAAGCCCCGTGCTGT	CTGAGCAACATGGACCACCACCAG	CGTCTGCTGGATCACTGTTG
Npr3	ACCATTGAGAGGCGAAATCA	AAGAGGAAAGCAACATCGGGAAGCA	TGGAATCTTCTCGCAGCTCT
Rpl32	TTCATCAGGCACCAGTCAGA	TGTGAAAATTAAGCGAAACTGGCGG	TTGTCAATGCCTCTGGGTTT

Probes were labeled with FAM (5') and TAMRA (3').

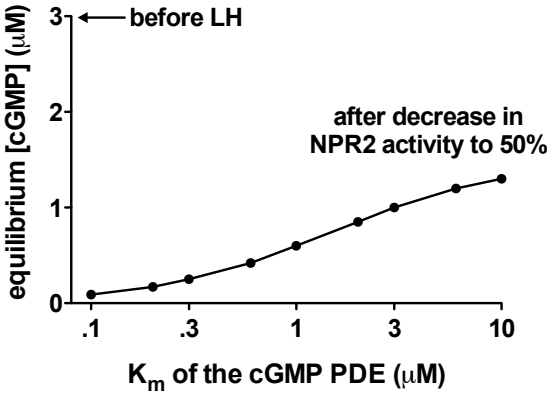

FIGURE S1. A simplified model to estimate how much the decrease in NPR2 activity in the follicle, to ~50% of the basal level at 20 minutes after LH application, could decrease the concentration of cGMP in the follicle. Starting from the pre-LH cGMP concentration of ~3 μM (Norris et al., 2010), the figure shows the concentration of cGMP that would be attained after a decrease in NPR2 activity to 50% of the pre-LH level, as a function of the K_m of the cGMP phosphodiesterase in these cells. The calculation used to make the graph is described below.

Before LH is applied, cGMP is being degraded at a rate that is equal to the rate at which it is being produced. After LH signaling decreases guanylyl cyclase activity and the concentration of cGMP begins to decrease, cGMP phosphodiesterase activity will also decrease, due to the decrease in its substrate. A new equilibrium cGMP concentration will be reached when the cGMP phosphodiesterase activity falls to 50% of its original value, such that rates of production and degradation of cGMP will again be equal. If the activity of the phosphodiesterase behaves as described by the Michaelis-Menten equation,

$$V/V_{max} = [cGMP]/(K_m + [cGMP]),$$

we can calculate the initial V/V_{max} (before LH) using the K_m value and the known initial concentration of cGMP, which is 3 μM. For example, if K_m = 1 μM,

$$V_o/V_{max} = 3/(K_m + 3) = 3/(1 + 3) = .75$$

Using this value for V_o/V_{max}, we can then calculate the concentration of cGMP when V/V_{max} falls to 50% of V_o/V_{max}:

$$V/V_{max} = .375 = [cGMP]/(1 + [cGMP])$$

$$.375 + .375\ [cGMP] = [cGMP]$$

$$.375 = [cGMP] - .375[cGMP]$$

$$.375 = .625\ [cGMP]$$

$$[cGMP] = .375/.625 = .6\ \mu M$$

Fig. S1 was generated using similar calculations to determine the equilibrium cGMP concentration for the particular K_m's shown. The graph shows that if the phosphodiesterase has a higher affinity for cGMP (lower K_m), it is capable of reducing the cGMP to a lower concentration.

Based on its sensitivity to sildenafil and tadalafil, ~75% of the cGMP phosphodiesterase activity in the non-LH-treated follicle is likely to be due to PDE5 (Vaccari et al., 2009), so we asked what would be predicted by this model if all of the cGMP phosphodiesterase activity was assumed to be due to PDE5. We disregarded factors that are not taken into account by the Michaelis-Menten equation (such as regulation of PDE5 by cGMP binding to allosteric sites and cGMP-dependent phosphorylation; see Francis et al., 2011). Although some of the values for the K_m of PDE5 made recombinantly are higher (Loughney et al., 1998; Corbin et al., 2000; Lin et al., 2000; Wang et al., 2006; Zoraghi et al., 2006), almost all of the values for the K_m of native PDE5 purified from mammalian tissues (aorta, platelets, trachea, lung) are in the range of ~0.2 to ~2 μM (e.g., Lugnier et al., 1986; Thomas et al., 1990; Robichon, 1991; Rousseau et al., 1994; Chulia et al., 1997; Kotera et al., 2000; Kameni-Tcheudji et al., 2007). PDE5 isolated from mammalian tissues is likely to be a better predictor of biological activity since its post-translational processing would have occurred normally.

With K_m values ranging from 0.2 to 2 μM, a decrease to 50% of the basal guanylyl cyclase activity is calculated to result in equilibrium cGMP concentrations of 0.17 to 0.85 μM. Thus, a 50% reduction in NPR2 activity could potentially account for the measured decrease in the follicle cGMP concentration from ~3 μM before LH treatment to ~0.5 μM 20 minutes later.

REFERENCES

Chulia, S., Ivorra, M.D., Martinez, S., Elorriaga, M., Valiente, M., Noguera, M.A., Lugnier, C., Advenier, C., D'Ocon, P., 1997. Relationships between structure and vascular activity in a series of benzylisoquinolines. British J. Pharmacol. 122, 409-416.

Corbin, J.D., Turko, I.V., Beasley, A., Francis, S.H., 2000. Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. Eur. J. Biochem. 267, 2760-2767.

Francis, S.H., Blount, M.A., Corbin, J.D., 2011. Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions. Physiol. Rev. 91, 651-690.

Kameni-Tcheudji, J.F., Keravis, T., Le Bec, A., Lebeau, L., Strub, J.M., van Dorsselaer, A., Lugnier, C., 2007. Vascular smooth muscle PDE5 is much more sensitive to N-alkylated zaprinast than platelet PDE5. BMC Pharmacology 7 (Suppl 1), P32.

Kotera, J., Fujishige, K., Michibata, H., Yuasa, K., Kubo, A., Nakamura, Y., Omori, K., 2000. Characterization and effects of methyl-2-(4-aminophenyl)-1,2-dihydro-1-oxo-7-(2-pyridinylmethoxy)-4-(3,4,5-trimethoxyphenyl)-3-isoquinoline carboxylate sulfate (T-1032), a

novel potent inhibitor of cGMP-binding cGMP-specific phosphodiesterase (PDE5). Biochem. Pharmacol. 60, 1333-1341.

Lin, C.-S., Lau, A., Tu, R., Lue, T. F., 2000. Expression of three isoforms of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in human penile cavernosum. Biochem. Biophys. Res. Commun. 268, 628-635.

Loughney, K., Hill, T.R., Florio, V.A., Uher, L., Rosman, G.J., Wolda, S.L., Jones, B.A,. Howard, M.L., McAllister-Lucas, L.M., Sonnenburg, W.K., Francis, S.H., Corbin, J.D., Beavo, J.A., Ferguson, K., 1998. Isolation and characterization of cDNAs encoding PDE5A, a human cGMP-binding, cGMP-specific 3',5'-cyclic nucleotide phosphodiesterase. Gene 216, 139-147.

Lugnier, C., Schoeffter, P., LeBec, A., Strouthou, E., Stoclet, J.C., 1986. Selective inhibition of cyclic nucleotide phosphodiesterases of human, bovine and rat aorta. Biochem. Pharmacol. 35, 1743-1751.

Norris, R.P., Freudzon, M., Nikolaev, V.O., Jaffe, L.A., 2010. Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. Reproduction 140, 655-662.

Robichon, A., 1991. A new cGMP phosphodiesterase isolated from bovine platelets is substrate for cAMP- and cGMP-dependent protein kinases: evidence for a key role in the process of platelet activation. J. Cell. Biochem. 47, 147-157.

Rousseau, E., Gagnon, J., Lugnier, C., 1994. Biochemical and pharmacological characterization of cyclic nucleotide phosphodiesterase in airway epithelium. Mol. Cell. Biochem. 140, 171-175.

Thomas, M.K., Francis, S.H., Corbin, J.D., 1990. Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. J. Biol. Chem. 265, 14964-14970.

Vaccari, S., Weeks, J.L., Hsieh, M., Menniti, F.S., Conti, M., 2009. Cyclic GMP signaling is involved in the LH-dependent meiotic maturation of mouse oocytes. Biol. Reprod. 81, 595-604.

Wang, H., Liu, Y., Huai, Q., Cai, J., Zoraghi, R., Francis, S.H., Corbin, J.D., Robinson, H., Xin, Z., Lin, G., Ke, H., 2006. Multiple conformations of phosphodiesterase-5: implications for enzyme function and drug development. J. Biol. Chem. 281, 21469-21479.

Zoraghi, R., Corbin, J.D., Francis, S.H., 2006. Phosphodiesterase-5 Gln^{817} is critical for cGMP, vardenafil, or sildenafil affinity: its orientation impacts cGMP but not cAMP affinity. J. Biol. Chem. 281, 5553-5558.

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RESEARCH ARTICLE

Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes

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ABSTRACT

In mammals, the meiotic cell cycle of oocytes starts during embryogenesis and then pauses. Much later, in preparation for fertilization, oocytes within preovulatory follicles resume meiosis in response to luteinizing hormone (LH). Before LH stimulation, the arrest is maintained by diffusion of cyclic (c)GMP into the oocyte from the surrounding granulosa cells, where it is produced by the guanylyl cyclase natriuretic peptide receptor 2 (NPR2). LH rapidly reduces the production of cGMP, but how this occurs is unknown. Here, using rat follicles, we show that within 10 min, LH signaling causes dephosphorylation and inactivation of NPR2 through a process that requires the activity of phosphoprotein phosphatase (PPP)-family members. The rapid dephosphorylation of NPR2 is accompanied by a rapid phosphorylation of the cGMP phosphodiesterase PDE5, an enzyme whose activity is increased upon phosphorylation. Later, levels of the NPR2 agonist C-type natriuretic peptide decrease in the follicle, and these sequential events contribute to the decrease in cGMP that causes meiosis to resume in the oocyte.

KEY WORDS: Guanylyl cyclase, Luteinizing hormone, Meiosis, Natriuretic peptide receptor, Ovarian follicle, Phosphorylation

INTRODUCTION

Oocytes in mammalian preovulatory follicles are held in meiotic prophase arrest by cyclic (c)GMP that is produced in the granulosa cells surrounding the oocyte, which then diffuses into the oocyte through gap junctions (Norris et al., 2009; Vaccari et al., 2009). Luteinizing hormone (LH), which is released from the anterior pituitary during each reproductive cycle, acts on a G-protein coupled receptor in the outer granulosa cells of the follicle (Bortolussi et al., 1977, Rajagopalan-Gupta et al., 1998) (see Fig. 1A), to relieve the inhibition of the prophase-to-metaphase transition. LH acts by lowering the levels of cGMP in the granulosa cells, thus reducing cGMP in the oocyte. Although this regulatory system is best understood in mice (Norris et al., 2009; Vaccari et al., 2009; Zhang et al., 2010; Kawamura et al., 2011; Robinson et al., 2012; Tsuji et al., 2012; Geister et al., 2013), there is evidence that similar mechanisms operate in other mammals (Törnell et al., 1991; Peng et al., 2013; Hiradate et al., 2014; Zhang et al., 2014), including women (Kawamura et al., 2011; Liu et al., 2014). The LH-induced resumption of the meiotic cell cycle leads into a series of events by which the oocyte matures to become a fertilizable egg (Conti et al., 2012; Clift and Schuh, 2013; Holt et al., 2013; Mehlmann, 2013; Hunzicker-Dunn and Mayo, 2014).

As a consequence of the initial cGMP decrease in the mural granulosa cells, cGMP diffuses out of the oocyte into the surrounding granulosa cells, and the decrease in cGMP in the oocyte re-initiates the meiotic cell cycle (Norris et al., 2009; Vaccari et al., 2009). The direct effect of the cGMP decrease in the oocyte is to relieve the inhibition of the cAMP phosphodiesterase PDE3A, which is competitively inhibited by cGMP. Thus, the cGMP reduction causes the levels of cAMP to decrease. cAMP, which is produced in the oocyte (Mehlmann et al., 2002, 2004; Horner et al., 2003; Hinckley et al., 2005; Ledent et al., 2005), maintains meiotic prophase arrest through activation of protein kinase A (PKA) (Bornslaeger et al., 1986; Kovo et al., 2006). PKA activity inhibits the CDC25B phosphatase and stimulates the WEE1B and MYT1 kinases, and this keeps the CDK1 kinase that controls the prophase-to-metaphase transition phosphorylated and inactive (Conti et al., 2012; Holt et al., 2013; Mehlmann, 2013). When LH signaling reduces oocyte levels of cGMP, and in turn cAMP, meiosis resumes.

cGMP is produced in the granulosa cells of mice by natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase-B (Zhang et al., 2010; Kawamura et al., 2011; Robinson et al., 2012; Tsuji et al., 2012; Geister et al., 2013). NPR2 appears to be the only relevant guanylyl cyclase in mouse follicles because follicle-enclosed oocytes in the ovaries of mice with non-functional NPR2 resume meiosis spontaneously (Zhang et al., 2010; Tsuji et al., 2012; Geister et al., 2013) and an inactivating mutation in NPR2 reduces the cGMP content of the follicle to an undetectable level (Geister et al., 2013). Gene expression analysis and enzyme assays have indicated that other guanylyl cyclases are present at levels much lower than those of NPR2 (Robinson et al., 2012). NPR2 is located throughout the granulosa cells of the follicle, in both the mural granulosa and the cumulus cells directly surrounding the oocyte; it is not expressed in the oocyte (Gutkowska et al., 1999; Zhang et al., 2010). The agonist of NPR2, C-type natriuretic peptide (CNP, released from a precursor protein that is encoded by the *Nppc* gene) is produced only in the mural granulosa cells (Zhang et al., 2010).

NPR2 is a single transmembrane-spanning enzyme that is activated by the binding of CNP to its extracellular domain (Potter et al., 2006; Potter, 2011). In order for the CNP activation signal to be transmitted to the catalytic domain, the juxtamembrane intracellular region of NPR2 must be phosphorylated on some

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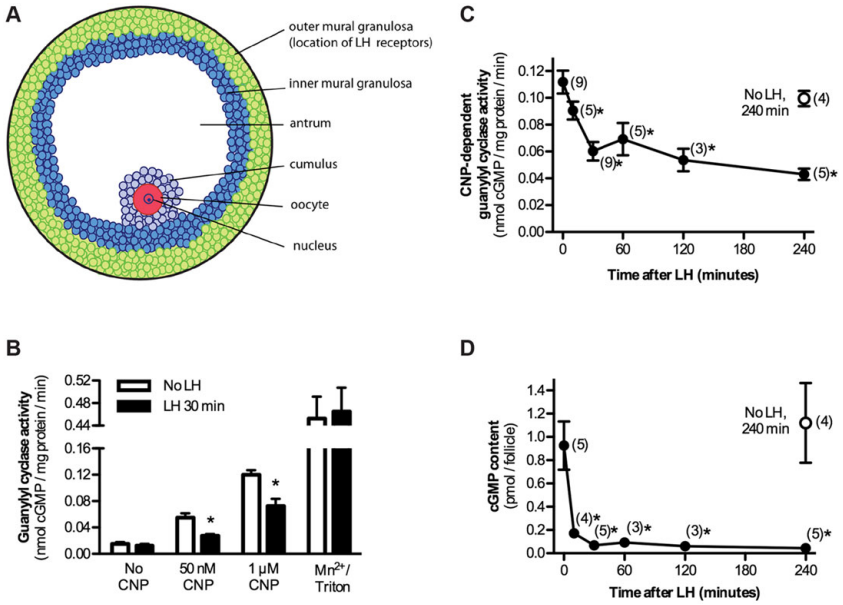

Fig. 1. Rapid decrease in follicle NPR2 guanylyl cyclase activity and cGMP content in response to LH signaling. (A) Diagram showing the cell layers of a rat ovarian follicle. (B) Guanylyl cyclase activity in follicle membranes in the presence or absence of CNP. Membranes were prepared from follicles with or without treatment with LH for 30 min. Each point shows the mean±s.e.m. for four separate membrane preparations, each assayed in triplicate. In the presence of 50 nM or 1 µM CNP, 30 min of treatment with LH caused a decrease in activity to ~50% of the initial level. 1% Triton X-100 and 5 mM $MgCl_2$ was used as a control condition that fully activates the enzyme (see text). (C) Time course of the LH-induced decrease in NPR2 activity, as determined in the presence of 1 µM CNP. The open circle represents measurements from follicle membranes incubated for 240 min in the absence of LH. Each point shows the mean±s.e.m. for (*n*) separate membrane preparations, each assayed in triplicate. (D) Time course of the LH-induced decrease in follicle cGMP content. Error bars are not visible for some points because they are smaller than the data point markers. C,D, *measurements that were significantly different (P<0.05) from the control without LH. Measurements at 30 and 240 min after LH exposure were not significantly different.

combination of five serine residues and two threonine residues that have been identified as regulatory (Potter, 1998; Potter and Hunter, 1998; Yoder et al., 2010, 2012). However, unlike many growth factor receptors, NPR2 phosphorylation is not increased upon binding to its agonist CNP (Potter, 1998). Thus, there are at least two separate mechanisms by which signaling pathways could increase or decrease the guanylyl cyclase activity of NPR2 – changing the amount of CNP or changing the level of receptor phosphorylation.

LH signaling is known to decrease the amount of CNP in rat and mouse ovaries (Jankowski et al., 1997; Kawamura et al., 2011; Robinson et al., 2012; Liu et al., 2014) and in human and porcine follicular fluid (Kawamura et al., 2011; Zhang et al., 2014); the decrease in the levels of CNP is associated with a decrease in *Nppc* mRNA (Kawamura et al., 2011; Tsuji et al., 2012; Liu et al., 2014). However, in the mouse ovary, where the kinetics are best characterized, the CNP decrease is first detected at 2 h (Robinson et al., 2012; Liu et al., 2014), whereas the decrease in cGMP is detected at 15 to 20 min (Norris et al., 2010; Liu et al., 2014). Guanylyl cyclase activity in mouse follicle membranes decreases to approximately half of the basal level at 20 min after LH application, and this is independent of any change in CNP (Robinson et al., 2012; Liu et al., 2014). Cultured human granulosa cells also show a rapid decrease in cGMP production, measured in the presence of a constant concentration of CNP (Liu et al., 2014).

The mechanism underlying this early decrease in guanylyl cyclase activity is unknown. Here, we show that the rapid reduction in NPR2 activity in rat follicles in response to LH signaling is caused by the dephosphorylation of NPR2, which is mediated by a process that requires the activity of the protein phosphatases of the phosphoprotein phosphatase (PPP) family, the most likely candidates being PPP1, PPP2 and/or PPP6. The rapid dephosphorylation of NPR2 is accompanied by a rapid phosphorylation of the cGMP phosphodiesterase PDE5 (also known as PDE5A), an enzyme whose activity is increased upon phosphorylation. Later, CNP levels decrease in the follicle, and these sequential events contribute to the decrease in cGMP that causes meiosis to resume in the oocyte.

RESULTS

LH signaling reduces NPR2 activity and cGMP content in rat ovarian follicles

Previous studies demonstrating an LH-induced decrease in guanylyl cyclase activity in ovarian follicles have been conducted using mice (Robinson et al., 2012), but the amount of protein that can be obtained from mouse follicles is small. We therefore tested whether a similar regulatory system operates in rats, from which an order of magnitude more follicle protein per animal can be obtained, making analysis of changes in phosphorylation feasible.

To test whether LH causes a decrease in NPR2 guanylyl cyclase activity in rat follicles, and to investigate the time course of the decrease as a basis for subsequent mechanistic studies, isolated preovulatory rat follicles were incubated for various times with or without LH. Because NPR2 is located in the plasma membrane,

Development (2014) 141, 3594-3604 doi:10.1242/dev.112219

the follicles were then homogenized to obtain a crude membrane fraction. The membranes were assayed for guanylyl cyclase activity, with and without the NPR2 agonist CNP; CNP-dependent activity indicates the activity of NPR2 (Fig. 1B,C). By 30 min after LH exposure, the CNP-dependent guanylyl cyclase activity decreased to ~50% of the initial level and stayed at this reduced level for at least 4 h, without any additional change (Fig. 1B,C). Approximately 40% of the decrease to the plateau level had occurred by 10 min (Fig. 1C). No change in CNP-dependent guanylyl cyclase activity was seen in follicles incubated for 4 h without LH (Fig. 1C). The cGMP content of the follicle also decreased rapidly in response to LH – most of the change had occurred by 10 min (Fig. 1D).

The LH-induced decrease in CNP-dependent guanylyl cyclase activity was not explained by a reduction in the affinity of NPR2 for CNP because the fraction by which the activity decreased in response to LH, when it was measured in the presence of 50 nM CNP, was similar to that measured in the presence of 1 µM CNP (Fig. 1B). LH had no effect on guanylyl cyclase activity when it was measured in the presence of 1% Triton X-100 and 5 mM $MnCl_2$ (Fig. 1B), a condition that activates guanylyl cyclase activity independently of natriuretic peptide and phosphorylation and that is indicative of guanylyl cyclase protein levels (Potter and Hunter, 1999; Abbey and Potter, 2003). Because the predominant guanylyl cyclase in ovarian follicles is NPR2, this indicates that the LH-induced decrease in CNP-stimulated activity is not due to a decrease in the amount of NPR2 protein.

LH signaling causes rapid dephosphorylation of NPR2

To investigate whether the rapid LH-induced decrease in NPR2 activity correlated with a decrease in NPR2 phosphorylation, we first considered the use of $^{32}PO_4$ metabolic labeling. However, because the expression level of NPR2 in native tissues is low, this approach has only been feasible for overexpressing cells (Potter, 1998). The NPR2 fraction of the total cell protein in ovarian follicles is ~5% of that in the 3T3 cells which had been transfected with NPR2 and used in previous $^{32}PO_4$ labeling studies, based on the relative levels of enzyme activity (Potter, 1998). To obtain the same amount of NPR2 protein used in the transfected 3T3 cell studies, this would have required ~10 mg of follicle membrane protein, corresponding to dissection of follicles from ~30 rats, for a single gel lane. Likewise, detection of changes in phosphorylation by mass spectrometry (e.g. Cargnello et al., 2012) would require much more NPR2 protein than is practical to obtain from rat follicles.

We then investigated whether an LH-induced shift in NPR2 migration could be detected using standard SDS-PAGE, as a previous study showed that dephosphorylation results in a small shift in the electrophoretic migration of the closely related guanylyl cyclase natriuretic peptide receptor 1 (NPR1) (Potter and Garbers, 1992). In standard SDS-PAGE gels, NPR2 from rat follicles migrated as a predominant band at the same position as rat NPR2 from stably transfected HEK-293T cells, at the typically observed size of ~130 kDa (Potter, 1998). The fuzziness of this band is primarily due to multiple glycosylation sites (Koller et al., 1993). There was also a minor band at ~115 kDa, which is thought to represent the polypeptide chain that has not been post-translationally modified (Koller et al., 1993; Potter, 1998) (Fig. 2A). However, treatment with LH did not result in a consistent shift in the electrophoretic migration of NPR2 under the conditions that we used (Fig. 2A).

As an alternative approach to investigate whether LH exposure led to dephosphorylation of NPR2, we used gels including Phos-tag acrylamide and $MnCl_2$. In these gels, phosphorylated proteins transiently interact with the Mn^{2+}-Phos-tag complex, which retards their migration relative to less-phosphorylated or non-phosphorylated forms (Kinoshita et al., 2006, 2012; McTague et al., 2012; Yu et al., 2013). NPR2 was purified from the membranes by using sequential immunoprecipitation and fractionation on gels containing Phos-tag acrylamide and was visualized by western blotting. In these gels, NPR2 from untreated follicles migrated as multiple bands, indicating the presence of multiple phosphorylated forms of the enzyme (Fig. 2B; see also Fig. 3A, Fig. 4A). Although molecular weight standards are not accurate indicators of relative molecular mass on a Phos-tag gel, they are useful as descriptive markers; the NPR2 bands migrated in a region that extended from above the 150 kDa marker to approximately the 250 kDa marker.

Controls that were performed using pre-immune serum (supplementary material Fig. S1), and co-migration of the immunoreactive bands from follicles with those from a HEK cell line that stably expressed NPR2 (Fig. 2A, Fig. 3A), validated the specificity of the antibody. The relative amount of NPR2 staining in a sample of follicle membranes was 2-7% of that in a sample from overexpressing HEK cells (normalized to the amount of membrane protein loaded per lane), and this corresponded closely to the relative amount of NPR2 activity (2-4% of that reported for this cell line, Robinson and Potter, 2011; Robinson and Potter, 2012), further confirming the antibody specificity. The use of Phos-tag gel migration as an indicator of the phosphorylation state of NPR2 was validated by showing that incubation of follicle membranes under conditions that promoted or inhibited phosphatase activity resulted in faster or slower migrating forms of NPR2 (Fig. 2E-G).

Exposure of the follicles to LH dramatically compressed the majority of the NPR2 into a predominant lower band, indicating dephosphorylation (Fig. 2B; see also Fig. 3A, Fig. 4A). The shift in migration was quantified by measuring the total amount of NPR2 immunostaining in the upper region relative to that in the region of the lower band (Fig. 2C,D). Most of the decrease in the relative amount of immunoreactive material in the upper region had occurred by 10 min after LH exposure, and a minimum level was reached at 30 min (Fig. 3A,B). The dephosphorylation persisted for at least 4 h (Fig. 3A,B), as did the reduction of NPR2 activity (Fig. 1C).

No decrease in NPR2 phosphorylation occurred in follicles that had been incubated for 4 h in the absence of LH (Fig. 3A,B). When LH was applied for 30 min and then washed out, the NPR2 dephosphorylation and activity measured 4 h later were the same as when LH was present continuously (two independent experiments, data not shown). Based on measurements of the total immunoreactive protein, no change in the total amount of NPR2 protein was detected when comparing membranes from follicles with or without treatment with LH (Fig. 3C). This conclusion is also supported by our earlier finding that LH did not affect the amount of guanylyl cyclase activity measured in the presence of Triton X-100 and $MnCl_2$ (Fig. 1B).

Most of the decrease in NPR2 phosphorylation had occurred by 10 min after application of LH (Fig. 3B), whereas only approximately 40% of the decrease in guanylyl cyclase activity had occurred by this time, compared with that measured at 30 min (Fig. 1C). The cause of the delay between the decreases in NPR2 phosphorylation and enzyme activity is unknown, but one possibility is that dephosphorylation of a particular serine or threonine residue that is crucial for the activity decrease occurs late in a series of dephosphorylation events, all of which are detected by the Phos-tag method. Consistent with this hypothesis, the individual phosphorylation sites in NPR2 have varying effects on the activity of the enzyme (Potter and Hunter, 1998; Yoder et al., 2012).

Development (2014) 141, 3594-3604 doi:10.1242/dev.112219

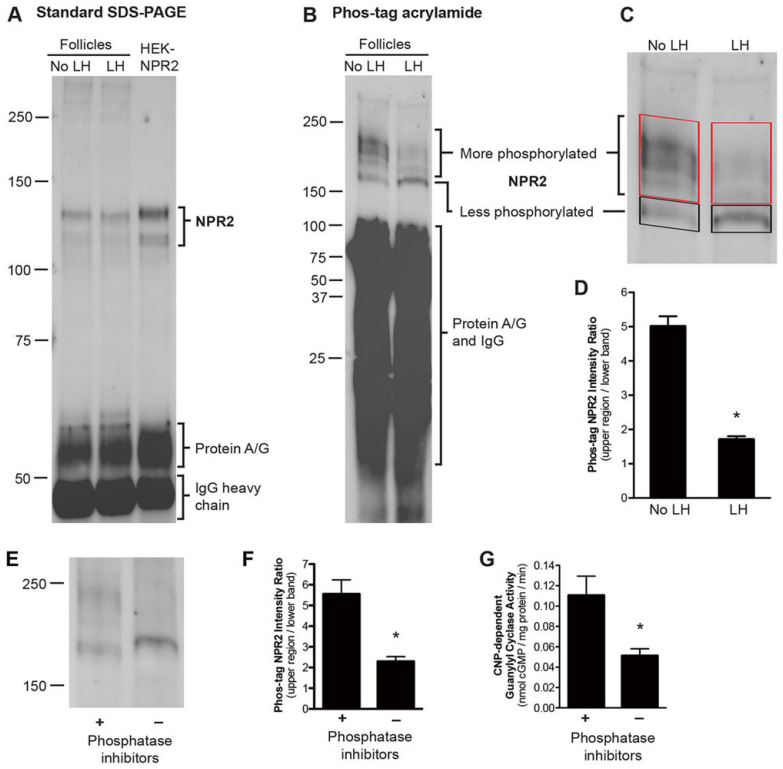

Fig. 2. Rapid dephosphorylation of NPR2 in follicle membranes in response to LH signaling. (A) A western blot of a standard SDS-PAGE gel that had been probed with NPR2 antiserum; for each lane, NPR2 was immunoprecipitated from 145 µg of membrane protein from follicles with or without treatment with LH for 20 min, or from 10 µg of membrane protein from HEK-293T cells that stably expressed NPR2. (B) A western blot of an SDS-PAGE gel containing 25 µM Phos-tag acrylamide that had been probed with NPR2 antiserum; NPR2 was immunoprecipitated from 175 µg of membrane protein from follicles with or without treatment with LH for 30 min. Molecular weight standards are shown for reference, but do not indicate relative molecular mass on a Phos-tag gel. (C) Method for quantifying the LH-induced shift in NPR2 migration on Phos-tag gels. Boxes show the upper region (more phosphorylated) and lower band (less phosphorylated) for which background-corrected total immunostaining intensity was measured. The upper region was defined as the region extending from just above the lower band to the top of the dense zone near the 250-kDa marker. (D) Ratios of the intensity of the upper region to that of the lower band; mean±s.e.m. for 20 blots similar to that shown in B. (E-G) Validation of the shift in Phos-tag gel migration as an indicator of NPR2 dephosphorylation. Follicle membranes were incubated at 30°C for 30 min, with or without phosphatase inhibitors (see supplementary Materials and Methods). In the absence of phosphatase inhibitors, NPR2 was dephosphorylated (E). (F) Quantification of the shift in NPR2 migration caused by dephosphorylating conditions (mean±s.e.m. for four experiments). (G) Dephosphorylating conditions resulted in a decrease in NPR2 guanylyl cyclase activity (mean±s.e.m. for three experiments). *P<0.05.

The LH-induced decrease in NPR2 activity is prevented by inhibiting NPR2 dephosphorylation with PPP-family protein phosphatase inhibitors

To test whether preventing the LH-induced dephosphorylation of NPR2 inhibits the decrease in NPR2 activity, and to begin to distinguish which of the approximately 30 known serine-threonine phosphatase catalytic subunits are required for dephosphorylation of NPR2, we treated the follicles with specific phosphatase inhibitors. Serine-threonine phosphatases belong to two main families – PPP or PPM (protein phosphatase Mg^{2+} or Mn^{2+} dependent); in addition, there is a smaller family (FCP) whose only known function is to dephosphorylate RNA polymerase II (Cohen, 2004; Swingle et al., 2007). Among these, only the PPP family is inhibited by the natural toxins cantharidin and okadaic acid (Swingle et al., 2007; Pereira et al., 2011). PPM family phosphatases (also known as PP2C) are not inhibited by 100 µM cantharidin (Li et al., 1993) or by 10 µM okadaic acid (Wang et al., 1995).

Pre-incubation of follicles for 1 h with cantharidin (100 µM) or okadaic acid (10 µM) prevented the LH-induced dephosphorylation and decrease in guanylyl cyclase activity of NPR2 (Fig. 4; supplementary material Fig. S2). These results indicate that the activity of PPP family phosphatases is required for the dephosphorylation of NPR2 in response to LH and that dephosphorylation is required for the LH-induced decrease in NPR2 activity. Among the PPP family phosphatases, our results with cantharidin argue against an important function for PPP3 (also known as PP2B or calcineurin) because PPP3 is insensitive to 100 µM cantharidin (Honkanen, 1993; Pereira et al., 2011).

These toxin results do not distinguish between other PPP family phosphatases because PPP1, PPP2, PPP4, PPP5 and PPP6 are all inhibited by the concentrations of cantharidin and okadaic acid that we applied to the follicles (Swingle et al., 2007; Pereira et al., 2011). Other toxins with greater specificities for particular PPP-family phosphatases (fostriecin, cytostatin and rubratoxin) were also tested and found to have no consistent effects under the conditions used

Development (2014) 141, 3594-3604 doi:10.1242/dev.112219

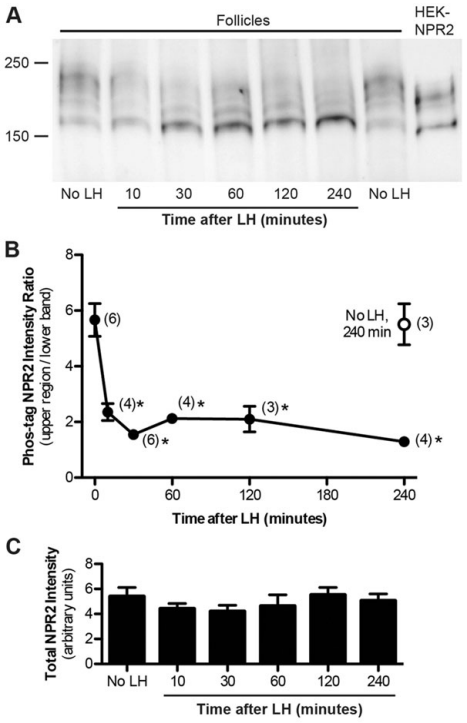

Fig. 3. Time course of the LH-induced dephosphorylation of NPR2. (A) Follicles were incubated with LH for various times. Crude membranes were then isolated and used for immunoprecipitation of NPR2 (from 190 µg of membrane protein per lane), Phos-tag gel electrophoresis and immunoblotting for NPR2. Samples from follicles without treatment with LH were prepared in parallel, at 30 min and 240 min after starting the incubation. The regions used for quantification are shown in supplementary material Fig. S3A. The right-hand lane sample was prepared using the same methods, instead using 10 µg of membrane protein from HEK-293T cells that stably expressed NPR2. (B) Time course of the LH-induced dephosphorylation of NPR2, quantified as shown in Fig. 2C. The open circle represents measurements from follicle membranes incubated for 240 min in the absence of LH. Each point shows the mean±s.e.m. for the number of experiments shown in parentheses. *P<0.05, measurements that were significantly different from the control without LH. Measurements at 30 and 240 min after LH exposure were not significantly different. (C) The total amount of NPR2 immunoreactive material in follicle membranes did not change during the first 4 h after treatment with LH. Measurements were made from the same set of blots used for B, by drawing a box around the entire NPR2 region of the blot (the sum of the red and black boxes shown in Fig. 2C).

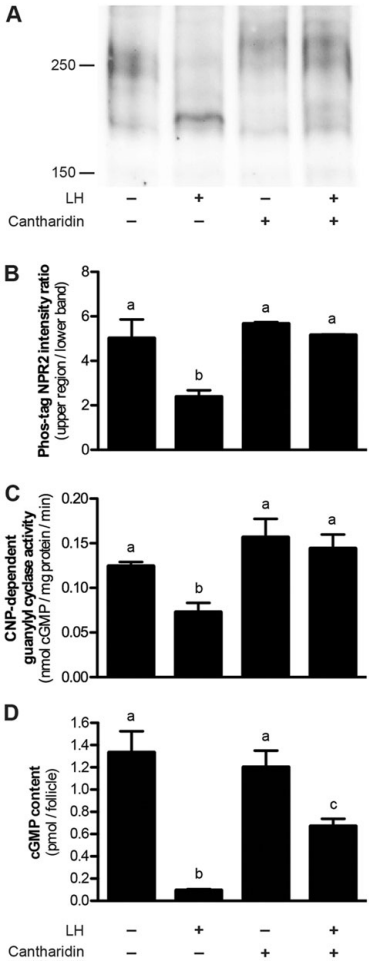

Fig. 4. Inhibition of the LH-induced dephosphorylation and inactivation of NPR2 and decrease in cGMP content upon the treatment of follicles with the PPP-family phosphatase inhibitor cantharidin. (A) Follicles were incubated with or without 100 µM cantharidin for 1 h, then with or without LH for 30 min. Crude membranes were isolated and used for immunoprecipitation, Phos-tag gel electrophoresis and immunoblotting for NPR2. In the presence of cantharidin, the ratio of NPR2 in the upper region and lower band did not change in response to LH, indicating that cantharidin inhibited the LH-induced dephosphorylation of NPR2. The regions used for quantification are shown in supplementary material Fig. S3B. (B) A graph showing the results of three experiments similar to that shown in A (mean±s.e.m.). (C) Membranes from follicles treated with or without cantharidin followed by LH, as described above, were assayed for NPR2 guanylyl cyclase activity. The graph shows the results from three experiments. (D) Follicles treated with or without cantharidin followed by LH, as described above, were assayed for cGMP content. The graph shows the results from seven experiments. Values not indicated by the same letter are significantly different (P<0.05).

(data not shown). However, these results were not definitive because we could not determine whether significant amounts of these toxins penetrated into the cytoplasm of the follicle cells.

The LH-induced decrease in follicle cGMP content is attenuated by inhibiting the decrease in NPR2 guanylyl cyclase activity

To determine the effect of preventing the LH-induced decrease in NPR2 guanylyl cyclase activity on the LH-induced cGMP decrease, we measured the cGMP content of follicles that had been pre-treated with 100 µM cantharidin for 1 h and then treated for 30 min with LH. Without cantharidin pre-treatment, LH caused cGMP content to decrease to 7% of the control level (Fig. 1D and Fig. 4D). Cantharidin pre-treatment attenuated the LH-induced decrease – cGMP content only decreased to 56% of the control level (Fig. 4D).

Development (2014) 141, 3594-3604 doi:10.1242/dev.112219

The effect of phosphatase inhibition on the LH-induced decrease in cGMP content is consistent with the conclusion that the LH-induced decrease in NPR2 phosphorylation and guanylyl cyclase activity contributes to the cGMP decrease. However, the finding that phosphatase inhibition did not completely prevent the LH-induced cGMP decrease, whereas it did completely prevent the LH-induced decrease in NPR2 phosphorylation and activity (Fig. 4A-C), indicates that LH must also induce another change in the follicle, such as an increase in cGMP phosphodiesterase activity. The finding that most of the cGMP decrease had occurred by the 10 min time point after treatment with LH (Fig. 1D), whereas only 40% of the NPR2 activity decrease had occurred by this time point (Fig. 1C), also suggests that LH induces an increase in cGMP phosphodiesterase activity in parallel with the decrease in NPR2 guanylyl cyclase activity.

LH signaling causes phosphorylation of the cGMP phosphodiesterase PDE5

An important component of the cGMP phosphodiesterase activity in mouse granulosa cells is contributed by PDE5 (Vaccari et al., 2009). PDE5 activity is stimulated by phosphorylation (Corbin et al., 2000; Rybalkin et al., 2002); therefore, we examined whether LH signaling increased PDE5 phosphorylation in rat follicles. In gels without Phos-tag, PDE5 migrated as a band at ~90 kDa, and treating follicles with LH did not change this migration (Fig. 5A). However, in Phos-tag-containing gels, PDE5 migrated as two separate bands, and treatment of the follicles with LH for 10 or 30 min increased the fraction of PDE5 in the upper band, indicating phosphorylation (Fig. 5B,C). PDE5 remained phosphorylated until at least 4 h after stimulation with LH (data not shown). *In vitro* phosphorylation of PDE5 in follicle lysates with the catalytic subunit of PKA also caused a shift of PDE5 to the upper band, confirming that the LH-induced shift was due to phosphorylation (Fig. 5D).

Although the effect of LH on the cGMP phosphodiesterase activity of PDE5 in rat follicles is unknown, the rapid phosphorylation of PDE5 in response to LH is expected to increase PDE5 activity based on *in vitro* studies (Corbin et al., 2000; Rybalkin et al., 2002). An increase in PDE5 activity could contribute to the LH-induced cGMP decrease in the follicle, acting in parallel with the decrease in the production of cGMP that results from the dephosphorylation of the NPR2 guanylyl cyclase.

Analysis of PPP family phosphatase gene expression in granulosa cells

To distinguish the PPP-family phosphatases that might contribute to the dephosphorylation of NPR2, we investigated which of these are expressed in granulosa cells. The rat genome contains 13 PPP-family genes, encoding seven subfamilies of PPP-family phosphatases (Pereira et al., 2011). We detected mRNA encoding all of these phosphatases, although the fractions of those encoding PPP4, PPP5 and PPP7 were each <2% of the total (Fig. 6). mRNA encoding PPP1, PPP2, PPP3 and PPP6 constituted 61%, 11%, 19% and 6% of the total, respectively. Although the amount of mRNA is not directly proportional to the amount of the protein they encode, and although the localization of a particular phosphatase could affect its functional significance, these measurements point to a role for PPP1, PPP2, PPP3 and/or PPP6, rather than PPP4, PPP5 or PPP7. Because our earlier findings with cantharidin argue against a function of PPP3 in the LH-induced decrease in NPR2 activity, PPP1, PPP2 and/or PPP6 are the phosphatases that are most likely to be important in this signaling cascade.

LH signaling gradually reduces the follicle content of the NPR2 agonist CNP, contributing to the decrease in cGMP production that triggers resumption of meiosis

The guanylyl cyclase assays described above were performed using membranes in the presence of a saturating concentration of CNP (1 µM), so the observed decreases in NPR2 activity occurred independently of any changes in CNP. However, previous studies in

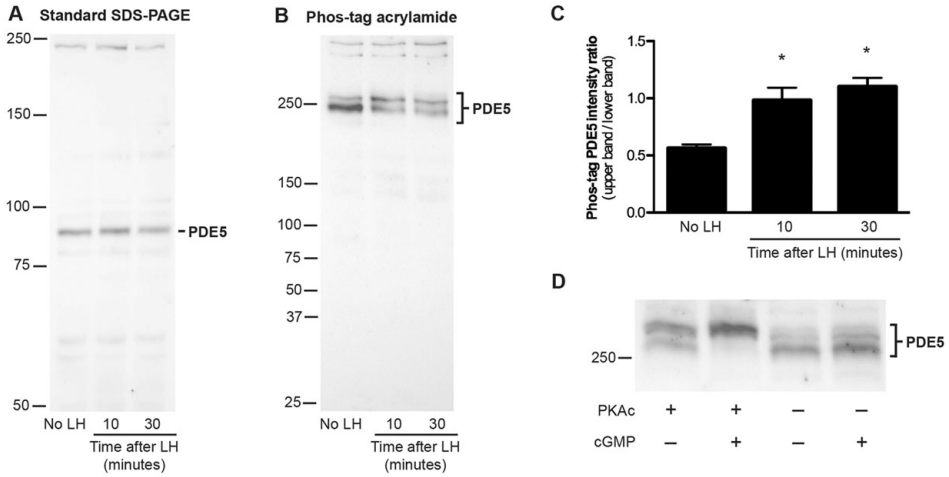

Fig. 5. Rapid phosphorylation of PDE5 in follicles in response to LH signaling. (A,B) Western blots of proteins from follicles with or without 10 or 30 min of treatment with LH, which were probed with an antibody against PDE5. (A) Standard SDS-PAGE gel, 30 µg protein per lane; (B) SDS-PAGE gel containing 25 µM Phos-tag acrylamide, 20 µg protein per lane. Molecular weight standards are shown for reference, but do not indicate relative molecular mass on a Phos-tag gel. (C) Ratios of the intensity of the upper band to that of the lower band; mean±s.e.m. for four blots similar to that shown in B. (D) Phos-tag gel separation and immunoblotting for PDE5 in follicle lysates that had been incubated with or without the catalytic subunit of PKA (PKA_c). Incubations were performed with or without 10 µM cGMP (see supplementary Materials and Methods); cGMP binds to an allosteric site on PDE5 and is required for PKA_c to phosphorylate PDE5 (Corbin et al., 2000; Rybalkin et al., 2002). In the presence of PKA_c and cGMP, the migration of PDE5 was retarded, indicating phosphorylation.

Development (2014) 141, 3594-3604 doi:10.1242/dev.112219

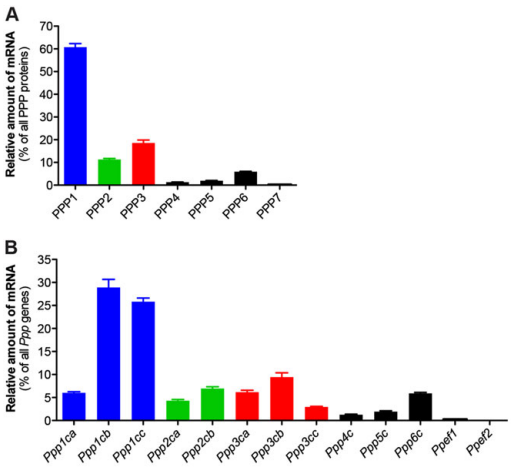

Fig. 6. Relative amounts of each PPP phosphatase catalytic subunit mRNA in isolated granulosa cells. (A) The graph shows the mean±s.e.m. for four independent preparations of granulosa cell RNA. The PPP1 bar shows the total for mRNA encoded by *Ppp1ca*, *Ppp1cb* and *Ppp1cc*. The PPP2 bar shows the total for *Ppp2ca* and *Ppp2cb*. The PPP3 bar shows the total for *Ppp3ca*, *Ppp3cb* and *Ppp3cc*. The PPP7 bar shows the total for *Ppef1* and *Ppef2*. (B) Data for each individual gene.

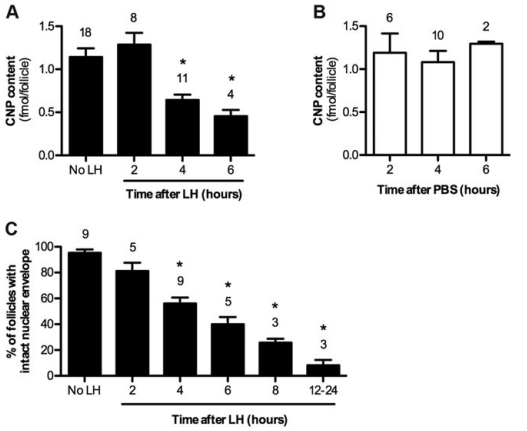

Fig. 7. Time course of the decrease in the CNP content of follicles, and the resumption of meiosis in response to LH signaling. (A) A time course of the decrease in CNP content of follicles that had been treated with LH. (B) There was no change in the CNP content of follicles that had been incubated for 2-6 h without LH. (C) A time course of nuclear envelope breakdown in follicles that had been treated with LH. Values indicate the mean±s.e.m. for the indicated number of experiments (above each bar). A total of 386 follicles were examined for C. A,C, the 'no LH' data combine results from follicles that had been incubated without LH for times corresponding to the times of LH exposure. **P*<0.05, measurements that are significantly different from controls without LH.

several species have shown that on a time scale of hours after hormone injection to activate LH receptors *in vivo*, CNP levels decrease (see Introduction). To integrate our findings regarding the dephosphorylation of NPR2 with this previous knowledge of another component of the regulatory system, we directly compared the kinetics of the decrease of CNP levels in the same experimental system that we had used for the phosphorylation studies. CNP content, as measured using ELISA, did not change during the initial 2 h after the application of LH to follicles, but by 4 h, CNP levels had decreased to 56% of the baseline level (Fig. 7A). CNP content in the follicles did not change during parallel incubations without LH (Fig. 7B). The LH-induced CNP decrease would further decrease NPR2 activity, beginning 2-4 h after the initial dephosphorylation. However, the magnitude of this subsequent activity decrease is unknown because the ELISA measurements detect the CNP peptide, as well as its biologically inactive precursor protein.

Because the significance of the decrease in guanylyl cyclase activity is to reduce cGMP and re-initiate meiosis in the oocyte, we also examined the time course of nuclear envelope breakdown (the first morphologically detectable event in the resumption of meiosis) following the addition of LH to isolated follicles in our culture system. The time by which 50% of the oocytes had undergone nuclear envelope breakdown was ~4 h, with a marginally significant decrease observed at 2 h; almost all oocytes had resumed meiosis 12-24 h after LH exposure (Fig. 7C). This time course is similar to that previously observed using a slightly different follicle culture system or after injection of LH into rats (Tsafriri, 1985). The effect of phosphatase inhibitors on LH-induced resumption of meiosis could not be determined because these inhibitors also act on phosphatases in the oocyte, causing meiosis to resume independently of LH (Rime and Ozon, 1990).

DISCUSSION

The results described here show that LH signaling decreases guanylyl cyclase activity in rat ovarian follicles by two sequential processes: rapid dephosphorylation of NPR2, detectable at 10 min after treatment with LH, and a slower decrease in the NPR2 agonist CNP, detectable at 4 h after treatment with LH (Fig. 8). Phosphorylation of the cGMP phosphodiesterase PDE5 at 10 min after LH exposure indicates that, in parallel, PDE5 activity may be rapidly increased. This would also contribute to the LH-induced cGMP decrease in the follicle, together with the decrease in the production of cGMP that results from the dephosphorylation of the NPR2 guanylyl cyclase. The decrease of cGMP in the follicle leads to the resumption of meiosis in the oocyte.

In addition, LH induces other changes in the follicle that would further decrease cGMP levels in the oocyte. One such change is a delayed decrease in cGMP generation by the inner granulosa (cumulus) cells of mouse follicles, detected 2-3 h after LH receptor stimulation, measured in the presence of a constant concentration of CNP (Robinson et al., 2012; Liu et al., 2014). Another factor that could contribute to the decrease in oocyte cGMP levels is that LH causes a transient decrease in gap junction permeability between the granulosa cells (Granot and Dekel, 1994; Sela-Abramovich et al., 2005; Norris et al., 2008). However, at least in mouse follicles, gap junction closure is not essential for the cGMP decrease (Norris et al., 2009), although it could be a 'fail-safe' mechanism if other mechanisms are defective.

How activation of the LH receptor is coupled to dephosphorylation of NPR2, and how dephosphorylation results in inactivation of NPR2, are unknown. The LH receptor activates G_s, as well as other G-proteins (Rajagopalan-Gupta et al., 1998; Hunzicker-Dunn and Mayo, 2014), and through one or more of these G-proteins, LH signaling must activate phosphatases and/or inactivate kinases, or

Development (2014) 141, 3594-3604 doi:10.1242/dev.112219

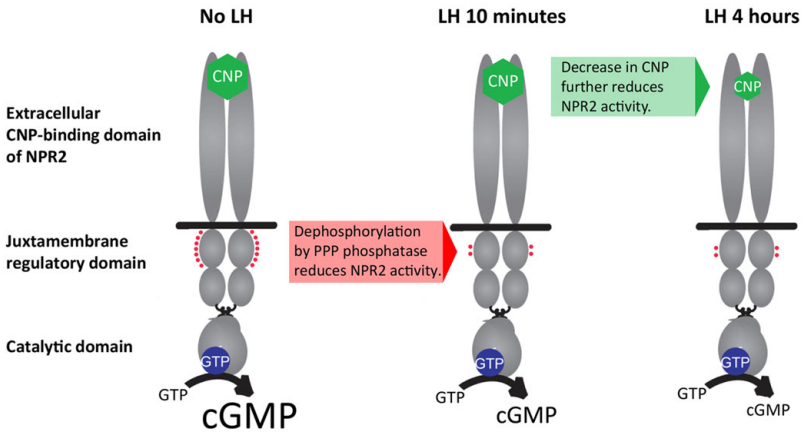

Fig. 8. LH signaling in rat ovarian follicles decreases NPR2 guanylyl cyclase activity by way of a rapid dephosphorylation of regulatory sites followed by a slower decrease of the levels of the agonist CNP. The functional domains of the homodimeric transmembrane protein NPR2 are shown in gray. Binding of CNP (green) to the extracellular domain and phosphorylation of seven juxtamembrane regulatory sites (red) both increase the catalytic activity of the enzyme. LH signaling acts by way of a PPP-family phosphatase to dephosphorylate some of these sites; dephosphorylation occurs by 10 min and persists for at least 4 h. By 4 h after LH exposure, the CNP content of the follicle decreases. Both of these changes decrease guanylyl cyclase activity, contributing to the decrease in cGMP that restarts meiosis.

possibly change the conformation of NPR2 such that it is a better or worse substrate for either enzyme. Although the kinases involved have not been identified, our results indicate that the phosphatases that are important for this dephosphorylation belong to the PPP family, and not to the PPM or FCP families. Among the PPP family, our findings using cantharidin argue against an important function for PPP3 (PP2B or calcineurin). Because PPP3 is activated by Ca^{2+}, this conclusion is consistent with the finding that deletion of $G\alpha_{q/11}$ in mouse granulosa cells, and the resulting prevention of LH-induced inositol phosphate accumulation, does not inhibit meiotic progression in response to LH (Breen et al., 2013). Taken together with our gene expression analysis, PPP1, PPP2 and/or PPP6 emerge as the most likely candidates for mediating the LH-induced dephosphorylation of NPR2.

Studies of rat granulosa cells in culture have shown that LH activation of G_s leads to the PKA-dependent activation of PPP2 and that PPP2 is associated with the MAP2D A-kinase anchoring protein (Flynn et al., 2008). One possible scenario, although not the only one, is that NPR2 could also be associated with MAP2D, such that it might be dephosphorylated by PPP2 in response to LH-G_s-PKA signaling. Because NPR2 contains multiple phosphorylation sites, and the sequences surrounding these sites differ, the regulation of other phosphatases (and kinases) might also be important in initiating or maintaining the dephosphorylation and inactivation of NPR2 in response to LH.

In particular, EGF receptor signaling is required for part of the LH-induced decrease in the cGMP content and the resumption of meiosis in mouse follicles; how much of the cGMP decrease depends on EGF receptor kinase activity is variable in different studies (Park et al., 2004; Vaccari et al., 2009; Norris et al., 2010; Hsieh et al., 2011; Liu et al., 2014). The release of EGF receptor ligands from the outer mural granulosa cell layers where the LH receptors are located provides a mechanism for paracrine signaling to cells in the interior of the follicle. In rats, the EGF receptor dependence of the LH-induced cGMP decrease has not been determined, but EGF receptor kinase activity is required over a period of hours for LH-induced resumption of meiosis (Ashkenazi et al., 2005; Reizel et al., 2010).

Signaling through various other hormones and growth factors also reduces the guanylyl cyclase activity of NPR2 in other cells, for example, vasopressin (Abbey and Potter, 2002), PDGF (Chrisman and Garbers, 1999; Abbey and Potter, 2003), lysophosphatidic acid (Abbey and Potter, 2003; Potthast et al., 2004), sphingosine-1-phosphate (Abbey-Hosch et al., 2005) and thyrotropin-releasing hormone (Thompson et al., 2014). In studies of sphingosine-1-phosphate acting on cultured fibroblasts that overexpress NPR2 (Abbey-Hosch et al., 2005), there is a correlation between dephosphorylation and the decrease in guanylyl cyclase activity. However, LH signaling in the ovarian follicle is the first example of a physiological pathway that is mediated by such a mechanism.

Other developmental processes that are regulated by the activity of NPR2 and the closely related natriuretic peptide receptor NPR1 could be controlled similarly. In particular, bone growth is affected by mutations in *Npr2* or the *Nppc* gene that encodes CNP; increased NPR2 activity results in longer bones, whereas decreased activity results in shorter bones (Chusho et al., 2001; Tamura et al., 2004; Yasoda et al., 2004; Olney, 2006; Miura et al., 2012; Geister et al., 2013). Natriuretic peptide receptors also function in the development of the nervous system (Ter-Avetisyan et al., 2014) and heart (Becker et al., 2014). Some of the actions of growth factors and hormones that affect chondrocyte differentiation, axon bifurcation and cardiomyocyte proliferation might involve the regulation of natriuretic peptide receptor phosphorylation and/or levels of natriuretic peptides, as seen for LH-mediated regulation of meiosis in the ovary.

MATERIALS AND METHODS

Isolation and culture of rat ovarian follicles

Ovaries were obtained from 25- to 26-day-old CD-Sprague-Dawley rats (Charles River Laboratories); procedures were approved by the animal care committee of the University of Connecticut Health Center. Rats were injected with 12 U of equine chorionic gonadotropin 48 h before ovary

collection to stimulate follicle growth and LH receptor expression. Preovulatory follicles, 700-900 µm in diameter, were dissected from the ovaries and cultured as previously described for mouse follicles (Norris et al., 2008) with some modifications (see supplementary Materials and Methods).

Cantharidin was obtained from Tocris Bioscience (R&D Systems) and prepared as a 50 mM stock in dimethylsulfoxide (DMSO). Okadaic acid was obtained from LC Laboratories and was prepared as a 1 mM stock in DMSO.

Preparation of crude membranes from rat follicles and measurement of guanylyl cyclase activity

Crude membranes were prepared from rat follicles, and guanylyl cyclase assays were conducted as previously described for mouse follicles (Robinson and Potter, 2011; Robinson et al., 2012). cGMP production was measured after a 9-min assay period and was approximately linear over this period (Robinson et al., 2012). Basal activity without CNP was subtracted from the activity in the presence of CNP to obtain the CNP-dependent activity. Additional details are described in the supplementary Materials and Methods.

Immunoprecipitation, Phos-tag acrylamide gel electrophoresis and western blotting

Immunoprecipitation was used to purify the low-abundance NPR2 protein from rat follicle membranes. NPR2 was immunoprecipitated by incubation with a rabbit polyclonal antiserum (6328) made against the ten C-terminal amino acids of NPR2 (Abbey and Potter, 2002). Phosphorylated forms of NPR2 were separated by electrophoresis on SDS-PAGE gels made with 6% acrylamide that had been co-polymerized with 25 µM Phos-tag-acrylamide (WAKO Chemicals) and 100 µM $MnCl_2$. Blots were probed with the 6328 antiserum. Phosphorylated forms of PDE5 were separated from follicle lysates, using Phos-tag gels as described for NPR2. The blots were probed with an affinity-purified rabbit polyclonal antibody made against a C-terminal sequence from human PDE5 (catalog no. 2395, Cell Signaling Technology). Additional details, as well as protocols for the generation of dephosphorylated NPR2 and phosphorylated PDE5 controls, are described in the supplementary Materials and Methods.

Images were analyzed using ImageJ software. As an indicator of changes in NPR2 phosphorylation, the intensity of the immunostaining was measured within boxes surrounding the upper region and the lower band (see Fig. 2C; supplementary material Fig. S3). The background intensity of each lane was collected from a box below the NPR2 signal and subtracted. The background-corrected intensity of the upper region was then divided by that of the lower band. This ratio method corrects for variability in the amount of protein that was immunoprecipitated and loaded in each lane. As a measure of total immunoreactive protein, we added the intensity of the upper region and the lower band and then subtracted the background intensity.

ELISA measurements of cGMP and CNP in follicles

The cGMP and CNP contents of rat follicles were measured as previously described (Norris et al., 2009; Robinson et al., 2012) by using ten follicles per sample and ELISA kits from Enzo Life Sciences (no. ADI-900-014 for cGMP) and Phoenix Pharmaceuticals (no. FEK-012-03 for CNP). Data were analyzed using Prism software (GraphPad).

Measurement of relative amounts of phosphatase mRNAs in granulosa cells

RNA was extracted from mural granulosa cells, mRNAs were reverse transcribed using random hexamers, and quantitative TaqMan analysis was performed as previously described (Robinson et al., 2012). Primer sequences are listed in supplementary material Table S1.

Statistics

Differences between a single treatment and control were analyzed by paired *t*-test using Prism software. Other data were analyzed by either repeated measures ANOVA using Prism (where sample sizes between groups were equal) or by repeated measures mixed models in IBM SPSS (v. 21.0). Post-hoc *t*-tests were corrected for multiple comparisons by the Holm–Bonferroni method (Holm, 1979). *P*-values<0.05 were considered to indicate a significant difference.

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Competing interests

The authors declare no competing financial interests.

Author contributions

All authors performed experiments or data analysis and contributed to the development of the concepts. J.R.E., L.R.P. and L.A.J. prepared the manuscript, and all authors edited the manuscript prior to submission.

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Supplementary material

Supplementary material available online at
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References

Abbey, S. E. and Potter, L. R. (2002). Vasopressin-dependent inhibition of the C-type natriuretic peptide receptor, NPR-B/GC-B, requires elevated intracellular calcium concentrations. *J. Biol. Chem.* **277**, 42423-42430.

Abbey, S. E. and Potter, L. R. (2003). Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. *Endocrinology* **144**, 240-246.

Abbey-Hosch, S. E., Smirnov, D. and Potter, L. R. (2005). Differential regulation of NPR-B/GC-B by protein kinase C and calcium. *Biochem. Pharmacol.* **70**, 686-694.

Ashkenazi, H., Cao, X., Motola, S., Popliker, M., Conti, M. and Tsafriri, A. (2005). Epidermal growth factor family members: endogenous mediators of the ovulatory response. *Endocrinology* **146**, 77-84.

Becker, J. R., Chatterjee, S., Robinson, T. Y., Bennett, J. S., Panakova, D., Galindo, C. L., Zhong, L., Shin, J. T., Coy, S. M. and Kelly, A. E. et al. (2014). Differential activation of natriuretic peptide receptors modulates cardiomyocyte proliferation during development. *Development* **141**, 335-345.

Bornslaeger, E. A., Mattei, P. and Schultz, R. M. (1986). Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. *Dev. Biol.* **114**, 453-462.

Bortolussi, M., Marini, G. and Dal Lago, A. (1977). Autoradiographic study of the distribution of LH(HCG) receptors in the ovary of untreated and gonadotrophin-primed immature rats. *Cell Tissue Res.* **183**, 329-342.

Breen, S. M., Andric, N., Ping, T., Xie, F., Offermans, S., Gossen, J. A. and Ascoli, M. (2013). Ovulation involves the luteinizing hormone-dependent activation of $G_{q/11}$ in granulosa cells. *Mol. Endocrinol.* **27**, 1483-1491.

Cargnello, M., Tcherkezian, J., Dorn, J. F., Huttlin, E. L., Maddox, P. S., Gygi, S. P. and Roux, P. P. (2012). Phosphorylation of the eukaryotic translation initiation factor 4E-transporter (4E-T) by c-Jun N-Terminal Kinase promotes stress-dependent P-body assembly. *Mol. Cell. Biol.* **32**, 4572-4584.

Chrisman, T. D. and Garbers, D. L. (1999). Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts. *J. Biol. Chem.* **274**, 4293-4299.

Chusho, H., Tamura, N., Ogawa, Y., Yasoda, A., Suda, M., Miyazawa, T., Nakamura, K., Nakao, K., Kurihara, T. and Komatsu, Y. et al. (2001). Dwarfism and early death in mice lacking C-type natriuretic peptide. *Proc. Natl. Acad. Sci. USA* **98**, 4016-4021.

Clift, D. and Schuh, M. (2013). Restarting life: fertilization and the transition from meiosis to mitosis. *Nat. Rev. Mol. Cell Biol.* **14**, 549-562.

Cohen, T. W. (2004). Overview of protein serine/threonine phosphatases. In *Protein Phosphatases* (ed. J. Arino and D. R. Alexander), pp. 1-20. Berlin: Springer-Verlag.

Conti, M., Hsieh, M., Zamah, A. M. and Oh, J. S. (2012). Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Mol. Cell. Endocrinol.* **356**, 65-73.

Corbin, J. D., Turko, I. V., Beasley, A. and Francis, S. H. (2000). Phosphorylation of phosphodiesterase-5 by cyclic nucleotide-dependent protein kinase alters its catalytic and allosteric cGMP-binding activities. *Eur. J. Biochem.* **267**, 2760-2767.

Flynn, M. P., Maizels, E. T., Karlsson, A. B., McAvoy, T., Ahn, J.-H., Nairn, A. C. and Hunzicker-Dunn, M. (2008). Luteinizing hormone receptor activation in

ovarian granulosa cells promotes protein kinase A-dependent dephosphorylation of microtubule-associated protein 2D. *Mol. Endocrinol.* **22**, 1695-1710.
Geister, K. A., Brinkmeier, M. L., Hsieh, M., Faust, S. M., Karolyi, I. J., Perosky, J. E., Kozloff, K. M., Conti, M. and Camper, S. A. (2013). A novel loss-of-function mutation in *Npr2* clarifies primary role in female reproduction and reveals a potential therapy for acromesomelic dysplasia, Maroteaux type. *Hum. Mol. Genet.* **22**, 345-357.
Granot, I. and Dekel, N. (1994). Phosphorylation and expression of connexin-43 ovarian gap junction protein are regulated by luteinizing hormone. *J. Biol. Chem.* **269**, 30502-30509.
Gutkowska, J., Jankowski, M., Sairam, M. R., Fujio, N., Reis, A. M., Mukaddam-Daher, S. and Tremblay, J. (1999). Hormonal regulation of natriuretic peptide system during induced oocyte follicular development in the rat. *Biol. Reprod.* **61**, 162-170.
Hinckley, M., Vaccari, S., Horner, K., Chen, R. and Conti, M. (2005). The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes. *Dev. Biol.* **287**, 249-261.
Hiradate, Y., Hoshino, Y., Tanemura, K. and Sato, E. (2014). C-type natriuretic peptide inhibits porcine oocyte meiotic resumption. *Zygote* **22**, 372-377.
Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* **6**, 65-70.
Holt, J. E., Lane, S. I. R. and Jones, K. T. (2013). The control of meiotic maturation in mammalian oocytes. *Curr. Top. Dev. Biol.* **102**, 207-226.
Honkanen, R. E. (1993). Cantharidin, another natural toxin that inhibits the activity of serine/threonine protein phosphatases types 1 and 2A. *FEBS Lett.* **330**, 283-286.
Horner, K., Livera, G., Hinckley, M., Trinh, K., Storm, D. and Conti, M. (2003). Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Dev. Biol.* **258**, 385-396.
Hsieh, M., Thao, K. and Conti, M. (2011). Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation. *PLoS ONE* **6**, e21574.
Hunzicker-Dunn, M. and Mayo, K. (2014). Gonadotropin signaling in the ovary. In *Knobil and Neill's Physiology of Reproduction*, 4th edn (ed. J. D. Neill). San Diego, CA: Elsevier (in press).
Jankowski, M., Reis, A. M., Mukaddam-Daher, S., Dam, T. V., Farookhi, R. and Gutkowska, J. (1997). C-type natriuretic peptide and the guanylyl cyclase receptors in the rat ovary are modulated by the estrous cycle. *Biol. Reprod.* **56**, 59-66.
Kawamura, K., Cheng, Y., Kawamura, N., Takae, S., Okada, A., Kawagoe, Y., Mulders, S., Terada, Y. and Hsueh, A. J. W. (2011). Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Hum. Reprod.* **26**, 3094-3101.
Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. and Koike, T. (2006). Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell. Proteomics* **5**, 749-757.
Kinoshita, E., Kinoshita-Kikuta, E. and Koike, T. (2012). Phos-tag affinity electrophoresis for protein kinase profiling. In *Protein Kinase Technologies* (ed. H. Mukai), pp. 13-34. New York: Humana Press.
Koller, K. J., Lipari, M. T. and Goeddel, D. V. (1993). Proper glycosylation and phosphorylation of the type A natriuretic peptide receptor are required for hormone-stimulated guanylyl cyclase activity. *J. Biol. Chem.* **268**, 5997-6003.
Kovo, M., Kandli-Cohen, M., Ben-Haim, M., Galiani, D., Carr, D. W. and Dekel, N. (2006). An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes. *Reproduction* **132**, 33-43.
Ledent, C., Demeestere, I., Blum, D., Petermans, J., Hamalainen, T., Smits, G. and Vassart, G. (2005). Premature ovarian aging in mice deficient for Gpr3. *Proc. Natl Acad. Sci. USA* **102**, 8922-8926.
Li, Y.-M., MacKintosh, C. and Casida, J. E. (1993). Protein phosphatase 2A and its [^{3}H]cantharidin/[^{3}H]endothall thioanhydride binding site: inhibitor specificity of cantharidin and ATP analogues. *Biochem. Pharmacol.* **46**, 1435-1443.
Liu, X., Xie, F., Zamah, A. M., Cao, B. and Conti, M. (2014). Multiple pathways mediate luteinizing hormone regulation of cGMP signaling in the mouse ovarian follicle. *Biol. Reprod.* **91**, 1-11.
McTague, J., Amyotte, N., Kanyo, R., Ferguson, M., Chick, C. L. and Ho, A. K. (2012). Different signaling mechanisms are involved in the norepinephrine-stimulated TORC1 and TORC2 nuclear translocation in rat pinealocytes. *Endocrinology* **153**, 3839-3849.
Mehlmann, L. M. (2013). Signaling for meiotic resumption in granulosa cells, cumulus cells, and oocyte. In *Oogenesis* (ed. G. Coticchio, D. F. Albertini and L. De Santis), pp. 171-182. London: Springer-Verlag.
Mehlmann, L. M., Jones, T. L. Z. and Jaffe, L. A. (2002). Meiotic arrest in the mouse follicle maintained by a G_s protein in the oocyte. *Science* **297**, 1343-1345.
Mehlmann, L. M., Saeki, Y., Tanaka, S., Brennan, T. J., Evsikov, A. V., Pendola, F. L., Knowles, B. B., Eppig, J. J. and Jaffe, L. A. (2004). The G_s-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science* **306**, 1947-1950.
Miura, K., Namba, N., Fujiwara, M., Ohata, Y., Ishida, H., Kitaoka, T., Kubota, T., Hirai, H., Higuchi, C. and Tsumaki, N. et al. (2012). An overgrowth disorder associated with excessive production of cGMP due to a gain-of-function mutation of the natriuretic peptide receptor 2 gene. *PLoS ONE* **7**, e42180.
Norris, R. P., Freudzon, M., Mehlmann, L. M., Cowan, A. E., Simon, A. M., Paul, D. L., Lampe, P. D. and Jaffe, L. A. (2008). 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. *Development* **135**, 3229-3238.
Norris, R. P., Ratzan, W. J., Freudzon, M., Mehlmann, L. M., Krall, J., Movsesian, M. A., Wang, H., Ke, H., Nikolaev, V. O. and Jaffe, L. A. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* **136**, 1869-1878.
Norris, R. P., Freudzon, M., Nikolaev, V. O. and Jaffe, L. A. (2010). Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. *Reproduction* **140**, 655-662.
Olney, R. C. (2006). C-type natriuretic peptide in growth: a new paradigm. *Growth Horm. IGF Res.* **16**, 6-14.
Park, J.-Y., Su, Y.-Q., Ariga, M., Law, E., Jin, S.-L. C. and Conti, M. (2004). EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* **303**, 682-684.
Peng, J.-Y., Xin, H.-Y., Han, P., Zhao, H.-B., Bai, L., An, X.-P. and Cao, B.-Y. (2013). Identification and gene expression analyses of natriuretic peptide system in the ovary of goat (*Capra hircus*). *Gene* **524**, 105-113.
Pereira, S. R., Vasconcelos, V. M. and Antunes, A. (2011). The phosphoprotein phosphatase family of Ser/Thr phosphatases as principal targets of naturally occurring toxins. *Crit. Rev. Toxicol.* **41**, 83-110.
Potter, L. R. (1998). Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptor B: dephosphorylation is a mechanism of desensitization. *Biochemistry* **37**, 2422-2429.
Potter, L. R. (2011). Regulation and therapeutic targeting of peptide-activated receptor guanylyl cyclases. *Pharmacol. Ther.* **130**, 71-82.
Potter, L. R. and Garbers, D. L. (1992). Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization. *J. Biol. Chem.* **267**, 14531-14534.
Potter, L. R. and Hunter, T. (1998). Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. *J. Biol. Chem.* **273**, 15533-15539.
Potter, L. R. and Hunter, T. (1999). A constitutively "phosphorylated" guanylyl cyclase-linked atrial natriuretic peptide receptor mutant is resistant to desensitization. *Mol. Biol. Cell* **10**, 1811-1820.
Potter, L. R., Abbey-Hosch, S. and Dickey, D. M. (2006). Natriuretic peptides, their receptors, and cyclic monophosphate-dependent signaling functions. *Endocr. Rev.* **27**, 2974-2985.
Potthast, R., Abbey-Hosch, S. E., Antos, L. K., Marchant, J. S., Kuhn, M. and Potter, L. R. (2004). Calcium-dependent dephosphorylation mediates the hyperosmotic and lysophosphatidic acid-dependent inhibition of natriuretic peptide receptor-B/guanylyl cyclase-B. *J. Biol. Chem.* **279**, 48513-48519.
Rajagopalan-Gupta, R. M., Lamm, M. L. G., Mukherjee, S., Rasenick, M. M. and Hunzicker-Dunn, M. (1998). Luteinizing hormone/choriogonadotropin receptor-mediated activation of heterotrimeric guanine nucleotide binding proteins in ovarian follicular membranes. *Endocrinology* **139**, 4547-4555.
Reizel, Y., Elbaz, J. and Dekel, N. (2010). Sustained activity of the EGF receptor is an absolute requisite for LH-induced oocyte maturation and cumulus expansion. *Mol. Endocrinol.* **24**, 402-411.
Rime, H. and Ozon, R. (1990). Protein phosphatases are involved in the in vivo activation of histone H1 kinase in mouse oocyte. *Dev. Biol.* **141**, 115-122.
Robinson, J. W. and Potter, L. R. (2011). ATP potentiates competitive inhibition of guanylyl cyclase A and B by the staurosporine analog, Go6976: reciprocal regulation of ATP and GTP binding. *J. Biol. Chem.* **286**, 33841-33844.
Robinson, J. W. and Potter, L. R. (2012). Guanylyl cyclases A and B are asymmetric dimers that are allosterically activated by ATP binding to the catalytic domain. *Sci. Signal.* **5**, ra65.
Robinson, J. W., Zhang, M., Shuhaibar, L. C., Norris, R. P., Geerts, A., Wunder, F., Eppig, J. J., Potter, L. R. and Jaffe, L. A. (2012). Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. *Dev. Biol.* **366**, 308-316.
Rybalkin, S. D., Rybalkina, I. G., Feil, R., Hofmann, F. and Beavo, J. A. (2002). Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J. Biol. Chem.* **277**, 3310-3317.
Sela-Abramovich, S., Chorev, E., Galiani, D. and Dekel, N. (2005). Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology* **146**, 1236-1244.
Swingle, M., Ni, L. and Honkanen, R. E. (2007). Small molecule inhibitors of ser/thr protein phosphatases: specificity, use and common forms of abuse. *Methods Mol. Biol.* **365**, 23-38.
Tamura, N., Doolittle, L. K., Hammer, R. E., Shelton, J. M., Richardson, J. A. and Garbers, D. L. (2004). Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. *Proc. Natl. Acad. Sci. USA* **101**, 17300-17305.

Ter-Avetisyan, G., Rathjen, F. G. and Schmidt, H. (2014). Bifurcation of axons from cranial sensory neurons is disabled in the absence of Npr2-induced cGMP signaling. *J. Neurosci.* **34**, 737-747.
Thompson, I. R., Mirczuk, S. M., Smith, L., Lessey, A. J., Simbi, B., Sunters, A., Baxter, G. F., Lipscomb, V. J., McGonnell, I. M. and Wheeler-Jones, C. P. et al. (2014). Homologous and heterologous desensitization of guanylyl cyclase-B signaling in GH3 somatolactotropes. *Cell Tissue Res.* **355**, 425-436.
Törnell, J., Billig, H. and Hillensjö, T. (1991). Regulation of oocyte maturation by changes in ovarian levels of cyclic nucleotides. *Human. Reprod.* **6**, 411-422.
Tsafriri, A. (1985). The control of meiotic maturation in mammals. In *Biology of Fertilization*, Vol. 1 (ed. C. B. Metz and A. Monroy), pp. 221-252. Orlando: Academic Press.
Tsuji, T., Kiyosu, C., Akiyama, K. and Kunieda, T. (2012). CNP/NPR2 signaling maintains oocyte meiotic arrest in early antral follicles and is suppressed by EGFR-mediated signaling in preovulatory follicles. *Mol. Reprod. Dev.* **79**, 795-802.
Vaccari, S., Weeks, J. L., Hsieh, M., Menniti, F. S. and Conti, M. (2009). Cyclic GMP signaling is involved in the Luteinizing Hormone-dependent meiotic maturation of mouse oocytes. *Biol. Reprod.* **81**, 595-604.
Wang, Y., Santini, F., Qin, K. and Huang, C. Y. (1995). A Mg^{2+}-dependent, Ca^{2+}-inhibitable serine/threonine protein phosphatase from bovine brain. *J. Biol. Chem.* **270**, 25607-25612.
Yasoda, A., Komatsu, Y., Chusho, H., Miyazawa, T., Ozasa, A., Miura, M., Kurihara, T., Rogi, T., Tanaka, S. and Suda, M. et al. (2004). Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.* **10**, 80-86.
Yoder, A. R., Stone, M. D., Griffin, T. J. and Potter, L. R. (2010). Mass spectrometric identification of phosphorylation sites in guanylyl cyclase A and B. *Biochemistry* **49**, 10137-10145.
Yoder, A. R., Robinson, J. W., Dickey, D. M., Andersland, J., Rose, B. A., Stone, M. D., Griffin, T. J. and Potter, L. R. (2012). A functional screen provides evidence for a conserved, regulatory, juxtamembrane phosphorylation site in guanylyl cyclase A and B. *PLoS ONE* **7**, e36747.
Yu, F.-X., Zhang, Y., Park, H. W., Jewell, J. L., Chen, Q., Deng, Y., Pan, D., Taylor, S. S., Lai, Z.-C. and Guan, K.-L. (2013). Protein kinase A activates the Hippo pathway to modulate cell proliferation and differentiation. *Genes Dev.* **27**, 1223-1232.
Zhang, M., Su, Y.-Q., Sugiura, K., Xia, G. and Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* **330**, 366-369.
Zhang, W., Yang, Y., Liu, W., Chen, Q., Wang, H., Wang, X., Zhang, Y., Zhang, M. and Xia, G. (2014). Brain natriuretic peptide and C-type natriuretic peptide maintain porcine oocyte meiotic arrest. *J. Cell. Physiol.* (in press).

SUPPLEMENTARY MATERIAL

Table S1. Primers and fluorescent probes used for qRT-PCR analysis of relative expression levels of PPP family phosphatases.

gene name	forward primer, 5'-3'	probe, 5'-3'	reverse primer, 5'-3'
Ppp1ca	AAGGGGAAGTATGGGCAGTT	AGGCCGTCCCATCACTCCACC	TTTCTTGGCTTTGGCAGAAT
Ppp1cb	ACCAGTATGGTGGGCTGAAT	CTGTCACTCCGCCTCGAACAGCTAA	TTCACCTTTTCTTCGGTGGA
Ppp1cc	CCCAACTACTGTGGCGAGTT	AATGCGGGCGCCATGATGAGT	AGCACATGAGGGTCTCATCC
Ppp2ca	CGTAGAGGCGAGCCACA	CTCGTCGTACCCCAGACTACTTCCTG	TTCATGGCAATACTGTACAAGG
Ppp2cb	CGTCGTGGAGAGCCTCAT	CCGGCGCACCCCAGACTACTTC	AGGTCCTGGGGAGGAATTTA
Ppp3ca	ACGCCAACCTTAACTCCATC	TCGCCTCAGAGACTAACGGCACAGA	TGCTGCTATTACTGCCGTTG
Ppp3cb	AGGAGAGTGAAAGCGTGCTG	AAGGGCCTGACTCCCACAGGGAT	CCAGCCAACACTCCACTAGG
Ppp3cc	TTGAAGAAGCCCGAGGTCTA	TGAGAGAATGCCACCCCGAAAAGAG	GTGTCTTTCCTGCATCATGG
Ppp4c	GGCAGCCATCTTAGAACTGG	TTCATCATCTTCGAGGCTGCACCC	GGATGCCACGTGTCTCTTG
Ppp5c	GACCAGATGGGAAACAAAGC	ACATCCACCTCCAGGGCTCCGAC	CACTGCTGTGAATTGGTGGA
Ppp6c	CGTCAACACGAGAGAACCAA	TCCGAGCAGTTCCAGATTCAGAACG	CGTGGTTCTGGGAGGAATAA
Ppef1	TGTGGAAACTTTTCAATGCTCA	ATGATTCCCAAATTGATGAGCTTGCC	TTGTTGGAGTCCATTGTGCT
Ppef2	CATCTGTGACCTTGCCAGAA	TTCAACAAGGACGGCCACATCGATA	AAGGCCTCCAGGAACTCATT
Rpl32	GAAAGAGCAGCACAGCTGGC	TCAGAGTCACCAATCCCAACGCCA	TCATTCTCTTCGCTGCGTAGC

Probes were labeled with FAM (5') and TAMRA (3').

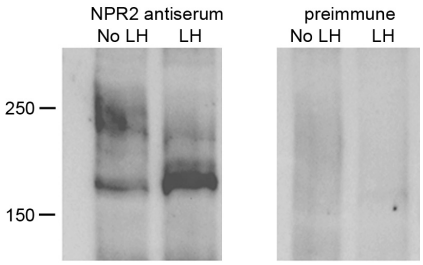

Figure S1. Validation of the specificity of the NPR2 antibody. Blot showing the lack of immunoreactivity when the immunoprecipitation and western blot of follicle membranes were done with preimmune serum instead of the 6328 antibody against a C-terminal peptide from NPR2. The follicles were treated with or without LH for 30 minutes.

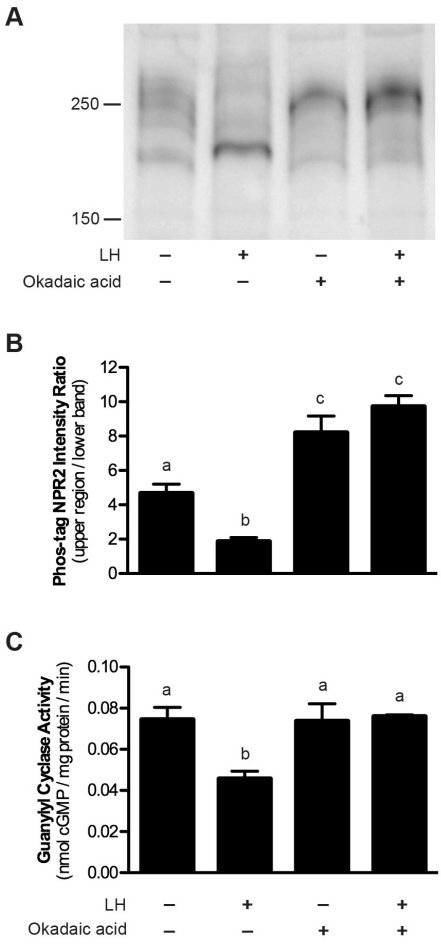

Figure S2. Inhibition of the LH-induced dephosphorylation and inactivation of NPR2 by treatment of follicles with the PPP family phosphatase inhibitor okadaic acid. (A) Follicles were incubated with or without 10 μM okadaic acid for one hour, then with or without LH for 30 minutes. Crude membranes were isolated and used for immunoprecipitation, Phos-tag gel electrophoresis, and immunoblotting for NPR2. In the presence of okadaic acid, basal phosphorylation of NPR2 increased, but LH did not change the ratio of NPR2 in the upper region and lower band, indicating that okadaic acid inhibited the LH-induced dephosphorylation of NPR2. **(B)** Graph showing the results of 4 experiments like that shown in **A** (mean ± s.e.m.). **(C)** Membranes from follicles treated with or without okadaic acid followed by LH, as described above, were assayed for NPR2 guanylyl cyclase activity (4 experiments). Values not indicated by the same letter are significantly different. We also attempted to determine the effect of okadaic acid on the LH-induced decrease in cGMP, but these experiments were not interpretable because okadaic acid alone caused cGMP to decrease in some of the trials.

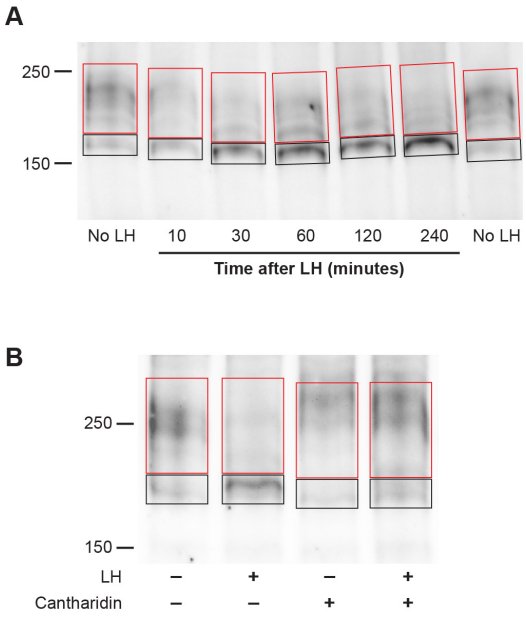

Figure S3. Blot images for figures 3A and 4A, with red boxes indicating the upper region (more phosphorylated) and lower band (less phosphorylated) for which immunostaining intensity was measured. (A) Figure 3A. **(B)** Figure 4A.

SUPPLEMENTARY MATERIALS AND METHODS

Isolation and culture of rat ovarian follicles

Preovulatory follicles, 700 - 900 μm in diameter, were dissected from the ovaries of rats that had been injected 48 hours previously with equine chorionic gonadotropin. Approximately 30 follicles were obtained per rat. The follicles were placed on Millicell culture inserts (PICMORG50, Millipore, Billerica, MA; 10-30 follicles per insert). MEMα medium (Invitrogen, Carlsbad, CA) was supplemented with 25 mM $NaHCO_3$, 3 mg/ml BSA, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 50 μg/ml streptomycin, and 75 μg/ml penicillin G. The follicles were cultured at 37°C in 5% CO_2 in air, and experimental procedures were started one to 4 hours after isolation. LH and other reagents were applied to the medium under the culture membrane, and 200-400 μl was also added to the top of the membrane to ensure rapid exposure of the follicles. Ovine LH and equine chorionic gonadotropin, purified from biological sources, were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). LH was used at a saturating concentration (10 μg/ml; approximately 350 nM). The kinetics of nuclear envelope breakdown in response to LH were determined by incubating isolated follicles with LH, and at various times afterwards, opening them with 30 gauge needles to release the cumulus-oocyte complex for observation of the presence or absence of the nucleus and nucleolus.

Preparation of crude membranes from rat follicles

To prepare crude membranes from rat follicles, 20-100 follicles were washed in PBS and then lysed in phosphatase inhibitor buffer (buffer A) containing 25 mM Hepes (pH 7.4), 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 20% glycerol, 1 μM microcystin-LR (Cayman Chemical Co., Ann Arbor, MI), and protease inhibitors (complete Mini, EDTA-free; Roche Applied Science), in a 0.1 ml glass homogenizer (Wheaton, Millville, NJ) on ice. The follicle wash procedure was done at room temperature and was started 3 minutes before the homogenizer was placed on ice. LH incubation times refer to the times at which the homogenizer was chilled. The homogenate (200-400 μl volume) was centrifuged at 10,000xg for 20

minutes at 4°C; the pellet was resuspended in buffer A using a probe sonicator. Protein content was determined by a BCA assay (Thermo Fisher Scientific, Rockford, IL). The crude membrane fraction contained approximately 10 μg of protein per follicle. Aliquots were frozen in liquid N_2 and stored at -80°C. Crude membranes were also prepared from HEK-293T cells stably expressing NPR2, from plates of cells at 70-80% confluency that had been serum starved for >2 hours.

Measurement of guanylyl cyclase activity in follicle membranes

Guanylyl cyclase assays were conducted as previously described (Robinson and Potter, 2011). Assays were performed at 37°C using 3-20 μg of follicle protein per assay tube, in the presence or absence of CNP (1 μM except as indicated). The reaction mixture contained 25 mM Hepes (pH 7.4), 50 mM NaCl, 0.1% BSA, 1 mM EDTA, 0.5 μM microcystin, 5 mM $MgCl_2$, as well as 1 mM of the allosteric activator, ATP, and 1 mM of the substrate, GTP. 5 mM creatine phosphate and 0.1 mg/ml creatine kinase were included in the reaction mixture to maintain ATP and GTP concentrations. 0.5 mM isobutylmethylxanthine was included to inhibit cGMP phosphodiesterase activity.

Immunoprecipitation of NPR2 from rat follicle membranes

Crude membrane samples (130-230 μg protein) were diluted to 400 μl in immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 10 mM NaH_2PO_4, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 μM microcystin-LR, and protease inhibitors. NPR2 was immunoprecipitated by incubation with 0.6 μl of 6328 rabbit polyclonal antiserum made against the 10 C-terminal amino acids of NPR2 (Abbey and Potter, 2002) for one hour at 4°C, and then with 25 μl of washed protein AG magnetic beads (Thermo Fisher Scientific, Rockford, IL) overnight at 4°C. The beads were washed and resuspended in Laemmli sample buffer with 75 mM dithiothreitol. Protein was eluted by heating at 70°C for 10 minutes. Approximately 50% of the membrane NPR2 was recovered after immunoprecipitation.

Phos-tag acrylamide gel electrophoresis and Western blotting

Phosphorylated forms of NPR2 or PDE5 were separated by electrophoresis on SDS-PAGE gels made with 6% acrylamide copolymerized with 25 μM Phos-tag-acrylamide (WAKO Chemicals USA, Richmond, VA) and 100 μM $MnCl_2$ (145 x 160 x 1.5 mm gel dimensions). Gels containing 6% acrylamide, but without Phos-tag-acrylamide or $MnCl_2$, were used for comparison. Immunoprecipitated NPR2, or lysates of follicles that had been washed in PBS and then sonicated in Laemmli sample buffer with 75 mM dithiothreitol, were electrophoresed at 25 mA/gel for 6-8 hours at 4°C. The gels were then incubated for 20 minutes in 400 ml transfer buffer (100 mM Tris, 192 mM glycine, no SDS or methanol) containing 1 mM EDTA to chelate Mn^{2+}, and then washed for 20 minutes in 400 ml transfer buffer alone to remove Mn^{2+}-EDTA.

Protein was transferred to a nitrocellulose membrane for 17-20 hours with 500 mA constant current at 4°C. The membrane was stained with Ponceau-S, and blocked with 0.1% Tween and 2% milk. Blots for NPR2 were probed overnight at 4°C with a 1:50,000 dilution of the 6328 antiserum (see main text), and then with a 1:500 dilution of Clean-Blot IP Detection Reagent coupled to HRP (Thermo Fisher Scientific, Rockford, IL). Blots for PDE5 were probed overnight with a 1:500 dilution of the 2395 antibody from Cell Signaling Technology (see main text) and then with a 1:5000 dilution of a goat-anti-rabbit antibody coupled to HRP (catalog # sc-2004; Santa Cruz Biotechnology Inc., Dallas, TX). Blots were developped using ECL Prime (GE Healthcare Bio-Sciences, Piscataway, NJ), and imaged using a charge-coupled device camera (G:Box Chemi XT4, Syngene, Frederick, MD).

In vitro dephosphorylation of NPR2 in follicle membranes

To confirm that the LH-induced acceleration of NPR2 migration in a Phos-tag gel was due to dephosphorylation (Fig. 2E,F), follicle membranes were incubated at 30°C for 30 min, either with phosphatase inhibitors (50 mM NaF + 2 mM EDTA + 1 μM microcystin-LR), or under conditions that

promoted phosphatase activity (no phosphatase inhibitors, and 2 mM $MgCl_2$; see Bryan and Potter, 2002). NPR2 was then immunoprecipitated and separated on a gel containing Phos-tag acrylamide; NPR2 was visualized by western blotting. To prepare membranes for these assays, follicles were homogenized in buffer A without microcystin-LR, to avoid irreversible modification of phosphatases. After centrifugation, the membranes were suspended in a buffer containing 25 mM Hepes, 50 mM NaCl, 20% glycerol, and protease inhibitors. After incubation under the indicated conditions, aliquots were frozen for immunoprecipitation and western blotting (Fig. 2 E,F), and for guanylyl cyclase assays (Fig. 2G).

Bryan, P. M. and Potter, L. R. (2002). The atrial natriuretic peptide receptor (NPR-A/GC-A) is dephosphorylated by distinct microcystin-sensitive and magnesium-dependent protein phosphatases. *J. Biol. Chem.* **277**, 16041-16047.

In vitro phosphorylation of PDE5 in follicle lysates

To confirm that the LH-induced retardation of PDE5 migration in a Phos-tag gel was due to phosphorylation (Fig. 5D), a lysate of follicles was incubated with the catalytic subunit of protein kinase A (PKA_c, kindly provided by Jackie Corbin, Vanderbilt University), following the procedure described by Rybalkin et al., 2002. The follicles were lysed in a glass homogenizer in a buffer containing 50 mM Tris, pH 7.5, 1.5 mM EDTA, 25 mM NaF, 0.2 mM Na vanadate, and protease inhibitors (Roche complete Mini) followed by sonication. Aliquots containing 100 μg protein were then incubated at 30°C for 30 minutes with 4 μM PKA_c, or with the buffer in which the PKA_c was dissolved. Reactions were performed with or without 10 μM cGMP, which is required for phosphorylation of PDE5 by PKA_c (Corbin et al., 2000). The samples were then spin-dialyzed into a buffer compatible with Phos-tag gel electrophoresis (50 mM Tris, pH 7.5, 25 mM NaF, 0.2 mM Na vanadate, and protease inhibitors), using a 0.5 ml, 10K Amicon Ultra centrifugal filter (EMD Millipore, Billerica, MA).

Publication 4: Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase regulates oocyte meiotic resumption and longitudinal bone growth.

Shuhaibar, L.C, Egbert, J.R., Edmund, A.B., Feil, R., Yee, S.P., Potter, L. R, Jaffe, L.A. (*in preparation*)

Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase regulates oocyte meiotic resumption and longitudinal bone growth.

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ABSTRACT

In mammals, the meiotic cell cycle of oocytes starts during embryogenesis and then pauses until luteinizing hormone (LH) acts to restart the cycle. This meiotic arrest is maintained by cGMP, which is produced in the granulosa cells by the natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase B. LH signaling decreases cGMP in the granulosa cells, and via equilibration through gap junctions, cGMP also decreases in the oocyte, thus releasing the meiotic arrest. Previous work has shown that one mechanism by which LH causes the cGMP decrease is by dephosphorylation and inactivation of NPR2, but it has not been directly tested whether NPR2 dephosphorylation is required for meiotic resumption. Studies of cells expressing point-mutated forms of NPR2 have identified seven serine and threonine phosphorylation sites that regulate NPR2 activity, but this mechanism has not been tested in physiological settings. Here, by use of a knock-in mouse in which seven juxtamembrane serine and threonine residues are changed to glutamates (NPR2-7E), such that NPR2 behaves as if it is constitutively phosphorylated, we show that dephosphorylation of these sites is required for the rapid resumption of meiosis in response to LH. In the NPR2-7E follicles, LH failed to decrease guanylyl cyclase activity, and the cGMP decrease was attenuated, thus causing a delay in meiotic resumption. Additionally, the NPR2-7E mutation resulted in increased longitudinal bone growth. Our results demonstrate that dephosphorylation of these seven serine and threonine regulatory sites of NPR2 is important for hormonal regulation of meiosis in oocytes as well as for bone growth.

INTRODUCTION

Meiosis in mammalian oocytes begins during embryonic development and is paused at prophase I until, beginning at puberty, luteinizing hormone (LH) that is secreted from the pituitary gland in each reproductive cycle acts to release the arrest. In human oocytes, this meiotic arrest can last up to 50 years. Meiotic arrest is maintained by the inhibitory action of cGMP, which is produced in the granulosa cells surrounding the oocyte and diffuses through gap junctions into the oocyte (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 high levels of cAMP that maintain the CDK1 kinase in a phosphorylated and inactive form, thus inhibiting the prophase-to-metaphase transition (Conti *et al*., 2012; Holt *et al*., 2013; Mehlmann, 2013). LH signaling induces meiotic resumption by lowering cGMP in the mural granulosa cells of the follicle and, as a consequence of diffusion through gap junctions, decreases cGMP in the oocyte (Norris, *et al*., 2009; Shuhaibar *et al*., 2015). Thus cAMP in the oocyte decreases and meiosis resumes.

cGMP is produced in the mural granulosa and cumulus cells by natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase B (Zhang *et al*., 2010; Kawamura *et al*., 2011; Robinson *et al*., 2012). NPR2 is a homodimeric transmembrane receptor activated by the C-type natriuretic peptide receptor (CNP). The receptor consists of an extracellular CNP-binding domain, a single-membrane spanning region, a juxtamembrane regulatory region, a kinase homology domain, and a guanylyl cyclase catalytic domain (Potter 2011, see Fig. 1A). NPR2 guanylyl cyclase activity is modulated by phosphorylation of serine and threonine sites in the juxtamembrane region (Potter and Garbers, 1992; Potter, 1998; Potter, 2011; Yoder *et*

al., 2012). Phosphorylation of these sites increases the CNP-dependent activity of NPR2 (Potter, 1998).

NPR2 activity is also essential for development of long bones and vertebrae. Deficiency of CNP or NPR2 causes dwarfism (Tamura *et al*., 2004; Tsuji and Kunieda, 2005; Tsuji *et al*., 2008; Geister *et al*., 2013). Conversely, overexpression of CNP or gain of function mutations of NPR2 causes disproportionately longer bones (Yasoda *et al.*, 2004; Miura *et al*., 2012; Hannema *et al*., 2013). This evidence indicates the pivotal role of the CNP/NPR2 pathway in the regulation of bone growth.

In the ovarian follicle, LH receptors are expressed in mural granulosa cells (Wang and Greenwald, 1993; Bortolussi *et al*., 1997). LH signaling, acting through a G-protein coupled receptor, rapidly reduces the production of cGMP by dephosphorylation and inactivation of NPR2 (Robinson *et al.* 2012; Egbert *et al*., 2014). Dephosphorylation of NPR2 is necessary for the fast decrease in NPR2 activity and cGMP in the follicle (Egbert *et al*., 2014). However, it has not been directly tested whether dephosphorylation of NPR2 is essential for the resumption of oocyte meiosis in response to LH. In addition, although it is known that NPR2 activity is required for normal bone growth, it is not known if the regulation of bone growth involves changes in the phosphorylation state of NPR2. Here we investigate if dephosphorylation of NPR2 is essential for the reinitiation of meiosis in the ovarian follicle, and for the growth of bones, using a transgenic mouse in which the seven juxtamembrane serine and threonine residues in NPR2 are changed to glutamates, such that NPR2 behaves as if it is constitutively phosphorylated.

We find that mutation of the seven serine and threonine sites in NPR2 to glutamate (NPR2-7E) completely inhibits the LH-induced decrease in guanylyl cyclase activity in the

granulosa cells, resulting in a delay in meiotic resumption. Additionally, the NPR2-7E mutations increased longitudinal bone growth. Our results indicate that dephosphorylation of NPR2 is necessary for the hormonal regulation of meiosis in the ovary and for the regulation of bone growth.

MATERIALS AND METHODS

Generation of a knock-in mouse with phosphomimetic glutamate mutations in NPR2

The general strategy for generating the *Npr2*-7E mouse line is outlined in Fig. 1B and the accompanying legend. More specific information is provided below.

A targeting vector for introducing the 7E-mutated form of *Npr2* was prepared by recombineering (Lee *et al*., 2001). Briefly, we first retrieved approximately 9.2 kb of the *Npr2* genomic sequence spanning introns 2 to 12 from the BAC vector, RP24-306K11 (Children's Hospital Oakland Research Institute), into a vector containing the negative selectable marker PGKdta, by gap repair. We then inserted a single LoxP site into exon 8 together with a unique restriction site, PmeI, by recombineering. An 800 bp genomic fragment spanning intron 7 to 9 with the 7E mutations S489E, S513E, T516E, S518E, S523E, S526E, and T529E, was prepared by PCR and inserted into the targeting vector to replace the single LoxP by recombineering. Unique site elimination, by PmeI digestion, was used to remove plasmids that did not undergo recombineering to generate the pNpr2-7E.dta vector (Noll *et al*., 2009). We then inserted a single LoxP with two unique restriction sites, SalI and AscI, in the 3' end into intron 8 by recombineering.

We then prepared a fragment containing a wild-type *Npr2* minigene with 200 bp of intron 8 and cDNA from exons 8 to 22 followed by IRESeGFP, Npr2 3'UTR and the Frt-PGK-Neo-Frt-LoxP cassette by a combination of PCR and conventional cloning. This fragment, which contains SalI and AscI, was then inserted 3' to the single LoxP site in the pNpr2-7E.dta vector by restriction digestion and ligation to generate the final targeting vector. This vector, which contains approximately 5.3 kb and 4 kb of 5' and 3' arms, respectively, was then linearized and electroporated into mouse ES cells derived from F1

(129Sv/C57BL6/J) blastocysts. Electroporated ES cells were cultured for 48 hour prior to drug selection using G418. Drug resistant colonies were picked and screened by long range PCR using primers corresponding to sequences outside the arms and specific to the 5'LoxP site, and eGFP fluorescence to identify targeted ES clones. These targeted ES clones were then further analyzed by PCR and sequencing to confirm the presence of the 7E mutation prior to using them for the generation of chimeric animals by ES cell-morula aggregation (Behringer et al., 2014, see Fig. S1, and Table S1).

Chimeric animals were then bred with *Hprt-Cre* mice to generate global knockin mice. Cre recombinase excises the *Npr2* wild-type mini-gene and IRES-eGFP sequences, leaving a single LoxP site in intron 7, and inducing expression of the *Npr2* gene with the 7E mutations (*Npr2*-7E). To obtain homozygous mice expressing the 7E mutations (*Npr2*-7E/7E), we crossed *Npr2*-7E/+ females with *Npr2*-7E/+ males.

Homozygous females and males are fertile, although future studies will be needed to investigate if they produce fewer pups than wild-type mice. Since fertility was not a limiting factor, pups were generated by breeding *Npr2*-7E/7E males and *Npr2*-7E/7E females, or *Npr2*-+/+ males and *Npr2*-+/+ females; heterozygous pairs were also used for some of the breeding. Mice used for the experiments described here had been crossed once with C57BL6/J mice. Animal care and all experiments were conducted as approved by the University of Connecticut Health Center Animal Care Committee.

Isolation and culture of mouse preovulatory follicles

Ovaries were obtained from pre-pubertal mice (22-24 days old). Antral follicles (≥320μm in diameter) were dissected and cultured for 24-30 hours on Millicell organotypic membranes (Norris *et al*., 2008). 10 ng/ml of follicle stimulating hormone (FSH) was

included in the culture medium to stimulate follicle growth and expression of LH receptors. LH was used at a concentration of 10 μg/ml. Ovine LH and ovine FSH, purified from biological sources, were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA).

For observations of nuclear envelope breakdown (NEBD) and cumulus expansion in follicle-enclosed oocytes, LH was applied to the medium in the dish holding the Millicell membrane, and oocytes within follicles on the Millicell membrane were observed for the presence or absence of a nucleus, nucleolus, and an expanded cumulus mass, at 1-hour intervals. For testing NEBD in isolated oocytes, follicles on the Millicell were opened using 30 gauge needles, and cumulus cells were removed using a glass micro pipette with an ~80μm diameter opening. Observations were made using a 20x/0.4 NA long-working distance objective. Photographs were taken using a Fotodiox Pro EF-EOS (M) camera.

Measurements of guanylyl cyclase activity and cGMP.

For guanylyl cyclase measurements, crude membranes were prepared and assayed as previously described (Robinson *et al*., 2012). For each membrane preparation, 80-100 antral follicles from 5-8 *Npr2*-7E/7E or *Npr2*-+/+ mice were isolated and cultured in the presence of FSH for 24 hours. The follicles were divided into 2 equal groups, and half were exposed to LH for 20 minutes, before preparing and freezing membrane samples. Assays were performed in the presence of 1 μM CNP, or 5mM $MnCl_2$ and 1% Triton X-100. 0.5 mM IBMX was included in the assay buffer to inhibit cyclic nucleotide phosphodiesterase activity.

To measure the effect of LH on cytosolic cGMP in follicles from *Npr2*-7E/7E mice, mice expressing the cGi500 FRET sensor for cGMP (Thunemann *et al*., 2013) were bred with

mice from the *Npr2*-7E line, to obtain mice with one copy of the cGi500 sequence, together with either the *Npr2*-7E/7E or *Npr2*-+/+ sequence. Follicles were imaged by confocal microscopy in a 200 μm-deep glass-bottomed chamber, before and after perfusion with LH, as previously described (Shuhaibar *et al*., 2015). CFP and YFP images were collected every 30-sec, and emission intensities from the mural granulosa region were measured from a 25μm-wide band just inside the basal lamina. Values for ratios "before LH" are averages of the 10 measurements before LH addition; values for ratios "20 min after LH" are averages of the 10 measurements between 15 and 20 min after LH perfusion through the chamber (see Shuhaibar *et al*., 2015).

Measurements of longitudinal bone growth

To measure tail growth in live mice, *Npr2*-7E/7E and *Npr2*-+/+ mice were housed in parallel, and tail length was measured every 3 weeks. Four litters with similar birth dates (±1-3 days) were included in this experiment, and the timing for each measurement was calculated with respect to the birth date of each individual litter. Tail length was measured from the base to the tip.

To measure vertebra lengths, 18-week old mice were sacrificed, and placed flat on their dorsal side on the stage of a Faxitron cabinet x-ray system. Tails were taped to the stage to hold them flat. Tail lengths were measured from the rostral end of caudal vertebra number 5 (CA5) to the tip of the final vertebra.

Statistics

Differences between multiple treatments conditions were analyzed by unpaired or paired t-tests or by one-way ANOVA followed by Tukey's multiple comparison test, using Prism software (GraphPad). Graph values that are indicated by an asterisk are significantly different from the control ($P<0.05$); values indicate mean ± s.e.m.

RESULTS

Generation of a knock-in mouse globally expressing phosphomimetic mutations in the seven juxtamembrane serines and threonines of NPR2.

To determine if dephosphorylation of NPR2 is required for the LH-induced decrease in NPR2 activity and the rapid decrease in cGMP in the oocyte, we generated knock-in mice with point mutations in the *Npr2* gene to substitute glutamate (E) for seven serine (S) and threonine (T) residues, to mimic a phosphorylated receptor that could not be dephosphorylated (Fig. 1A). These seven sites in the juxtamembrane domain of NPR2 were previously identified as regulators of NPR2 activity based on studies using HEK-293 cells (Yoder et al., 2012). Mice globally expressing the 7E mutations were produced by crossing mice with the *Npr2-7E* targeted allele with *Hprt-Cre* mice (Tang et al., 2002) (Fig 1B, bottom). Homozygous knock-in mice globally expressing the mutation, referred through this chapter as NPR2-7E/7E, showed no obvious physical defects and were fertile. However, as will be described below, they showed a delay in oocyte meiotic resumption as well as increased longitudinal bone growth.

The LH-induced decrease in NPR2 guanylyl cyclase activity in ovarian follicle membranes is prevented by the NPR2-7E mutations.

Previous studies in mouse and rats have demonstrated that one mechanism by which LH causes the cGMP decrease in the ovarian follicle is by dephosphorylation and inactivation of NPR2 (Robinson *et al.*, 2012; Egbert *et al*., 2014). To investigate if dephosphorylation of the serine and threonine sites mutated in the NPR2-7E mouse is required for the LH-induced

decrease in guanylyl cyclase activity, we prepared membranes from isolated antral follicles treated with or without LH for 20 min as previously described (Robinson *et al*., 2012). Membranes were assayed for guanylyl cyclase activity, with and without the NPR2 agonist CNP; CNP-dependent activity indicates the activity of NPR2 (Fig. 2A).

In wild-type follicles treated with LH for 20 min, the CNP-dependent guanylyl cyclase activity decreased to ~47% of the initial level (Fig. 2A; 1B, left). Little or no change in CNP-dependent guanylyl cyclase activity was detected in membranes from NPR2-7E/7E follicles that had been treated for 20 min with LH (Fig. 2A, B right). These findings indicate that dephosphorylation 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 activates the NPR2 guanylyl cyclase nearly maximally, independently of CNP or phosphorylation. Activities measured with Mn/Triton are indicative of guanylyl cyclase protein levels (Potter and Hunter, 1998; Potter and Hunter, 1999; Abbey and Potter, 2003). Based on these assays neither the 7E mutations nor the LH treatment changed the NPR2 protein content of the follicles.

The LH induced decrease in cGMP in the mural granulosa cells of ovarian follicles is attenuated by the NPR2-7E mutations.

LH signaling induces resumption of oocyte meiosis by decreasing cGMP in the ovarian follicle (Norris *et al*., 2009; Vaccari *et al.*, 2009). This LH-induced decrease in cGMP begins in the mural granulosa cells, where both LH receptors and NPR2 are expressed, and

later propagates into the oocyte (Shuhaibar *et al*., 2015). To examine the effect of the NPR2-7E mutations on the LH-induced cGMP decrease in mural granulosa cells, we measured changes in cGMP in live ovarian follicles from mice globally expressing the cGi500 sensor for cGMP (Thunemann *et al*., 2013) along with the NPR2-7E mutations. Binding of cGMP to the cGi500 sensor decreases Förster resonance energy transfer (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). Follicles from cGi500/*Npr2*-7E/7E and cGi500/*Npr2*-+/+ mice were imaged by confocal microscopy before and after perfusion with LH.

In the mural granulosa cells of *Npr2*-7E/7E and wild-type follicles, the CFP/YFP ratios before LH treatment were similar, indicating that the 7E mutations do not alter the basal concentration of cGMP in granulosa cell cytosol (Fig. 3A, B). However, in *Npr2*-7E/7E follicles, the LH-induced decrease in CFP/YFP ratio was attenuated (Fig. 3A,B), indicating a smaller decrease in cGMP after treatment with the hormone. After 20 min of exposure to LH, the CFP/YFP ratio in the *Npr2*-7E/7E follicles had decreased by ~21%, compared to ~34% in wild-type follicles (Fig. 3C). Because follicles with the 7E mutations did not show a significant LH-induced decrease in NPR2 guanylyl cyclase activity (Fig. 2A, B), the partial decrease in cytosolic cGMP suggested that LH signaling leads to additional changes in the follicle that also act to decrease cGMP, such as an increase in cGMP phosphodiesterase activity (Egbert *et al*., 2014).

Dephosphorylation of serine and threonine residues in the NPR2 receptor is required for the LH-induced resumption of oocyte meiosis.

To investigate whether preventing the LH-induced decrease in guanylyl cyclase activity in the NPR2-7E/7E follicles affects meiotic resumption in the oocyte and events preceding ovulation, 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 and oocytes showed normal morphology and underwent cumulus expansion by 8 hours after stimulation with LH (Fig. 4A). 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. 4B). In control wild-type follicles, NEBD occurred between 2 and 6 hours after LH exposure. In contrast, in NPR2-7E/7E follicles, no NEBD occurred in the first 6 hours following treatment with LH.

However, by 8 hours after LH application, NEBD had occurred in ~40% of NPR2-7E/7E follicle-enclosed oocytes, and in ~80% by 12 hours. Compared to the time course of NEBD in the wild-type, the time course of NEBD in the NPR2-7E/7E follicles showed a 5-hour delay. The 7E mutation had no effect on meiotic resumption in response to isolating oocytes from the follicle (Fig. 4C). 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 isolated from the source of inhibitory cGMP from the granulosa cells.

During cumulus expansion preceding ovulation, gap junction communication between the oocyte and cumulus cells is disrupted (Gilula *et al*., 1978). The LH-induced cumulus expansion occurred similarly in NPR2-7E/7E and wild-type follicles (Fig. 4D), suggesting

that gap junction disruption during cumulus expansion might contribute to why meiosis eventually resumes in the NPR2-7E/7E follicles.

Dephosphorylation of the NPR2 receptor functions in the regulation of longitudinal bone growth.

To investigate if the inability to dephosphorylate NPR2 has an effect on longitudinal bone growth, we measured tail lengths in live NPR2-7E/7E and wild-type mice from age 3 to 15 weeks. Tails from NPR2-7E/7E mice were significantly longer than those from wild-type mice at all ages examined (Fig. 5A).

To determine the structural basis for the longer tails, the NPR2-7E/7E and wild-type controls were sacrificed at 18 weeks for x-ray imaging. The number of tail vertebrae was the same for tails of each genotype, but for the NPR2-7E/7E tails, each vertebra was longer (Fig. 5B). Measurements of caudal vertebra 5 (CA5) of *Npr2*-7E/7E mice showed an 18 ± 0.1% increase in length compared to wild-type (Fig. 5C). These findings indicate that dephosphorylation of NPR2 is important for regulation of longitudinal bone growth, and suggest that a hormonal pathway in growth plate chondrocytes may regulate NPR2 guanylyl cyclase activity like LH regulates NPR2 activity in granulosa cells.

DISCUSSION

By generating a transgenic mouse with mutations in phosphorylation sites in the juxtamembrane domain of NPR2, such that the receptor behaves as if it is constitutively phosphorylated and active, we show that luteinizing hormone signaling leading to oocyte meiotic resumption requires dephosphorylation of NPR2 in granulosa cells. Additionally, we investigated the effect of the NPR2 mutations on bone, and determined that dephosphorylation of NPR2 is a regulator of longitudinal bone growth. These findings indicate that regulation of NPR2 activity by dephosphorylation is an important mechanism for the hormonal control of oocyte meiotic resumption and longitudinal bone growth.

Our studies in the mouse ovary show that dephosphorylation of the seven serine and threonine sites in NPR2 is required for the rapid decrease in guanylyl cyclase activity induced by LH in the ovarian follicle. After mutation of these regulatory phosphorylation residues to glutamate, there was little or no decrease in guanylyl cyclase activity in response to LH. These results are consistent with guanylyl cyclase measurements in membranes from 293-HEK cells transfected with NPR2 with these same point mutations (Yoder *et al*., 2012). In transfected HEK cells, the guanylyl cyclase activity of the NPR2-7E protein is resistant to inhibition by calcium and protein kinase C (Yoder *et al*., 2012).

Although follicles with the 7E mutation showed little or no LH-induced decrease in guanylyl cyclase activity, LH did cause a significant decrease in cytosolic cGMP. The decrease in cGMP in *Npr2*-7E/7E follicles was less than that seen in wild-type follicles. A likely explanation of this residual cGMP decrease in 7E follicles is that LH signaling also increases phosphodiesterase activity (Egbert *et al*., 2014; Egbert *et al*., unpublished results).

Our findings show that inhibition of the LH-induced decrease in guanylyl cyclase activity and cGMP in *Npr2*-7E/7E follicles delays oocyte meiotic resumption in response to LH. However, after 6-8 hours, oocytes in *Npr2*-7E/7E follicles eventually resume meiosis as indicated by nuclear envelope breakdown. A likely factor that contributes to this eventual resumption of meiosis is the disconnection of gap junctions upon expansion of cumulus cells (Gilula *et al*., 1978). Our results indicate that although the 7E mutations prevent the LH-induced decrease in NPR2 activity and attenuate the drop in cGMP, they do not disrupt cumulus expansion. Because the inhibitory cGMP is produced in the granulosa cells and diffuses through gap junctions into the oocyte to maintain meiotic arrest, disruption of the communication between the oocyte and granulosa cells would remove the source of cGMP and, as a consequence, release the cGMP-inhibition of PDE3A in the oocyte. Another factor that could contribute to eventual resumption of meiosis in the *Npr2*-7E/7E follicles is the LH-induced decrease in the NPR2 agonist CNP, which is detectable after 2 hours of treatment with the hormone (Kawamura *et al*., 2011; Robinson *et al*., 2012). This decrease in CNP would decrease guanylyl cyclase activity, reducing the concentration of cGMP in the follicle and contributing to the release of meiotic arrest in the oocyte.

Although the *Npr2*-7E/7E females are fertile, is has not been determined whether they produce the same number of pups. During maturation, the oocyte acquires the structural and physiological competence for egg activation initiated by the sperm (Chiba *et al*., 1990; Ducibella *et al*., 1990; Ducibella and Buetow, 1994). Oocyte maturation involves development of sensitivity for IP_3-induced calcium release from the ER and exocytosis of cortical granules (Ducibella *et al*., 1990; Mehlmann and Kline, 1994; Mehlmann *et al*., 1996; Wakai *et al*, 2012). Oocytes that are fertilized at the MI stage are more susceptible to

polyspermy, due to compromised cortical granule exocytosis of materials that modify the surface coat (Ducibella and Buetow, 1994).

The 5-hour delay in LH-induced nuclear envelope breakdown in oocytes in *Npr2*-7E/7E follicles suggests that they would be approximately at metaphase I instead of metaphase II at the time of ovulation. Additionally, they would be delayed in terms of the development of the ability to release calcium and undergo the exocytosis of cortical vesicles that accompanies meiotic progression, since these processes are also triggered by the cAMP decrease in the oocyte (Mehlmann and Kline, 1994). As a consequence of incomplete cytoplasmic maturation, oocytes fertilized at metaphase I are susceptible to polyspermy (Ducibella and Buetow, 1994). Thus, if sperm reached the maturing oocyte immediately after ovulation, fertilization of *Npr2*-7E/7E oocytes could result in polyspermy and failure of embryonic development. Further experiments will be conducted to address this question, in which *Npr2*-7E/7E or *Npr2*-+/+ females, of the same age, will be mated with *Npr2*-+/+ males and the cumulative number of pups recorded (Breen *et al*., 2013).

Our measurements of tail and vertebrae lengths showed that the NPR2-7E mutation results in increased longitudinal bone growth. This suggests that as in the ovary, dephosphorylation of NPR2 plays a role in controlling its activity and regulating development of chondrocytes in the growth plate of bones. Studies using a chondrocyte cell line indicated that the fibroblast growth factor receptor 3 (FGFR3) signaling antagonizes the CNP/NPR2 signaling pathway, thus inhibiting bone growth, making it a potential target for regulating activity of NPR2 in bones (Ozasa *et al.* 2005). The FGFR3 is expressed in restricted layers of chondrocytes within the growth plate, negatively regulating the rate of proliferation and differentiation of chondrocytes (Ornitz, 2005; Minina *et al.,* 2005; de Frutos *et al*., 2007).

Like the LH receptor in the ovarian follicle, the FGFR3 could be regulating proliferation and differentiation of chondrocytes in adjacent regions of the growth plate, perhaps by diffusion of cGMP.

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REFERENCES

Abbey, S. E. and Potter, L. R. (2003). Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. *Endocrinology* **144**, 240-246.

Behringer, R., Gertsenstein, M., Vintersten, K. and Nagy, A. (2014) Manipulating the mouse embryo: A laboratory manual. 4th Ed. Cold Spring Harbor Laboratory Press. 502 pp

Bortolussi, M., Marini, G. and Dal Lago, A. (1977). Autoradiographic study of the distribution of LH(HCG) receptors in the ovary of untreated and gonadotrophin-primed immature rats. *Cell Tissue Res.* **183**, 329-342.

Breen, S.M., Andric, N., Ping, T., Xie, F., Offermans, S., Gossen, J.A., Ascoli, M. (2013). Ovulation involves the luteinizing hormone-dependent activation of G(q/11) in granulosa cells. *Mol. Endocrinol.* **27**, 1483-1491.

Chiba, K., Kado, R.T., Jaffe, L.A. (1990). Development of calcium release mechanisms during starfish oocyte maturation. *Dev Biol*. **140**, 300-306.

Conti, M., Hsieh, M., Zamah, A. M. and Oh., J. S. (2012). Novel signaling mechanisms during oocyte maturation and ovulation. *Mol. Cell. Endocrinol.* **356**, 65-73.

De Frutos, C.A., Vega, S., Manzanares, M., Flores, J.M., Huertas, H., Martínez-Frías, M.L., Nieto, M.A. (2005) Snail1 is a transcriptional effector of FGFR3 signaling during chondrogenesis and achondroplasias. *Dev Cell.* **13**,872-883.

Ducibella, T., Buetow, J. (1994). Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Dev Biol*. **165,** 95-104.

Ducibella, T., Duffy, P., Reindollar, R., Su, B. (1990). Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo. *Biol Reprod*. **43,** 870-876.

Egbert, J.R., Shuhaibar. L.C., Edmund, A.B., Van Helden, D.A., Robinson, J.W., Uliasz, T.F., Baena, V., Geerts, A., Wunder, F., Potter, L.R., Jaffe, L.A. (2014) Dephosphorylation and inactivation of the NPR2 guanylyl cyclase in the granulosa cells contributes to the LH-induced cGMP decrease that causes resumption of meiosis in rat oocytes. *Development* **141,** 3594-3604.

Geister, K. A., Brinkmeier, M. L., Hsieh, M., Faust, S. M., Karolyi, I. J., Perosky, J. E., Kozloff, K. M., Conti, M. and Camper, S. A. (2013). A novel loss-of-function mutation in *Npr2* clarifies primary role in female reproduction and reveals a potential therapy for acromesomelic dysplasia, Maroteaux type. *Hum. Mol. Genet.* **22**, 345-357.

Gilula, N.B., Epstein, M.L., and Beers, W.H. (1978). Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. *J. Cell Biol*. **78**, 58-75.

Hannema, S.E., van Duyvenvoorde. H.A., Premsler, T., Yang, R.B., Mueller, T.D., Gassner, B., Oberwinkler, H., Roelfsema, F., Santen, G.W., Prickett, T., Kant, S.G., Verkerk, A.J., Uitterlinden, A.G., Espiner, E., Ruivenkamp, C.A., Oostdijk, W., Pereira, A.M., Losekoot, M., Kuhn, M., Wit, J.M. (2013). An activating mutation in the kinase homology domain of the natriuretic peptide receptor-2 causes extremely tall stature without skeletal deformities. *J. Clin. Endocrinol. Metab.* **98,** E1988-1998.

Holt, J. E., Lane, S. I. R. and Jones, K. T. (2013). The control of meiotic maturation in mammalian oocytes. *Curr. Top. Dev. Biol.* **102**, 207-226.

Kawamura, K., Cheng, Y., Kawamura, N., Takae, S., Okada, A., Kawagoe, Y., Mulders, S., Terada, Y. and Hsueh, A. J. W. (2011). Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Hum. Reprod.* **26**, 3094-3101.

Lee, E.C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D.A., Court, D.L., Jenkins, N.A. and Copeland, N.G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**, 56–65.

Mehlmann, L. M. (2013). Signaling for meiotic resumption in granulosa cells, cumulus cells, and oocyte. In *Oogenesis* (ed. G. Coticchio, D. F. Albertini and L. De Santis), pp. 171-182. London: Springer-Verlag.

Mehlmann, L.M., Kline, D. (1994). Regulation of intracellular calcium in the mouse egg: calcium release in response to sperm or inositol trisphosphate is enhanced after meiotic maturation. *Biol. Reprod.* **51,** 1088-1098.

Mehlmann, L.M., Mikoshiba, K., Kline, D. (1996). Redistribution and increase in cortical inositol 1,4,5-trisphosphate receptors after meiotic maturation of the mouse oocyte. *Dev Biol.* **180,** 489-498.

Minina, E., Schneider, S., Rosowski, M., Lauster, R., Vortkamp, A. (2005). Expression of Fgf and Tgfbeta signaling related genes during embryonic endochondral ossification. *Gene Expr Patterns*. **6**, 102-109.

Miura, K., Namba, N., Fujiwara, M., Ohata, Y., Ishida, H., Kitaoka, T., Kubota, T., Hirai, H., Higuchi, C., Tsumaki, N., Yoshikawa, H., Sakai, N., Michigami, T. and Ozono, K. (2012). An overgrowth disorder associated with excessive production of cGMP due to a gain-of-function mutation of the natriuretic peptide receptor 2 gene. *PLoS ONE* **7**, e42180.

Noll, S., Hampp, G., Bausbacher, H., Pellegata, N.S., and Kranz, H. (2009). Site-directed mutagenesis of multi-copy-number plasmids: Red/ET recombination and unique restriction site elimination. *Biotechniques* **46**, 527-533.

Norris, R.P., Freudzon, M., Mehlmann, L.M., Cowan, A.E., Simon, A.M., Paul, D.L., Lampe, P.D., Jaffe, L.A. (2008). Luteinizing hormone causes MAPK-dependent phosphorylation and closure of Cx43 gap junctions in mouse ovarian follicles. *Development* **135**, 3229-3238.

Norris, R. P., Ratzan, W. J., Freudzon, M., Mehlmann, L. M., Krall, J., Movsesian, M. A., Wang, H., Ke, H., Nikolaev, V. O. and Jaffe, L. A. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* **136**, 1869-1878.

Ornitz, D.M. (2005). FGF signaling in the developing endochondral skeleton. *Cytokine Growth Factor Rev*. **16**, 205-213.

Ozasa, A., Komatsu, Y., Yasoda, A., Miura, M., Sakuma, Y., Nakatsuru, Y., Arai, H., Itoh, N., Nakao, K. (2005). Complementary antagonistic actions between C-type natriuretic peptide and the MAPK pathway through FGFR-3 in ATDC5 cells. *Bone* **36**, 1056-1064.

Potter, L. R. (1998). Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptor B: Dephosphorylation is a mechanism of desensitization. *Biochemistry* **37**, 2422-2429.

Potter, L. R. (2011). Regulation and therapeutic targeting of peptide-activated receptor guanylyl cyclases. *Pharmacol. Ther.* **130**, 71-82.

Potter, L. R. and Garbers, D. L. (1992). Dephosphorylation of the guanylyl cyclase-A receptor causes desensitization. *J. Biol. Chem.* **267**, 14531-14534.

Potter, L. R. and Hunter, T. (1998). Identification and characterization of the major phosphorylation sites of the B-type natriuretic peptide receptor. *J. Biol. Chem.* **273**, 15533-15539.

Potter, L. R. and Hunter, T. (1999). A constitutively "phosphorylated" guanylyl cyclase-linked atrial natriuretic peptide receptor mutant is resistant to desensitization. *Mol. Biol. Cell* **10**, 1811-1820.

Robinson, J. W., Zhang, M., Shuhaibar, L. C., Norris, R. P., Geerts, A., Wunder, F., Eppig, J. J., Potter, L. R. and Jaffe, L. A. (2012). Luteinizing hormone reduces the activity of the NPR2 guanylyl cyclase in mouse ovarian follicles, contributing to the cyclic GMP decrease that promotes resumption of meiosis in oocytes. *Dev. Biol.* **366**, 308-316.

Russwurm, M., Mullershausen, F., Friebe, A., Jäger, R., Russwurm, C., Koesling, D. (2007). Design of fluorescence resonance energy transfer (FRET)-based cGMP indicators: a systematic approach. *Biochem. J.* **407**, 69-77.

Shuhaibar, L.C., Egbert, J.R., Norris, R.P., Lampe, P.D., Nikolaev, V.O., Thunemann, M., Wen, L., Feil, R., Jaffe, L.A. (2015). Intercellular signaling via cyclic GMP diffusion through gap junctions in the mouse ovarian follicle. *Proc. Natl. Acad. Sci. U. S. A*. **12**, 5527-5532.

Tang, S.H., Silva, F.J., Tsark, W.M., Mann, J.R. (2002). A Cre/loxP-deleter transgenic line in mouse strain 129S1/SvImJ. *Genesis* **32,** 199-202.

Tamura, N., Doolittle, L.K., Hammer, R.E., Shelton, J.M., Richardson, J.A., Garbers, D.L. (2004). Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. . *Proc. Natl. Acad. Sci*. U. S. A. **101**, 17300-17305.

Thunemann, M., Wen, L., Hillenbrand, M., Vachaviolos, A., Feil, S., Ott, T., Han, X., Fukumura, D., Jain, R. K., Russwurm, M., de Wit, C., Feil, R. (2013) Transgenic mice for cGMP imaging. *Circ. Res.* **113,** 365-371.

Tsuji, T., Kunieda, T. (2005). A loss-of-function mutation in natriuretic peptide receptor 2 (Npr2) gene is responsible for disproportionate dwarfism in cn/cn mouse. *J. Biol. Chem.* **280**, 14288-14292.

Tsuji ,T., Kondo, E., Yasoda, A., Inamoto, M., Kiyosu, C., Nakao, K., Kunieda, T. (2008). Hypomorphic mutation in mouse Nppc gene causes retarded bone growth due to impaired endochondral ossification. *Biochem. Biophys. Res. Commun.* **376**, 186-190.

Vaccari, S., Weeks, J. L., Hsieh, M., Menniti, F. S. and Conti, M. (2009). Cyclic GMP signaling is involved in the LH-dependent meiotic maturation of mouse oocytes. *Biol. Reprod.* **81**, 595-604.

Wakai, T., Vanderheyden, V., Yoon, S.Y., Cheon, B., Zhang, N., Parys, J.B., Fissore, R.A. (2012). Regulation of inositol 1,4,5-trisphosphate receptor function during mouse oocyte maturation. *J. Cell. Physiol.* **227,** 705-717.

Wang, X., Greenwald, G. S., (1993). Hypophysectomy of the cyclic mouse. II. Effects of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chroionic gonadotropin receptors, and steroidogenesis. *Biol Reprod*. **48,** 595-605.

Yasoda, A., Komatsu, Y., Chusho, H., Miyazawa, T., Ozasa, A., Miura, M., Kurihara, T., Rogi, T., Tanaka, S., Suda, M., Tamura, N., Ogawa, Y. and Nakao, K. (2004). Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.* **10**, 80-86.

Yoder, A. R., Stone, M. D., Griffin, T. J. and Potter, L. R. (2010). Mass spectrometric identification of phosphorylation sites in guanylyl cyclase A and B. *Biochemistry* **49**, 10137-10145.

Yoder, A. R., Robinson, J. W., Dickey, D. M., Andersland, J., Rose, B. A., Stone, M. D., Griffin, T. J. and Potter, L. R. (2012). A functional screen provides evidence for a conserved, regulatory, juxtamembrane phosphorylation site in guanylyl cyclase A and B. *PLoS One* **7**, e36747.

Zhang, M., Su, Y.-Q., Sugiura, K., Xia, G., and Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science* **330**, 366-369.

FIGURE LEGENDS

Figure 1. Design of knockin a mouse with seven phosphomimetic glutamate mutations in the transmembrane guanylyl cyclase NPR2. (A) Diagram of NPR2 showing the seven serine and threonine phosphorylation sites that were changed to glutamate (E) in the NPR2-7E mouse. The functional domains of the homodimeric transmembrane protein NPR2 are shown in gray. Binding of CNP (green) to the extracellular domain and phosphorylation of seven serine and threonine sites (red) activate the guanylyl cyclase activity. Dephosphorylation of these regulatory sites results in a decrease in guanylyl cyclase activity; the 7E mutation results in a protein that cannot be dephosphorylated. (B) Strategy for making the NPR2-7E mouse. A sequence encoding the 7E point mutations (red asterisks), located in exon 8 and 9, was knocked into the endogenous *Npr2* gene by homologous recombination. Global expression of the 7E mutation was accomplished by crossing mice expressing the targeted *Npr2* allele to *Hprt-Cre* mice. Cre-recombinase-mediated excision removed the mini-cDNA containing wild type exons 8 to 22 flanked by the two LoxP sites leading to expression of the 7E mutation.

Figure 2. Inhibition of the LH-induced decrease in guanylyl cyclase activity in mouse follicles by the NPR2-7E mutations. (A) Guanylyl cyclase activity in wild-type and NPR2-7E/7E follicle membranes, measured in the presence of 1 μM CNP. Membranes were prepared from follicles with or without treatment with LH for 20 min. Each value shows the mean ± s.e.m. for four separate membrane preparations. The asterisk indicates that LH treatment significantly decreased the CNP-dependent guanylyl cyclase activity in wild-type follicles ($P<0.05$). LH treatment did not cause a significant change in CNP-dependent activity

in NPR2-7E/7E follicles (n.s.). (B) NPR2-7E/7E data from (A) normalized to the No LH value for each experiment. This analysis also showed that there is no significant change in NPR2 activity when LH is applied to NPR2-7E/7E follicles. Additional experiments are in progress to investigate if the apparent small decrease is real. (C) Guanylyl cyclase activity in wild-type and NPR2-7E/7E follicles from the same membrane preparations as in (A), measured in the presence of 5 mM Mn^{2+} and 1% TritonX-100. Mn/Triton fully activates NPR2, independent of phosphorylation of the regulatory sites or CNP, and this activity provides a measure of total NPR2 protein (see main text). LH-treatment did not decrease the Mn/Triton-dependent guanylyl cyclase activity, indicating that the amount of NPR2 protein was unchanged under these conditions (n.s.). There was also no significant difference in Mn/Triton-dependent guanylyl cyclase activity comparing wild-type and NPR2-7E/7E membranes .

Figure 3. Partial inhibition of the LH-induced cGMP decrease in NPR2-7E/7E follicles. Changes in cytosolic cGMP levels were measured in mural granulosa cells of follicles expressing the cGi500 sensor for cGMP, comparing wild-type (left) and NPR2-7E/7E (right). (A) Records from representative follicles before and after perfusion of LH. A decrease in the CFP/YFP ratio indicates a decrease in cGMP. (B) Measurements before and at 20 min after treatment with LH (mean ± s.e.m for 7 and 9 follicles respectively). (C) The CFP/YFP decrease after 20 min treatment with LH was smaller in NPR2-7E/7E than in wild-type follicles; 21±1.6% and 34±1.7% respectively. Asterisks indicate values that are significantly different from the control without LH ($P<0.05$).

Figure 4. Delay of LH-induced meiotic resumption in NPR2-7E/7E follicle-enclosed oocytes. (A) Images from a representative NPR2-7E/7E follicle before treatment with LH, showing the oocyte arrested in prophase I (left), and at 8 hours after treatment with LH, showing the expanded cumulus mass around the oocyte (right). (B) Time course of nuclear envelope breakdown in follicle-enclosed oocytes from NPR2-7E/7E and wild-type mice. LH was applied to the medium in the dish holding the Millicell membrane, and oocytes within follicles on the Millicell membrane were observed for the presence or absence of a nuclear envelope and nucleolus at 1-h intervals. At 8 and 12 hours after LH, the oocytes were not visible within the follicle due to the expanded cumulus; therefore follicles were opened to isolate and score the oocyte. The graph shows the mean ± s.e.m. for 4-6 sets of measurements at each time point (only one measurement at 12 hours). (C) Time course of nuclear envelope breakdown after isolating oocytes from NPR2-7E/7E and wild-type follicles. The graph shows the mean for one set of measurement for each genotype. (D) Time course of cumulus expansion in NPR2-7E/7E and wild-type follicles treated with LH. Follicles were treated as for (B) and the presence of an expanded cumulus mass was determined at 1-hour intervals. The graph shows mean ± s.e.m. for 2 and 5 experiments with wild-type and NPR2-7E/7E follicles, respectively. Additional experiments are in progress to increase the number of trials for figures B-D.

Figure 5. Increased bone growth in NPR2-7E/7E mice. (A) Tail lengths measured from live NPR2-7E/7E and wild-type mice (mean ± s.e.m, 10 and 13 mice respectively). There were approximately half females and half males in each group; no difference in tail lengths for females and males was detected. (B) X-ray images of representative tails from 18-week

old NPR2-7E/7E and wild-type mice; from the set of animals shown in (A), imaged after euthanizing. The vertical line marks the start of the tail at caudal vertebra number 5 (CA5). Numbers indicate the length of the tails measured from the x-rays (start of CA5 to tip of tail), mean ± s.e.m. (C) CA5 vertebra lengths (mean ± s.e.m.) measured from x-ray images as shown in (B). Asterisks indicate values that are significantly different from the wild-type control ($P<0.05$).

Figure 1

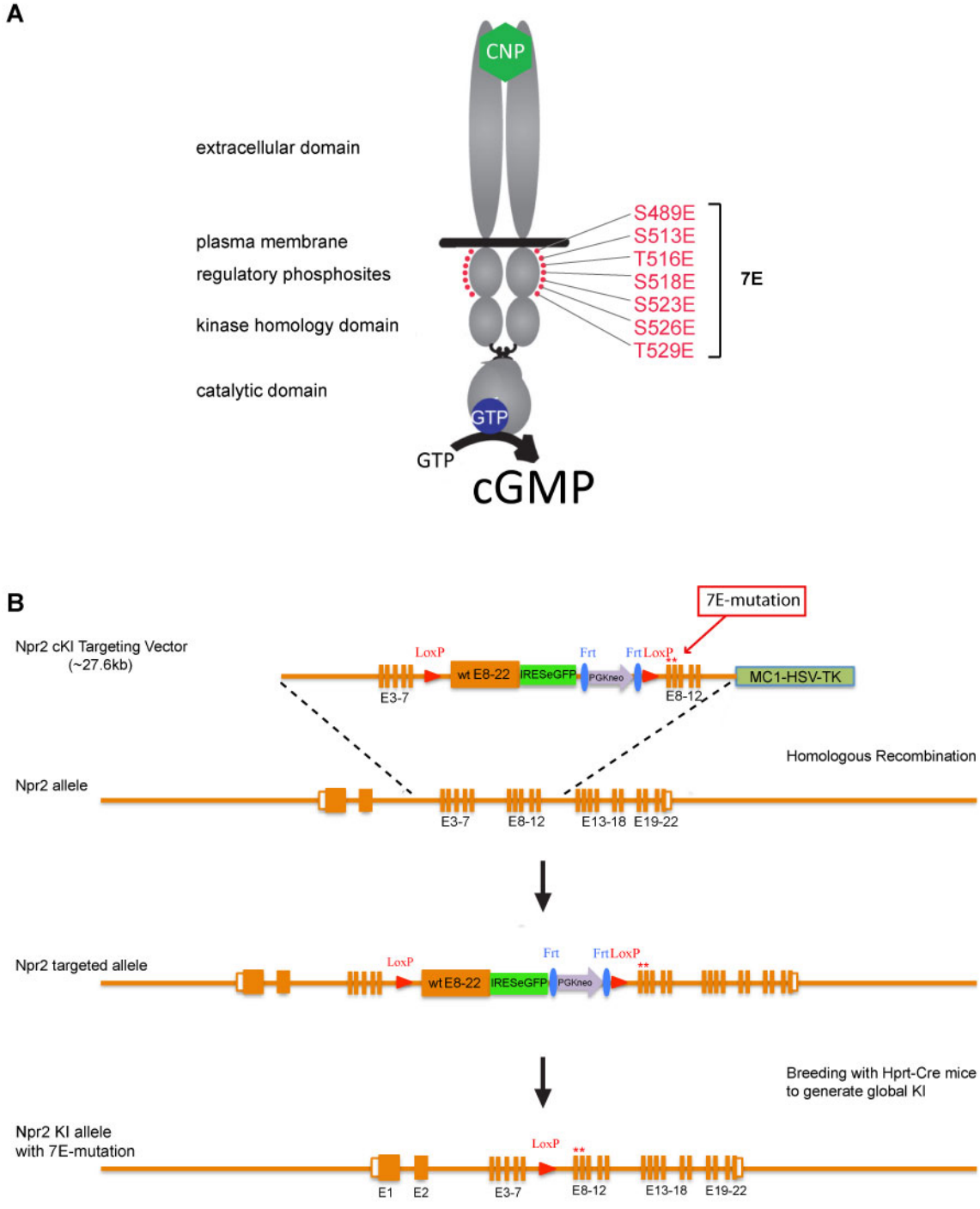

A
CNP
extracellular domain
plasma membrane
regulatory phosphosites
kinase homology domain
catalytic domain
S489E
S513E
T516E
S518E
S523E
S526E
T529E
7E
GTP
GTP
cGMP
B
7E-mutation
Npr2 cKI Targeting Vector
(~27.6kb)
LoxP
wt E8-22
IRESeGFP
Frt
PGKneo
Frt LoxP
MC1-HSV-TK
E3-7
E8-12
Homologous Recombination
Npr2 allele
E3-7
E8-12
E13-18
E19-22
Npr2 targeted allele
LoxP
wt E8-22
IRESeGFP
Frt
PGKneo
Frt LoxP
Breeding with Hprt-Cre mice
to generate global KI
Npr2 KI allele
with 7E-mutation
LoxP
E1
E2
E3-7
E8-12
E13-18
E19-22

Figure 2

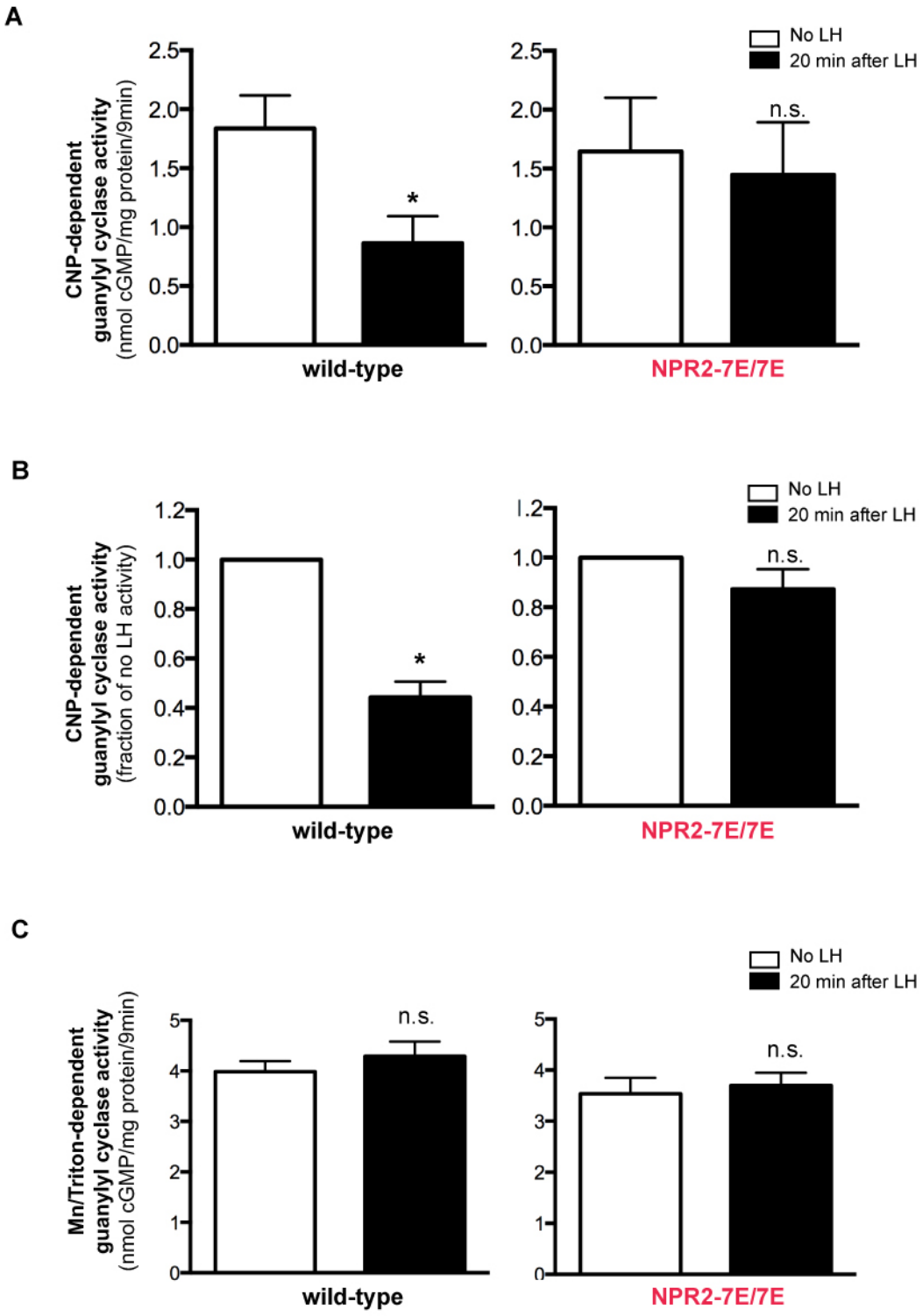

A
CNP-dependent guanylyl cyclase activity (nmol cGMP/mg protein/9min)
2.5
2.0
1.5
1.0
0.5
0.0
*
wild-type
n.s.
NPR2-7E/7E
No LH
20 min after LH
B
CNP-dependent guanylyl cyclase activity (fraction of no LH activity)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
wild-type
n.s.
NPR2-7E/7E
No LH
20 min after LH
C
Mn/Triton-dependent guanylyl cyclase activity (nmol cGMP/mg protein/9min)
5
4
3
2
1
0
n.s.
wild-type
n.s.
NPR2-7E/7E
No LH
20 min after LH

Figure 3

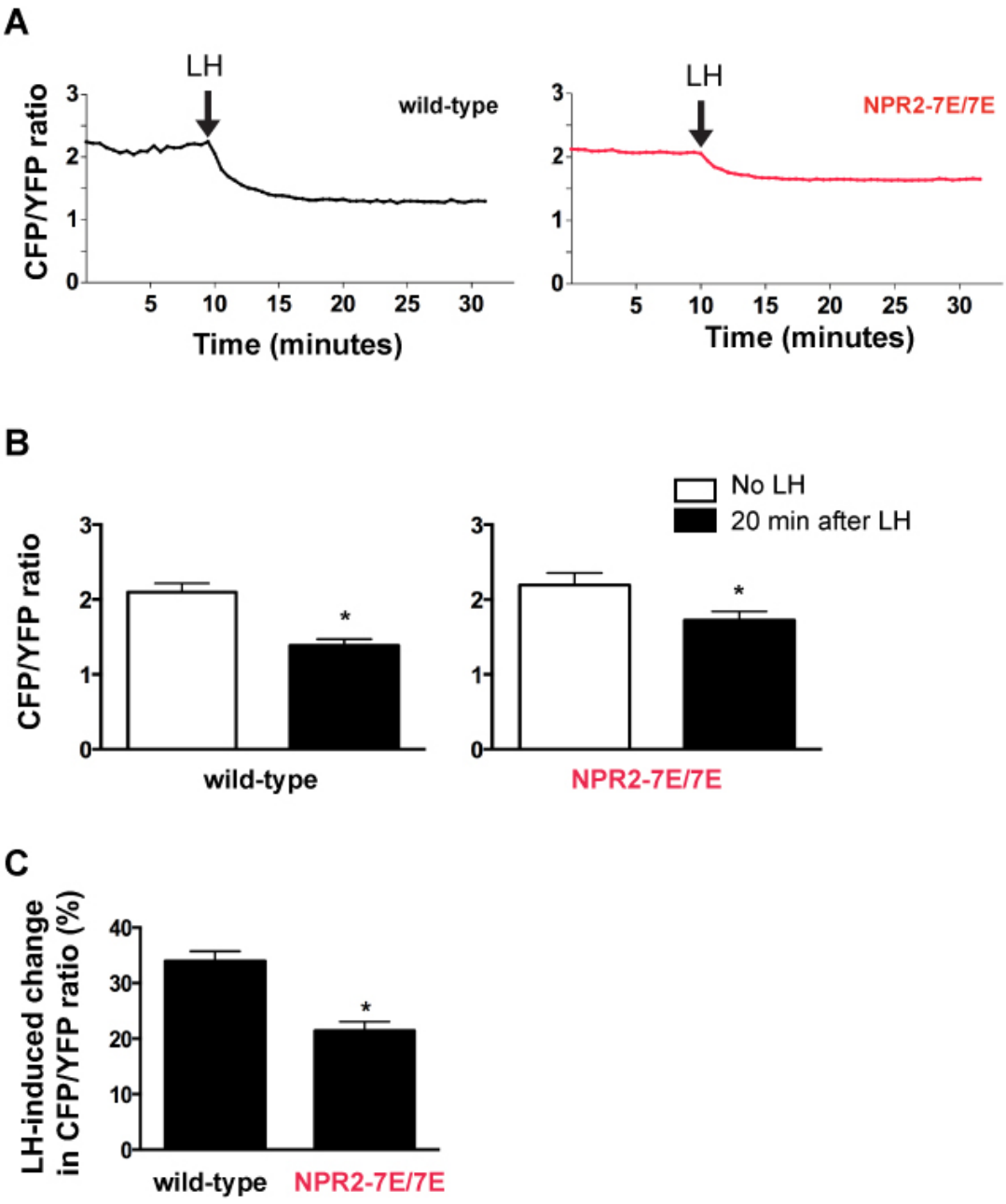

A
LH
wild-type
CFP/YFP ratio
Time (minutes)
LH
NPR2-7E/7E
Time (minutes)
B
No LH
20 min after LH
CFP/YFP ratio
wild-type
NPR2-7E/7E
C
LH-induced change in CFP/YFP ratio (%)
wild-type
NPR2-7E/7E

Figure 4

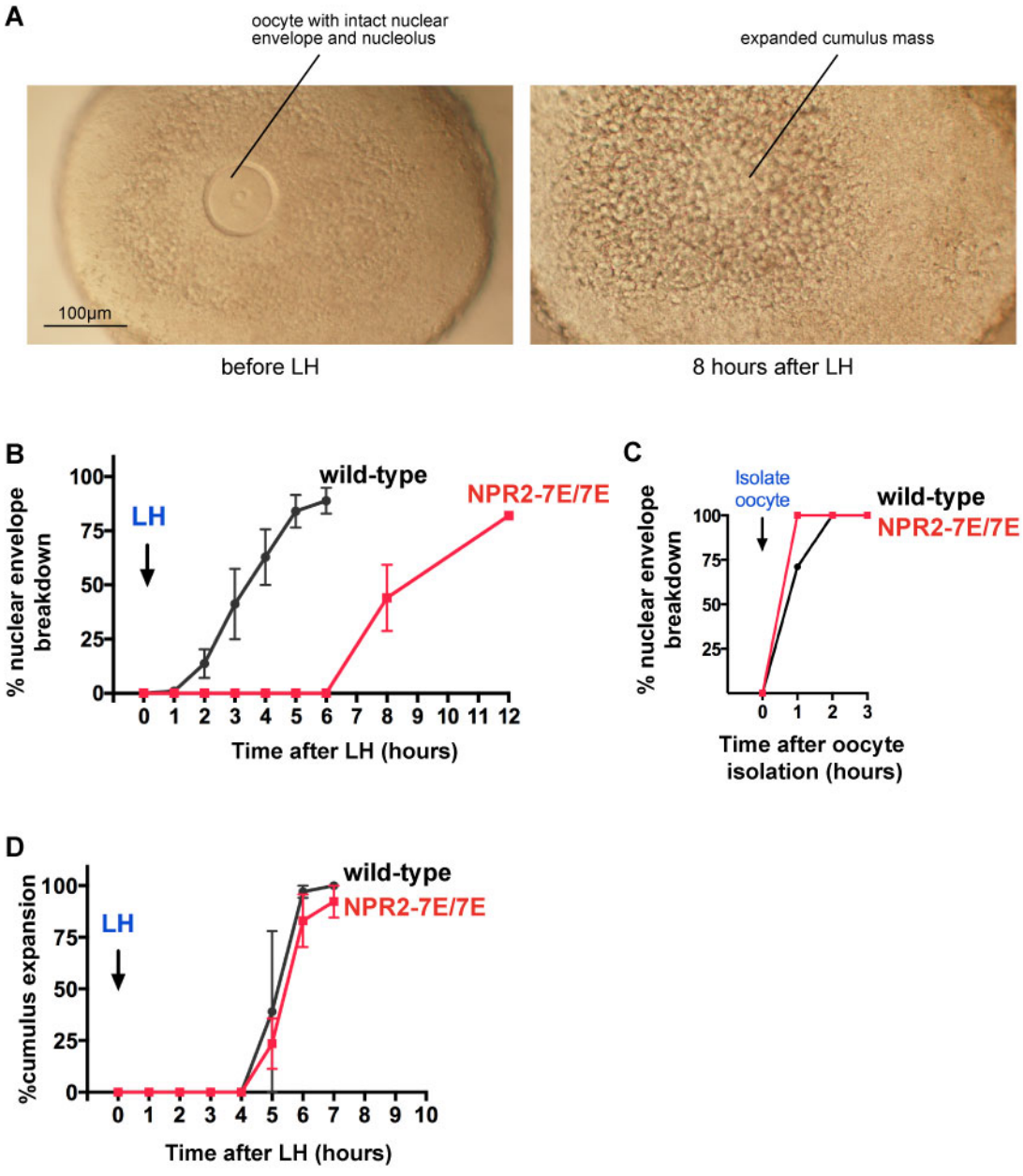
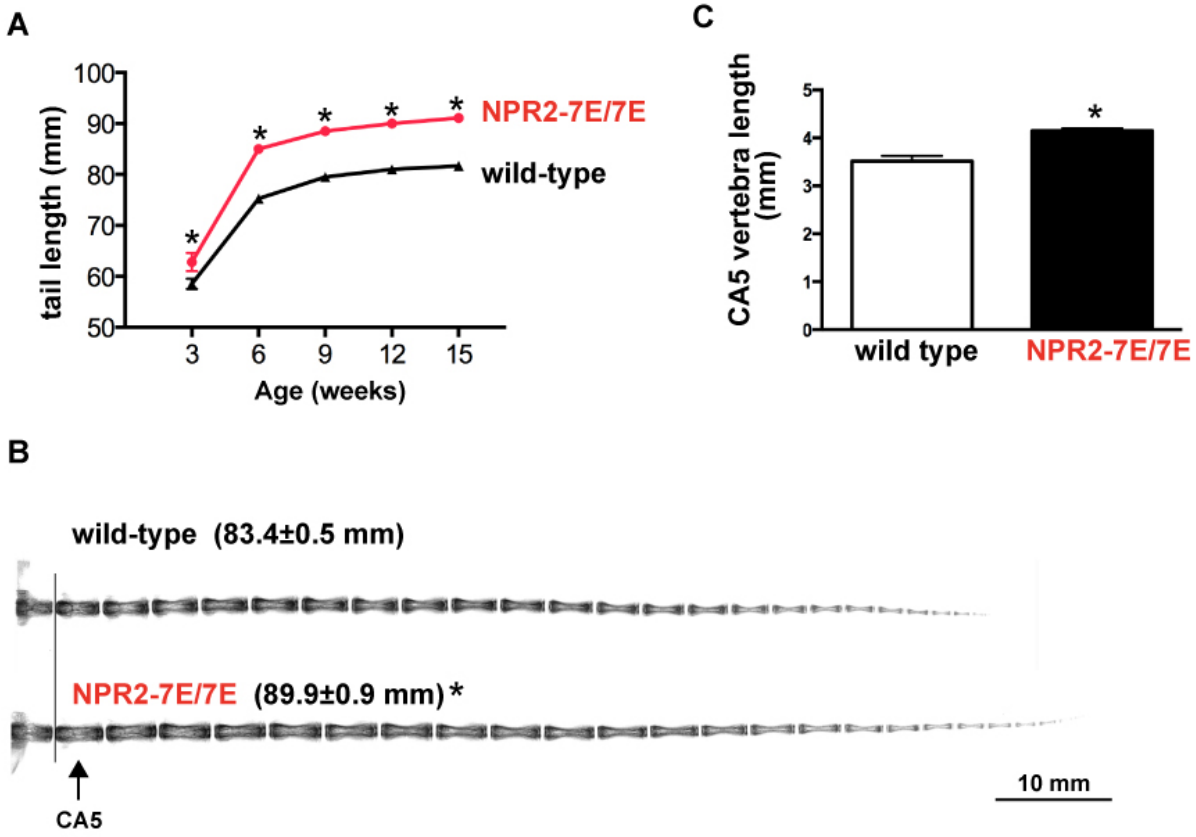
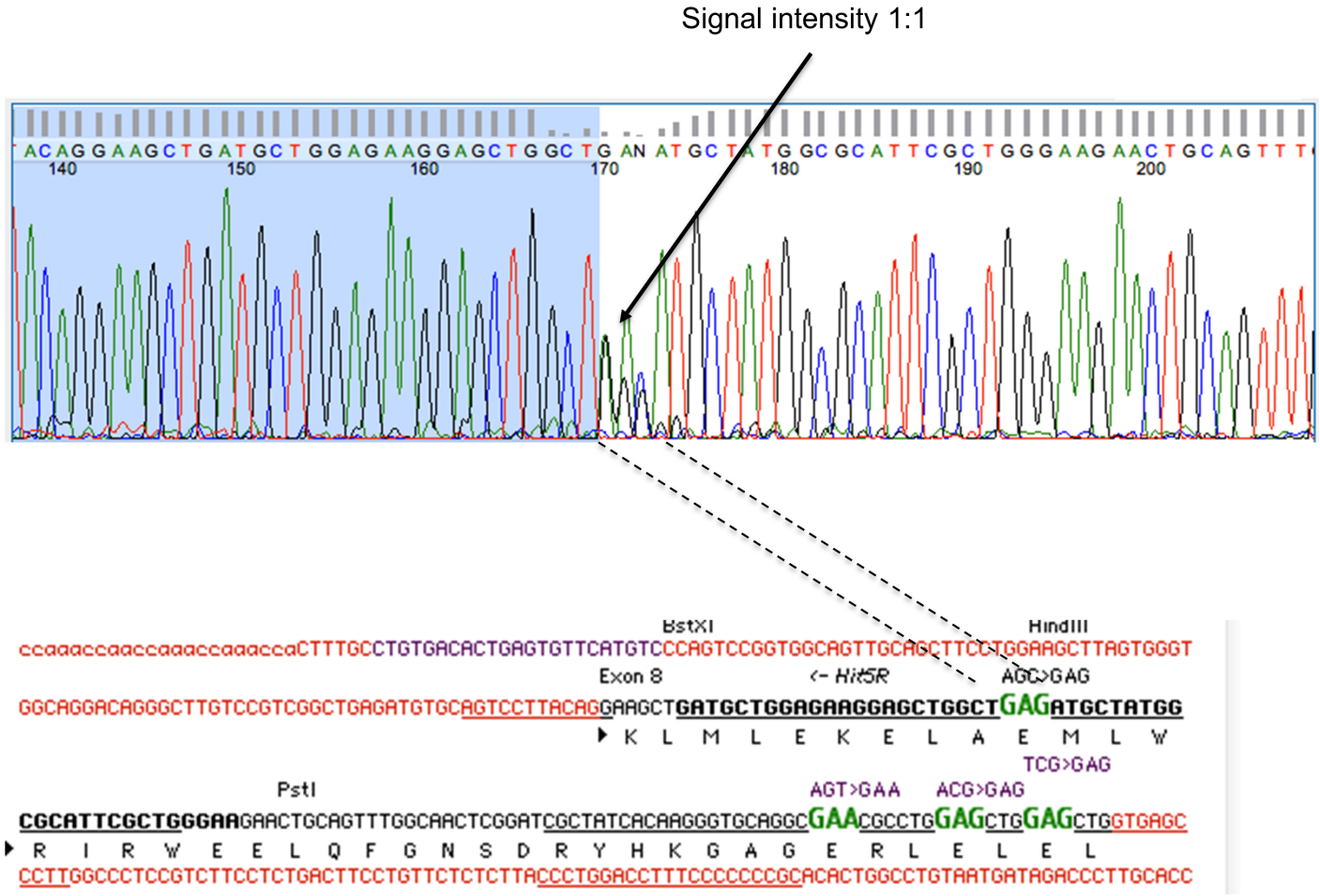

A
oocyte with intact nuclear envelope and nucleolus
expanded cumulus mass
100µm
before LH
8 hours after LH
B
wild-type
NPR2-7E/7E
LH
% nuclear envelope breakdown
Time after LH (hours)
C
Isolate oocyte
wild-type
NPR2-7E/7E
% nuclear envelope breakdown
Time after oocyte isolation (hours)
D
wild-type
NPR2-7E/7E
LH
%cumulus expansion
Time after LH (hours)

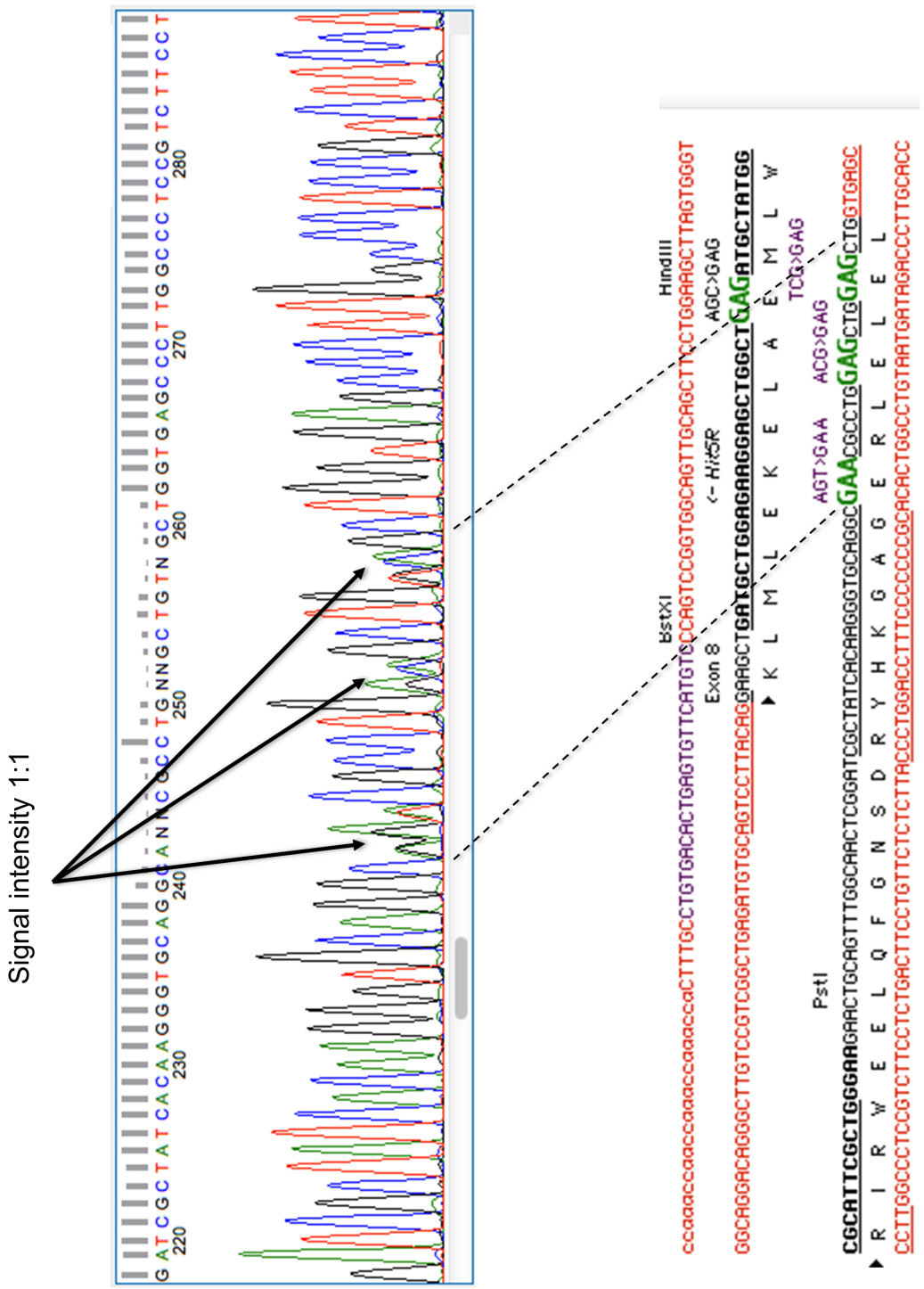
Figure 5

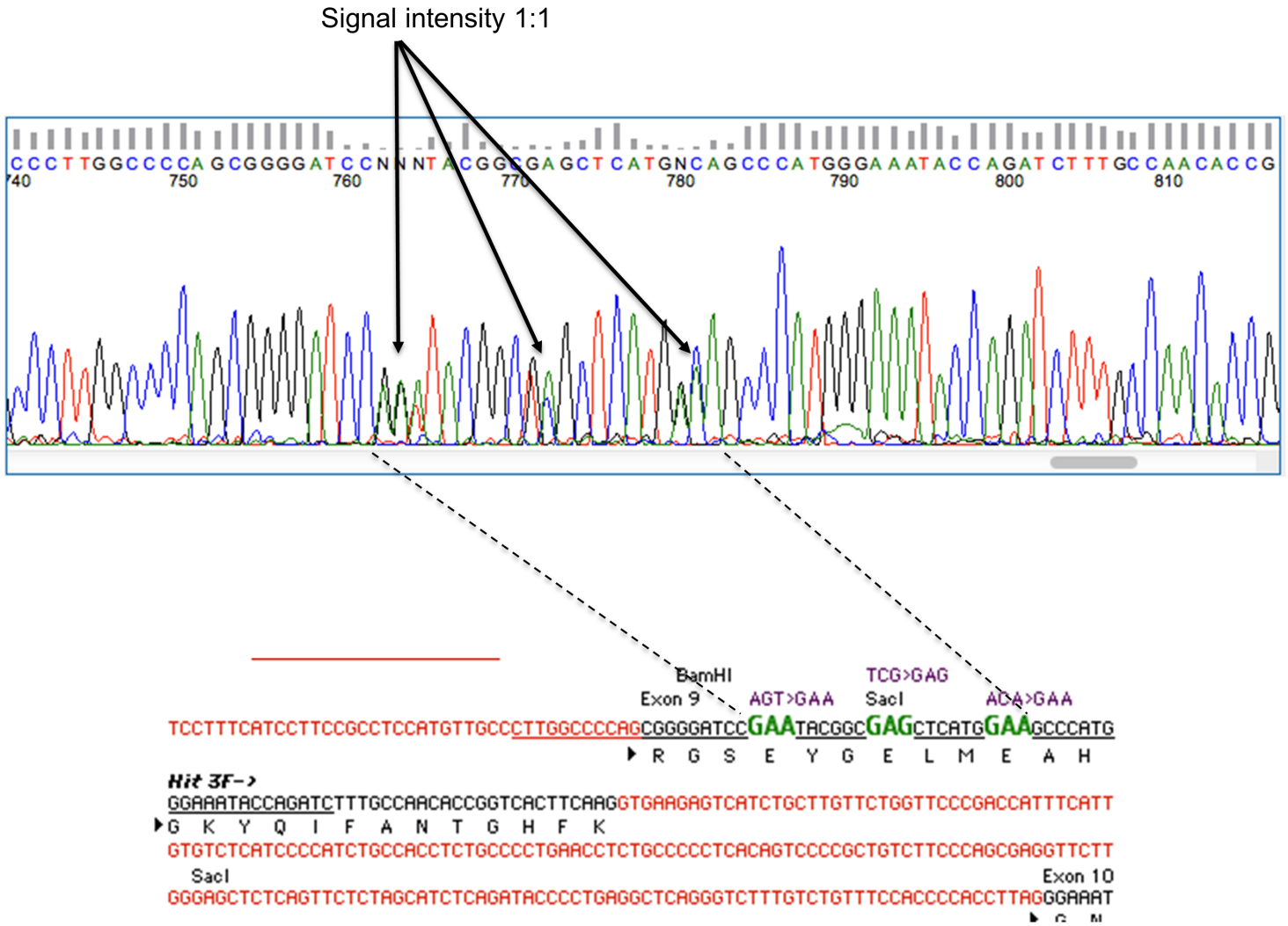

A
tail length (mm)
100
90
80
70
60
50
3
6
9
12
15
Age (weeks)
NPR2-7E/7E
wild-type
C
CA5 vertebra length (mm)
5
4
3
2
1
0
wild type
NPR2-7E/7E
B
wild-type (83.4±0.5 mm)
NPR2-7E/7E (89.9±0.9 mm)*
CA5
10 mm

Supplementary Information.

Fig. S1. Sequencing results from an *Npr2*-7E/+ mouse confirming the point mutations in the seven serine and threonine sites of the *Npr2* gene. The sequencing chromatographs are shown at the top and the matching nucleotide and amino-acid sequences at the bottom. Nucleotides at these seven sites were changed to the GAA or GAG (green) codons for glutamate (E). Arrows point to the seven mutation sites and show a 1:1 signal intensity ratio between the wild-type nucleotide and the mutated nucleotide, as expected for a heterozygote.


Signal intensity 1:1
ACAGGAAGCTGATGCTGGAGAAGGAGCTGGCTGANATGCTATGGCGCATTCGCTGGGAAGAACTGCAGTTT
140
150
160
170
180
190
200
BstXI
HindIII
ccaaaccaaccaaaccaaaccaCTTTGCCTGTGACACTGAGTGTTCATGTCCCAGTCCGGTGGCAGTTGCAGCTTCCTGGAAGCTTAGTGGGT
Exon 8
<- Hit5R
AGC>GAG
GGCAGGACAGGGCTTGTCCGTCGGCTGAGATGTGCAGTCCTTACAGGAAGCTGATGCTGGAGAAGGAGCTGGCTGAGATGCTATGG
K L M L E K E L A E M L W
TCG>GAG
PstI
AGT>GAA
ACG>GAG
CGCATTCGCTGGGAAGAACTGCAGTTTGGCAACTCGGATCGCTATCACAAGGGTGCAGGCGAACGCCTGGAGCTGGAGCTGGTGAGC
R I R W E E L Q F G N S D R Y H K G A G E R L E L E L
CCTTGGCCCTCCGTCTTCCTCTGACTTCCTGTTCTCTCTTACCCTGGACCTTTCCCCCCCGCACACTGGCCTGTAATGATAGACCCTTGCACC


Signal intensity 1:1
BstXI
HindIII
Exon 8
<- HisSR
AGC>GAG
AGT>GAA
ACG>GAG
TCG>GAG
PstI


Signal intensity 1:1
740
750
760
770
780
790
800
810
BamHI
Exon 9
AGT>GAA
TCG>GAG
SacI
ACA>GAA
TCCTTTCATCCTTCCGCCTCCATGTTGCCCTTGGCCCCAGCGGGGATCCGAATACGGCGAGCTCATGGAAGCCCATG
R G S E Y G E L M E A H
Hit 3F->
GGAAATACCAGATCTTTGCCAACACCGGTCACTTCAAGGTGAAGAGTCATCTGCTTGTTCTGGTTCCCGACCATTTCATT
G K Y Q I F A N T G H F K
GTGTCTCATCCCCATCTGCCACCTCTGCCCCTGAACCTCTGCCCCCTCACAGTCCCCGCTGTCTTCCCAGCGAGGTTCTT
SacI
Exon 10
GGGAGCTCTCAGTTCTCTAGCATCTCAGATACCCCTGAGGCTCAGGGTCTTTGTCTGTTTCCACCCCACCTTAGGGAAAT

Name	Primer sequence
LoxF (forward)	5'- CATCCTAGGTATTTATCTTGC
LoxR (reverse)	5'- TAAAAACTACTTCTTTTTTAAAAAATCCTTA

Table S1. PCR primers for genotyping *Npr2*-7E mice. Primers result in a 399bp band in wild-type mouse or 496bp band in *Npr2*-7E mouse.