New Insights into Molecular Mechanisms Regulating Oocyte Development

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Table of Contents

Abstract

Fertilization and embryonic development depend on the acquisition of developmental competence by the oocyte, which is defined by the achievement of growth and meiotic maturation. Therefore, learning the fundamental biology of these two crucial events under the physiological conditions is essential for understanding oocyte developmental abnormalities which directly manifest as infertility. In this thesis, I addressed both events of oocyte growth and meiotic maturation. Manuscript I is focused on the possible role of Yes-associated protein (YAP) ―the key effector of the Hippo signalling pathway which acts as a transcriptionl co-activator and is well known for being a powerful promoter of organ size and development ―in the process of oocyte growth. We report that YAP is expressed in the oocyte throughout the entire window of oocyte development but is constitutively excluded from the nucleus and thus inactive as a transcriptional co-factor. We also show that this nuclear exclusion, which causes YAP not to play a significant transcription-based physiological role in promoting oocyte growth, is induced by multiple phosphorylation-dependent and -independent mechanisms acting cooperatively within the oocyte. In manuscript II, we move on from the growth phase and focus on the next stage of oocyte development, meiotic maturation. It is known that during oocyte growth in mammals, the surrounding granulosa cells elaborate many specialized actin-rich filopodia, termed transzonal projections (TZPs), that contact the oocyte plasma membrane and enable transmission of essential signals and metabolites from the granulosa cells towards the oocyte. During meiotic maturation, the TZPs are lost, freeing the germ cell from these granulosa-cell regulatory influences prior to fertilization. Here we define the signalling pathway that regulates TZPs maintenance and loss. We show that prior to fully grown oocyte maturation, high levels of cyclic GMP (cGMP) in the neighbouring granulosa cells (cumulus cells) maintain TZPs.

However, during maturation, activation of epidermal growth factor-like receptor (EGFR) signalling within the cumulus cells decreases the cGMP level. Lack of cGMP in turn enables Rho-associated kinase (ROCK), a key cytoskeleton organizer, to trigger retraction of the TZPs likely via actomyosin contractility. Our results reveal a new role for both EGFR and cGMP in regulating cumulus cell-oocyte communication and identify the molecular mechanism underpinning TZP loss during maturation. The findings in this thesis provide new insights into molecular basis of oocyte normal and healthy development which could be applied in the clinical methods and therapeutic innovations aiming to preserve fertility.

Résumé

Les processus de fertilisation et de développement embryonnaire dépendent de l'acquisition d'une compétence développementale par l'ovocyte. Cette compétence est obtenue lorsque la croissance et la maturation méiotique de l'ovocyte sont achevées. Par conséquent, approfondir notre compréhension de la biologie fondamentale de ces deux événements cruciaux en conditions physiologiques est essentiel. Cela pourrais permettre une meilleure compréhension des anomalies du développement ovocytaire, qui se manifestent directement par l'infertilité. Dans cette thèse, je présente à la fois les événements impliqués dans la croissance des ovocytes, ainsi que ceux impliqués dans la maturation méiotique. Dans mon manuscrit, je me concentre sur le rôle éventuel de la protéine YAP (Yes-associated protein), un facteur clé dans la voie de signalisation Hippo, qui agit comme un coactivateur de transcription. De plus, la protéine YAP est un régulateur important de la taille et du développement de l'organe lors de la croissance des ovocytes. Nous mettons en évidence que la protéine YAP est exprimée dans l'ovocyte tout au long de son développement, mais est entièrement exclue du noyau- elle est donc inactive lors de l'activation de la transcription. Cette exclusion nucléaire empêche YAP de jouer un rôle physiologique important sur la transcription dans le cadre de la promotion de la croissance ovocytaire. Nous montrons que cette exclusion est induite par des mécanismes à la fois dépendants et indépendants de la phosphorylation, qui fonctionnent ensembles au sein de l'oocyte. Dans le manuscrit II, je passe de la première étape du développement ovocytaire, la croissance, à la deuxième étape, la maturation méiotique. Il est reconnu que lors de la croissance des ovocytes chez les mammifères, les cellules de granulosa environnantes élaborent de nombreux filopodes spécialisés riches en actine, appelés projections trans-zonales (TZPs). Ces projections entrent en contact avec la membrane plasmique de l'ovocyte, et permettent la

transmission de signaux et de métabolites essentiels des cellules de granulosa à l'ovocyte. Au cours de la maturation méiotique, les TZPs disparaissent, libérant ainsi la cellule germinale des signaux venant des cellules granulosa avant la fécondation. Nous définissons ici la voie de signalisation qui régule à la fois la maintenance et la disparation des TZPs. Nous montrons qu'avant la maturation complète des ovocytes, des taux élevés de GMP cyclique (cGMP) dans les cellules de granulosa voisines (les cellules du cumulus) maintiennent les TZPs. Cependant, lors de la maturation de l'ovocyte, l'activation de la voie de signalisation du récepteur de type EGF, ou Epidermal Growth Factor (EGFR) dans les cellules du cumulus diminue le niveau de cGMP. L'absence de cGMP permet à son tour à la kinase-Rho (Rho-associated kinase, ou ROCK), un organisateur clé du cytosquelette, de déclencher la rétraction des TZPs, probablement grâce à la contractilité de l'actomyosine. Nos résultats révèlent un nouveau rôle à la fois pour EGFR et cGMP dans la régulation de la communication entre les cellules cumulus et l'ovocyte. Ils permettent l'identification du mécanisme moléculaire à l'origine de la perte de TZPs lors de la maturation ovocytaire. Les résultats de cette thèse fournissent de nouvelles informations sur les bases moléculaires du développement physiologique des ovocytes, et pourraient éventuellement être appliqués aux méthodes cliniques et aux innovations thérapeutiques visant à préserver la fertilité.

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Contribution to Original Knowledge

Multiple evidences have shown that oocyte appropriate development, which includes full growth and timely meiotic maturation, is an essential perquisite for female fertility. The research presented in this thesis reveals novel insights into molecular basis of oocyte normal and healthy development from both aspects of growth and meiotic maturation.

In the course of this thesis, we first target the initial phase of oocyte development, growth.
It is well known that YAP acts as a crucial key protein which promotes growth in a broad range of somatic cell types and controls organ size. However, it is completely unknown whether YAP also promotes growth in female germ cell, oocyte, or not. In Chapter 2 (Manuscript I) we show, for the first time, that YAP is functionally inactive throughout the entire window of oocyte growth and thus does not play any significant physiologic role in the context of oocyte development. We further indicate the molecular mechanisms which keep YAP constantly inactive during the oocyte growth period.

We next move on to the second and final phase of oocyte development, meiotic maturation.
Here, we investigate the underlined molecular mechanisms which regulate the dynamics of germline-soma physical contact throughout oocyte development. It is known that filopodia-like structures termed TZPs are the only means of physical contact between the oocyte and its surrounding granulosa cells during folliculogenesis. It has also been shown that TZPs are maintained around the fully grown oocyte prior to meiotic maturation, but start to disappear as the oocyte initiates meiotic maturation. However, the molecular mechanisms behind TZPs maintenance and loss are totally unclear. In Chapter 3 (Manuscript II) we show, for the first time,

that activation of cGMP-ROCK axis within the cumulus granulosa cells maintains TZPs around the fully grown oocyte. We also identify that activation of EGF-ERK-PDE5 pathway in the cumulus granulosa cells, at the onset of meiotic maturation, suppresses cGMP-ROCk axis and this causes actomyosin contractility and therefore TZPs retraction.

Altogether, the results discovered in this work shed light on a part of the physiology of oocyte normal development whose disturbance causes pathological conditions resulting in female infertility.

Publications and Contribution of Authors

1- Abbassi L, Malki S, Cockburn K, Macaulay A, Robert C, Rossant J, Clarke HJ. Multiple Mechanisms Cooperate to Constitutively Exclude the Transcriptional Co-Activator YAP from the Nucleus During Murine Oogenesis. Biol Reprod. 2016 May; 94(5): 102.
doi: 10.1095/biolreprod.115.137968. PMID: 26985001. (Manuscript I)

2- El-Hayek S, Yang Q, Abbassi L, FitzHarris G, Clarke HJ. Mammalian Oocytes Locally Remodel Follicular Architecture to Provide the Foundation for Germline-Soma Communication. Curr Biol. 2018 Apr ;28(7):1124-1131.
doi: 10.1016/j.cub.2018.02.039. PMID: 29576478

3- Abbassi L, El-Hayek S, Yang Q, Mondadori R, Bordignon V, Clarke HJ. Depletion of cyclic GMP in the somatic compartment triggers retraction of filopodia mediating germline-soma communication in females. (Manuscript II; in preparation)

Contribution of Authors:

1-Abbassi L, Malki S, Cockburn K, Macaulay A, Robert C, Rossant J, Clarke HJ (2016). Contribution: The candidate, Abbassi L, performed all the experiments presented in this manuscript, except for the immunofluorescence and immunohistochemistry illustrated in Figures 15D, and 16, which were performed by Cockburn K/Rossant J and Malki S, respectively. Macaulay A/Robert C provided bovine specimens used for the western blot experiment in Figure 17B. Abbassi L performed all data analysis and figures preparation. Abbassi L and, Clarke HJ provided the intellectual contribution, designed the experiments and wrote the manuscript.

2- El-Hayek S, Yang Q, Abbassi L, FitzHarris G, Clarke HJ (2018).

Contribution: El-Hayek S and Clarke HJ provided conceptualization and methodology, El-Hayek S, Yang Q, and Abbassi L performed the experiments and collected data. Clarke HJ, El-Hayek S, and FitzHarris G wrote, reviewed and edited the manuscript. Clarke HJ and FitzHarris provided resources.

3- Abbassi L, El-Hayek S, Yang Q, Mondadori R, Bordignon V, Clarke HJ

Contribution: The candidate, Abbassi L, performed all the experiments presented in this manuscript, except for the Lucifer Yellow microinjection (applied for the experiment illustrated in Figure 25G) which is performed by Qin Yang. Mondadori R/Bordignon V provided and cultured the porcine specimens for the experiment indicated in Figure 22D. Abbassi L performed all data analysis and figures preparation. Abbassi L and, Clarke HJ provided the intellectual contribution, designed the experiments and wrote the manuscript.

List of Figures

List of Abbreviations

AKT	protein kinase B
AMH	anti-Mullerian hormone
APC	anaphase-promoting complex
AR	amphiregulin
ARX	aristaless related homeobox
ATP	adenosine triphosphate
BAX	BCL-2-associated x protein / BCL-2-like protein 4
BCL-XL	B-cell lymphoma-extra large
BMP	bone morphogenic protein
BSA	bovine serum albumin
BSA	bovine serum albumin
BTC	betacellulin
CAMP	cyclic adenosine monophosphate
CASPASE	cysteine-aspartic proteases
CBX2	chromobox protein homolog 2
CDH1	cadherin 1
CDK1	cyclin-dependent kinase 1
CGMP	cyclic guanosine monophosphate
CHD1	chromodomain-helicase-DNA-binding protein 1
CNP	C-type natriuretic peptide ligand
COC	cumulus oocyte complex

CREB	cAMP response element-binding protein
DAX1	dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DHH	desert hedgehog
EGF	epidermal growth factor
EGFR	EGF receptor
EMX2	empty spiracles homeobox 2
EPI	epiregulin
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FDSP	farnesyl diphosphate synthase
FGF8B	fibroblast growth factor 8B
FGF9	fibroblast growth factor 9
FOXO3	forkhead 2 box O3
FSH	follicle stimulating hormone
FSHR	FSH receptor
GDF9	growth differentiation factor 9
GnRH	gonadotropin releasing hormone
GOC	granulosa-oocyte complex
GPR	G-protein coupled receptor
GV	germinal vesicle
GVBD	germinal vesicle breakdown
H3acK9	histone H3 acetylated on K9

HAS2	hyaluronan synthase 2
HDL	high density lipoprotein HDL
HPG	hypothalamic–pituitary–gonadal
IGF1R	insulin-like growth factor I receptor
IGF-I	insulin-like growth factor 1
IL-1β	interleukin 1 beta
IR	immune response
IRR	IR-related receptor
ITS	insulin transferrin selenium
KFG	keratinocyte growth factor
KIT	mast/stem cell growth factor receptor
KL	KIT ligand
KSOM	potassium simplex optimization embryo culture medium
LATS1/2	large tumor suppressor 1 and 2
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LHX9	LIM homeobox 9
LMB	Leptomycin B
MAPK	mitogen-activated protein kinase
MEM	minimal essential medium
MII	metaphase II
MOB1A/B	MOB kinase activator 1A and B
MPF	maturation-promoting factor

MST1/2	mammalian STE20-like protein kinases 1 and 2
mTmG	membrane-Tomato/membrane-Green
mTORC	mammalian target of rapamycin complex 1
NF2	neurofibromatosis type 2
NGF	nerve growth factor
NOBOX	newborn ovary homeobox protein
NPPC	natriuretic peptide C
NPR2	natriuretic peptide receptor 2
NSN	non-surrounded nucleolus
PBS	phosphate-buffered saline
PBST	tween- PBS
PCR	polymerase chain reaction
PDE3A	phosphodiesterase 3A
PDE5	phosphodiesterase 5
PDGF	platelet-derived growth factor
PDGFRA	platelet-derived growth factor receptor alpha
PGC	primordial germ cell
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PTEN	phosphatase and tensin homolog
PTGS2	prostaglandin-endoperoxide synthase 2
RFP	red fluorescent protein

ROCK	Rho-associated protein kinase
SAV1	salvador family WW domain-containing protein 1
SF1	steroidogenic factor 1
SMAD	sma-and mad-related
SN	surrounded nucleolus
SOHLH	spermatogenesis and oogenesis specific basic helix-loop-helix
SOX9	SRY-Box 9
SRY	sex-determining region Y
TAZ	transcriptional coactivator with a PDZ-binding domain
TEAD	TEA domain family member
TGFβ	transforming growth factor β
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
TNFα	tumor necrosis factor alpha
TSC	tumor suppressor tuberous sclerosis complexes
TZP	transzonal projections
VEGF	vascular endothelial growth factor
WNT	wingless-Type MMTV integration site family
WT1	Wilms Tumor 1
WWTR1	WW domain containing transcription regulator 1
YAP	yes-associated protein
ZP	zona pellucida

Chapter I

INTRODUCTION

1.1 Concept of reproduction and significance of reproductive biology

The term “Reproduction” describes the act of creating offspring from their parent(s) through asexual or sexual processes. In asexual reproduction an organism duplicates itself through mitotic division and without depositing any genetic content from a second organism. Thus, these organisms usually do not have different sexes. Cloning, budding, fragmentation and sporogenesis are of different types of asexual reproduction. In contrast, sexual reproduction essentially needs the combination of two specialized cells, termed gametes, through a process named fertilization. Each of the two parents contributes one haploid gamete which contains half of the genetic content of the offspring diploid cells. As a result, offspring genetic features are derived from both parents. Oocyte, the female gamete, and the spermatozoa, the male gamete, are produced through a specialized form of cell division termed meiosis which occurs in all sexually reproducing organisms including animals, plants, and fungi.

The science of reproductive biology looks at the cellular, molecular, and genetic mechanisms regulating reproduction at both physiological and pathological levels and develops novel techniques to assist fertility disorders. Research in this area is mainly focused on regulation of sex hormones, cryopreservation of gametes and embryos, in vitro fertilization, intra-cytoplasmic sperm injection, micromanipulation of embryos, pre-implantation genetic diagnosis, embryo implantation and development, sex determination and reproductive biotechnologies, such as cloning, which altogether aim to improve cooperative interaction between the basic, translational, and clinical research.

1.2 Female reproductive system

Ovaries, oviducts, uterus, vagina, vulva, mammary glands and breasts are the main and basic structures of mammalian female reproductive system. These organs are designed to generate and transport the oocyte, produce sex hormones, promote the process of fertilization, support the growing foetus during pregnancy, and nourish the newborn during infancy. The female reproductive system is dynamic in mammals and undergoes morphological changes due to hormonal fluctuations during the oestrus cycle in rodent or menstrual cycle in human, pregnancy, and menopause (Heffner and Schust 2010, Dintzis and Treuting 2011, Jones and Lopez 2013).

Ovaries

Ovaries, paired pelvic organs equivalent to male testes, are central to the process of female reproduction (Tulsiani 2012). Ovaries are responsible to generate and release fully grown mature oocytes ready for fertilization as well as to biosynthesize and secrete high levels of female steroid sex hormones such as oestrogen and progesterone (Heffner and Schust 2010).

Oviducts

Oviducts, also known as uterine tubes or fallopian tubes, are a pair of muscular hollow tubes which extend from the uterus to the ovaries. Near the ovary, the oviduct terminates in a funnel-shaped section termed the infundibulum, which is lined with small finger-like structures called fimbriae. The responsibility of these fimbriae is to sweep over the ovarian cortex to pick up the ovulated oocyte, and then transfer it into the infundibulum lumen. The inner layer of each oviduct is covered with cilia whose beating, in cooperation with contractions of the oviduct wall smooth muscles, transport the oocyte towards the uterus. Accordingly, oviduct function is to

conduct the oocyte and the sperm to meet each other, serve as the site of fertilization, and following fertilization support the early embryonic development and carry the early embryo towards the uterus for implantation (Heffner and Schust 2010, Dintzis and Treuting 2011, Stewart and Behringer 2012).

Uterus

Mice have a bicornuate uterus with two lateral horns and a single body whereas rats have a duplex bicornuate uterus with two lateral horns and humans have a simplex pear-shaped uterus without any horns (Dintzis and Treuting 2011). The uterus consists of three anatomically and functionally distinct parts including corpus or body, lower segment and cervix. Uterine functions include: (1) production of prostaglandin F2α which is an essential factor for regulating ovarian cycles in domestic animals; (2) provision of an appropriate environment for spermatozoa movement, storage, and maturation; (3) supporting the foetus normal growth and development during pregnancy; and (4) pushing the foetus through the birth canal at parturition (Bartol, Wiley et al. 1999, Grainger 1999).

Vagina

The vagina is a muscular tube that extends from the uterine cervix to the vulva. Vagina passes the menstrual flow, functions as the receptacle for the penis during sexual intercourse, serves as a temporary receptacle for semen, transports sperm to the uterus, and forms a part of the birth canal which stretches during delivery (Heffner and Schust 2010, Jones and Lopez 2013).

Vulva

The external female sex organs are collectively known as vulva. Vulva comprises the opening of the vagina and urethra, the clitoris and the labia (Heffner and Schust 2010, Jones and Lopez 2013).

Breasts

The breasts are female specialized organs which are formed of mammary glands, milk ducts, and adipose tissue. Growth and development of mammary glands as well as lactation as their main function are directed by the guidance of steroid sex hormones (Jones and Lopez 2013).

1.3 Morphology, anatomy and histology of the ovary

Ovaries are small paired structures which are spherical in rodents and oval in humans. Ovarian surface is smooth in sexually immature females. However, after puberty when the females become sexually mature the surface of the ovary turns nodular due to development of follicles and formation of corpus lutea during repetitive oestrus/menstrual cycles. Rodents ovaries are attached to the dorsal wall of the pelvic cavity and completely enveloped within a thin transparent membranous structure called ovarian bursa which physically separates the ovary from its adjacent environment. However, human ovaries are directly open to the pelvic cavity and attached to the pelvis lateral walls. (Dintzis and Treuting 2011)

In terms of histology, the whole ovarian cortex is covered by a layer of simple squamous or cuboidal epithelium, called germinal epithelium. Beneath the epithelium is a layer of dense connective tissue, the tunica albuginea, which separates epithelium from the cortex. The cortex

lies internal to the tunica albuginea and houses the functional units of the ovary, follicles (Blaustein 1978, Clement 1987, Bacha Jr and Bacha 2012, Tulsiani 2012). The appearance of the cortex is associated with the female age such that during the infancy it is tightly packed with primordial follicles while during the reproductive period it mostly contains follicles and corpora lutea at different stages of development or regression. The presence of scarred remnants of both atretic follicles and corpora lutea on the ovarian cortex is another morphological feature of the cortex throughout the female reproductive lifespan (Blaustein 1978). Ovarian medulla, situated underneath the cortex and in the center of the ovary, is composed of connective tissue richly supplied with blood vessels, lymph vessels and nerves. The main ovarian vessels and nerves enter or leave the ovary through the hilus region (Blaustein 1978, Clement 1987, Bacha Jr and Bacha 2012, Tulsiani 2012).

1.4 Sex determination and ovary development

Gonadal primordia are paired organs that form as a result of the outgrowth of the coloemic epithelium, which lines the body cavity, particularly at the site of covering the ventromedial mesonephros. In mouse, this event occurs between 10.5–11.5 days post coitum (dpc) under the indirect regulation of *Sf1*, *Wt1*, *Lhx9*, *Cbx2*, *Emx2*, *Igf1r*/*Ir*/*Irr* genes (Fig. 1) (Kreidberg, Sariola et al. 1993, Luo, Ikeda et al. 1994, Miyamoto, Yoshida et al. 1997, Katoh-Fukui, Tsuchiya et al. 1998, Schedl and Hastie 1998, Birk, Casiano et al. 2000). The gonad primordium is a bipotential organ which is capable of developing to either a testis or an ovary (Brennan and Capel 2004). In most mammals, it is the presence or absence of the Y chromosome which determines the fate of the gonad so that it differentiates into testis or ovary, which itself defines male or female secondary sex characteristics of the embryo.

In fact, in mammals, differentiation into ovaries is the default fate of the gonads, since the presence and expression of the Y-linked gene *Sry* (male sex determining gene) triggers the testes fate and male pathway. Therefore, the absence of *Sry* gene on the X chromosomes leads the XX gonads to differentiate into ovaries, by default. Consistent with this, lack of *Sry* in XY mice triggers ovary fate while induction of *Sry* expression in XX mice promotes the testis pathway (Gubbay, Collignon et al. 1990, Lovell-Badge and Robertson 1990, Koopman, Gubbay et al. 1991). Interestingly, it has been reported that 10% of XY human patients with sex reversal indications carry mutant forms of *Sry* gene (Hawkins, Taylor et al. 1992). It has also been shown that the first distinction between XX and XY gonads is *Sry* gene expression which occurs at 10.5 dpc and is present only during 10.5–12.5 dpc (Hacker, Capel et al. 1995, Albrecht and Eicher 2001, Bullejos and Koopman 2001, Lovell-Badge, Canning et al. 2002).

There is not much known about the molecular mechanisms regulating *Sry* expression; however, it is known that WT1+KTS, GATA4 and FOG2 are involved in upregulation of *Sry* transcription (Hammes, Guo et al. 2001, Tevosian, Albrecht et al. 2002). Following the initiation of SRY expression and function, SOX9 as a direct downstream target of SRY which can play the exact same roles as SRY (De Santa Barbara, Bonneaud et al. 1998, Arango, Lovell-Badge et al. 1999, Daneau, Pilon et al. 2002), FGF9 as an essential factor for establishing *Sox9* expression (Colvin, Green et al. 2001, Schmahl, Kim et al. 2004) and finally DAX1 as a factor which acts either downstream of or in a parallel with SRY (Muscatelli, Strom et al. 1994) work together to promote the first steps of male pathway which is sertoli cell-lineage differentiation (Fig. 1). Eventually *Dhh* expressed by Sertoli cells (Bitgood, Shen et al. 1996, Clark, Garland et al. 2000, Yao, Whoriskey et al. 2002) along with *Pdgfrα* (Brennan, Tilmann et al. 2003) and *Arx*

(Kitamura, Yanazawa et al. 2002) expressed by testis interstitium induce the rapid morphological changes of the XY gonad that define early testis formation (Fig. 1) (Brennan and Capel 2004).

In contrast, no major structural changes is obvious in the XX gonads until around the birth time (18.5 dpc), close to onset of primordial follicles formation (Fig. 1) (Brennan and Capel 2004). As mentioned above, gonads normally differentiate to ovaries in the absence of the Y chromosome carrying *Sry* gene. It has been shown that Factor Z which acts as the most upstream effector of the molecular cascade suppressing male development is inhibited by SRY in male gonads. Therefore, in XX gonads with a mutated form of factor Z the entire male pathway might be activated in spite of the *Sry* absence (Eicher and Washburn 1986, Goodfellow and Lovell-Badge 1993, McElreavey, Vilain et al. 1993, Jiménez, Sánchez et al. 1996). So far, no factor has been found to overwhelm the effect of Factor Z in promoting female pathway (Brennan and Capel 2004); however, *Wnt4* and *Fst* have been identified as the only two genes which play certain roles in early ovarian development (Fig. 1) (Vainio, Heikkilä et al. 1999, Menke and Page 2002). A Summary of the above-mentioned genetic pathways involved in determination of gonadal sexual fate is depicted in Figure 1.

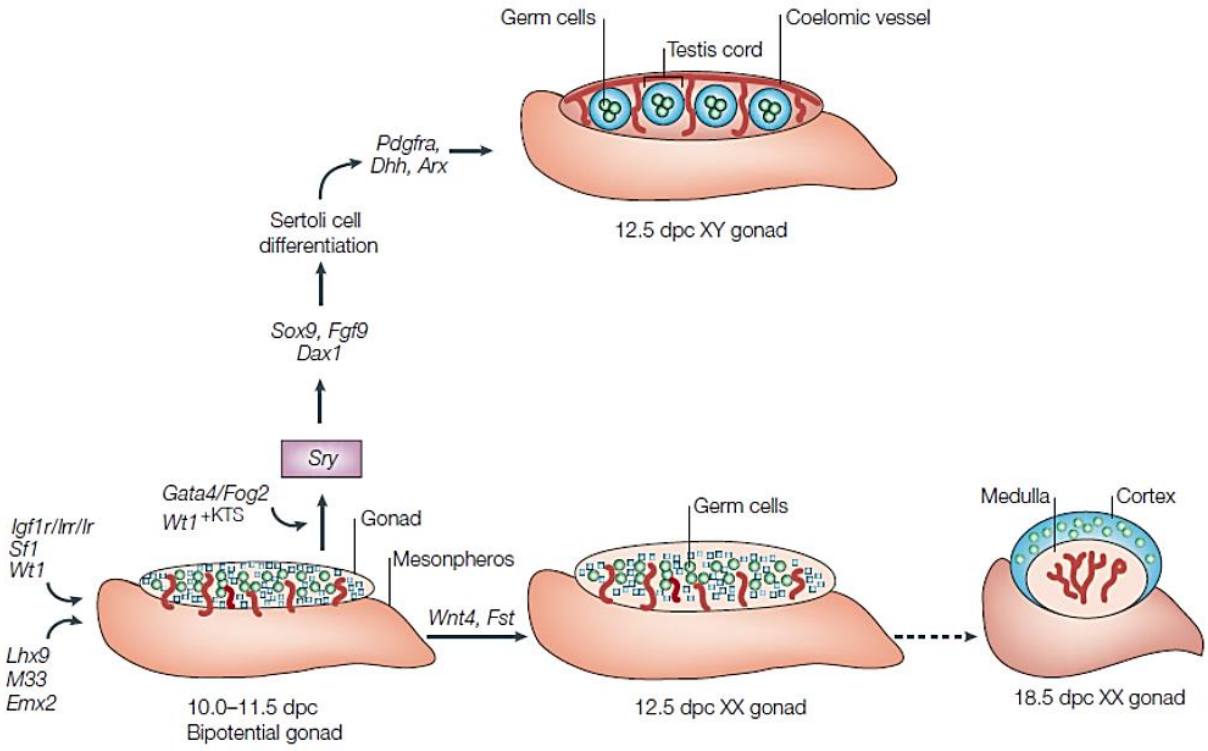

Figure 1: Schematic overview of the molecular mechanisms regulating mammalian sex determination.

The balance between multiple pro-death and pro-survival factors (SF1, WT1, LHX9, M33, EMX2, IGF1R/IR/IRR) results in the outgrowth of the bipotential gonad, at 10.5-11.5 days post coitum (dpc). It is during 10.5-12.5 dpc that cooperation between GATA4/FOG2 and WT1+KTS induces *Sry* gene expression, in case that the gonad is male. Following SRY function, three SRY downstream factors including SOX9, FGF9 and DAX1 induce sertoli cell-lineage differentiation as the early stage of male pathway. Subsequently, DHH expressed by Sertoli cells, and PDGFRA and ARX expressed by testis interstitium make rapid and chief morphological changes within the XY gonad which emerge as the early signs of testis formation at 12.5 dpc. In contrast to male, female gonads do not undergo major structural changes up until the very late stages of embryonic life, almost around the birth time, when primordial follicles start to develop within the ovaries. So far, WNT4 and FST are the only factors which have been identified to be implicated in female germ cells survival as well as early steps of ovarian development (Adapted from (Brennan and Capel 2004)).

1.5 Descriptive overview of oogenesis and folliculogenesis events

1.5.1 From primordial germ cells to primordial follicle formation

Oocytes originally differentiate from primordial germ cells (PGCs) which are the embryonic source of both male and female gametes (Fig. 2) (Pesce, Klinger et al. 2002). In the mouse embryo, PGC generation occurs at 7.5 dpc, in the extra embryonic mesoderm, under the signalling regulation of TGFβ family members, particularly BMP2, BMP4, and BMP8b (Lawson and Hage 1994, Ying, Liu et al. 2000, Ying and Zhao 2001). At 10.5 dpc, around the period that sex determination begins (Bowles and Koopman 2010), PGCs migrate to the undifferentiated gonad, the genital ridge, where they undergo a wave of mitotic divisions and become oogonia (Pepling and Spradling 1998). Eventually, around 13.5 dpc the colonized oogonia undergo a wave of mitosis with incomplete cytokinesis which leaves intercellular bridges (ring canals) among daughter cells and results in the formation of structures termed germ cell cysts or nests (Fig. 2) (Pepling and Spradling 2001, Pepling 2006). The cysts are then invaded and fully surrounded by mitotically arrested somatic cells called pre-granulosa (Pepling and Spradling 2001). It is also shown that a small fraction of germ cells die within each cyst while others survive and continue their normal process of development (Pepling and Spradling 2001).

Following germ cells cysts emergence and prior to follicle formation, oogonia stop proliferation, initiate meiosis, and become primary oocytes (Ginsburg, Snow et al. 1990). Oocytes proceed in meiosis up to the diplotene stage of prophase I where they enter a prolonged resting period called meiotic arrest (Hunt and Hassold 2008). In mouse, it is around the birth that the germ cell cysts undergo programmed breakdown (Fig. 2). Throughout the two-day window of cysts breakdown, two-thirds of the oocyte population undergoes random necrosis or apoptosis and might transfer

their cellular components such as nutrients, mRNAs, or organelle to the surviving cells before dying. This loss results in a significant decrease in the overall number of germ cells which ultimately develop to potential mature oocytes (Pepling and Spradling 2001). Following cyst breakdown, surviving oocytes become enveloped by the pre-granulosa cells and the primordial follicles which are the most immature form of ovarian follicles form (Fig. 2). Generation of the primordial follicles takes place before birth (during the second trimester of embryonic life) in human while immediately after birth, by day 2 postpartum at the latest, in the mouse newborn (Fig. 2) (Barnett, Schilling et al. 2006, Bristol-Gould, Kreeger et al. 2006, Pepling 2006). The timeline of different stages of primordial follicles formation as well as the list of genes involved in regulating each stage are depicted in Figure 2 (Pelosi, Forabosco et al. 2015).

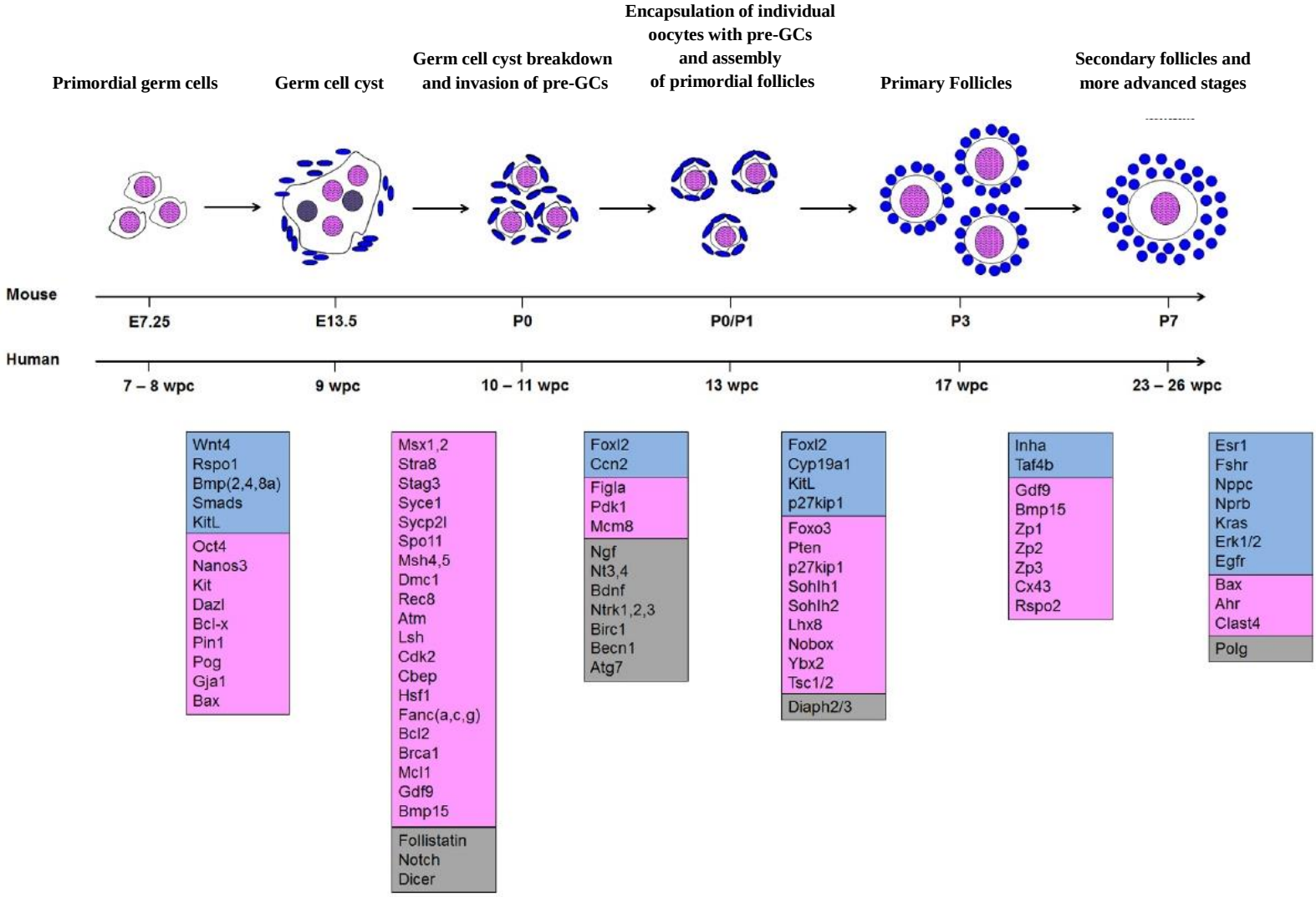

Figure 2: From primordial germ cells to primordial follicle formation

At a specific period of time during the fetal life, germ cells become interconnected via ring canals which integrates adjacent germ cells and result in the creation of cluster-like structures named germ cell cysts or nests. However, before birth in human and right after birth in mouse, the germ cell cysts break down and individual germ cells become released and surrounded by pre-granulosa cells which lead to the formation of primordial follicles. Apoptosis of 66% of the germ cells at the onset of cysts breakdown causes a dramatic reduction in the final number of oocytes and therefore primordial follicles. Genes associated with each transition are listed in the boxes. Blue boxes include the genes expressed in granulosa cells; pink boxes include the genes expressed in germ cells; genes included in the gray boxes are either expressed in both cell types (oocytes and granulosa cell) or their specific cell of expression is not yet known. Time is referred as either E (embryonic day) or P (postnatal day) in mouse, and as wpc (weeks post-conception) in human. (Adapted and modified from (Pelosi, Forabosco et al. 2015)).

1.5.2 Primordial follicle activation, and transition to primary stage

Each primordial follicle is composed of a single small oocyte (approximately 12 and 36 μm in diameter, in mouse and human respectively) enclosed by an incomplete layer of squamous (flattened) pre-granulosa cells (Fig. 2) (Gondos and Zemjanis 1970, Skinner 2005, Griffin, Emery et al. 2006). Primordial follicles are present in a stromal-interstitial tissue with no theca or mesenchymal cell around them (Rajah, Glaser et al. 1992). However, not only primordial follicles, but also each individual follicle throughout the entire folliculogenesis is surrounded by a basement membrane that separates granulosa cells from the external environment. It is also suggested that this basement membrane enhances morphological and biochemical differentiation as well as proliferation of the granulosa cells (Amsterdam, Rotmensch et al. 1989, Richardson, Davies et al. 1992, Luck 1994). Another role of the basement membrane in healthy follicles is to exclude capillaries, blood cells and neurites from the granulosa cells during follicle development, until it breaks down at the onset of ovulation (Irving-Rodgers and Rodgers 2000).

Females are born with a limited non-renewable pool of primordial follicles which constitute the entire source of oocytes that may potentially develop and ovulate during the reproductive lifespan (Skinner 2005). After birth, primordial follicles, which are in a dormant or quiescent mode with their oocytes meiotically and pre-granulosa cells mitotically arrested, exit the arrested pool and enter the growth phase, periodically. This event is referred to as primordial follicle activation (Faddy and Gosden 1995, McGee and Hsueh 2000, Fortune 2003). One of the early steps in primordial to primary follicle development is the morphological transformation of squamous pre-granulosa cells to cuboidal granulosa cells (Lintern-Moore and Moore 1979). Transition of primordial to primary follicle is also accompanied by an increase in the oocyte

diameter as well as its complete coverage by a single layer of cuboidal granulosa cells (Fig. 2) (Lintern-Moore and Moore 1979, Hirshfield 1991, Rajah, Glaser et al. 1992, Fortune 2003). Even though in theory every primordial follicle is intrinsically competent to become activated, grow, mature, and ovulate, this does not necessarily occur in reality. In fact, each primordial follicle may undergo one of these four possible fates: to remain dormant; to die out at its dormant stage; to initiate growth but later degenerate through atresia; or to fully develop and ovulate (Greenwald 1972, Skinner 2005, Tingen, Bristol-Gould et al. 2009). When the ovarian reserve of primordial follicles is depleted, the female reproductive life terminates and menopause occurs (Skinner 2005).

Primordial follicle activation is a very dynamic and closely regulated process (Sánchez and Smitz 2012); however, the molecular mechanism controlling the selective activation of primordial follicles is not well understood and this is due to the difficulties in studying and tracking the slow and prolonged process of recruitment of a large cohort of small follicles from the resting pool (Hirshfield 1989, McGee and Hsueh 2000).

Nonetheless, it has been shown that PI3K pathway, which is implicated in regulating cell growth, proliferation, survival, and migration (Cantley 2002) and is reported as one of the most frequently mutated proteins in different types of human cancers (Chalhoub and Baker 2009, Spoerke, O'Brien et al. 2012, Tran, Brettingham-Moore et al. 2013), is functional in oocytes and plays crucial roles in primordial follicles activation, both *in vivo* (Reddy, Shen et al. 2005) and *in vitro* (Li, Kawamura et al. 2010). In fact, stimulated PI3K phosphorylates and activates its downstream effector AKT, a serine/threonine protein kinase which normally promotes cell

proliferation (Fig. 3) (Cantley 2002). Consistently, phosphatase and tensin homolog (PTEN) as a negative regulator of PI3K/Akt pathway, inhibits cell proliferation (Fig.3) (Vlahos, Matter et al. 1994, Maehama and Dixon 1998, Cantley and Neel 1999, Cantley 2002). Therefore, the absence of PTEN activates PI3K/AKT pathway (Reddy, Liu et al. 2008) which in turn phosphorylates and inhibits the function of its downstream component FOXO3a (forkhead box O3). FOXO3a is a transcription factor that while dephosphorylated and activated enters the cell nucleus and stimulates the expression of the genes involved in cell cycle arrest (Fig. 3) (Brunet, Bonni et al. 1999). Therefore, either lack of *Pten* which allows for the FOXO3 translocation from the nucleus to the cytoplasm or *Foxo3* deletion cause premature depletion of primordial follicles due to their global activation (Castrillon, Miao et al. 2003, John, Gallardo et al. 2008, Jagarlamudi, Liu et al. 2009).

Interestingly, *in vitro* treatment of neonatal mouse ovaries with PTEN inhibitor along with a PI3K activator, successfully stimulated the PI3K-Akt pathway within the oocytes of dormant primordial follicles which in turn resulted in FOXO3 nuclear exclusion and thereby activation of the primordial follicles. Strikingly, following transplantation of the treated ovarian tissues under kidney capsules of ovariectomized mice, the awaken primordial follicles started to grow and developed up to preovulatory stage and generated mature oocytes, which were even capable of being fertilized and create viable, healthy, and fertile offspring. The similar scenario also happened in human primordial follicles when ovarian cortical fragments were cultured with PTEN inhibitor and xenografted under the kidney capsules of ovariectomized immunodeficient mice: quiescent primordial follicles became activated, entered growth, and developed to

preovulatory follicles containing mature oocytes, which albeit, were not fertilized due to ethical issues (Li, Kawamura et al. 2010).

In contrast, primordial follicles quiescence is associated with the presence of another signalling pathway engaging TSC1&2 (tumor suppressor tuberous sclerosis complexes 1&2) and their downstream component, mTORC1 (mammalian target of rapamycin complex 1). In fact, within the oocyte, upon the removal of AKT inhibitory effect, TSC1&2 become active and in turn suppresse mTORC1 function which leads to the maintenance of primordial follicle arrest (Fig. 3). It is demonstrated that oocyte conditional deletion of *Tsc1* or *Tsc2* triggers activation of the entire pool of primordial follicles around mouse puberty which leads to premature loss of ovarian reserve at early adulthood (Adhikari and Liu 2010). Therefore, it could be concluded that the synergistic effects of TSC1&2/mTORC1 pathway from one side and PTEN/PI3K from the other side play a crucial role in defining the quiescence or activation fates of the primordial follicles (Sánchez and Smitz 2012).

Additionally, several *in vivo* and *in vitro* studies have shown that the interaction between KIT ligand, expressed by the granulosa cells, and its receptor KIT, expressed by the oocyte, is essential and sufficient to induce primordial follicle activation (Packer, Hsu et al. 1994, Yoshida, Takakura et al. 1997, Parrott and Skinner 1999, Nilsson and Skinner 2004, Jin, Han et al. 2005, Thomas, Ismail et al. 2008). More specifically, KIT signalling acts as a crucial upstream regulator of intraoocyte PI3K/AKT pathway to trigger oocyte growth (Fig. 3) (Reddy, Shen et al. 2005, Liu, Rajareddy et al. 2006, Reddy, Liu et al. 2008). On the other hand, it has been shown that mTORC1 signalling within the granulosa cells is required for KITL expression. Thus,

suppression of mTORC1 signalling in the granulosa cells causes the loss or insufficiency of KITL production and secretion, and therefore lack of KIT activation on the oocyte surface. Consequently, this causes the intraoocyte PI3K/AKT signalling to remain unstimulated and thus inactive which itself leads to the maintenance of primordial follicle dormancy state (Fig. 3) (Zhang, Risal et al. 2014).

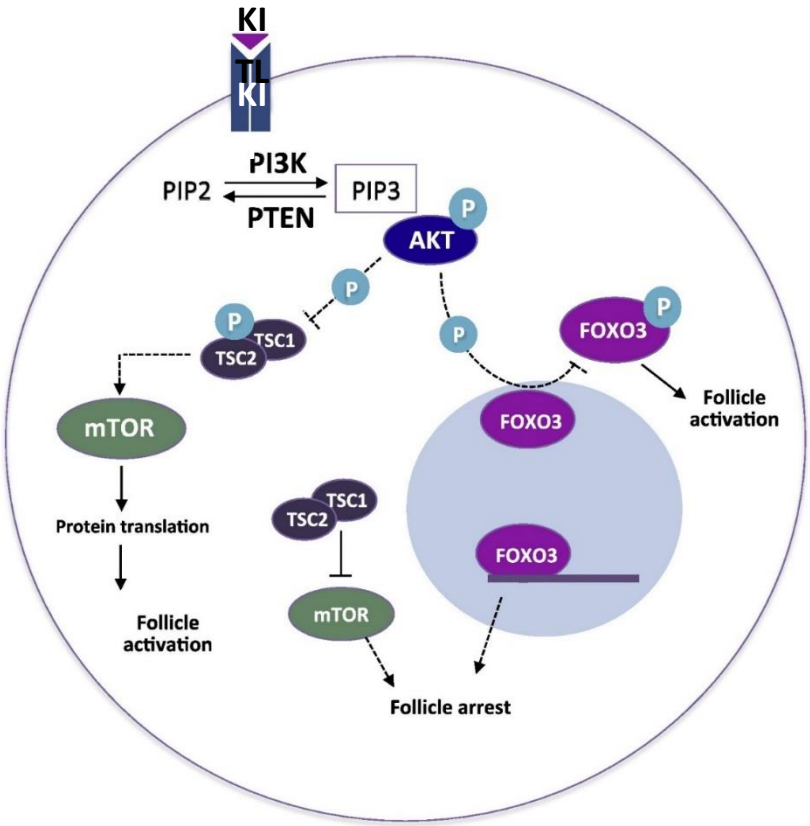

Figure 3: Signalling pathways involved in primordial follicles activation.

KITL and KIT coupling activates PI3K pathway in the oocyte which in turn phosphorylates and activates AKT. Activated AKT promotes two pathways: a) phosphorylates transcription factor FOXO3 which causes its nuclear export and termination of follicle arrest. b) phosphorylates and inactivate TSC, a negative regulator of mTOR. This allows mTOR to become active and induce production of proteins which are associated with follicle activation. Upon activation of PTEN, as PI3K suppressor, PI3K/AKT pathway beomes off and primordial follicle remains at its resting state (Adapted and modified from (Sánchez and Smitz 2012)).

Based on the fact that oocyte orchestrates the rate of follicle growth (Eppig, Wigglesworth et al. 2002) it could be concluded that KITL binding to oocyte KIT receptors may activate the molecular events within the oocyte which not only induce oocyte growth but also trigger the oocyte to produce and secrete specific paracrine factors that bind their receptors on the adjacent granulosa cells and initiate their proliferation (Kidder and Vanderhyden 2010). This implies that the initial activation of primordial follicles is triggered by granulosa cells but further promoted by the oocyte itself (Zhang, Risal et al. 2014).

Another granulosa cell-related factor that is involved in regulating primordial follicles activation is AMH (anti-Mullerian hormone), a member of the TGFβ superfamily, whose responsibility is to maintain a balance between the population of primordial follicles which enter growth or remain quiescent. *Amh* null mice exhibit an accelerated rate of primordial follicles recruitment which leads to precocious loss of ovarian reserve. This proves the potential role of AMH in suppressing the activation of primordial follicles in mice. (Durlinger, Kramer et al. 1999, Durlinger, Gruijters et al. 2002, Fortune 2003).

(SOHLH)1/2 (spermatogenesis and oogenesis specific basic helix-loop-helix and NOBOX (newborn ovary homeobox protein) as germ cell- and oocyte-specific transcription factors, respectively, are also key regulators of follicle transition from primordial to primary stage. NOBOX deficiency not only causes follicles to get stuck at the primordial stage but also reduces transcription level of many oocyte genes which are essential for promotion of oocyte growth (Rajkovic, Pangas et al. 2004). Similarly, both *Sohlh1* and *Sohlh2* null mutant mice exhibit disruption in follicle development from primordial to primary phase (Pangas, Choi et al. 2006,

Choi, Yuan et al. 2008). Furthermore, studies have shown that lack of SOHLH1causes down-regulation of *Nobox* and subsequently many other genes whose transcription is under the control of NOBOX protein (Pangas, Choi et al. 2006).

Apart from the granulosa or oocyte specific factors which are involved in primordial follicle activation, there are also some extra-follicular components, such as growth factors including NGF (nerve growth factor) (Dissen, Hill et al. 1996, Dissen, Romero et al. 2001, Dissen, Garcia-Rudaz et al. 2009) and KFG (keratinocyte growth factor) (Kezele, Nilsson et al. 2005) as well as certain members of the TGFβ family including BMP4 (Nilsson and Skinner 2003) and BMP7 (Lee, Otsuka et al. 2001) which take part in the regulation process of primordial follicles activation. These factors are derived from either theca cells or ovarian interstitial cells and function commonly on both oocyte and granulosa cells aiming to awaken the dormant follicle as a whole.

1.5.3 Progression of follicular growth

There are two main indexes for the follicle growth: oocyte enlargment and granulosa cells proliferation (Gougeon 2010). Oocytes increase in size rapidly and reach their maximum volume early during follicle development. However, the granulosa cells continue mitotic divisions extensively even after the oocyte has fully grown and stopped increasing in size (Hirshfield 1991).

At the early satges of follicle development, as the primary follicle grows to the secondary stage, its small oocyte becomes bigger and the single layer of granulosa cells proliferate to two ore more compelete layers of granulosa cells (Fig. 4). It has been reported that very small growing

follicles are deprived of an independent blood supply. However, it is around the early secondary stage that the medium-sized follicles start to be served by one or two arterioles, leading to the formation of an anastomotic network immidiately outside the basement membrane (Bassett 1943). The more follicles grow, the more this network becomes extensive such that eventually each ripe and mature follicle is richly supported by its own independent blood supply (Hirshfield 1991). The physiological significance of this event is the direct exposure of the follicles to factors circulating in the blood (Gougeon 2003).

Another change in the follicular structure which occurs at the onset of primary stage is the formation of a gelly coat, termed zona pellucida, all around the oocyte plasma membrane which physically separates the oocyte from its adjacent granulosa cells. (Wassarman and Litscher 2013). This subject will be discussed in more details in the next pages.

It is also around the early secondary stage of the follicle development that some stroma cells close to the basement membrane of the medium-sized secondary follicles become organized, sit parallel to each other and form the thecal layer (Fig. 4) (Gougeon , Mossman and Duke 1973). As the follicle enlarges, the surrounding theca cells stratify and differentiate into two subsets: the outer layer which is named the theca externa and is composed of cells that are similar to the undifferentiated theca cells; and the inner layer, called theca interna, which consists some fibroblast-like precursor cells, termed epitheloid cells, that morphologically resemble steroid-secreting cell (Gougeon 1996, Gougeon 1998). Based on the morphological features, only when the granulosa cells proliferate up to three-six layers the distinction of theca layers occurs. Upon

the appearance of epithelioid cells, secondary follicles transit to the preantral stage (Gougeon 1996).

When the growing follicles reach a certain size (200-500 mm in diameter depending on the species (Picton, Briggs et al. 1998)), supplies provided by the thecal capillaries are no longer sufficient to fully nourish the granulosa cells. This is due to the thickening of the granulosa cells layers as well as the higher nutritional requirements of these cells (Redding, Bronlund et al. 2007). It is around this stage that small spaces filled with follicular fluid start to form between the granulosa cells (Fig. 4) and serve as a source of oxygen, buffering molecules, carbohydrates, amino acids, growth factors, hormones and other essential molecules (Hirshfield 1991, Sutton, Gilchrist et al. 2003). As the follicle growth progresses, these spaces become bigger and join each other and finally aggregate into a single, large, fluid-filled cavity named antrum (Fig. 4) (Hirshfield 1991). Upon antrum appearance, the follicle is designated as antral follicle (Li and Chian 2017). It is shown that this follicular fluid is basically diffused from thecal blood plasma and becomes filtered by passing through a molecular barrier named "blood-follicle barrier" which is impermeable to proteins with molecular weight above 850 kDa (Shalgi, Kraicer et al. 1973). As a result, molecular components of the fullicular fluid are noticeably different from the blood plasma. For example, glucose and lipid levels are lower in the follicular fluid in comparison to the blood plasma (Hirshfield 1991).

As the antrum expands, it separates granulosa cells into two locally and functionally differentiated populations: the mural granulosa cells which line the follicle wall underneath the basement membrane and are characterised by their role in steroid synthesis; and the cumulus

granulosa cells which are closely associated with the oocyte and regulate its growth and maturation. At this point, follicle is at the preovulatory or graafian stage and ready to undergo ovulation. (Fig. 4) (Picton, Briggs et al. 1998, Collado-Fernandez, Picton et al. 2013).

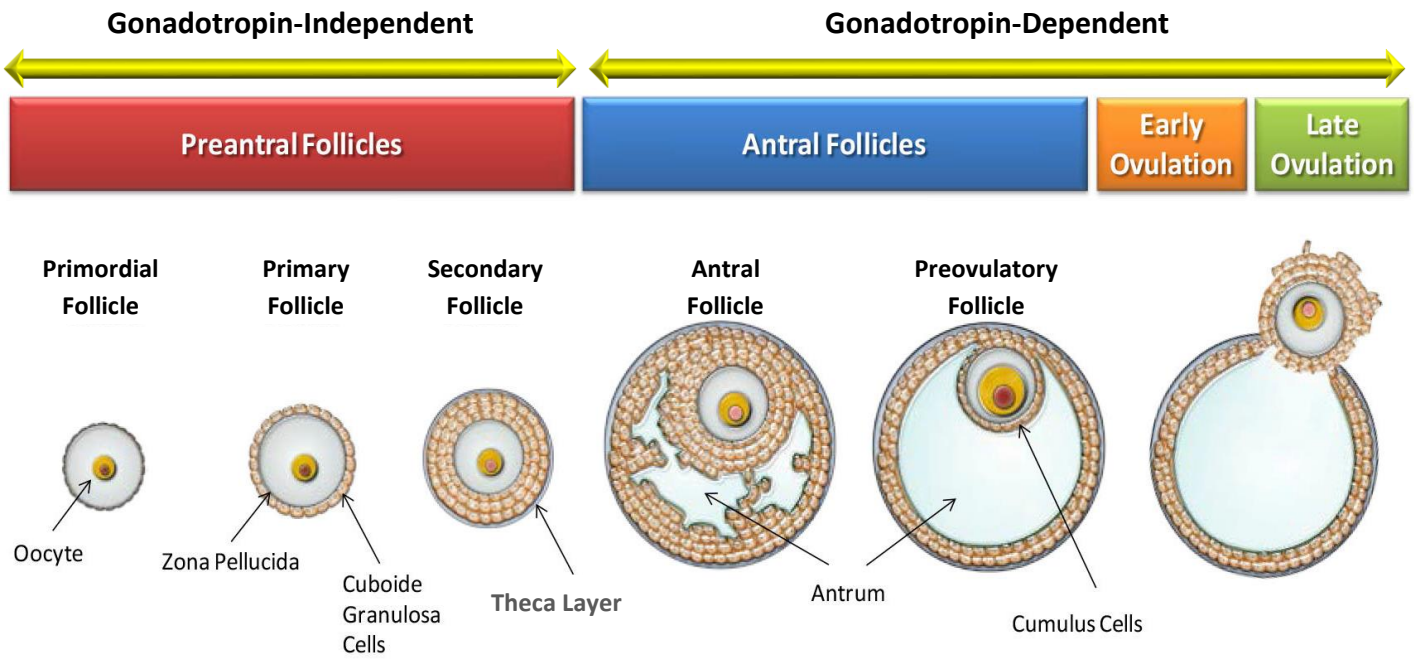

Figure 4: Follicle development

Each primordial follicle contains a small oocyte incompletely surrounded by a single layer of flattened granulosa cells and is isolated from the extra-follicular environment by the basal lamina. As the resting primordial follicle becomes activated, the oocyte initiates growth and granulosa cells not only proliferate to fully cover the oocyte but also transform from flat to cuboidal shape and primary follicle forms. Oocyte becomes larger in size and granulosa cells divide rapidly and give rise to a multilayered secondary follicle. At this stage, oocyte secretes extracellular glycoproteins forming a layer termed the zona pellucida which coats the oocyte cortex and segregates it from the neighbour granulosa cells. Simultaneously, a group of ovarian stromal cells align on the outer side of the basement membrane and deposit the theca layer. As the follicle further develop, small spaces filled with follicular fluid form between granulosa cells which then aggregate into a single, large, fluid-filled cavity known as antrum and now the follicle transits to the antral stage. As the antrum expands, it segregates granulosa cells into two distinct populations: mural and cumulus granulosa cell. Now the follicle is called preovulatory and is ripe enough to ovulate. Under LH surge, the ovulating follicle ruptures and excretes the oocyte with a complement of cumulus cells. Preantral stages of folliculogenesis progress gonadotropin independently while antral stages and ovulation tightly rely on gonadotropins. (Adapted and modified from (Sales, Lobo et al. 2013)).

Although follicles can undergo atresia at any time during development, the majority of them experience atretic degeneration during the antral stage since granulosa cells differentiation renders the follicle susceptible to apoptosis. Eventually, arrest of the granulosa cell proliferation and their programmed death causes the entire follicle to break down. (Boone, Yan et al. 1995, Boone and Tsang 1997). It is not exactly known which receptors or extracellular signals promote apoptosis within the granulosa cells; nonetheless, many intraovarian factors have been reported to be implicated in the processes of follicle survival (such as FSH, IGF-I, IL-1β, EGF, Bcl-2, Bcl-X_L) or death (such as inhibin, Bax, FasL, TNF-α, Caspase) (Hussein 2005). Therefor, it could be implied that the balance between these pro-survival and pro-death molecules is decicive for the follicle fate and determines if it grows further or degenerate (Matsuda-Minehata, Inoue et al. 2006).

As mentioned above, at the antral stage most follicles undergo atresia and only a few of them, called dominant follicles, are selected to survive and continue their growth up to the preovulatory stage (Fig. 4). Although the precise mechanisms regulating dominant follicle selection are not well clarified, multiple intraovarian components have been suggested to govern the process of selection in favor of follicles with higher production of oestrogen and more developmental competence (Ginther, Beg et al. 2001, Mihm and Evans 2008, Kidder and Vanderhyden 2010).

At the onset of ovulation, the cumulus cells expand (a process which will be discussed in the next pages), the oocyte resumes meiotic maturation, and finally the mature oocyte escorted by the expanded cumulus cells ovulates and leaves the ovary (Fig. 4) (Collado-Fernandez, Picton et al. 2013). After ovulation, the follicle remnant transforms into a richly vascularized endocrine

structure, the corpus luteum (CP), whose main function is to produce progesterone aiming to maintain the uterus endometrium during pregnancy (Kidder and Vanderhyden 2010).

1.6 Role of gonadotropins in regulating folliculogenesis

1.6.1 Introduction to gonadotropins

Gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are glycoproteins and components of the hypothalamic–pituitary–gonadal (HPG) axis. Gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus stimulates the anterior pituitary gland to synthesize and release gonadotropins whose target organs are testes and ovaries (Cattanach, Iddon et al. 1977, Pierce and Parsons 1981, Mason, Hayflick et al. 1986, Pernasetti, Vasilyev et al. 2001, Kumar, Agno et al. 2003). Function of both gonadotropins within the ovary is mediated through their interaction with their specific receptors [LH receptor (LHR) and FSH receptor (FSHR)], which are members of the seven-transmembrane domain G protein-coupled receptor family (McFarland, Sprengel et al. 1989, Sprengel, Braun et al. 1990, Richards 1994).

LHR is mainly expressed on theca interna cells but also present on the mural granulosa cells of the preovulatory follicles. (Zeleznik, Midgley Jr et al. 1974, Erickson, Wang et al. 1979, Segaloff, Wang et al. 1990, Camp, Rahal et al. 1991, Piquette, Lapolt et al. 1991, Themmen and Huhtaniemi 2000) and its coupling with LH promotes androgen synthesis (Fortune and Armstrong 1977, Richards, Jahnsen et al. 1987, Smyth, Gosden et al. 1994). On the other hand, FSH interaction with FSHR which is exclusively expressed on the surface of granulosa cells, induces aromatase activity within these cells (Dorrington, Moon et al. 1975, Erickson, Wang et

al. 1979, Hickey, CHen et al. 1988, Fitzpatrick and Richards 1991, Whitelaw, Smyth et al. 1992). Activated aromatase then synthesizes estrogen from the androgen which has been already transferred from the theca cells to the granulosa cells. This synergism between theca and granulosa cells in estrogen biosynthesis is referred to as the "two-cell two-gonadotrophin" model (Fig. 5) (Liu and Hsueh 1986).

1.6.2 Initiation of follicular growth

There are conflicting data regarding the involvement of gonadotropins in activation of resting follicles. In 1994, Hirshfield proposed that gonadotropins might play a role in activation of primordial follicles in 5-7 days old rats (Hirshfield 1994). It has also been reported that lack of gonadotropin-releasing hormone (GnRH) in a transgenic mouse model causes a decrease in the population of early growing follicles; however, this reduction could be reversed to the normal level by short-term injection of FSH analogue (Halpin and Charlton 1988). Consistently, it has been shown that hypophysectomy in rats slows down the rate of ovarian reserve depletion (Meredith, Kirkpatrick-Keller et al. 1986), while keeping LH concentration above the normal physiologic level over a prolonged period of time, in certain transgenic mice, accelerates the process of primordial follicles loss (Flaws, Abbud et al. 1997). Furthermore, studies have indicated that unilateral ovariectomy of old rats manifests as an increase in the serum FSH levels as well as promotion in the loss of primordial follicles (Meredith, Dudenhoeffer et al. 1992). This observation is similar to human studies which report parallel reduction in both serum FSH concentration (Lee, Lenton et al. 1988) and ovarian reserve size (Gougeon, Ecochard et al. 1994) in aged women. Putting these data together, it could be interpreted that gonadotropins may be engaged in awakening dormant follicles.

However, on the other hand, there are several other findings which suggest that gonadotropins do not act as the main stimulator of the quiescent primordial follicles. For example, Briggs et al showed in 1997 that *Fshr* is not transcripted in the human primordial follicles (Oktay, Briggs et al. 1997). Moreover, it has been is shown in mice, rats, cattle, and baboons that dormant follicles are able to become activated *in vitro* even in the absence of FSH supplement (Gougeon and Busso 2000). Another piece of evidence demonstrates that following transplantation of human ovarian cortical fragments under the kidney capsules of immunodeficient mice suffering from hypogonadism, resting follicles start to grow in spite of being deprived from FSH (Oktay, Newton et al. 1998). In addition, in the transgenic mouse models null for either *Fshβ* subunit (Kumar, Wang et al. 1997) or *Fshr* (Dierich, Sairam et al. 1998) primordial follicles can enter the growth phase and develop successfully up to the preantral stage. Likewise, in women with natural *Fshβ* subunit mutation, follicular development proceeds normally up to the point of dominant follicle selection (Barnes, Namnoum et al. 2002). Collectively, based on the above findings, it could be concluded that primordial follicles activation is independent of gonadotropins engagement.

Apart from the gonadotropins direct signalling, several local ovarian factors have been identified which are produced and secreted by either dormant follicles themselves or their adjacent growing follicles which can potentially activate primordial follicles. Perhaps, this justifies why primordial follicles could become activated even in the absence of gonadotropins. Nonetheless, considering the fact that gonadotropins stimulate growing follicles to synthesize these local factors, it could still be interpeted that gonadotropins are indirectly involved in the process of primordial follicles

activation which itself clarifies why elevated levels of circulating gonadotropins could promote depletion of ovarian reserve.

Taken together, although data regarding the involvement of gonadotropins in regulating primordial follicles activation are very conflicting and controversial, and even though molecular mechanisms through which local factors induce primordial follicles activation remain unclear, there is a general consensus that this activation process is likely controlled by various factors which act through multiple directions and target the oocyte or the granulosa cells to eventually awaken the resting follicle as a whole unit (Gougeon 2003).

1.6.3 Progression from primary to secondary stage

In both mouse and human, in spite of FSHR expression on the granulosa cells of early growing follicles (O'shaughnessy, Dudley et al. 1996, Oktay, Briggs et al. 1997), follicle development from primary to secondary stage can occur in the absence of gonadotropins (Fortune and Eppig 1979). Instead, this transition is essentially dependent on the presence and function of local intraovarian paracrine factors which are produced and released by either oocytes, granulosa cells, or theca cells (Kol and Adashi 1995).

1.6.4 Progression from preantral to antral phase

Follicle development from preantral to early antral stage is still primarily and predominantly dependent on locally secreted intraovarian factors; however, both *in vivo* and *in vitro* studies have shown that this transition is responsive to gonadotropins. Furthermore, in *vivo* studies in mice have shown that following injection of gonadotropins analogues, the transcription pattern

of certain genes (*Nobox, Oct4, Bmp15, Gdf9, Oogenesin1 and Oogenesin2*) which are involved in the process of oogenesis has been unaffected in primordial, primary, and secondary follicles in comparison with non-injected mice. However, the expression profile of the above genes were altered in antral follicles of the injected mice versus the control group (Monti and Redi 2009). Moreover, it has been demonstrated that FSH addition to the follicle culture environment is essential for *in vitro* antrum formation (Cortvrindt, Smitz et al. 1996, Cortvrindt, Smitz et al. 1997). Consistently, *in vivo* studies have shown that *Fshβ* and *Fshr* null mice are infertile due to the failure in antral follicle development (Dierich, Sairam et al. 1998, Burns, Yan et al. 2001).

1.6.5 Termination of follicular growth and ovulation

Late stages of folliculogenesis are strictly relied on FSH function. Furthermore, in the mural granulosa cells, FSHR signalling stimulates the expression of LHR whose subsequent coupling with LH plays the key role in initiation of the ovulatory process.

Lack of any of these two receptors in the transgenic mouse models has uncovered the crucial function of gonadotropins within the ovary throughout the final stages of follicle development as well as ovulation (Sánchez and Smitz 2012). For instance, in LHR deficient mice, follicle growth is arrested at the antral phase which in turn leads to infertility due to the insufficient estrogen level and anovulation (Zhang, Poutanen et al. 2001). In addition, injection of LHR mutant mice with high doses of FSH analogue cannot compensate for LHR deficiency and push the follicle development towards the final stages and induce ovulation (Pakarainen, Zhang et al. 2005). Therefore, LHR signalling is essential for two events: completion of follicle development prior to ovulation; initiation of ovulation process.

Lack of FSH-β in transgenic mouse models causes the process of folliculogenesis to be blocked before the antral follicle formation which results in anovulation and thus infertility. Comparing to the wildtype group, granulosa cells of these mice produce elevated levels of *Fshr* mRNA but reduced levels of aromatase. *Lhr* transcripts are also detected in these mice, but their proportion is much lesser comparing to the control groups (Burns, Yan et al. 2001).

FORKO mice (follitropin receptor knockout mice), which are free of all forms of FSH receptors, are also infertile due to the blockage of follicullogenesis prior to the antral stage as well as uterine atrophy (Dierich, Sairam et al. 1998). Because of the impaired follicle growth in these mutants, even the largest follicles have less than five layers of granulosa cells. Although the aromatase level of granulosa cells are normal in these mice, there is an absolute lack of estrogen biosynthesis which suggests the possible role of FSH in induction of aromatase activity (Danilovich, Babu et al. 2000). However, it is not clear whether FSH-β or FSHR deficiencies disturb normal follicle development via decreasing proliferation or increasing apoptosis in the granulosa cells (Sánchez and Smitz 2012). Furthermore, it has been reported that the expression level of cyclin D2, a factor which regulates granulosa cells division under FSH influence, undergoes a moderate reduction in FSH-β knockout mice but not in FORKO models (Dierich, Sairam et al. 1998). Also, women with natural mutation in *Fsh-β* and *Fshr* genes exhibit similar phenotype to the correspondent transgenic mouse models (Layman and McDonough 2000). Interestingly, women in menopause as well as those who suffer from hypergonadotropic hypogonadism share some common symptoms with FORKO mice.

Regardless of the above *in vivo* data, there are also *in vitro* evidences indicating that the absence of FSH in the follicle culture system results in folliculogenesis arrest, lack of antrum formation, and eventually follicle death, which could all be simply prevented by adding FSH supplement to the culture environment (Nayudu and Osborn 1992, Cortvrindt, Smitz et al. 1996). Apart from FSH "presence" in the culture, its "proper dose" is also critical to guarantee the optimal development of the follicles. For example, it has been shown that following antrum formation, decrease in the FSH concentration within the culture system improves the efficiency of cumulus cells differentiation (Sánchez, Adriaenssens et al. 2010, Sánchez, Romero et al. 2012). Consistently, addition of low doses of FSH to the culture system of primate follicles, albeit after antrum formation, allows the follicles to develop more competently and the oocytes to reach larger sizes. (Xu, Lawson et al. 2011). In contrast, *in vitro* studies in both mice and primates have shown that high FSH doses above the physiological borders disturb granulosa cells differentiation and function during transition from preantral to antral stages (Sánchez, Adriaenssens et al. 2010, Xu, Lawson et al. 2011). Therefore, not only gonadotropins presence, but also their appropriate dosage are critical to ensure normal development of antral and preovulatory follicles (Sánchez and Smitz 2012).

Taken together, it is deduced while early folliculogenesis is gonadotropin independent, FSH tightly regulates follicle development from the antral stage, and LH acts as a significant stimulus for initiation of ovulation process (Fig. 4) (Zhang, Poutanen et al. 2001).

1.7 Role of sex steroid hormones in regulating folliculogenesis

Gonadotropins main function is to trigger follicles to produce steroid hormones, androgens and estrogens, which subsequently bind and activate their specific receptors, androgen receptor (AR) and estrogen receptor (ER), respectively. In fact, based on the previously mentioned "two-cell, two-gonadotropin concept", LH-stimulated theca cells produce androgens, whereas FSH-provoked granulosa cells uptake this androgen and recruit it as a substrate to produce estrogens (Fig. 5) (Dorrington and Armstrong 1979, Hillier, Whitelaw et al. 1994).

Androgen-AR coupling promotes granulosa cells proliferation and prevents follicular atresia. *Ar* global deletion in mice causes a decrease in the rate of antral follicles development and ovulation as well as an increase in the rate of granulosa cells programmed death; these mice are subfertile and eventually end up with premature ovarian failure (Hu, Wang et al. 2004, Shiina, Matsumoto et al. 2006). AR is expressed not only in the granulosa cells but also in the oocytes. However, based on study carried on both granulosa cell- and oocyte-specific AR knockout mice, it was revealed that the majority of reproductive phenotypes exhibited by mice with *Ar* global deletion are in fact associated with the lack of AR in the granulosa cells but not the oocyte. Therefore, it could be concluded that granulosa cell-specific AR is a critical factor to ensure follicle normal development (Sen and Hammes 2010).

Estradiol, which acts as the main ovarian estrogen, is synthesized via aromatase activity within the preovulatory granulosa cells. It is well established that estradiol interaction with its receptors present in the granulosa cells nuclei and cytosol (Richards 1975, Saiduddin and Zassenhaus 1977) not only regulates the proliferation rate of granulosa cells, but also defines their

responsiveness level to gonadotropins (Pencharz 1940, Goldenberg, Vaitukaitis et al. 1972, Richards, Ireland et al. 1976, Richards, Jonassen et al. 1979).

Most of the present knowledge regarding the estrogen function, in the context of follicular development, have been achieved by studying transgenic mouse models depleted of both estrogen receptors, ERα and ERβ. In fact, ERα knockout (ERKO) mice exhibit elevated levels of estradiol and LH but normal FSH concentration. These mice are infertile due to the disruption of folliculogenesis at early antral stage which reflects the critical role of estradiol in driving follicle growth in response to gonadotropin (Lubahn, Moyer et al. 1993, Emmen and Korach 2003). In contrast, in ERβ knockout (BERCO) mice follicles development proceeds normally, antral follicles form successfully, and the females are fertile. However, these mice do not ovulate in response to LH analogue injection (Emmen and Korach 2003, Couse, Yates et al. 2005).

Hence, normal biosynthesis of androgens and estradiol as a consequence of efficient cooperation among follicular compartments is essential to assure follicle survival, development and ovulation.

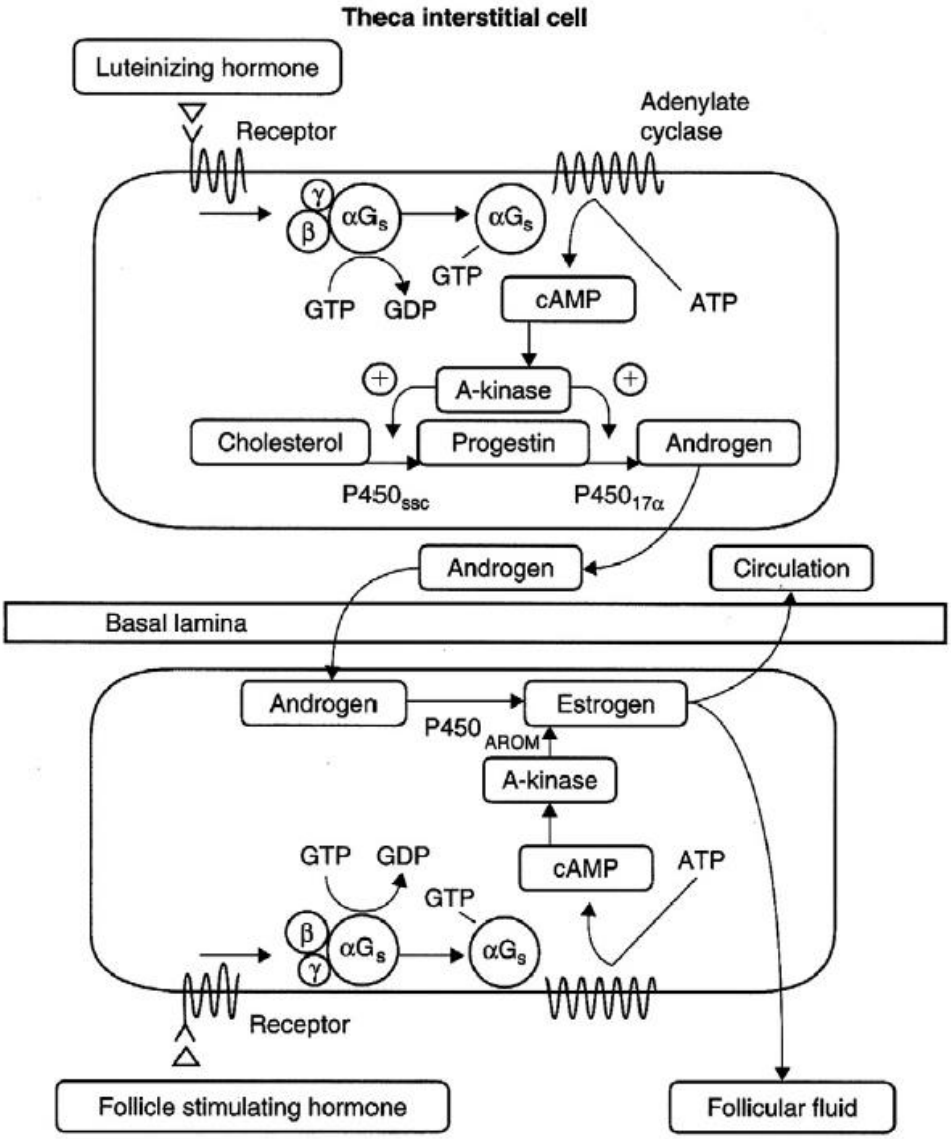

Figure 5: The two cell-two gonadotrophin concept

Under the influence of LH, the theca cells produce androgen from cholesterol. Androgen diffuses into the mural granulosa cell and acts as a substrate for FSH-activated aromatase enzyme to synthesize follicular estradiol which will then circulate in the blood flow and follicular fluid. This cooperation between the two cell types, (theca and granulosa cells) and the two gonadotropins (FSH and LH) to produce follicular estradiol is referred to as 'two cell-two gonadotrophin' model in the physiology of ovarian function (Adapted from (Erickson and Shimasaki 2001)).

1.8 Oocyte acquisition of developmental competence

In order to be fertilized and create a viable healthy embryo that is capable of full-term development terminating with live birth, the oocyte needs to achieve developmental competence which includes two phases: growth and meiotic maturation. Oocyte growth is a slow and long-term process which takes place within the follicle, whereas meiotic maturation is a much briefer process that occurs at the time of ovulation (Sánchez and Smitz 2012, Clarke 2018). Both oocyte growth and meiotic maturation include nuclear and cytoplasmic events.

1.8.1 Oocyte growth

1.8.1.1 Cytoplasmic events

During growth, oocyte diameter increases from 10 to 80 μm in mouse and from 35 to 120 μm in human which corresponds to more than 500- and 100-fold increase in the oocyte volume, in mouse and human respectively. As mentioned above, growth is a gradual and prolonged process whose length is ~ 3 weeks in mouse and 3 to 4 months in human (De Leon, Johnson et al. 1983, Eppig and O'brien 1996, Picton, Briggs et al. 1998, Sánchez and Smitz 2012).

Oocyte enlargement is a reflection of continuous synthesis and storage of many macromolecules including RNAs and proteins (a 300- and 38-fold increase in the levels of mouse oocyte's total RNA and protein, respectively, has been reported during oocyte growth (Schultz, Letourneau et al. 1979, Sternlicht and Schultz 1981)), different organelles including mitochondria and Golgi apparatus, and multiple cell structures such as lipid globules, cortical granules, and cytoplasmic vesicles. Production and accumulation of all these components within the oocyte are vital for the future embryo to survive and develop during the interval between fertilization and implantation

(Moore and Lintern-Moore 1974, Moore, Lintern-Moore et al. 1974, Moore and Lintern-Moore 1978, De Leon, Johnson et al. 1983, Bachvarova, De Leon et al. 1985, Eichenlaub-Ritter and Peschke 2002, Mahrous, Yang et al. 2012, Sánchez and Smitz 2012, Svoboda, Franke et al. 2015); because, for instance, the embryonic genome does not become robustly active until a specific stage of embryo development, depending on the species; or it has been shown that embryonic cells are not able to generate new mitochondria before the blastocyst implantation (Li, Zheng et al. 2010, Svoboda, Franke et al. 2015). These observations prove that early embryo survival and development are essentially relied on the oocyte supplies, highlighting the need for oocyte growth to provide maternal reserves.

1.8.1.2 Nuclear events

In parallel with cytoplasmic changes during growth, oocyte nucleus also undergoes certain changes (De La Fuente and Eppig 2001, Su, Sugiura et al. 2007). For example, whereas transcriptional activity is at its peak throughout the earliest stages of oocyte growth, a global transcriptional silencing and even selective mRNA degradation occur when the mouse oocyte reaches 65 micrometer in diameter, which is almost prior to its full size (Wassarman and Letourneau 1976, Schultz and Wassarman 1977, Bachvarova, De Leon et al. 1985, Bouniol-Baly, Hamraoui et al. 1999, De La Fuente, Viveiros et al. 2004, Su, Sugiura et al. 2007).

Epigenetic modifications such as DNA methylation also impact oocyte genome during the growth period (Lucifero, Mann et al. 2004, Smallwood, Tomizawa et al. 2011, Tomizawa, Nowacka-Woszuk et al. 2013, Stewart, Veselovska et al. 2015). Moreover, coincident with transcriptional silencing, oocyte chromatin configuration reorganizes from a non-surrounded

nucleolus (NSN) state in which the chromatin is decondensed and dispersed throughout the nucleu to a surrounded nucleolus (SN) state in which the chromatin becomes partially condensed and forms apparent loops along the nuclear wall (Mattson and Albertini 1990, Wickramasinghe, Ebert et al. 1991, Debey, Szöllösi et al. 1993, Christians, Boiani et al. 1999). This chromatin remodelling is a signature of oocyte growth termination in multiple species (McGaughey, Montgomery et al. 1979, Albertini 1987, Führer, Mayr et al. 1989, Lefevre, Gougeon et al. 1989, Parfenov, Potchukalina et al. 1989, Hinrichs, Schmidt et al. 1993, Schramm, Tennier et al. 1993, Sui, Liu et al. 2005, Jin, Lee et al. 2006, Russo, Martelli et al. 2007, Sun, Li et al. 2009, Comizzoli, Pukazhenthi et al. 2011) and is reported to be closely associated with the status of oocyte developmental competence. Indeed, during the early stages of growth, oocytes exhibit NSN configuration and high transcriptional activity, but weak developmental competence while reaching the full-size, the oocytes start to demonstrate SN configuration and low transcriptional activity, but advanced developmental competence (Zuccotti, Giorgi Rossi et al. 1998, Bouniol-Baly, Hamraoui et al. 1999, Pesty, Miyara et al. 2006). It should be noted that although there is also an association between the oocyte transcriptional activity and chromatin configuration, these two events are not necessarily causally linked (De La Fuente 2006, Abe, Inoue et al. 2010, Andreu-Vieyra, Chen et al. 2010).

1.8.2 Oocyte meiotic maturation

Oocyte needs to become nuclearly and cytoplasmically competent in order to be able to initiate nuclear and cytoplasmic events of meiotic maturation. Nuclear competence is acquired first, when the oocyte reaches about 80% of its final size (Szybek 1972, Durinzi, Saniga et al. 1995) and later on, as the oocyte reaches its maximum size, the cytoplasmic competence is also

acquired. Therefore, an oocyte whose nucleus is ready to enter the nuclear events of meiotic maturation is not necessarily competent to initiate the cytoplasmic events of meiotic maturation as well (Sánchez and Smitz 2012).

1.8.2.1 Nuclear events

Meiosis is a unique and specialized type of cell division which includes two rounds: meiosis I and meiosis II. The outcome of a full meiosis is segregation of half of the genetic content of the original diploid cell into the produced haploid gamete: oocyte or sperm (Adhikari and Liu 2014). The oocyte is meiotically arrested at prophase I from the fetal life and it is only shortly before ovulation that it resumes meiosis I in response to LH surge (Conti, Hsieh et al. 2012, Holt, Lane et al. 2013, Adhikari and Liu 2014).

Throughout the whole period of being prophase I-arrested, the oocyte is characterized by a large nucleus, termed germinal vesicle (GV), which is enclosed by the nuclear envelope. Under the influence of LH surge, the nuclear envelope disintegrates and GV breakdown (GVBD) takes place, and this is considered as the first morphological sign of meiosis I resumption (Fig. 6). Subsequently, meiosis I spindle fibers assemble, homologous pairs of chromosomes become aligned and attach to the spindle microtubules at their kinetochores while being oriented along the equatorial plate. Here the oocyte is considered to be at metaphase I (MI). Next, in response to actin induced tension, the spindles carrying paired chromosomes migrate to the oocyte cortex and this is the hallmark of the metaphase I completion (Fig. 6) (Kudo, Wassmann et al. 2006).

Meiosis I terminates by the extrusion of the first polar body. From each pair of the chromosomes, one homologue is allocated to the polar body and the other homologue remains in the oocyte, waiting to enter the second meiotic division and associate with the newly assembled spindles at metaphase II (MII) (Fig. 6). When the oocyte reaches MII, once more it becomes meiotically arrested and remains so until the onset of fertilization which triggers meiosis II resumption and completion by the emergence of the second polar body. In terms of meiotic maturation, prophase I- and metaphase II- arrested oocytes are considered immature and mature, respectively. (Li and Albertini 2013, Chaigne, Campillo et al. 2015). Also, in the present thesis, meiotic arrest refers to prophase I-arrest and correspondently meiotic resumption refers to meiosis I re-initiation.

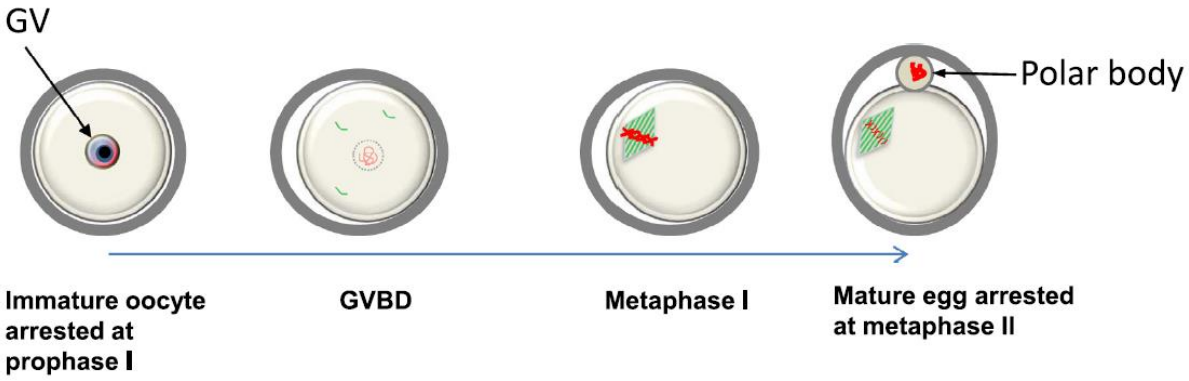

Figure 6: Nuclear maturation

From the fetal life, the oocyte is arrested at prophase I and is considered meiotically immature. Prophase I-arrested oocyte is marked by a large nucleus, germinal vesicle (GV), which contains decondensed chromatin. LH surge at the onset of ovulation re-activates meiosis I and this is marked by nuclear membrane fragmentation leading to germinal vesicle breakdown (GVBD). Subsequently, the chromosomes (red) undergo full condensation and the first meiotic spindles form (green). Thickened and shortened chromosomes line up on the spindles and then the whole complex moves to the oocyte margin which represents completion of metaphase I. Polar body appearance and deposition of one set of the homologous chromosomes in it assigns termination of meiosis I. The other set of homologous chromosomes remains in the oocyte, and upon initiation of meiosis II attaches to the newly formed spindles, again at the oocyte periphery. At this stage which is termed metaphase II, the oocyte enters the second round of meiotic arrest and remains so until fertilization triggers meiosis II resumption (Adapted from (Adhikari and Liu 2014)).

1.8.2.1.1 Molecular mechanisms maintaining meiotic arrest

It is well established that the high concentration of second messenger cyclic AMP (cAMP) is the key factor for preventing meiotic resumption within the immature fully grown oocytes (Cho, Stern et al. 1974, Dekel and Beers 1978, Downs, Daniel et al. 1989, Conti, Andersen et al. 2002, Nogueira, Albano et al. 2003). It is also thought that the immature fully grown oocytes are supplied with a high level of cAMP via two routes: i) mainly through the synthesis of endogenous cAMP which occurs downstream of the activated G-protein coupled receptor 3 (Fig. 7) (Mehlmann 2005, Vaccari, Horner et al. 2008) and ii) through the direct import of cAMP molecules from the neighbouring cumulus granulosa cells, via the gap junction channels (Sela-Abramovich, Edry et al. 2006).

1.8.2.1.1.1 Regulation of meiotic arrest downstream of cAMP

The question which is raised here is that through which molecular mechanism (s) cAMP prevents oocyte meiotic maturation? In fact, cAMP does so by suppressing the maturation-promoting factor (MPF) through cAMP-dependent protein kinase A (PKA) activity (Fig. 7) (Maller and Krebs 1977, Maller and Krebs 1980, Bornslaeger, Mattei et al. 1986). MPF is a complex composed of cyclin-dependent kinase 1 (CDK1) (encoded by *Cdc2*) and cyclin B1 (encoded by *Ccnb1*) (Brunet and Maro 2007), and its function is highly dependent on phosphorylation or dephosphorylation of two specific sites on CDK1, Thr14 and Tyr15. Indeed, Wee1 kinases inhibit MPF by phosphorylating CDK1 while CDC25 phosphatases activate MPF by dephosphorylating CDK1 (Fig. 7) (Lew and Kornbluth 1996, Lincoln, Wickramasinghe et al. 2002). Activation of both Wee1 kinases and CDC25 phosphatases is directly regulated by PKA (Fig. 7) (Han, Chen et al. 2005, Zhang, Zhang et al. 2008, Pirino, Wescott et al. 2009, Oh, Han et

al. 2010). Specifically, phosphorylation of CDC25B by PKA allows it to bind 14-3-3 protein and therefore remain cytoplasmic and inactive (Zhang, Zhang et al. 2008, Pirino, Wescott et al. 2009). On the other hand, PKA-mediated phosphorylation of Wee1B, which is an oocyte-specific kinase, stimulates Wee1B function (Han, Chen et al. 2005, Oh, Han et al. 2010). To conclude, cAMP-dependent PKA activity via direct regulation of Wee1 kinases and CDC25 phosphatases plays the key role in maintaining MPF complex inactive and thus keeping the oocyte meiotically arrested. In addition to the inhibitory effect of cAMP, it has also been shown that MPF inactivation in immature oocytes may be partially due to the degradation of Cyclin B induced by anaphase-promoting complex (APC)-CDH1 (Holt, Weaver et al. 2010, Bacha Jr and Bacha 2012).

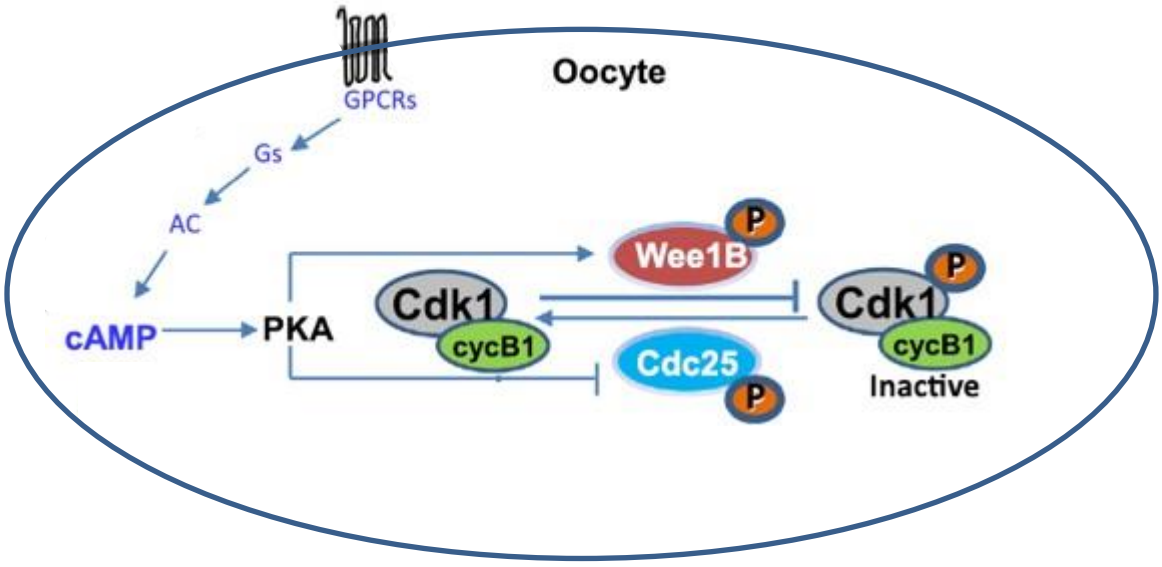

Figure 7: cAMP inhibition of meiotic maturation

The presence of a high level of cAMP, synthesized downstream of the G-protein coupled receptors signalling within the fully grown oocytes, is the crucial element for inhibiting meiosis resumption. cAMP does so by activating PKA which subsequently stimulates Wee1B and disables CDC25B via phosphorylating them both. As a result of Wee1b activity and CDC25B inactivity, CDK1 remains suppressed and this prevents cell cycle re-entry. (adapted and modified from (Adhikari and Liu 2014)).

1.8.2.1.2 Role of cGMP in the maintenance of high cAMP levels

So far, I have described how high cAMP levels prevent meiotic resumption. I now turn to discuss the molecular mechanism which keep cAMP at a high level to ensure meiotic arrest. Studies over the past decades had come to the consensus that there is a factor within the follicle whose presence prevents meiotic maturation while its absence induces oocyte spontaneous meiotic resumption (Pincus and Enzmann 1935, Edwards 1965, Tsafriri, Pomerantz et al. 1976, Sato and Koide 1984). However, recent data clearly show that cyclic guanosine monophosphate (cGMP) is the key factor in the maintenance of oocyte meiotic arrest (Vaccari, Weeks et al. 2009). More precisely, it has been shown that cGMP produced in the granulosa cell compartment of the pre-ovulatory follicle is transferred to the oocyte where it suppresses the activity of the cAMP-hydrolytic enzyme, phosphodiesterase (PDE3A), to ensure high cAMP concentration which consequently maintains oocyte meiotic arrest (Fig. 8) (Norris, Ratzan et al. 2009).

As mentioned above, granulosa cells are the source of cGMP production within the pre-ovulatory follicles. In fact, mural cells of the pre-ovulatory follicles express high level of *Nppc*, which subsequently becomes translated to the precursor of C-type natriuretic peptide ligand (CNP) and released into the follicular fluid. CNP then binds and activates its receptor, natriuretic peptide receptor 2 (NPR2), on the surface of mural and cumulus cells in autocrine and paracrine manners, respectively (Fig. 8). Activation of NPR2 signalling stimulates cGMP production in both granulosa cell types. Then, through the gap junctional communication between the mural and cumulus cells as well as between the cumulus cells and the oocyte, cGMP flows from the somatic compartment to the oocyte (Fig. 8). Within the oocyte, as its final destination, cGMP inhibits PDE3A activity and therefore keeps cAMP at a high level allowing for the meiotic

maturation to remain arrested (Fig. 8) (Törnell, Brännström et al. 1984, Zhang, Tao et al. 2005, Zhang, Tao et al. 2005).

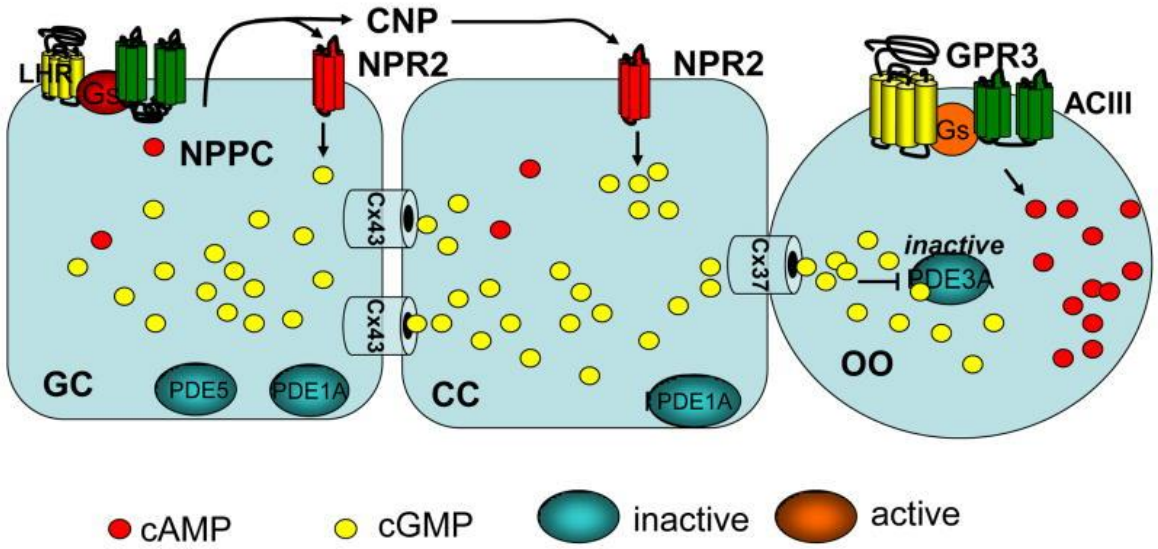

Figure 8: Molecular network regulating oocyte meiotic arrest

Within the pre-ovulatory follicles high level of CNP is synthesized and released by the mural granulosa cells. CNP then binds its receptor, NPR2, on the surface of both mural and granulosa cells which itself results in cGMP production in both of these cells. cGMP transfers to the fully grown oocyte via gap junctions and suppresses PDE3A activity to prevent cAMP degradation. This results in the maintenance of high cAMP levels within the oocyte which prevents meiotic resumption. (GC: Granulosa Cell, CC: Cumulus Cell, OO: oocyte) (Adapted from (Conti, Hsieh et al. 2012)).

It is known that FSH and estradiol amplify the expression of *Nppc* and *Npr2* in the granulosa cells, which highlights the unique contribution of these two hormones in the maintenance of oocyte meiotic arrest prior to ovulation. (Zhang, Su et al. 2010, Kawamura, Cheng et al. 2011, Zhang, Su et al. 2011, Lee, Zhang et al. 2013). Notably, the oocytes themselves are also actively engaged in the regulation of their own meiotic arrest. In fact, oocyte secretion of paracrine factors including GDF9, BMP15 and FGF8B promotes the expression of *Npr2* in the cumulus cells (Sela-Abramovich, Edry et al. 2006, Norris, Ratzan et al. 2009, Vaccari, Weeks et al. 2009, Zhang, Su et al. 2010, Richard and Baltz 2014). Moreover, via secreting paracrine factors, oocytes further participate in self-regulation of their own meiotic arrest by elevating the expression level of Inosine-5′-monophosphate dehydrogenase (IMPDH), an essential enzyme for the cGMP biosynthesis within the cumulus cells (Wigglesworth, Lee et al. 2013). Conclusively, oocytes stay meiotically arrested before ovulation under the control of not only the granulosa cells but also themselves. This cooperation between the two compartments of the preovulatory follicle controls the appropriate timing of meiotic resumption and prevents it from occurring before the oocyte fully grows and achieves the cytoplasmic competence (Clarke 2018).

1.8.2.1.2 Molecular mechanisms inducing meiotic resumption

1.8.2.1.2.1 LH surge triggering meiotic resumption

Although the pre-ovulatory LH surge is responsible to re-initiate oocyte meiotic maturation, LH does not directly affect the oocyte, simply because LH receptors do not exist on the oocyte membrane (Peng, Hsueh et al. 1991, Van Tol, Van Eijk et al. 1996, McGee and Hsueh 2000). In fact, LHRs are exclusively expressed in theca and mural granulosa cells and activation of those present on the surface of mural granulosa cells are responsible to launch a series of signalling

pathways (discussed below) which result in meiotic resumption and ovulation (Richards, Ren et al. 2017).

Following the interaction between LH and LHR on the surface of mural granulosa cells, LHR signalling becomes activated within the mural granulosa cells which causes a significant reduction in pre-ovulatory follicle cGMP content (Fig. 9) via three different processes: inhibition of NPPC synthesis (Kawamura, Cheng et al. 2011, Robinson, Zhang et al. 2012, Lee, Zhang et al. 2013), dephosphorylation and therefore deactivation of NPR2 (Norris, Ratzan et al. 2009, Vaccari, Weeks et al. 2009, Robinson, Zhang et al. 2012, Egbert, Shuhaibar et al. 2014), and phosphorylation and thus activation of cGMP-specific phosphodiesterase type 5 (PDE5) which results in cGMP degradation (Egbert, Uliasz et al. 2016). There are also evidences showing that activation of the epidermal growth factor receptor (EGFR) signalling within the granulosa cells (described in the next pages) may reduce cGMP levels, similar to the LHR effect (Norris, Ratzan et al. 2009, Vaccari, Weeks et al. 2009).

As a result of cGMP drop in the somatic compartment, cGMP levels also fall in the oocyte allowing PDE3A to become active and degrade cAMP (Fig. 9) (Schultz, Montgomery et al. 1983, Vivarelli, Conti et al. 1983, Richard, Tsafriri et al. 2001). Loss of oocyte intracellular cAMP prevents PKA activity which in turn causes both Wee1B and CDC25B dephosphorylation (Solc, Saskova et al. 2008, Oh, Han et al. 2010) letting CDK1 to become active and trigger meiotic resumption. From the other side, loss of PKA activity results in CHD1 degradation causing cyclin B1 accumulation which may also contribute to maturation re-initiation (Mitra and Schultz 1996, Reis, Chang et al. 2006, Reis, Madgwick et al. 2007).

Even though LH surge is the physiological factor which triggers meiotic resumption *in vivo*, fully grown oocyte removal from the pre-ovulatory follicle under *in vitro* conditions, causes the same effect as LH and induces spontaneous meiotic resumption (Pincus and Enzmann 1935). Furthermore, studies have shown that addition of exogenous NPPC to the culture environment of cumulus-oocyte complexes maintains oocytes meiotic arrest (Franciosi, Coticchio et al. 2014). Collectively, it could be interpreted that either *in vivo* or *in vitro* deprivation of cumulus-oocytes complexes from the source of NPPC, the essential signal for cGMP-production, manifests as meiotic resumption.

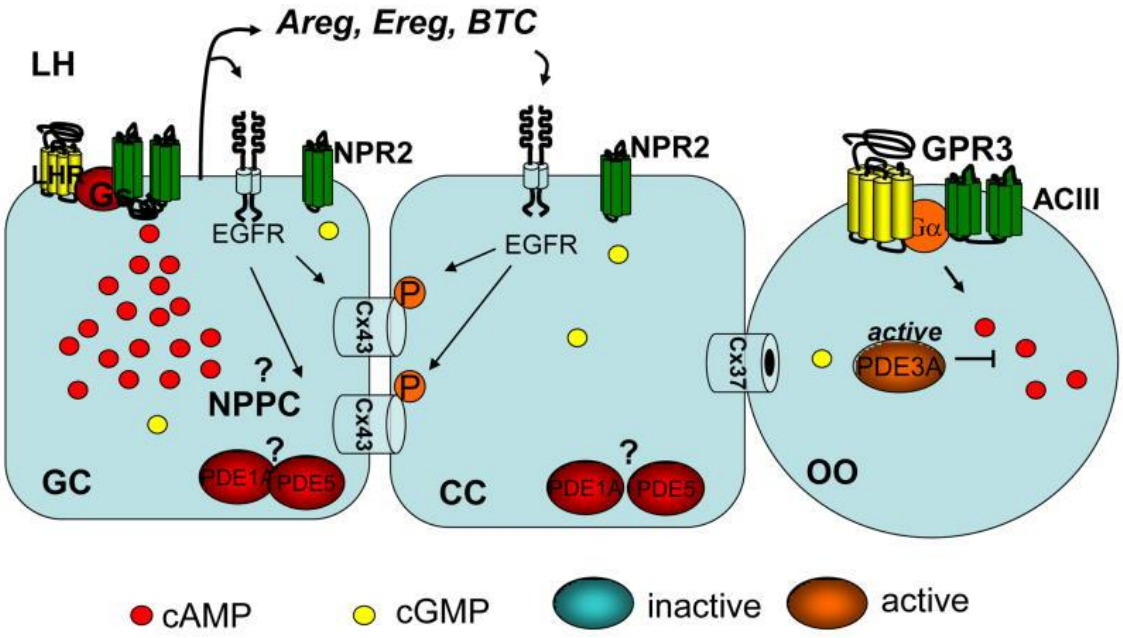

Figure 9: Molecular network triggering meiotic resumption

Interaction between LH and LH receptor on the surface of mural cells, launches multiple molecular events which together result in a significant decrease in the population of cGMP molecules. Consequently, less cGMP is transferred to the oocyte which allows for the PDE3A activity and thus cAMP degradation which in turn triggers meiotic resumption. (Adapted from (Conti, Hsieh et al. 2012)).

1.8.2.1.2.2 EGF-ERK1/2 mediation of LH signalling

It has long been known that addition of exogenous EGF to the culture system of antral follicles induces oocyte meiosis (Dekel and Sherizly 1985) and even enhances the quality of *in vitro* matured oocytes resulting in a higher rate of embryo development (De La Fuente, O'Brien et al. 1999). However, whether EGF physiologically exists in the pre-ovulatory follicles or not was still uncertain for many years (Westergaard and Anhdersen 1989, Reeka, Berg et al. 1998, Hsieh, Zamah et al. 2009).

Based on the recent studies performed in various species including mouse, rat, monkey, chicken, pig, mare and human, we now know that following the stimulation of LHR signalling and through its downstream cAMP-dependent PKA activity, a wave of instant but temporary production of EGF-like growth factors takes place within the mural cells of pre-ovulatory follicles (Fig. 10). These factors include Amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) which share an EGF-like motif and are able to bind to EGFR (also known as ErbB1) and other members of the ErbB receptor family (Park, Su et al. 2004, Sekiguchi, Mizutani et al. 2004, Ashkenazi, Cao et al. 2005, Fru, Cherian-Shaw et al. 2007, Wang, Li et al. 2007, Chen, Zhou et al. 2008, Lindbloom, Farmerie et al. 2008, Inoue, Miyamoto et al. 2009, Zamah, Hsieh et al. 2010). These EGF-like growth factors are released from mural granulosa cells into the follicular fluid and bind EGF receptors expressed at the surface of mural and cumulus cells in autocrine and paracrine fashion, respectively (Fig. 10) (Chabot, St-Arnaud et al. 1986, El-Danasouri, Frances et al. 1993, Singh, Rutledge et al. 1995, Göritz, Jewgenow et al. 1996, Hill, Hammar et al. 1999, Zwick, Hackel et al. 1999, Gall, Chene et al. 2004, Park, Su et al. 2004).

In both mural and cumulus cells, activated EGFR signalling transduces its message to the entire cell through phosphorylating and therefore activating ERK1/2 pathway (Fig. 10) (Keel, Hildebrandt et al. 1995, Cameron, Foster et al. 1996, Su, Wigglesworth et al. 2002, Su, Denegre et al. 2003, Tajima, Dantes et al. 2003, Sela-Abramovich, Chorev et al. 2005, Li, Liang et al. 2008). So far, studies have shown two routes through which activated ERK1/2 contribute to meiotic resumption: by inducing expression of prostaglandin E2 (PGE2) in both mural and cumulus cells which additionally elevates the biosynthesis of EGF-like factors within mural cells and consequently multiplies LH effect (Fig. 10) (Ben-Ami, Freimann et al. 2006); by preventing cGMP transfer to the oocyte via closing gap junctions (Fig. 10) (Sela-Abramovich, Chorev et al. 2005, Norris, Freudzon et al. 2008). Whether other molecular mechanisms may exist downstream of EGFR-activated ERK1/2 that participate in stimulating meiotic re-entry is remained to be investigated (Richards and Ascoli 2018).

Furthermore, under the influence of activated ERK, the expression level of certain genes including *Ptgs2*, *Has2*, and *Tnfaip6* becomes increased within the cumulus cells which consequently promotes the process of cumulus expansion (Fig. 10) (Sirois, Simmons et al. 1992, Fülöp, Salustri et al. 1997, Davis, Lennard et al. 1999, Varani, Elvin et al. 2002, Ochsner, Day et al. 2003, Sugiura, Su et al. 2009).

Cumulus expansion is another key event which occurs in parallel with meiotic maturation around the onset of ovulation and in response to LH surge (Sánchez and Smitz 2012). This process is characterized by two main changes in the structure of the oocyte-cumulus complex. First, cumulus cells which are organized in the form of compact layers around the oocyte prior to the

LH surge, become physically dispersed and distanced from the oocyte in response to the LH surge. This requires morphological changes in the cumulus cells which are mediated by cytoskeletal remodelling (Wert and Larsen 1989, Šutovský, Fléchon et al. 1993, Šutovský, Fléchon et al. 1995). Second, an extracellular matrix mainly composed of glycosaminoglycan hyaluronan becomes secreted from the cumulus cells and assembled around the oocyte. This mucifies the ovulated mass of the oocyte and expanded cumulus cells and thus facilitates its later transportation along the oviduct (Odor 1960, Eppig 1981, Salustri, Yanagishita et al. 1989, Chen, Russell et al. 1993, Fülöp, Salustri et al. 1997, Salustri, Camaioni et al. 1999, Lam, Gieseke et al. 2000).

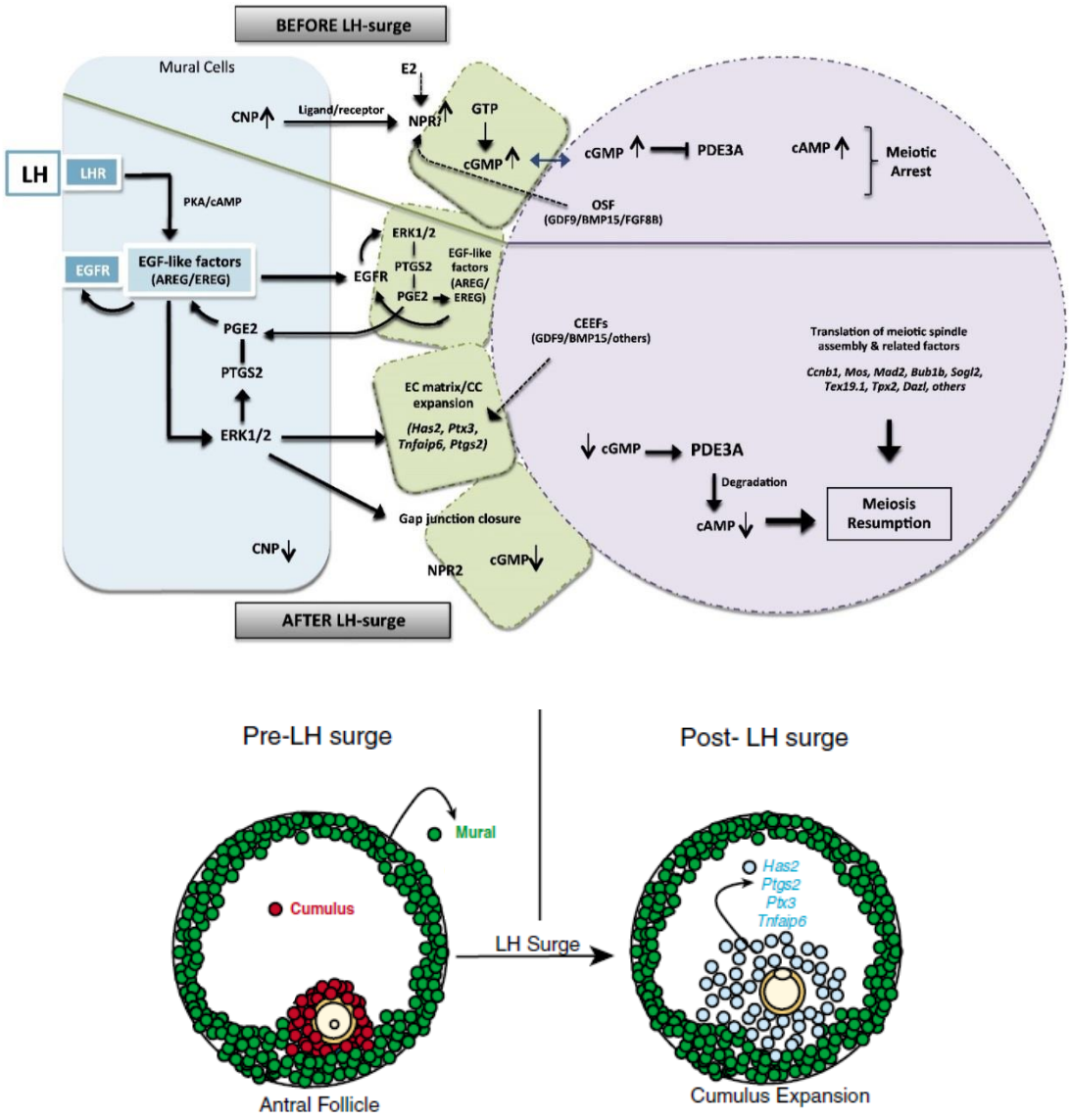

Figure 10: EGF-ERK transduction of LH effect

At the time of ovulation and within the mural granulosa cells, LH surge through cAMP dependent PKA activity induces the synthesis of EGF-like growth factors including AREG and EREG. These factors then become released into the follicular fluid and bind their receptors, EGFR, on the surface mural and cumulus granulosa cells. EGFR signalling activates ERK1/2 pathway in both cell types which itself inserts three main effects: i) induces the expression of *Has2*, *Tnfaip*, *Ptx3* and *Ptgs2* genes within the cumulus cells which triggers cumulus expansion; ii) causes the gap junctions closure which prevents cGMP transfer to the oocyte and thus contributes to meiotic resumption; iii) elevates PGE2 production in both mural and cumulus granulosa cells which in turn stimulates more synthesis and secretion of EGF-like growth factors and thus amplifies LH signalling (Adapted from (Sánchez and Smitz 2012) [upper figure] and (Diaz, Wigglesworth et al. 2007) [lower figure]).

1.8.2.2 Cytoplasmic events

Oocyte cytoplasmic maturation comprises many aspects, including most notably regulation of the mRNAs population. On one hand, some transcripts whose translated proteins takes part in the maintenance of meiotic arrest, such as CDH1, may become degraded or translationally impaired. On the other hand, some transcripts whose translated proteins are required for meiotic resumption, become translationally activated (Cui, Li et al. 2007, Su, Sugiura et al. 2007, Chen, Melton et al. 2011, Holt, Tran et al. 2011). A genome-wide analysis of oocyte translational activity during oocyte meiotic maturation has shown that while nearly 7600 transcripts undergo translation throughout this period, many other transcripts remain silent either due to degradation or translational inhibition (Chen, Melton et al. 2011). Inappropriate maintenance, degradation, or silencing of the maternal transcripts within the cytoplasm lead to oocyte incompetency to pass through the normal process of meiotic maturation.

1.9 Germline-Soma bidirectional communication

Oocyte survival, function and development during all the stages of postnatal fulliculogenesis are dependent on close communication with the surrounding granulosa cells. This contact is not unidirectional, necessarily from the granulosa cells side to the oocyte. In fact, there are pieces of evidence which indicate that oocyte is actively involved in regulating all aspects of granulosa cells development (Brower and Schultz 1982, Eppig 1991, Eppig, Chesnel et al. 1997, De La Fuente and Eppig 2001, Eppig 2001). Therefore, follicle development as a whole relies on bidirectional communication between the germline and somatic compartments.

1.9.1 Means of germline-soma communication

At least two means of intercellular communication set up this crosstalk between the oocyte and the granulosa cells: gap junctions and paracrine factors (Vanderhyden 2002).

1.9.1.1 Gap junctions

Gap junctions establish a unique form of communication between the oocyte and its immediate neighbouring granulosa cells through which granulosa cells send essential metabolites and regulatory signals to the oocyte aiming to control its growth and maturation throughout its development (Heller and Schultz 1980, Kidder and Vanderhyden 2010, Winterhager and Kidder 2015).

Gap junctions are channels made up of trans-membrane proteins, termed connexins, which physically link two neighbour cells and allow them to directly exchange molecules smaller than 1 kDa between themselves (Koval, Molina et al. 2014, Evans 2015). Connexins belong to a family whose members are encoded by nearly 20 different genes. Depending on the type of connexins used in the structure of a gap junction, the features of the developed cell-cell contact varies (White, Paul et al. 1995).

Multiple types of connexins are produced by oocytes and granulosa cells; however, connexins 37 and 43 have been reported as the most predominant ones present in the structure of oocytes and granulosa cells gap junctions, respectively (Simon, Goodenough et al. 1997, Ackert, Gittens et al. 2001, Gittens, Mhawi et al. 2003, Gittens, Barr et al. 2005). Ovarian follicles of mouse models lacking connexion 43 are incapable of developing beyond the early preantral stage while

connexion 37 deficiency impairs the normal processes of ovulation and luteinisation, and both mutant mouse models demonstrate severe infertility (Simon, Goodenough et al. 1997, Juneja, Barr et al. 1999). These findings indicate that the existence of functional gap junctional coupling between granulosa cells themselves and also between granulosa cells and oocytes are essentially required to ensure normal and healthy follicle development and thus fertility.

1.9.1.1.1 gap junctions are located at the tips of transzonal projections

Before the initiation of follicle growth, at the primordial stage, there is no physical space between the oocyte and its neighbouring granulosa cells. Thus, the plasma membranes of the two cell types directly face each other and gap junction channels as well as adherens junctions assemble at the contact site of the membranes and mediate the cell-cell attachment. The predominant proteins which participate in building up adherens junctions are E-cadherin from the oocyte side and N-cadherin from the granulosa cell side (Mora, Fenwick et al. 2012, Clarke 2018).

As the primordial follicle exits the dormancy state and enters the growth phase, the oocyte secrets an extracellular matrix named the zona pellucida which coats all around the oolemma (Fig. 11) (Chiquoine 1960, Wassarman and Litscher 2013, Wassarman and Litscher 2013). The zona pellucida, composed of either three (in mice) or four (in primates) types of glycoproteins named ZP1, ZP2, ZP3, ZP4, thickens in parallel with oocyte increase in size and eventually reaches a maximum thickness of 7 μm in mice and 15 μm in human. The biological roles of zona pellucida are to physically protect the oocyte and pre-implantation embryo from any environmental damage, to mediate sperm contact with the oocyte, and to prevent polyspermy at

fertilization (Wassarman 1988, Wassarman and Mortillo 1991, Dean 1992, Green 1997, Avella, Xiong et al. 2013, Li and Albertini 2013).

However, the formation of the zona pellucida also imposes a physical barrier between the oocyte and the surrounding granulosa cells which keeps them separated from each other. To overcome this barrier, granulosa cells project thin cytoplasmic filaments termed transzonal projections (TZPs) which pass through the thickness of the zona pellucida and anchor on the oocyte plasma membrane (Fig. 11). In terms of morphology, dimensions, and structure, TZPs resemble filopodia (Albertini, Combelles et al. 2001, Li and Albertini 2013) and have been identified for decades (Hadek 1965) not only in all studied mammals including dog, sheep, cow, pig, and human (De Smedt and Szöllösi 1991, Albertini and Rider 1994, Motta, Makabe et al. 1994, de Lesegno, Reynaud et al. 2008, Yi, Nagyova et al. 2008, Makita and Miyano 2014) but also in various non-mammalian vertebrates such as starfish (Perry, Gilbert et al. 1978, Schroeder 1981, Browne and Werner 1984). This highlights the evolutionary conservation of these structures as the only means of physical contact between the oocyte and granulosa cells (Clarke 2018).

Gap junctions are assembled at the tips of TZPs (Fig. 11), and this allows TZPs to play their most crucial role which is providing a platform for direct exchange of molecules between the oocyte and granulosa cells (Albertini, Combelles et al. 2001, Li and Albertini 2013). This also suggests that, during the oocyte growth period, gap junctional communication between the two cell types is fully dependent on the existence and function of TZPs. Not only gap junctions, but also adherens junctions are located at the tips of TZPs (Fig. 11). This enables TZPs to play a second important role which is attaching the plasma membrane of the granulosa cells to that of

the oocyte, thus holding the "granulosa-oocyte complex" as an integrated unit and preventing it from falling apart (Mora, Fenwick et al. 2012).

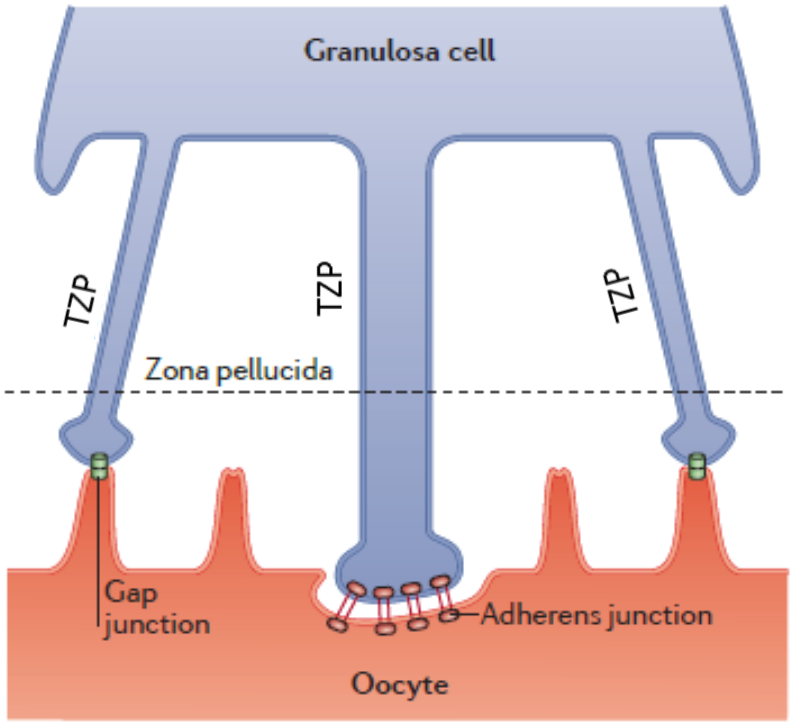

Figure 11: Germline-soma physical contact

Formation of the zona pellucida layer between the oocyte and its surrounding granulosa cells physically separates the two cell types. To overcome this barrier, granulosa cells project thin cytoplasmic filaments termed TZPs (transzonal projections) which pass through the thickness of the zona pellucida and contact the oocyte plasma membrane. Adherens and gap junctions present at the tips of TZPs ensure communication and attachment between the oocyte and its neighbouring granulosa cells. (Adapted and modified from (Li and Albertini 2013)).

So far, two types of TZPs have been reported based on their cytoskeleton structure: actin- and tubulin- rich TZPs. The majority of TZPs population is represented by actin-rich TZPs while tubulin-rich TZPs are only rarely detected (Clarke 2018). The backbone of actin rich TZPs is made up of the parallel bundles of actin filaments which align to the TZP length and highlights their structural resemblance to filopodia (Faix, Breitsprecher et al. 2009). Therefore, the actin filament binding component, phalloidin, is typically used as a marker to detect these type of TZPs (Barrett, Shea et al. 2010, Li and Albertini 2013, Macaulay, Gilbert et al. 2014, Makita and Miyano 2014, McGinnis and Kinsey 2015).

The molecular mechanism regulating TZPs formation or maintenance is not well understood. Discoveries in 1998 showed that lack of growth-differentiation factor (GDF9), an oocyte-derived paracrine factor targeting granulosa cells, induces a decrease in TZPs numbers and also disturbs the normal pattern of TZPs alignment (Carabatsos, Elvin et al. 1998). Recently, it has been revealed that, under the regulation of GDF9 signalling, TZP numbers increase significantly during the oocyte growth period, which provides more germline- soma communication and thus enables more nutrient transfer from the granulosa cells to the oocyte to support its growth (El-Hayek, Yang et al. 2018). However, the fate of TZPs during oocyte meiotic maturation remains to be studied.

1.9.1.2 Paracrine factors

Germline-soma communication is not limited to the direct transfer of small molecules through the gap junction channels. In fact, intrafollicular paracrine factors secreting from one cell type

and interacting with their matched receptors present on the opposite cell type also play important roles in this germline-soma crosstalk (Kidder and Vanderhyden 2010).

Although it is well-established that granulosa cells support oocyte development by transferring several essential nutrients and regulatory molecules to it via gap junctions (Fig. 12), very limited paracrine factors have been reported to be released from the granulosa cells and signal to the oocyte (Kidder and Vanderhyden 2010). Among these few factors, the most well-known is KIT ligand (KITL), which binds its tyrosine kinase receptor, KIT, expressed on the surface of the oocyte (Fig. 12) (Manova, Nocka et al. 1990, Hutt, McLaughlin et al. 2006, Thomas and Vanderhyden 2006, Kidder and Vanderhyden 2010). This interaction subsequently activates a series of molecular events within the oocyte which trigger initiation of its growth, as discussed earlier (Parrott and Skinner 1999, Jin, Han et al. 2005).

As also explained before, germline-soma communication within the ovarian follicle is not exclusive to a unidirectional contact from granulosa cells side to the oocyte. Indeed, oocyte actively synthesize and secret paracrine factors, most studied GDF9 and BMP15, which couple with their receptors on the surface of the granulosa cells (Fig. 12). This coupling regulates many different aspects of granulosa cells development and function including KITL expression as well as steroidogenesis (McGrath, Esquela et al. 1995, Dube, Wang et al. 1998, Elvin, Clark et al. 1999, Teixeira Filho, Baracat et al. 2002, Kidder and Vanderhyden 2010). This subject will be explained in more details in the following pages.

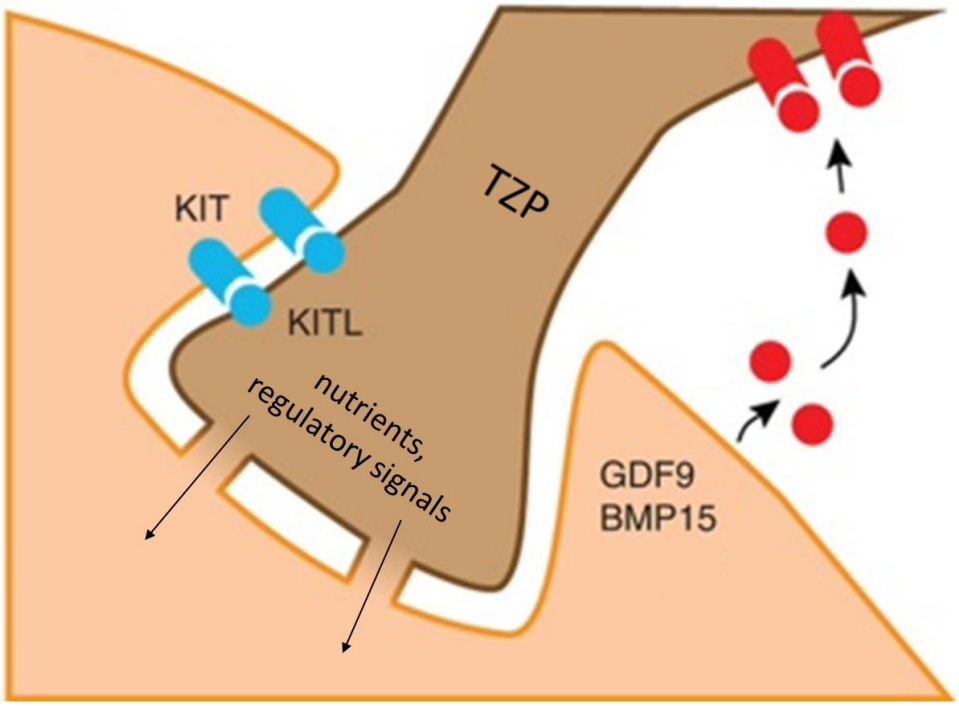

Figure 12: Germline-soma bidirectional communication

Bidirectional communication between the oocyte and its surrounding granulosa cells is mediated by two means: i) gap junctions which allow for the direct transfer of nutrients and regulatory signals from the granulosa cells to the oocyte in order to regulate oocyte normal development; ii) paracrine factors, most known GDF9 and BMP15, which are produced and secreted by the oocyte and bind their matched receptors on the surface of the granulosa cells. This ligand-receptor interaction regulates multiple aspects of granulosa cells development.

Furthermore, binding of the Kit ligand (KITL) produced by the granulosa cells to its receptor KIT expressed on the oocyte membrane triggers the initiation of oocyte growth. (Adapted and modified from (Clarke 2018)).

1.9.2 Role of granulosa cells in controlling oocyte development

1.9.2.1 Granulosa cells regulation of oocyte growth

1.9.2.1.1 Growth Initiation

It has long been known that the initiation of oocyte growth within the dormant primordial follicle is induced by its surrounding granulosa cells (Lintern-Moore and Moore 1979). More recently, it was specifically demonstrated that KIT ligand (KITL) released from the granulosa cells and binding its receptor KIT on the oocyte surface is responsible for triggering oocyte growth, through activation of PI3K/AKT signaling pathway in the oocyte (Reddy, Shen et al. 2005, Liu, Rajareddy et al. 2006, Reddy, Liu et al. 2008).

1.9.2.1.2 Growth progression

There is some uncertainty regarding the role of KITL in promoting oocyte growth. For example, even though it has been shown that FSH induces *Kitl* expression within the granulosa cells of preantral follicles, oocytes are still able to grow independently of FSH (Demeestere, Streiff et al. 2012). It has also been shown although function-blocking antibodies against KITL disturb the normal process of follicle development, they still allow for the continuation of oocyte growth (Yoshida, Takakura et al. 1997, Parrott and Skinner 1999). This implies that KITL antibodies probably impair follicle development by preventing the activation of KIT receptors which are expressed on the ovarian interstitial cells in addition to the oocyte (Yoshida, Takakura et al. 1997, Eppig 2001). On the other hand, some studies have indicated that addition of KITL to the culture system accelerates oocyte *in vitro* growth (Packer, Hsu et al. 1994), as well as that the absence of KITL in mutant mouse models not only interferes with follicle development but also decreases oocyte growth (Huang, Manova et al. 1993, Bedell, Brannan et al. 1995). Still other

data suggest that KITL does not affect oocyte growth but improves its cytoplasmic maturation (Reynaud, Cortvrindt et al. 2000).

Altogether, these observations imply an important but complex interaction between KITL released from the granulosa cells and KIT receptor expressed on the oocyte throughout follicle development, although the precise role of KITL in progression of oocyte growth remains unknown. Apart from KITL-mediated paracrine signalling, granulosa cells regulate oocyte growth through gap junctional communications, as well. In fact, since oocyte is a large spherical shaped cell, the ratio of its plasma membrane area to its cytoplasm volume is very low. Therefore, oocyte plasma membrane may not provide an adequate surface for the entrance of all essential nutrients to the oocyte and the exit of all metabolic wastes form it. Here, the plasma membrane of the granulosa cells unites with the plasma membrane of the oocyte via gap junctions and this event results in an increase in the overall exchange surface of the oocyte allowing for more nutrient uptake and waste excretion (Eppig 1991).

Moreover, gap junctions are responsible to establish metabolic cooperation between the oocyte and surrounding granulosa cells (Eppig 1991). Indeed, granulosa cells play crucial roles in supporting oocyte metabolism (Zamboni 1970, Gilula, Reeves et al. 1972, Brower and Schultz 1982) because oocytes themselves are incapable of covering all their metabolic needs (Biggers, Whittingham et al. 1967, Buccione, Cecconi et al. 1987). In other words, while oocyte growth is a highly energy-consuming process and thus insufficient energy in the form of ATP decreases its developmental competence (Van Blerkom, Davis et al. 1995, Igarashi, Takahashi et al. 2005, Zhang, Wu et al. 2006), mammalian oocytes lack the required machinery for metabolizing

glucose to produce ATP as their main source of energy and thus cannot independently afford their energy requirements. Fortunately, oocytes are able to directly use pyruvate, the mediating product of glycolysis, to generate ATP (Biggers, Whittingham et al. 1967, Rushmer and Brinster 1973, Eppig and Steckman 1976, Tsutsumi, Yano et al. 1990, Fagbohun and Downs 1992, Tsutsumi, Satoh et al. 1992, Zuelke and Brackett 1992, Downs and Mastropolo 1994, Sugiura, Pendola et al. 2005, Johnson, Freeman et al. 2007, Harris, Leese et al. 2009). Here again, granulosa cells play a supportive role by metabolizing glucose, producing pyruvate, delivering it to the oocyte through gap junctions, and thus allowing the oocyte to make its required ATP from the imported pyruvate (Donahue and Stern 1968, Brinster 1971, Leese and Barton 1985, Tsutsumi, Yano et al. 1990, Tsutsumi, Satoh et al. 1992, Zuelke and Brackett 1992, Rieger and Loskutoff 1994, Hérubel, El Mouatassim et al. 2002, Sugiura, Pendola et al. 2005).

As described before, during its growth, oocyte actively synthesizes and accumulates transcripts, proteins, and organelles. This requires a large supply of different types of amino acids within the oocyte. However, oocytes are incapable of importing specific types of amino acids including alanine, glycine, and proline from the extracellular environment to their cytoplasm. In contrast, granulosa cells are well equipped with an efficient transport system which allows them to uptake and possess the amino acids which oocyte lacks. Therefore, granulosa, once more, support the oocyte by transferring these certain amino acids to the oocyte via gap junctions, (Colonna and Mangia 1983, Haghighat and Van Winkle 1990) to ensure optimal development of the oocyte and future embryo.

The oocyte is also metabolically dependent on coupling with granulosa cells due to its inefficiency in generating cholesterol. Indeed, the expression levels of the essential enzymes for cholesterol biosynthesis such as farnesyl diphosphate synthase (*Fdps*) and cytochrome P450 *(Cyp)-51* are low in the oocyte, preventing it from self-producing cholesterol (Su, Sugiura et al. 2008). Additionally, the oocyte fails to import extracellular cholesterol because of lacking receptors for high density lipoprotein (HDL) cholesterol (Trigatti, Rayburn et al. 1999, Sato, Kawamura et al. 2003). Therefore, oocyte is neither able to synthesize cholesterol *de-novo* nor is able to uptake cholesterol from the extracellular environment, even though it needs large amounts of cholesterol to assure healthy embryo development (Comiskey and Warner 2007), particularly considering the fact that the pre-implantation embryo is not able to produce cholesterol (Pratt, Keith et al. 1980, Pratt 1982).

Granulosa cells, in contrast, are highly efficient in cholesterol synthesis and thus fulfill oocyte requirement by transferring cholesterol molecules to the oocyte (Su, Sugiura et al. 2008). It is thought that, unlike pyruvate and amino acids, cholesterol is not transported from the granulosa cells to the oocyte through gap junctions, but more likely through the sites where plasma membrane of the two cell types are closely opposed to each other allowing for the direct conduction of cholesterol from the granulosa cells to the oocyte (Clarke 2018). In summary, it is concluded that oocyte full growth is crucially dependent on the constant and efficient communication with the adjacent granulosa cells, which ensures deposition of pyruvate, amino acids and cholesterol in the oocyte in preparation for normal embryo development.

1.9.2.2 Granulosa cells regulation of oocyte meiotic maturation

The detailed roles of granulosa cells in the maintenance of oocyte meiotic arrest as well as induction of meiotic resumption were discussed in the previous pages. Below, a brief summary of the mechanisms through which granulosa cells regulate oocyte meiotic maturation is described.

Within the primordial follicles, oocytes are arrested at the prophase of the first meiotic division and remain meiotically arrested until the LH surge which occurs closely before ovulation (Conti, Hsieh et al. 2012, Adhikari and Liu 2014). Prior to LH surge, mural granulosa cells of the antral follicles synthesize and secret C-type natriuretic peptide (CNP) (Zhang, Su et al. 2010, Kawamura, Cheng et al. 2011, Zhang, Su et al. 2011, Franciosi, Coticchio et al. 2014) into the follicular fluid which reaches and binds its receptor, natriuretic peptide receptor (NPR2) on the surface of both mural and cumulus granulosa cells. Activated NPR2 produces cyclic guanosine monophosphate (cGMP) which then inflows to the oocyte through the gap junctions (Norris, Ratzan et al. 2009, Zhang, Su et al. 2010). Within the oocyte, cGMP supresses phosphodiesterase 3A (PDE3A) which is a potent hydrolyzer of cyclic adenosine monophosphate (cAMP). As a result of cGMP-induced deactivation of PDE3A, cAMP remains at a high level within the oocyte (Vaccari, Weeks et al. 2009) which in turn keeps cyclin-dependent kinase 1 (CDK1) inactive preventing the oocyte from re-entry into the cell cycle (Holt, Lane et al. 2013).

However, around the ovulation time, preovulatory follicle faces a peak in the level of LH releasing from pituitary gland (Conti, Hsieh et al. 2012, Holt, Lane et al. 2013, Adhikari and Liu 2014, Coticchio, Dal Canto et al. 2015). Subsequently, LH binds and activates its receptor LHR

which is expressed on the mural granulosa cells but not cumulus granulosa cells or oocytes. LHR signalling results in a remarkable and rapid decrease in the cGMP level, which initiates from the mural cells, next observed in the cumulus cells and eventually happens in the oocyte (Shuhaibar, Egbert et al. 2015). Loss of cGMP in the somatic compartment of the pre-ovulatory follicles occurs via three different routes: degrading the existing cGMP population by activating cGMP hydrolyzer, phosphodiesterase 5A (PDE5A) (Egbert, Uliasz et al. 2016), suppressing *Nppc* expression (Kawamura, Cheng et al. 2011, Robinson, Zhang et al. 2012, Liu, Xie et al. 2014) and inhibiting NPR2 activity (Egbert, Shuhaibar et al. 2014, Shuhaibar, Egbert et al. 2016). The loss of cGMP in the oocyte is the consequence of cGMP molecules outflow from the higher gradient (oocyte) to the lower gradient (granulosa cells) to balance the cGMP concentration between the two cell types (Clarke 2018) . As the oocyte loses its cGMP supply, PDE3A becomes active and degrades cAMP (Schultz, Montgomery et al. 1983, Vivarelli, Conti et al. 1983, Richard, Tsafriri et al. 2001) leading to CDK1 activation and re-initiation of the meiotic maturation (Holt, Lane et al. 2013).

As also discussed before, activation of LHR signalling within the mural granulosa cells provokes the secretion of EGF-like growth factors from these cells which bind and activate their receptor EGFR on the surface of both mural and cumulus granulosa cells. On the other hand, the role of EGFR signalling in triggering meiotic maturation is also well known. Here, this question is raised that whether LHR signalling triggers meiotic maturation directly and independently, or indirectly through EGFR cascade. However, the answer to this question is still uncertain and remains to be studied, even though there are some recent pieces of evidence that suggest probably the two pathways in cooperation with each other promote meiotic resumption (Conti,

Hsieh et al. 2012, Egbert, Shuhaibar et al. 2014, Liu, Xie et al. 2014, Shuhaibar, Egbert et al. 2015, Jaffe and Egbert 2017).

1.9.2.3 Granulosa cells regulation of oocyte transcriptional activity

Quantitative analyses of the oocyte global transcriptional activity have demonstrated that the presence of the surrounding granulosa cells is required to ensure the normal stage-specific pattern of oocyte transcription throughout its development. In fact, granulosa cells induce the elevation and subsequent suppression of the transcription in growing and fully-grown oocytes, respectively, and this accurate pattern is disturbed if the oocytes are isolated from their companion granulosa cells. However, the molecular mechanisms through which granulosa cells influence the transcription rhythm of oocyte genome are not yet known although the involvement of gap junctional communications is being investigated (De La Fuente and Eppig 2001).

1.9.3 Role of oocyte in controlling granulosa cells development and function

The initial concept of oocyte impact on granulosa cell development arose from studies in which oocyte removal from the pre-ovulatory follicle triggered rapid luteinisation of the remaining granulosa cells, an event that under the physiological conditions takes place only after the oocyte leaves the follicle through the ovulation process. These observations suggested that oocyte presence prevents luteinisation, raising the possibility that the oocyte might secrete a factor(s) that contributes to regulation of some processes within the granulosa cells (El-Fouly, Cook et al. 1970, Nekola and Nalbandov 1971, Hubbard and Erickson 1988). Later, taking advantage of oocytectomy technique, which refers to the oocyte removal from the oocyte-cumulus complex *in vitro*, several oocyte-secreted factors have been identified that signal to the granulosa cells in a

paracrine fashion (Buccione, Cecconi et al. 1987, Vanderhyden, Cohen et al. 1993, Vanderhyden and Tonary 1995, Vanderhyden and Macdonald 1998) including the factors which suppress the expression of LH receptors on the granulosa cells thus likely lead to inhibition of granulosa cells luteinisation (Eppig, Wigglesworth et al. 1997). However, no trace of gap junctional signalling from the oocyte towards the granulosa cells has been found so far (Gilchrist, Lane et al. 2008, Su, Sugiura et al. 2008).

Interestingly, oocyte derived paracrine factors GDF9 and BMP15 show very similar behaviour to that of oocyte in regulating granulosa cells activities. This allows them to be considered as potential candidates for transferring oocyte signals to the granulosa cells (McGrath, Esquela et al. 1995, Dube, Wang et al. 1998, Elvin, Clark et al. 1999, Teixeira Filho, Baracat et al. 2002). Similar to other members of the TGFβ superfamily, GDF9 and BMP15 are dimeric pro-peptides which bind and activate their corresponding serine–threonine kinase receptors. Downstream of receptor activation, the signal is transmitted throughout the target cell via SMAD signalling (Kaivo-oja, Jeffery et al. 2006, Peng, Li et al. 2013, Mottershead, Sugimura et al. 2015). Both GDF9 and BMP15 are expressed in the oocytes throughout the entire period of follicle development, from the primary stage up until ovulation (McGrath, Esquela et al. 1995, Dube, Wang et al. 1998).

Most of our knowledge regarding the specific roles of these factors in folliculogenesis is generated from the observations in transgenic mouse models. In *Gdf9* null mutant mice, follicles are blocked at the primary stage, and do not develop further leaving the females anovulatory and thus infertile. These phenotypes are also correspondent with the changes in the normal expression pattern of the key genes involved in follicle development. For example, it is reported

that transcription level of *Kitl* is decreased in the ovaries of these mutant mice (Dong, Albertini et al. 1996, Elvin, Clark et al. 1999, Elvin, Yan et al. 1999) which supports the role of GDF9 in KITL production and therefore highlights the involvement of oocyte in regulating its own development (Joyce, Pendola et al. 1999, Joyce, Clark et al. 2000).

In comparison with *Gdf9* deficient mice, *Bmp15* null females are only sub-fertile, suggesting that lack of BMP15 results in milder reproductive issues compared to that of GDF9 (Yan, Wang et al. 2001). Moreover, natural mutations in both genes have been reported in sheep. However, while either $Gdf9^{-/-}$ or $Bmp15^{-/-}$ females are infertile with folliculogenesis blockade at the primary stage, $Gdf9^{+/-}$ or $Bmp15^{+/-}$ demonstrate increased ovulation rate and are more efficient in reproduction (Galloway, McNatty et al. 2000, Mottershead, Sugimura et al. 2015). There is also evidence from human studies that *Gdf9* or *Bmp15* mutations are associated with female infertility (Chang, Qiao et al. 2016). Interestingly, it has even been shown that addition of recombinant rat GDF9 to the culture of human ovarian cortical fragments increased the number of primordial follicles which entered growth and developed up to the secondary stage. These findings suggest that GDF9 could be clinically used as a simple supplement to human ovarian tissue culture in order to improve the success rate of *in vitro* follicle development (Hreinsson, Scott et al. 2002).

Studies using specific antibodies or inhibitors against different components of GDF9 and BMP15 signaling pathways have revealed more information about how oocytes regulate granulosa cell function. GDF9 and BMP15 can both act as mitogenic factors that stimulate granulosa cells proliferation from the early stages of fulliculogenesis and cause the formation of multiple layers of granulosa cells (Hayashi, McGee et al. 1999, Otsuka, Yao et al. 2000, Vitt, Hayashi et al.

2000) and they both induce granulosa cell differentiation into the two subpopulations of cumulus and mural granulosa cells (Glister, Groome et al. 2003, Diaz, Wigglesworth et al. 2007).

Moreover, GDF9 particularly affects cumulus cells in two different ways: i) prevents FSH-induced estradiol synthesis and release via disabling aromatase enzymatic activity; ii) inhibits expression of LH receptors under the influence of FSH. BMP15, on the other hand, supports cumulus cells survival and also suppresses FSH effect on these cells by inhibiting their expression of FSHR (Elvin, Clark et al. 1999, Vitt, Hayashi et al. 2000, Otsuka, Yamamoto et al. 2001). Cooperation of GDF9 and BMP15 is also required for suppression of progesterone synthesis under the FSH stimulation within the granulosa cells, which not only protects the follicle against apoptosis but also prevents the precocious luteinisation process (Elvin, Clark et al. 1999, Yoshino, McMahon et al. 2006).

As mentioned before, oocyte-derived factors are also essential for the occurrence of cumulus expansion. GDF9 specifically increases the expression level of cumulus expansion associated genes, *Has2, Tnfaip6, Ptx3, and Ptgs2* within these cells (Elvin, Yan et al. 1999, Varani, Elvin et al. 2002), and other studies have also indicated the involvement of both GDF9 and BMP15 in the process of cumulus expansion (Dragovic, Ritter et al. 2005, Gui and Joyce 2005, Yoshino, McMahon et al. 2006).

So far, I discussed the significance of oocyte development and its dependency on functional communication with the surrounding granulosa cells. I now introduce a recently identified cascade, Hippo, which is specifically known for its role in controlling cell growth and development. The engagement of this pathway in regulation of oocyte growth constitutes the first part of this research thesis.

1.10 Overview of the Hippo Signaling Pathway

Hippo signalling is particularly known for its extensive involvement in regulating cell growth and proliferation. It was originally revealed in Drosophila through mosaic genetic screens but later identified as a highly evolutionarily conserved pathway across a wide range of species (Harvey, Zhang et al. 2013, Piccolo, Dupont et al. 2014, Varelas 2014, Moroishi, Hansen et al. 2015). The core of mammalian Hippo pathway is formed by serial phosphorylating events where Mammalian STE20-like protein kinases 1 and 2 (MST1/2) phosphorylate and activate Large tumor suppressor 1 and 2 (LATS1/2) (Chan, Nousiainen et al. 2005), which subsequently phosphorylate and inhibit their two downstream effectors: Yes-associated protein (YAP) and its highly related paralogue, Transcriptional coactivator with a PDZ-binding domain (TAZ) (Fig. 13) (Zhao, Wei et al. 2007, Lei, Zhang et al. 2008). The promotion of this cascade is facilitated by the mediation of Salvador family WW domain-containing protein (SAV) and MOB kinase activator (MOB1A/B) which bind and act as the co-factors of MST1/2 and LATS1/2, respectively (Fig. 13) (Callus, Verhagen et al. 2006, Praskova, Xia et al. 2008).

As transcriptional co-activators, YAP and TAZ are considered to be the key components of the Hippo pathway. When the Hippo pathway is on, YAP/TAZ become phosphorylated and remain

cytoplasmic and thus transcriptionally inactive. However, when the pathway is off, YAP/TAZ dephosphorylation allows them to accumulate in the nucleus and initiate their transcriptional co-activity (Fig. 13) (Piccolo, Dupont et al. 2014). Even though the role of TAZ in the canonical Hippo pathway is similar to YAP (Lei, Zhang et al. 2008), its features and activities have not been as deeply studied. Thus, description of YAP functions will be the main focus of the next few pages.

1.10.1 YAP regulation of gene expression

No specific DNA-binding domain has been identified in YAP; therefore, it apparently cannot directly attach to DNA to regulate gene expression. However, it could bind certain transcription factors and act as a coactivator to enhance their function. The four members of the TEA domain (TEAD) family (TEAD1-4) are the main transcription factors that YAP interact with (Fig. 13) (Ota and Sasaki 2008, Zhao, Ye et al. 2008, Liu-Chittenden, Huang et al. 2012, Diepenbruck, Waldmeier et al. 2014, Zanconato, Forcato et al. 2015). YAP-TEAD complex stimulates transcription of target genes, including *Myc*, whose translated proteins are mostly and directly involved in regulating cell cycle S-phase entry and mitosis (Piccolo, Dupont et al. 2014, Zanconato, Forcato et al. 2015).

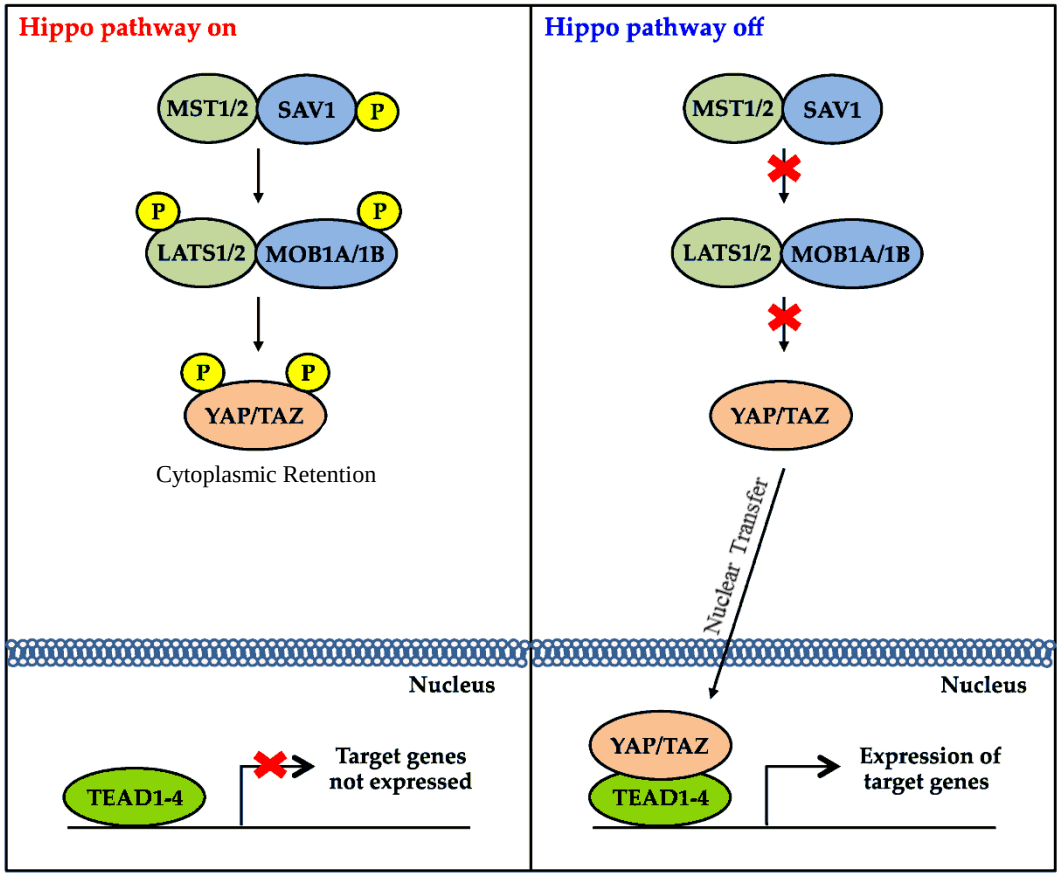

Figure 13: Hippo pathway at a glance

(Adapted and modified from (Juan and Hong 2016))

1.10.2 YAP regulation of cell growth

Studies in mice have shown that the mutations which disturb the Hippo pathway and thus lead to YAP dephosphorylation and activation, manifest as cell overgrowth. Multiple lines of evidence indicate that either MST1/2 or SAV deficiencies or YAP overabundance in hepatocytes causes liver overgrowth and tumorigenesis (Camargo, Gokhale et al. 2007, Dong, Feldmann et al. 2007, Zhou, Conrad et al. 2009, Lee, Lee et al. 2010, Lu, Li et al. 2010, Song, Mak et al. 2010). Similarly, in cardiomyocytes, either YAP ectopic expression or Hippo pathway impairment due to the lack of MST1/2, SAV, or LATS1/2, result in uncontrollable division of cardiomyocytes which is evident by oversized heart (Song, Mak et al. 2010, Heallen, Zhang et al. 2011, von Gise, Lin et al. 2012, Xin, Kim et al. 2013). Furthermore, pancreas-specific deletion of *Mst1/2* induces overgrowth of pancreas exocrine compartment (George, Day et al. 2012, Gao, Zhou et al. 2013) while Mst1/2 absence in the intestinal epithelium allows for overactivation of YAP which subsequently elevates the proliferation rate of intestinal stem cells and imposes colon cancer (Zhou, Zhang et al. 2011).

In addition to its role in regulation of cell numbers, YAP is also involved in controlling cell size, and these two processes take place through independent circuits downstream of YAP. For example, it has been shown in HEK293 cells that overexpression of a mutant form of YAP (YAP5SA) which is not able to become phosphorylatable by LATS1/2 and thus constantly dephosphorylated and active, induces an increase in both cell size (hypertrophy) and cell number (hyperplasia) (Mugahid, Kalocsay et al. 2018). In contrast, deletion of *Yap* from HEK293A cells remarkably reduces the cell volume while deletion of *Lats1/2* results in significantly bigger cells comparing to the wild type (Plouffe, Lin et al. 2018). Furthermore, studies have shown that

mechanical overload in skeletal muscle cells stimulates physiologic overexpression of YAP which manifests as hypertrophy of theses cells (Goodman, Dietz et al. 2015). Interestingly, in cardiomyocyte samples from hypertrophic cardiomyopathy (HCM) patients, expression levels of YAP mRNA and protein are increased, while MST1 and phosphorylated (inactive) form of YAP are decreased significantly. Consistently, HCM mouse models exhibit increase in YAP protein level but decrease in YAP phosphorylation. Moreover, in mouse cultured cardiomyocytes, overexpression of human YAP leads to increase in cell size and whereas YAP knockout decreases the cell size (Wang, Mao et al. 2014). Altogether, these findings indicate that YAP is actively involved in regulation of both cell size and cell proliferation, situating it as a crucial protein in controlling tissue and organ size.

Rationale, Objective, and Hypothesis

1- Based on the facts that a) YAP plays a crucial role in promoting cell growth and organ development in a wide range of somatic cell types, and b) oocyte increases in size dramatically throughout its development, we investigate the activity and role of YAP in regulating oocyte growth which has not been yet studied. We hypothesize that YAP may play a role(s) in promoting the growth of oocyte, as it does in many somatic cell types.

2- Knowing that a) oocyte growth is a critical phase of its development which is essential for female fertility b) oocyte growth is highly dependent on receiving certain nutrients from its surrounding granulosa cells via gap junctions c) gap junctions are placed at the tips of filopodia-like structures termed TZPs which are the only means of physical attachment between the oocyte and its neighbouring granulosa cells d) TZPs are present around the meiotically immature fully grown oocyte but disappear as the fully grown oocyte enters meiotic maturation, we set out to study the molecular mechanisms regulating TZPs maintenance and loss which is completely unknown. Since TZPs maintenance and retraction occur simultaneously with oocyte meiotic arrest and resumption, respectively, we hypothesize that the same molecular pathway which regulates oocyte meiotic state may also regulate TZPs dynamics.

Chapter 2

MANUSCRIPT I

Multiple Mechanisms Cooperate to Constitutively Exclude the Transcriptional Co-Activator YAP from the Nucleus During Murine Oogenesis

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Preface

YAP, as a core component and the main downstream effector of the Hippo pathway, is well known for its roles in the context of "growth and development". In fact, YAP is a transcriptional co-activator that upon reaching the genome within the nucleus, in cooperation with other factors, induces the expression of specific genes which promote cell growth and proliferation as well as organ size and development. Dephosphorylation of YAP at the specific residue of serine-112 in mouse and -127 in human is its entry permit to the nucleus and thus enables it to perform its transcriptional co-activity (Basu, Totty et al. 2003, Dong, Feldmann et al. 2007, Zhao, Wei et al. 2007, Piccolo, Dupont et al. 2014, Oku, Nishiya et al. 2015, Zanconato, Forcato et al. 2015). From another point of view, oocyte developmental competence which is the necessary condition for female fertility is firmly linked with the concept of oocyte growth, since the oocyte normally undergoes more than 100-fold increase in size during its journey of development (Eppig and O'brien 1996, Picton, Briggs et al. 1998, Sánchez and Smitz 2012, Clarke 2018). This background knowledge provides a rationale to hypothesize that YAP may also play a role(s) in promoting the growth of oocyte, similar to many somatic cell types. To test this, the following objectives were designed in mice:

Ia) Systematically investigate expression, serine-112 phosphorylation, and subcellular localization of YAP during pre- and postnatal oocyte development.

Ib) Identify the molecular mechanisms which regulate YAP subcellular localization during oocyte growth.

This work is discussed in Chapter 2 (Manuscript I) which contains the published work with slight modifications.

Significance statement

The growing incidence of female infertility linked to age, disease or disease therapies has sparked intensive efforts to develop clinical interventions that can promote normal oocyte development *in vivo* or *in vitro*. Recent studies have shown that subjecting ovarian fragments to certain therapeutic interventions that are reported to trigger the translocation of YAP to oocyte nuclei can induce oocytes of primordial follicles to enter the growth phase, and even newborn infants have been obtained in mouse (Kawamura, Cheng et al. 2013, Cheng, Feng et al. 2015). This identifies a potential method of fertility preservation or rescue in women. Yet, no study has so far investigated the physiological role of nuclear YAP in oocyte development of any species. However, our results which were obtained under *in vivo* conditions demonstrate the robust exclusion of YAP from oocyte nucleus throughout pre- and post-natal oogenesis. Moreover, we found that YAP is constitutively restricted to the cytoplasm of granulosa cells at different stages of follicle development. Therefore, our work provides strong evidences that nuclear YAP does not play a physiological role neither in oocyte growth nor in follicle development. Accordingly, our data call for caution in developing techniques based on YAP nuclear activation to induce human oocyte growth.

Abstract

Reproduction depends on the generation of healthy oocytes. Improving therapeutic strategies to prolong or rescue fertility depends on identifying the inter- and intracellular mechanisms that direct oocyte development under physiological conditions.Growth and proliferation of multiple cell types is regulated by the Hippo signaling pathway, whose chief effectors are the transcriptional co-activator YAP and its paralogue WWTR1. To resolve conflicting results concerning the potential role of Hippo in mammalian oocyte development, we systematically investigated the expression and localization of YAP in mouse oocytes. We report that YAP is expressed in the germ cells beginning as early as Embryonic Day 15.5 and subsequently throughout pre- and postnatal oocyte development. However, YAP is restricted to the cytoplasm at all stages. YAP is phosphorylated at serine-112 in growing and fully grown oocytes, identifying a likely mechanistic basis for its nuclear exclusion, and becomes dephosphorylated at this site during meiotic maturation. Phosphorylation at serine-112 is regulated by a mechanism dependent on cyclic AMP and protein kinase A, which is known to be active in oocytes prior to maturation. Growing oocytes also contain a subpopulation of YAP, likely dephosphorylated, that is able enter the oocyte nucleus, but it is not retained there, implying that oocytes lack the cofactors required to retain YAP in the nucleus. Thus, although YAP is expressed throughout oocyte development, phosphorylation-dependent and -independent mechanisms cooperate to ensure that it does not accumulate in the nucleus. We conclude that nuclear YAP does not play a significant physiological role during oocyte development in mammals.

Introduction

Reproduction depends on the generation of healthy oocytes that are able to develop as embryos following fertilization. Identifying inter- and intracellular mechanisms that control and direct oocyte development has been a focus of intensive research, with the aim of applying this knowledge to design and improve recent therapeutic innovations, including activation of oocytes in primordial follicles to enter the growth pool (Li, Kawamura et al. 2010, Adhikari, Gorre et al. 2012, Monniaux, Clément et al. 2014, Novella-Maestre, Herraiz et al. 2015, Sun, Su et al. 2015), growth of oocytes in vitro (Smitz, Dolmans et al. 2010, Telfer and McLaughlin 2011, Telfer and Zelinski 2013, Brito, Lima et al. 2014, Shea, Woodruff et al. 2014, Woodruff 2015), and generation of oocytes from pluripotent stem cells (Hayashi, Ogushi et al. 2012, Handel, Eppig et al. 2014), whose common goal is to preserve fertility. For example, after the key role of PTEN (phosphatase and tensin homolog deleted on chromosome 10) activity in the oocyte in maintaining its quiescent state in primordial follicles was uncovered (Reddy, Liu et al. 2008, Jagarlamudi, Liu et al. 2009), pharmacological inhibitors of PTEN were successfully used to activate growth of primordial follicles (Li, Kawamura et al. 2010, Adhikari, Gorre et al. 2012, Novella-Maestre, Herraiz et al. 2015), thereby generating a potential supply of oocytes that can be used for fertilization. The widespread introduction and efficient application of these new reproductive therapies, particularly in the context of increasingly common conditions such as primary ovarian insufficiency and reproductive aging, will depend crucially, however, on a more complete and comprehensive understanding of the function of different signaling pathways during oocyte differentiation, growth, and meiotic maturation. The Hippo pathway, originally identified in Drosophila and named for the overgrowth phenotype induced by mutation in genes encoding its members, is an evolutionarily conserved regulator of a wide range of cellular

functions, including growth and proliferation, stem cell activity, and tumorigenesis (Harvey, Zhang et al. 2013, Piccolo, Dupont et al. 2014, Varelas 2014, Hansen, Moroishi et al. 2015, Moroishi, Hansen et al. 2015). Three protein complexes make up the Hippo core in mammalian cells: 1) MST1/2 (mammalian STE20-like protein kinase) and SAV (Salvador family WW domain-containing protein), 2) their substrates LATS1/2 (large tumor suppressor) and MOB1A/B (MOB kinase activator), and 3) their substrates YAP (Yes-associated protein) and its paralogue WWTR1 (WW domain containing transcription regulator; also known as TAZ [transcriptional coactivator with a PDZ-binding domain]). In contrast to its conserved core components, a wide range of extra- and intracellular signals, including but not limited to G-protein coupled receptors, WNTs, and changes in the state of actin polymerization, can regulate the activity of the Hippo pathway.

YAP and WWTR1, the key effectors of Hippo signaling, are transcriptional co-activators. Each can be phosphorylated by the LATS kinases on multiple sites. In their nonphosphorylated form, YAP and WWTR1 are able to accumulate in the nucleus. Neither possesses a known DNA-binding domain, however, so their nuclear accumulation depends on physical association with DNA-binding proteins, principally members of the TEA domain (TEAD) family (Ota and Sasaki 2008, Zhao, Ye et al. 2008, Liu-Chittenden, Huang et al. 2012, Diepenbruck, Waldmeier et al. 2014, Zanconato, Forcato et al. 2015). The YAP/WWTR1-TEAD complex is thought to activate transcription of target genes, although only a small number of such targets have so far been identified (Ota and Sasaki 2008, Zhao, Ye et al. 2008, Liu-Chittenden, Huang et al. 2012, Harvey, Zhang et al. 2013, Diepenbruck, Waldmeier et al. 2014, Piccolo, Dupont et al. 2014, Zanconato, Forcato et al. 2015). In contrast, phosphorylation of YAP on serine (S) 127

(human)/S112 (mouse) or WWTR1 on S89 prevents their nuclear accumulation (Basu, Totty et al. 2003, Dupont, Morsut et al. 2011, Hansen, Moroishi et al. 2015). YAP and WWTR1 phosphorylated at these sites instead become associated with 14-3-3 proteins and thereby anchored in the cytoplasm (Kanai, Marignani et al. 2000, Basu, Totty et al. 2003, Oku, Nishiya et al. 2015). Nonphosphorylated YAP and WWTR1 can also be anchored in the cytoplasm through interaction with the angiomotin (AMOT), a plasma membrane-associated protein (Wang, Huang et al. 2011, Zhao, Li et al. 2011). Cytoplasmic YAP and WWTR1 may serve specific functions, such as by binding to and sequestering βcatenin in the cytoplasm (Imajo, Miyatake et al. 2012); these functions have been little explored, however, and it is noteworthy that cytoplasmic YAP and WWTR1 can be phosphorylated via LATS1/2 at additional sites leading to their degradation (Zhao, Li et al. 2010, Varelas 2014). Thus, LATS1/2- dependent phosphorylation of YAP and WWTR1 plays a central role in regulating the Hippo pathway. Recent studies have shown that subjecting ovarian fragments to mechanical or pharmacological interventions that inactivate the Hippo pathway can trigger human primordial follicles to enter the growth phase (Kawamura, Cheng et al. 2013, Cheng, Feng et al. 2015). Moreover, when the fragments were transplanted into patients, healthy live births were obtained, confirming that the experimental treatment induced normal oocyte development (Kawamura, Cheng et al. 2013). Immunohistochemical studies using mice revealed that YAP was localized in the nuclei of the growing oocytes, suggesting that inactivation of the Hippo pathway in the oocyte itself might be the mechanism by which growth was induced. However, the antibody employed to assess YAP expression in oocytes, although widely used, is not specific to YAP when used in immunohistochemistry or immunofluorescence (Hirate, Cockburn et al. 2012, Finch, Passman et al. 2015). Notably, this antibody recognizes nuclear antigens in cells that lack YAP (Finch,

Passman et al. 2015). Moreover, YAP has also been reported to be restricted to the cytoplasm in oocytes (Sun, Pepling et al. 2015). Conversely, WWTR1, although thought to be coregulated with YAP (Piccolo, Dupont et al. 2014, Varelas 2014) was localized in the nucleus of growing oocytes as well as granulosa cells (Sun, Pepling et al. 2015). Thus, the potential role of the Hippo pathway in regulating oocyte development remains uncertain. Focusing on YAP because the specificity of the available antibodies has been verified (Hirate, Cockburn et al. 2012, Finch, Passman et al. 2015), we used immunoblotting and immunohistochemistry to systematically investigate its expression, phosphorylation, and intracellular distribution during pre- and postnatal oocyte development. We find that phosphorylation-dependent and -independent mechanisms cooperate to ensure that YAP does not accumulate in the nuclei of oocytes at any stage of development, indicating that nuclear YAP does not play a significant physiological role during mammalian oogenesis.

Materials and Methods

Ethical Approval

Experiments at McGill University and at the Hospital for Sick Children Research Institute were carried out following the policies of the Canadian Council on Animal Care and were approved by the animal care committees of the Research Institute of the McGill University Health Centre and the Toronto Centre for Phenogenomics, respectively. Experiments at the Carnegie Institute were performed in compliance with ethical regulations and approved by the Institutional Animal Care and Use Committee of the Carnegie Institution for Science. No animals were handled on the premises of Laval University; the Canadian guidelines were followed by the abattoir that provided the bovine ovaries.

Animals

CD-1 mice were obtained from Charles River Canada. Nf2þ/þ and Nf2/ mice were maintained and genotyped as described (Cockburn, Biechele et al. 2013). Bovine oocytes were collected from 2- to 6-mm follicles, and oocytes displaying homogenous cytoplasm, a complete cumulus cloud with no signs of atresia, and a diameter greater than 120 μm were selected. To obtain mouse fetal ovaries, male and 6- to 8-wk-old female 129/SvJae mice were caged as individual pairs and the female was examined daily for the presence of a vaginal plug in the morning. The day of the plug appearance was designated Embryonic Day 0.5 (E0.5). Collection of Oocytes and Embryos To obtain cumulus-oocyte complexes (COCs) containing immature fully grown oocytes arrested at prophase I of meiosis, ovaries were dissected from 19-day-old female CD-1 mice and transferred to Hepes-buffered minimum essential medium with Earle salts (MEM-H; pH 7.2) (Life Technologies) supplemented with sodium pyruvate (0.25 mM; Sigma Chemicals), penicillin G (63 mg/L) (Sigma), streptomycin (50 mg/L) (Sigma), and BSA (1 mg/ml) (Sigma) at 378C. Dibutyryl cyclic AMP (dbcAMP) (0.1 mg/ml) (Sigma) was added to the medium to maintain the oocytes in meiotic arrest. The ovarian follicles were punctured using a 30-gauge needle to isolate the enclosed COCs. Granulosa-oocyte complexes (GOCs) containing growing oocytes were collected from 12-day-old female pups using enzymatic methods as previously described (Demeestere, Streiff et al. 2012). Where required, granulosa- or cumulus-free oocytes were obtained by mechanically stripping the granulosa cells from the GOC or COC (El-Hayek, Demeestere et al. 2014, El-Hayek and Clarke 2015). Embryos were produced and collected as described (Clarke, Oblin et al. 1992). Cell Culture and Drug Treatment Complexes, oocytes, and embryos were incubated at 378C in a humidified atmosphere of 5% O2, 5% CO2, 90% N2 in bicarbonate-buffered MEM (complexes and oocytes) or KSOM (embryos) as described

(Demeestere, Streiff et al. 2012). Dibutyryl cyclic AMP (D0627; Sigma) was prepared at 10 mg/ml in water and used at 0.2 mg/ml. Roscovitine (R7772; Sigma) was prepared at 40 mM in dimethyl sulfoxide and used at 100 µM. KT5720 (420320; Millipore) was prepared at 2 mM in dimethyl sulfoxide and used at 30 µM. Leptomycin B (L2913; Sigma) was prepared at 20 µM in ethanol and used at 20 nM.

Reverse Transcription and PCR

RNA purification from freshly collected oocytes, cDNA synthesis, and RTPCR reaction were performed as described (Allard, Yang et al. 2005). Actb was used as a positive control, and a reaction without template was used as the negative control.

Primer pairs are listed as below (forward primer given first, followed by reverse primer):

Actb: 5'-GGCTGTATTCCCCTCCATCG-3'; 5'-CCAGTTGGTAA CAATGCCATGT-3'.

Yap1: 5'-CCGTTTCTCCTGGGACACTC-3'; 5'- TGCTCCAGTGTAGGCAACTG-3'.

Immunoblotting

Immunoblotting was performed as previously described (Yang, Allard et al. 2010). The primary antibodies used were directed against YAP (4912; Cell Signaling Technologies), phospho-S127 YAP (113008, Cell Signaling Technologies), phospho- S133 CREB (9198; Cell Signaling Technologies), MAPK3/1 (sc-94; Santa Cruz Biotechnology), and tubulin (T8203; Sigma). All primary antibodies were used at 1:1000 dilution. Blots were scanned using a Storm phosphorimager (Amersham), and the intensity of the signals was quantified using Image J software (National Institutes of Health).

Immunofluorescence

Oocytes, GOCs, or COCs were fixed in freshly prepared 2% (w/v) paraformaldehyde (Fisher Scientific) in phosphate-buffered saline containing 0.1% Triton X-100 followed by washing and storing in blocking buffer (PBS containing 0.1% Triton X-100 and 3% bovine serum albumin). Samples were incubated overnight at 48C in primary antibodies—mouse anti-YAP (H00010413-M01; Abnova) or (101199; Santa Cruz Biotechnology) and rabbit histone H3-acetyl-K9 (9671; Cell Signaling Technologies)—diluted 1:100 in blocking buffer. The next day, samples were washed twice for 10 min each in blocking buffer at room temperature, then transferred to blocking buffer containing secondary antibody diluted 1:100 in blocking buffer that also contained 5 lM DRAQ5 (4084S; New England Biolabs). YAP was detected using Alexa546-conjugated donkey anti-mouse (A10036; Life Technologies). Acetylated histone H3 was detected using Alexa488-conjugated goat anti-rabbit (A11008; Life Technologies). To mount the samples, a 9 3 0.12 mm spacer (GBL654008; Sigma) was attached to a glass microscope slide. A 2 µl drop of PBS was placed in the center of the spacer and covered with 20 µl of mineral oil. Samples were then transferred into the drop of PBS and a cover slip was placed on top. Samples were imaged using a Zeiss LSM510 or Quorum spinning disk confocal microscope.

Immunohistochemistry

To prepare cryosections, ovaries were fixed in freshly prepared 2% (w/v) paraformaldehyde (EMS Biosciences) in PBS at 48C for 2 h, then washed through a sucrose gradient (10%, 20%, 30% in PBS), embedded in Tissue-Tek OCT (Sakura Finetek), and stored at 808C. Sections were cut at 8 lm thickness and rehydrated for 10 min in PBS. Immunostaining was performed using Vector Mouse on Mouse Immunodetection Kit (2201), following the manufacturer's directions

except that the PBS was supplemented with 0.05% Triton X-100 and 0.15% glycine. Slides were counterstained using 40,6- diamidino-2-phenylindole and covered with coverslips using Vectashield (Vector) as an antifading solution. Slides were examined using a TCS-SP5 laser-scanning confocal microscope (Leica), and images were analyzed using LAS AF (Leica Microsystems CMS GmbH) and Imaris (Bitplane). Primary antibodies used were rabbit polyclonal anti-MVH (1:1000 dilution, ab13840; Abcam) and mouse anti-YAP (1:100 dilution, 101199; Santa Cruz Biotechnology). Paraffin-embedded sections were prepared and used for immunohistochemistry as previously described (El-Hayek and Clarke 2015), using the same primary antibodies and dilutions as for immunofluorescence. YAP was detected using Alexa488-conjugated rabbit anti-mouse (1:500 dilution, A11059; Life Technologies). Images were recorded using an LSM 510 confocal microscope (Zeiss).

Statistical Analysis

Quantitative data were analyzed using the Student t-test or ANOVA. A Pvalue, 0.05 was considered significant.

Results

YAP Is Expressed Throughout Pre- and Postnatal Oocyte Development but Is Excluded from the Nucleus

To determine whether YAP is expressed in oocytes, we used growing oocytes obtained from primary and secondary follicles and fully grown oocytes obtained from antral follicles. The granulosa cells can easily be removed from oocytes at these stages of development, allowing a purified cell population to be analyzed. Using RT-PCR with primers specific for *Yap*, we detected a product of the expected size in both the growing and the fully grown oocytes (Fig. 14A). We then used immunoblotting to test whether YAP protein was present. Because fully grown oocytes contain more total protein than partially grown oocytes, we loaded a larger number of growing oocytes into the gels so that we would obtain approximately equal amounts of total protein at the two stages. Equal loading was confirmed by the similar signal intensities observed for MAPK3/1, which is expressed throughout oocyte growth (Harrouk and Clarke 1995, de Vantéry Arrighi, Campana et al. 2000) (Fig. 14B). We observed that YAP protein was expressed in both growing and fully grown oocytes (Fig. 14B). Moreover, the signal intensities at the two stages were similar, which suggests that the amount of YAP as a fraction of total cellular protein does not change substantially during oocyte growth (Fig. 14C). We then examined whether YAP was present in the oocyte nucleus, as would be expected if it promotes oocyte growth via its canonical function as a transcriptional co-activator. The antibody we used for immunoblotting (4912; Cell Signaling), although very effective for that application, is not suitable for immunolocalization studies because it recognizes nuclear. antigens in situ that are not YAP (Hirate, Cockburn et al. 2012, Finch, Passman et al. 2015). We used instead an antibody (H00010413-M01; Abnova) whose specificity for YAP in immunofluorescence has

been established (Hirate, Cockburn et al. 2012, Cockburn, Biechele et al. 2013). In preliminary experiments, we confirmed that this antibody recognized a single species in immunoblots and stained the nuclei of trophectodermal cells but not of the inner cell mass in mouse blastocysts (Fig. 21A and 21B), consistent with previous reports (Hirate, Cockburn et al. 2012, Cockburn, Biechele et al. 2013). Using an antibody against histone H3 acetylated on K9, we also verified that we could detect nuclear antigens in oocytes (Fig. 21C). When we stained growing and fully grown oocytes using the YAP antibody, however, although we observed a strong fluorescent signal in the cytoplasm at both stages (Fig. 15A), little or no fluorescence was detectable in oocyte nuclei at either stage. We also observed the same staining pattern using a different YAP antibody (101199; Santa Cruz) (data not shown). This result suggested that YAP is largely excluded from the nuclei of growing and fully grown oocytes. We were concerned that the experimental intervention of removing the granulosa cells surrounding the oocyte prior to fixation might have altered YAP localization. Therefore, we immunostained intact GOCs containing growing oocytes and COCs containing fully grown oocytes. As observed using the granulosa cell-free oocytes, fluorescence was detectable in the oocyte cytoplasm but not in the nucleus in both GOCs and COCs (Fig. 15B). It was also possible that removing the oocyte from the follicular environment might have altered YAP localization or that YAP was present in the nucleus at a stage of oocyte development not represented in the samples that we had collected. Therefore, we also immunostained tissue sections of paraffin-fixed ovaries after verifying that we could detect nuclear acetylated histone H3 in these sections (Fig. 21D). Oocytes within primordial, primary, secondary, and antral follicles all displayed strong cytoplasmic YAP fluorescence. In contrast, nuclear YAP fluorescence was weak or undetectable at all stages (Fig. 15C). We conclude that YAP is mainly excluded from oocyte nuclei at all stages of postnatal

development. We then examined prenatal oogenesis. We obtained ovarian sections from mice at E13.5, E15.5, and E18.5 and from 2-dayold pups, and stained these for Mouse Vasa Homologue (MVH) to identify germ cells and YAP to assess its localization in these cells. YAP was barely detectable in the germ cells at E13.5. At later stages, including when primordial follicles were present, YAP was present in the cytoplasm but undetectable in the nucleus (Fig. 16). Hence, YAP appears to be predominantly localized in the cytoplasm throughout female germ cell development in the mouse.

YAP in Oocytes Is Phosphorylated at S112

The exclusion of YAP from the nucleus throughout oogenesis implies that a robust mechanism restricts it to the cytoplasm. In other cell types, the intracellular localization of YAP is regulated by phosphorylation. In particular, phosphorylation of S112 (S127 in human) has been identified as a critical determinant because this modification enables YAP to associate with 14-3-3 proteins that anchor it in the cytoplasm (Basu, Totty et al. 2003, Zhao, Wei et al. 2007). To test whether YAP in oocytes is phosphorylated at S112, we obtained growing and fully grown oocytes free of granulosa cells and subjected them to immunoblotting using a well-characterized antibody that is specific for S112-phosphorylated YAP. We detected S112-phosphorylated YAP in both growing and fully grown oocytes (Fig. 17A). The phosphospecific antibody also detected a species of the appropriate molecular weight in bovine oocytes (Fig. 17B), suggesting that phosphorylation of YAP on S112 (or its equivalent) is a conserved property of mammalian oocytes. Two immunoreactive bands were present in the blot of mouse fully grown oocytes, whereas only the faster-migrating band was detectable in growing oocytes. This may reflect phosphorylation of additional sites on YAP in fully grown oocytes. Unexpectedly, whereas growing and fully grown

oocytes contain approximately the same amount of total YAP when equal amounts of cellular protein are analyzed (Fig. 14B), less S112-phosphorylated YAP was detectable in growing oocytes (Fig. 17A). This implies that growing oocytes contain both phosphorylated YAP and a subpopulation of YAP that is not phosphorylated on S112.

Protein Kinase A Regulates S112 Phosphorylation of YAP in Oocytes

We next sought to identify the mechanism responsible for YAP phosphorylation. S112 phosphorylation is typically regulated by the Hippo pathway, and the membrane-associated FERM-domain protein, neurofibromatosis-2 (NF2), is required for Hippo signaling in a broad range of cell types (Zhang, Su et al. 2010, Liu-Chittenden, Huang et al. 2012). Notably, in mouse blastocysts lacking Nf2, YAP accumulates in the nuclei of the inner cell mass whereas it is exclusively cytoplasmic in these cells in wild-type blastocysts (Cockburn, Biechele et al. 2013), indicating that NF2 regulates YAP in the early embryo. When we examined oocytes in which Nf2 had been deleted, however, YAP remained largely excluded from the nucleus (Fig. 15D). Crucially, we could detect no difference in the nucleocytoplasmic distribution of YAP in the presence or absence of Nf2. Although we did not directly examine phosphorylation in these experiments, this result indicates that, in contrast to the embryo, NF2 does not regulate YAP in oocytes. The cAMP-dependent protein kinase A regulates YAP phosphorylation in a small number of cell types (Yu, Zhao et al. 2012, Kim, Kim et al. 2013, Yu, Zhang et al. 2013). Because protein kinase A activity is high in growing and fully grown oocytes (Nogueira, Albano et al. 2003, Kovo, Kandli-Cohen et al. 2006, Conti, Hsieh et al. 2012, Zeng, Ren et al. 2013, Nishimura, Fujii et al. 2014), we hypothesized that it might play a key role in regulating S112 phosphorylation of YAP. To test this, we first removed fully grown oocytes from the follicle,

which causes protein kinase A activity in the oocyte to rapidly fall, and allowed them to undergo maturation in vitro. We found a dramatic reduction in the amount of S112-phosphorylated YAP in oocytes that had matured to metaphase II (Fig. 17C). The small amount that remained migrated more slowly than YAP in immature (germinal vesicle-stage) oocytes, consistent with the possibility that other sites on the protein became phosphorylated during maturation. Crucially, the loss of phosphorylated YAP was not due to degradation of the protein, whose quantity remained stable during maturation (Fig. 17C). In contrast, when we incubated fully grown oocytes overnight with dbcAMP, which maintains high protein kinase A activity, YAP remained phosphorylated on S112 (Fig. 17D). The loss of S112-phosphorylated YAP in mature oocytes could be due to the decrease in protein kinase A activity or to the process of meiotic maturation. To resolve this point, we isolated fully grown oocytes and incubated them in the presence of an inhibitor of cyclin-dependent kinase (CDK) activity, roscovitine. These oocytes thus possessed low protein kinase A activity, as a result of being removed from the follicle, but were unable to mature. Strikingly, S112-phosphorylated YAP was substantially diminished in the roscovitine-treated oocytes (Fig. 17C), and this was not due to degradation of the protein (Fig. 17C). Taken together, these results indicate that protein kinase A activity regulates S112 phosphorylation of YAP in fully grown oocytes. We then tested whether protein kinase A also regulates S112 phosphorylation of YAP in growing oocytes. We first isolated GOCs containing growing oocytes and incubated them overnight in the presence or absence of dbcAMP, after which the oocytes were removed from the GOC and immunoblotted. Unexpectedly, YAP remained phosphorylated on S112 even in the absence of dbcAMP (Fig. 18A). To understand this result, we considered prior observations that the granulosa cells supply the oocyte with cGMP, which inhibits phosphodiesterase (PDE) 3A and thereby helps to maintain high intra-

oocyte cAMP and protein kinase A activity (Norris, Ratzan et al. 2009, Zhang, Su et al. 2010, Richard and Baltz 2014). It was possible that the granulosa cells in the cultured GOCs had supplied the oocyte with enough cGMP to maintain high protein kinase A activity in the absence of external dbcAMP. Therefore, we removed the granulosa cells from the growing oocytes prior to overnight incubation. Even in the absence of the granulosa cells, however, YAP remained phosphorylated on S112 in the growing oocytes (Fig. 18B). These results could indicate that S112 phosphorylation of YAP in growing oocytes does not require protein kinase A activity or that, in contrast to fully grown oocytes, growing oocytes are able to maintain high protein kinase A activity in vitro in the absence of an extracellular source of cAMP (i.e., dbcAMP). To test the role of protein kinase A directly, we isolated GOCs containing growing oocytes and incubated them overnight in KT5720, a cell-permeable inhibitor of protein kinase A that acts by blocking its ATP-binding site. Incubation in the presence of KT5720 reduced the phosphorylation of CREB, a known substrate of protein kinase A, by about 50% (Fig. 18C). KT5720 also induced a quantitatively similar reduction in the amount of S112-phosphorylated YAP (Fig. 18D). These results imply that protein kinase A regulates S112 phosphorylation of YAP in growing oocytes as well as in fully grown oocytes.

Dephosphorylated YAP Enters the Nuclei of Growing Oocytes but Is Unable to Accumulate

Because a portion of the YAP in growing oocytes was not phosphorylated on S112, as discussed above, we were surprised that it was not detectable in the nuclei at this stage. Moreover, when we incubated either growing or fully grown oocytes under conditions that reduced S112 phosphorylation of YAP, we did not detect nuclear YAP at either stage (Fig. 19A and 19B). These results suggested that, even when YAP was not phosphorylated on S112, it was unable to

accumulate in the oocyte nucleus. To understand the basis for this nuclear exclusion, we incubated growing oocytes in the presence of leptomycin B, an inhibitor of nuclear export. Under these conditions, we observed a robust accumulation of YAP in the oocyte nuclei (Fig. 19A). These results confirm that our fixation and processing conditions permitted nuclear YAP to be detected when it was present. More importantly, they indicate that a portion of the oocyte YAP, likely that which is not phosphorylated on S112, is transported to the nuclei in growing oocytes. However, unless trapped there using an export inhibitor, the nonphosphorylated YAP is not retained and instead rapidly returns to the cytoplasm. In striking contrast, when we treated fully grown oocytes with leptomycin B, together with roscovitine to prevent nuclear membrane breakdown, YAP did not accumulate in the nucleus (Fig. 19B). This was not due to an unanticipated effect of the roscovitine because the drug did not block nuclear accumulation of YAP in growing oocytes (data not shown). Rather it appears that nonphosphorylated YAP is not transported to the nucleus in fully grown oocytes. Thus, fully grown oocytes possess an additional mechanism not present in growing oocytes that prevents YAP from accumulating in the nucleus.

YAP Is Primarily Cytoplasmic in the Granulosa Cells

Although the primary focus of our study was the oocyte, we were also able to examine the intracellular distribution of YAP in the granulosa cells of the follicle. In intact GOCs and COCs (Fig. 15B) as well as in ovarian tissue sections (Fig. 15C), we consistently observed cytoplasmic staining of the granulosa cells. In contrast, we never detected nuclear granulosa cell staining in follicles at any stage. These results indicate that YAP is largely excluded from the nucleus of the granulosa cells throughout postnatal follicular growth.

Discussion

We have investigated the potential role of YAP during oocyte development. We find that YAP is expressed as early as Day 15.5 of embryonic development and continues to be expressed during all stages of oocyte development up to and including meiotic maturation. Throughout all these stages, however, YAP is predominantly present in the cytoplasm and is largely excluded from the nucleus. These results identify and map for the first time the expression and intracellular localization of YAP during pre- and postnatal oogenesis in vertebrates. They also strongly suggest that nuclear YAP does not regulate mammalian oocyte development under physiological conditions. Our results reveal that multiple mechanisms cooperate to prevent YAP accumulation in the oocyte nucleus. First, phosphorylation of S112 enables YAP to associate with 14-3-3 proteins, which in other cell types anchors it in the cytoplasm (Kanai, Marignani et al. 2000, Basu, Totty et al. 2003). Protein kinase A has recently been identified as an effector of S112 phosphorylation, through its activity to phosphorylate the LATS kinases (Kim, Kim et al. 2013, Yu, Zhang et al. 2013). High protein kinase A activity is a characteristic property of growing and fully grown mammalian oocytes as well as those of nonmammalian species (Nogueira, Albano et al. 2003, Kovo, Kandli-Cohen et al. 2006, Conti, Hsieh et al. 2012, Zeng, Ren et al. 2013, Nishimura, Fujii et al. 2014). In rodents, this activity is maintained by cyclic AMP, whose synthesis is stimulated by a constitutively active G-protein coupled receptor (GPR3 in mice; GPR12 in rats) (Mehlmann, Jones et al. 2002, Mehlmann, Saeki et al. 2004, Freudzon, Norris et al. 2005). Although less is known of cAMP and protein kinase A levels at earlier stages of oogenesis, adenyl cyclase was recently detected in mouse oocytes as early as E15.5 (Wang, Teng et al. 2015). This concords strikingly with our observation that YAP is cytoplasmic even in oocytes at this stage. Crucially, it also suggests that protein kinase A activity may be high

throughout postmitotic oogenesis. Moreover, oocytes express numerous members of the 14-3-3 family of proteins (De, Marcinkiewicz et al. 2012). Thus, it is likely that much of the YAP in growing and fully grown oocytes, because it is phosphorylated at S112, is associated with 14-3-3 proteins that anchor it in the cytoplasm. Second, although phosphorylation at S112 would likely anchor YAP in the cytoplasm, we observed using the nuclear export inhibitor, leptomycin B, that some YAP can enter the nucleus in growing oocytes. Leptomycin B also promotes YAP nuclear localization in other cell types (Ren, Zhang et al. 2010, Dupont, Morsut et al. 2011). Because a portion of the YAP in growing oocytes is not phosphorylated at S112, we speculate that this nonphosphorylated YAP can enter the nucleus. Yet this YAP is rapidly exported back to the cytoplasm. Therefore, oocytes are unable to retain YAP in the nucleus. YAP does not possess a known DNA-binding domain and relies on binding partners that possess DNA-binding activity to remain in the nucleus. YAP principally associates with the TEAD family of proteins (Zhao, Ye et al. 2008), although other binding partners have been identified (Mo, Park et al. 2014, Piccolo, Dupont et al. 2014). Importantly, binding to TEAD is required to retain YAP in the nucleus (Diepenbruck, Waldmeier et al. 2014). Growing oocytes express mRNAs encoding several TEAD proteins but the expression of the encoded proteins has not been reported (Kaneko, Cullinan et al. 1997). We suggest that these partners may be expressed too weakly to retain a detectable quantity of YAP in the nucleus or that posttranslational modifications of YAP prevent stable association with them (Mo, Meng et al. 2015). As a result, YAP returns to the cytoplasm. Thus, in the absence of a mechanism to retain it in the nucleus, dephosphorylated YAP accumulates in the cytoplasm by default. It would be valuable to examine the localization of YAP in a recently described Yap^{S112A} mutant (Chen, Zhang et al. 2015). Third, YAP failed to accumulate in the nuclei of fully grown oocytes even when we induced its dephosphorylation

and blocked nuclear export. This was not due to an unanticipated effect of the roscovitine used to maintain an intact nucleus in fully grown oocytes because the drug did not prevent YAP nuclear accumulation in leptomycin B-treated growing oocytes. Rather, it appears that in fully grown oocytes, even nonphosphorylated YAP does not enter the nucleus. This suggests that a third mechanism excludes YAP from the nucleus at this stage. Although the nature of this mechanism remains unknown, it might be speculated that the transcriptionally inactive state of fully grown oocytes is accompanied by a modification of the nuclear membrane that prevents entry of YAP. Alternatively, S112 phosphorylation might be required for YAP to associate with the 14-3-3 proteins, but no longer needed once YAP has become anchored in the cytoplasm. Thus, it might be that phosphorylated YAP associates with 14- 3-3 proteins and subsequently becomes dephosphorylated. Multiple intracellular mechanisms thus cooperate to ensure that YAP does not accumulate in the oocyte nucleus (Fig. 20).

What function might be served by nuclear exclusion? On the one hand, it may be that, if abundant in the nucleus, YAP would impair oocyte development. The link between inactivation of the Hippo pathway and tumorigenesis suggests that nuclear YAP can regulate cell-cycle progression (Varelas 2014, Moroishi, Hansen et al. 2015). In oocytes, the cell cycle becomes arrested at late G2 before they are assembled into primordial follicles and does not resume until meiotic maturation. Nuclear exclusion of YAP might be important to ensure that the cell cycle does not resume precociously. Intriguingly, global deletion of *Lats1* causes perinatal germ-cell apoptosis and precocious growth of the oocytes that remain (Sun, Pepling et al. 2015). As *Lats1* deletion would be expected to favor nuclear localization of YAP, this result supports the notion that normal oocyte development depends on excluding YAP from the nucleus. It is also possible

that cytoplasmic YAP serves a function during oocyte development, perhaps by sequestering molecules away from the nucleus (Imajo, Miyatake et al. 2012). The apparent abundance of YAP, as indicated by intense immunofluorescent signal, is consistent with a cytoplasmic function. Targeted deletion of *Yap1* in the oocyte could test its role directly. As discussed in the Introduction, the YAP paralogue, WWTR1, has been detected using immunohistochemistry in the nuclei of oocytes at all stages of growth (Sun, Pepling et al. 2015). YAP and WWTR1 share substantial sequence identity, including the region that binds to 14-3-3 proteins, and are thought to be coregulated (Varelas 2014). WWTR1 also contains a serine at position 89, corresponding to S112 in YAP. These structural similarities suggest that WWTR1 in oocytes is likely to be phosphorylated at S89 and anchored in the cytoplasm. Hence, its apparent nuclear localization is unanticipated. WWTR1 lacks certain domains that are present in YAP, however, including an N-terminal proline-rich region and an SH3 (Src homology 3)- binding motif. Although these domains have not been implicated in YAP intracellular localization, it is possible that their absence in WWTR1 permits nuclear accumulation even when the protein is phosphorylated. Alternatively, if a fraction of the WWTR1 in oocytes is nonphosphorylated, a WWTR1-specific mechanism might retain the nonphosphorylated form in the nucleus. Further studies of the phosphorylation state and localization of WWTR1 within the oocyte should help to resolve these apparently conflicting results.

Our results demonstrate that YAP is excluded from the nucleus throughout oocyte development beginning at prenatal stages. Moreover, although we did not systematically study YAP expression in the somatic compartment of the follicle, we found no stage at which it was nuclear in the granulosa cells. These results provide strong evidence that nuclear YAP does not play a

physiological role in oocyte or follicular development. Yet, previous studies have unambiguously demonstrated that experimental interventions that repress Hippo signalling trigger oocyte and follicular growth (Kawamura, Cheng et al. 2013, Cheng, Feng et al. 2015). How may these be reconciled? It was proposed that these interventions repressed the Hippo pathway by inducing actin rearrangements, which in other cell types can induce YAP nuclear localization (Richardson 2011). Thus, experimental inactivation of the Hippo pathway in the oocyte or somatic cell compartment may trigger oocyte and follicular growth even if nuclear YAP does not regulate these processes in a physiological context. Our results nonetheless indicate that maintaining the normal intracellular distribution and function of YAP should be an important consideration during the development and evaluation of conditions designed to recapitulate normal oocyte and follicle development.

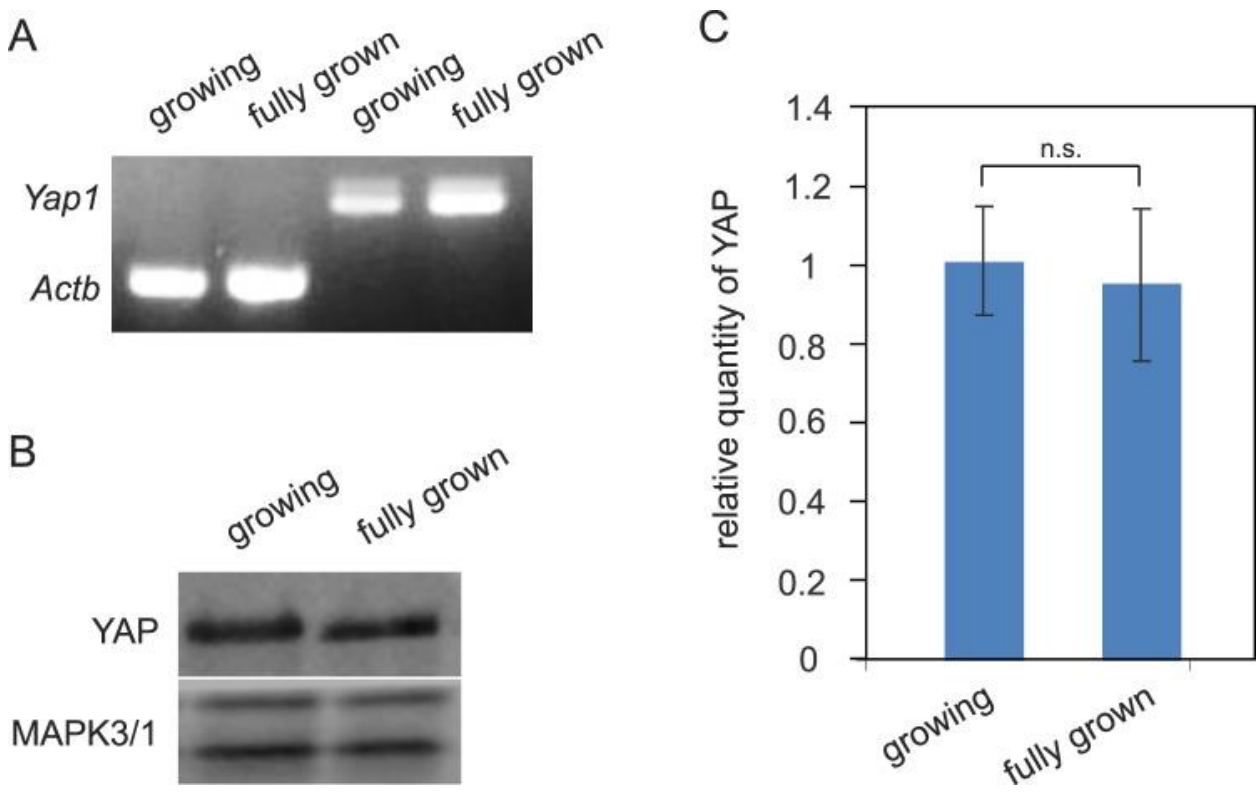


Figure 14: Expression of YAP in oocytes

A) Messenger RNA was extracted from growing and fully grown oocytes. *Yap1* and *Actb* were detected using RTPCR. B) Growing and fully grown oocytes were subjected to immunoblotting using antibodies against YAP and MAPK3/1; 150 growing oocytes and 80 fully grown oocytes were loaded.

C) Quantification of immunoblots. YAP signal was normalized to MAPK3/1 signal. The ratio of YAP: MAPK3/1 in growing and fully grown oocytes did not significantly (n.s.) differ (Student t-test).

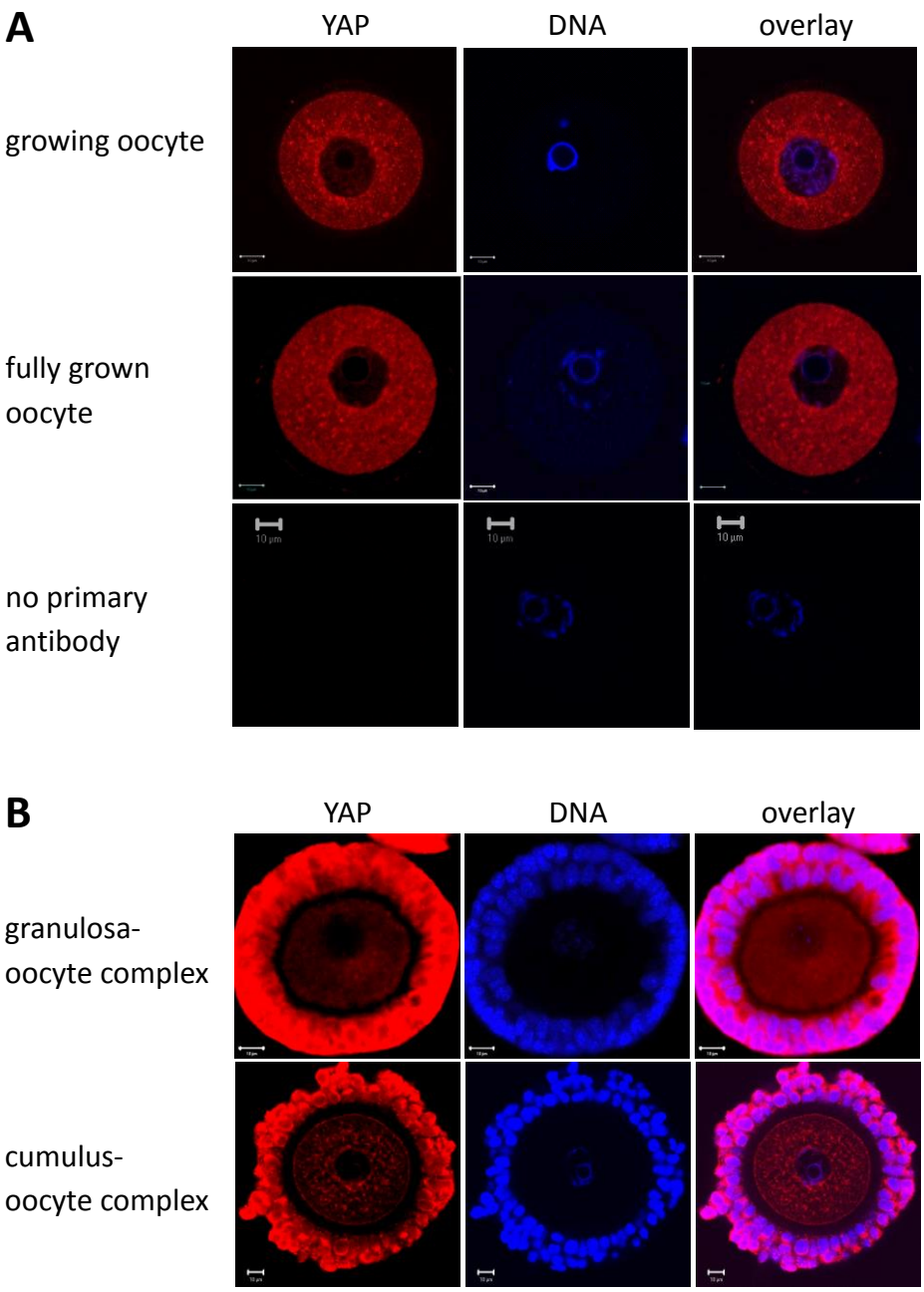
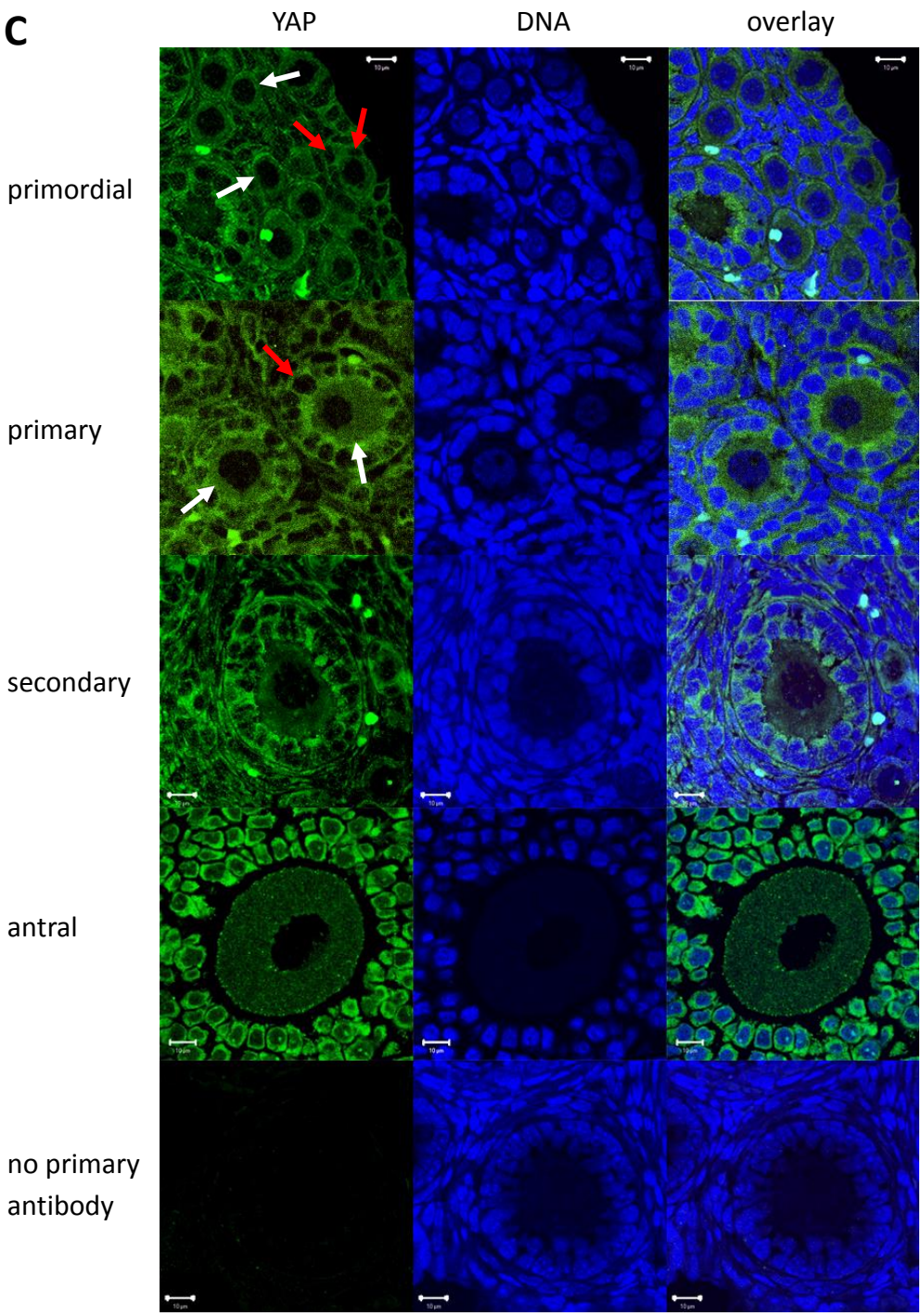
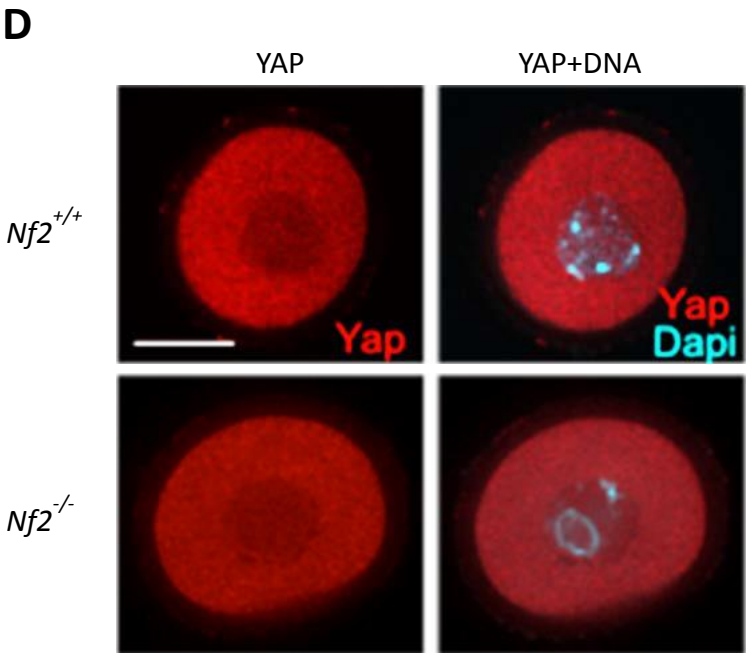


Figure 15: Intracellular localization of YAP in oocytes

A, B) Growing and fully grown oocytes (A) or granulosa-oocyte complexes (GOCs) and cumulus oocyte complexes (COCs) (B) were stained using anti-YAP. DNA was stained using DRAQ5. Bottom panel in A shows staining when primary antibody was omitted. YAP is present in the cytoplasm but is undetectable in the nucleus. The GOC in B is overexposed to reveal YAP in oocytes. Bar ¼ 10 µm.



C) Paraffin sections were stained using anti-YAP. DNA was counterstained using DRAQ5. Representative examples of oocytes in primordial, primary, secondary, and antral follicles are shown. White arrows indicate oocytes. Red arrows indicate granulosa cells. YAP is present in the cytoplasm but is undetectable in the nucleus. Bar ¼10 µm.



D) Oocytes of *Nf2*$^{-/-}$ and *Nf2*$^{+/+}$ littermates were stained using anti-YAP. No difference in staining pattern is detectable between the genotypes. Bar ¼ 50 μm.

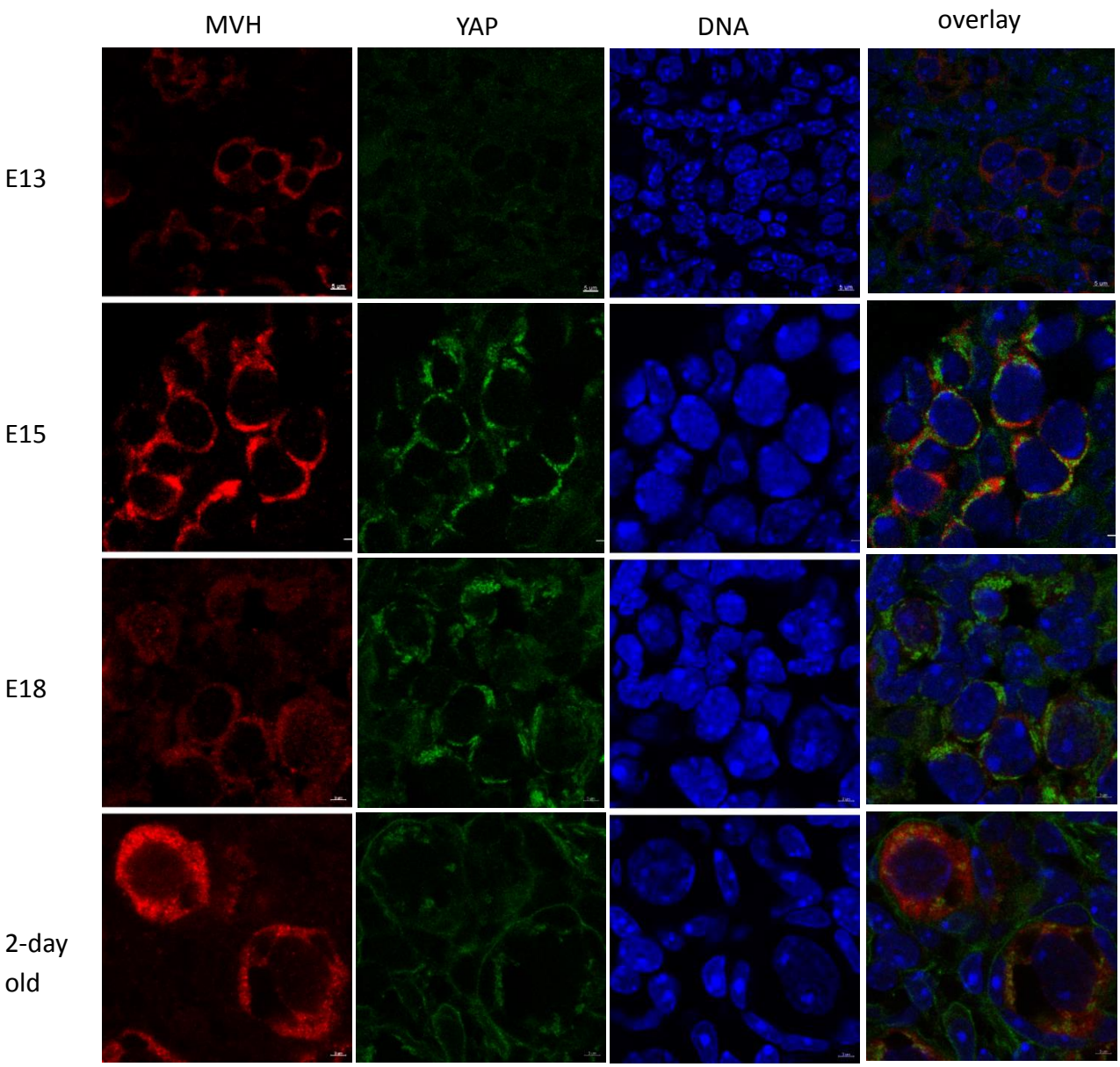


Figure 16: Intracellular localization of YAP in pre- and perinatal oocytes

Frozen sections of ovary obtained at the indicated stages of embryonic (E) development or 2 days after birth were stained using anti-MVH to identify germ cells, anti-YAP, and DRAQ5. YAP is barely detectable at E13.5 but is present in oocyte cytoplasm at E15.5, E18.5, and newborns. YAP is not detectable in the oocyte nucleus at any stage. Bar ¼ 5 µm.

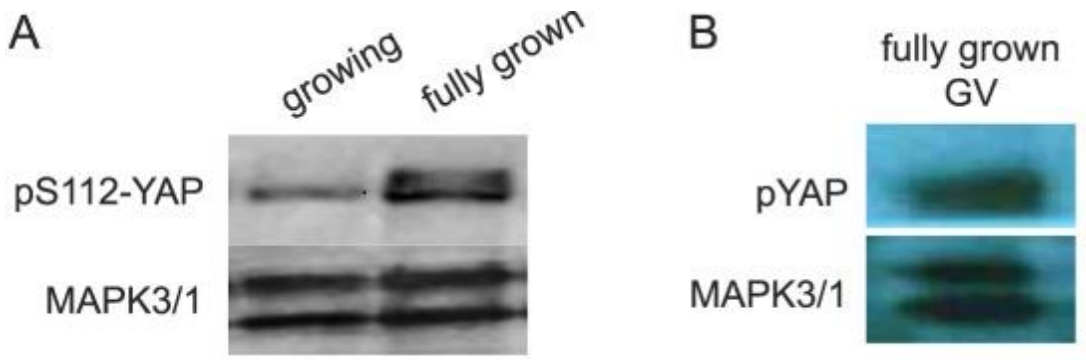
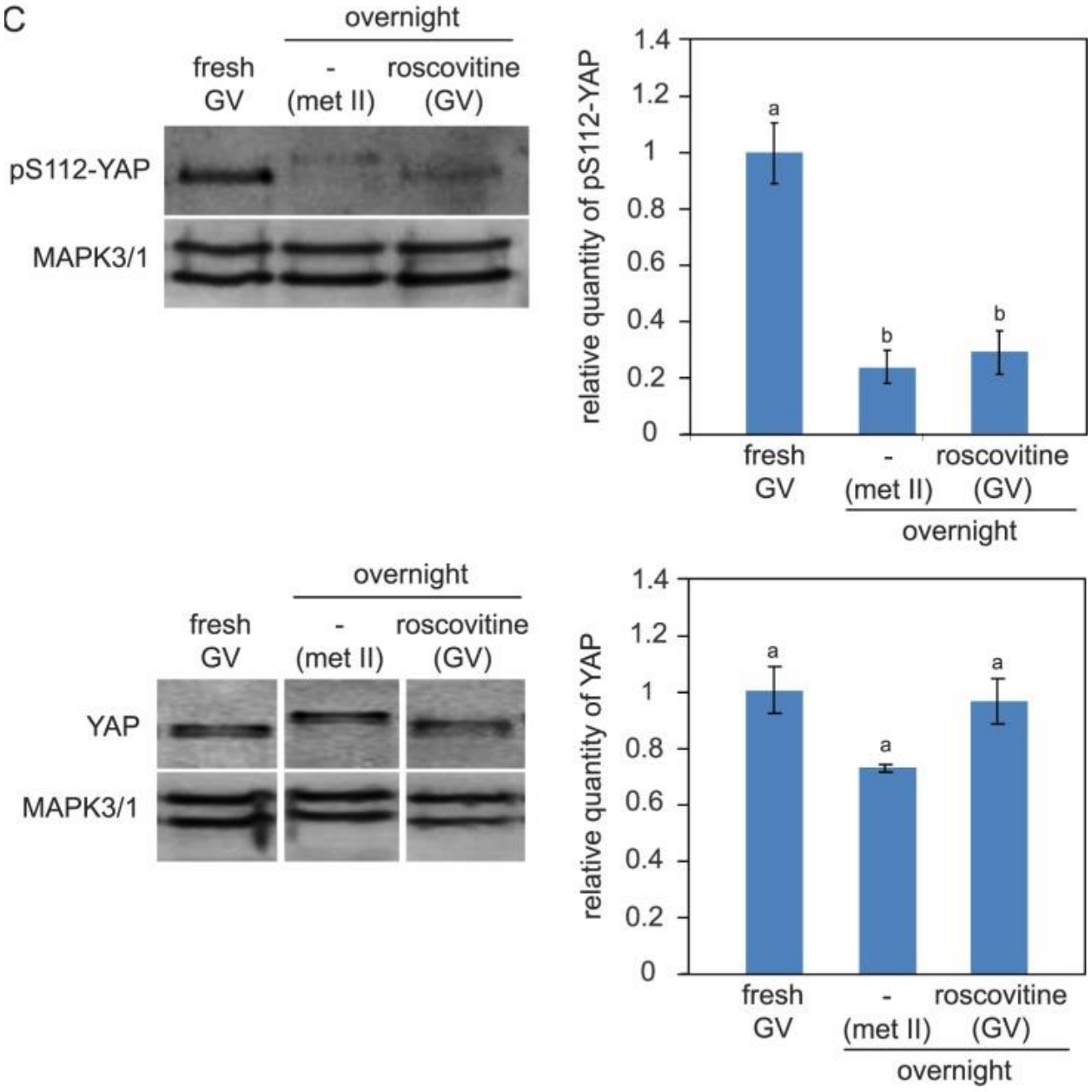
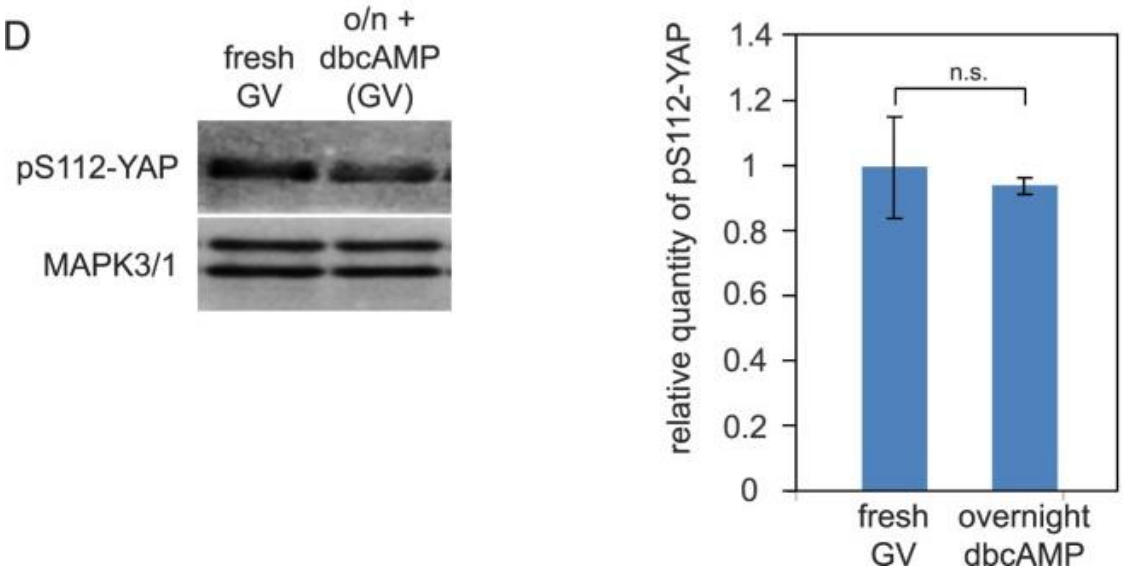


Figure 17: Phosphorylation of YAP on S112 in oocytes

A) Growing (150) and fully grown (80) oocytes were subjected to immunoblotting using antiphosphorylated S112-YAP antibody and anti-MAPK3/1. The slow-migrating band in fully grown oocytes may be phospho-S112 YAP carrying additional modifications or an unrelated protein.



B) Bovine oocytes (40) were immunoblotted as in A. The position of the serine corresponding to S112 in mouse is not certain. The experiment was performed twice. C) Fully grown oocytes were collected and one portion (fresh GV) was reserved immediately for immunoblotting while the remaining oocytes were incubated overnight in the absence or presence of roscovitine. Oocytes that reached metaphase II in the absence of roscovitine or remained at the GV stage in its presence were used. Phospho-S112-YAP (upper panel) or total YAP (lower panel) and MAPK3/1 were detected as in A. In the total YAP blot, intervening lanes present in the gel have been removed from the micrograph, as indicated by the white lines between lanes. Phospho-S112-YAP is lost during maturation and in GV oocytes incubated in roscovitine.



D) Fully grown oocytes were collected and one portion (GV) was reserved immediately for immunoblotting while the remaining oocytes were incubated overnight in the presence of dbcAMP. Phospho-S112-YAP remains in oocytes incubated in the presence of dbcAMP. In all histograms, YAP signal was normalized to MAPK3/1 signal of the same sample and results were analyzed using the Student t-test (A, D) or ANOVA (C) where different superscripts indicate a statistically significant difference (P, 0.05); n.s., not significantly different.

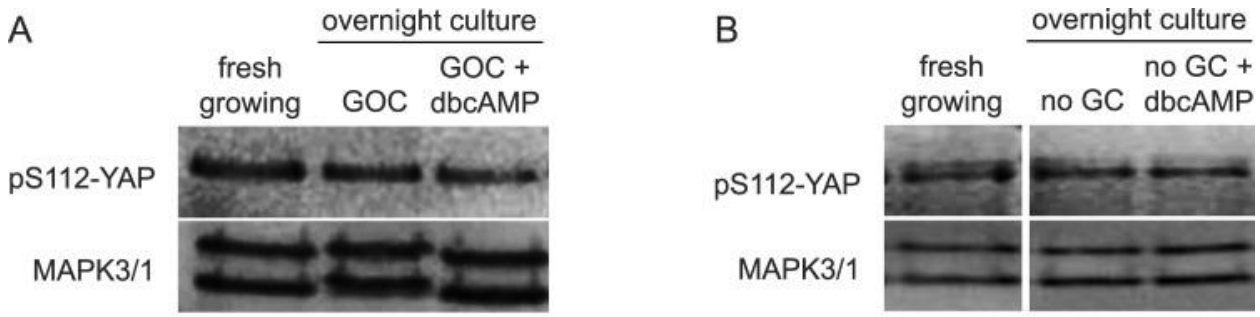
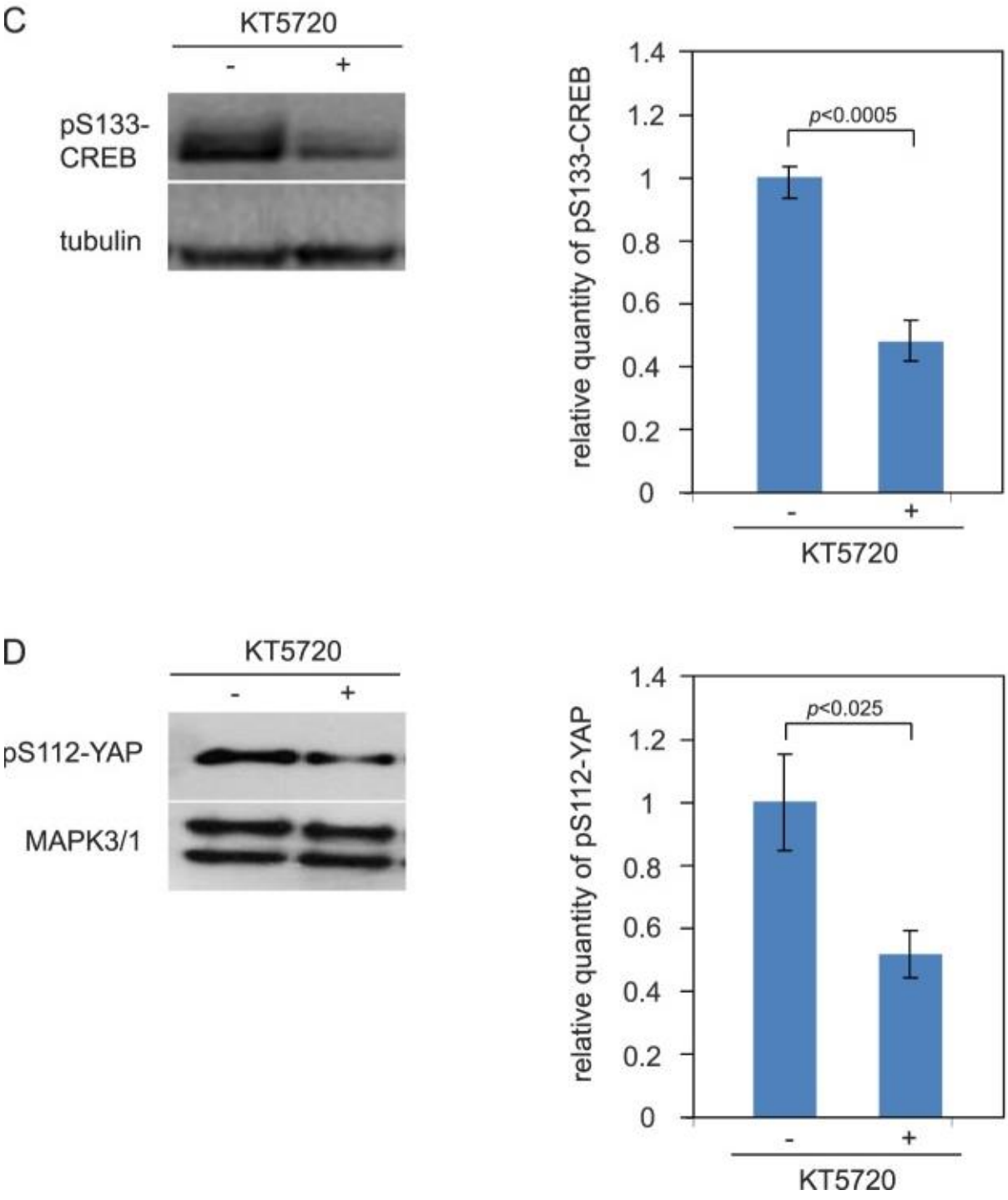


Figure 18: Regulation of S112-YAP phosphorylation in growing oocytes

A, B) Granulosa-oocyte complexes (GOCs) were collected. The oocyte was isolated from one portion and reserved for immunoblotting. The remaining GOCs (A) or oocytes isolated from the GOCs (B) were incubated overnight in the absence or presence of dbcAMP, after which the oocyte used for immunoblotting. Both pS112-YAPand MAPK3/1 were detected. Dibutyryl cyclic AMP is not required to maintain pS112-YAP during overnight incubation.



C, D) Growing oocytes were incubated overnight in the absence or presence of the protein kinase A inhibitor, KT5720, then subjected to immunoblotting to detect pS133-CREB (C) or pS112-YAP (D). Tubulin was used as a loading control in C because the molecular weights of CREB and MAPK3/1 are similar. Both pS133-CREB and pS112-YAP were decreased in the presence of KT5720 (Student t-test, P , 0.05).

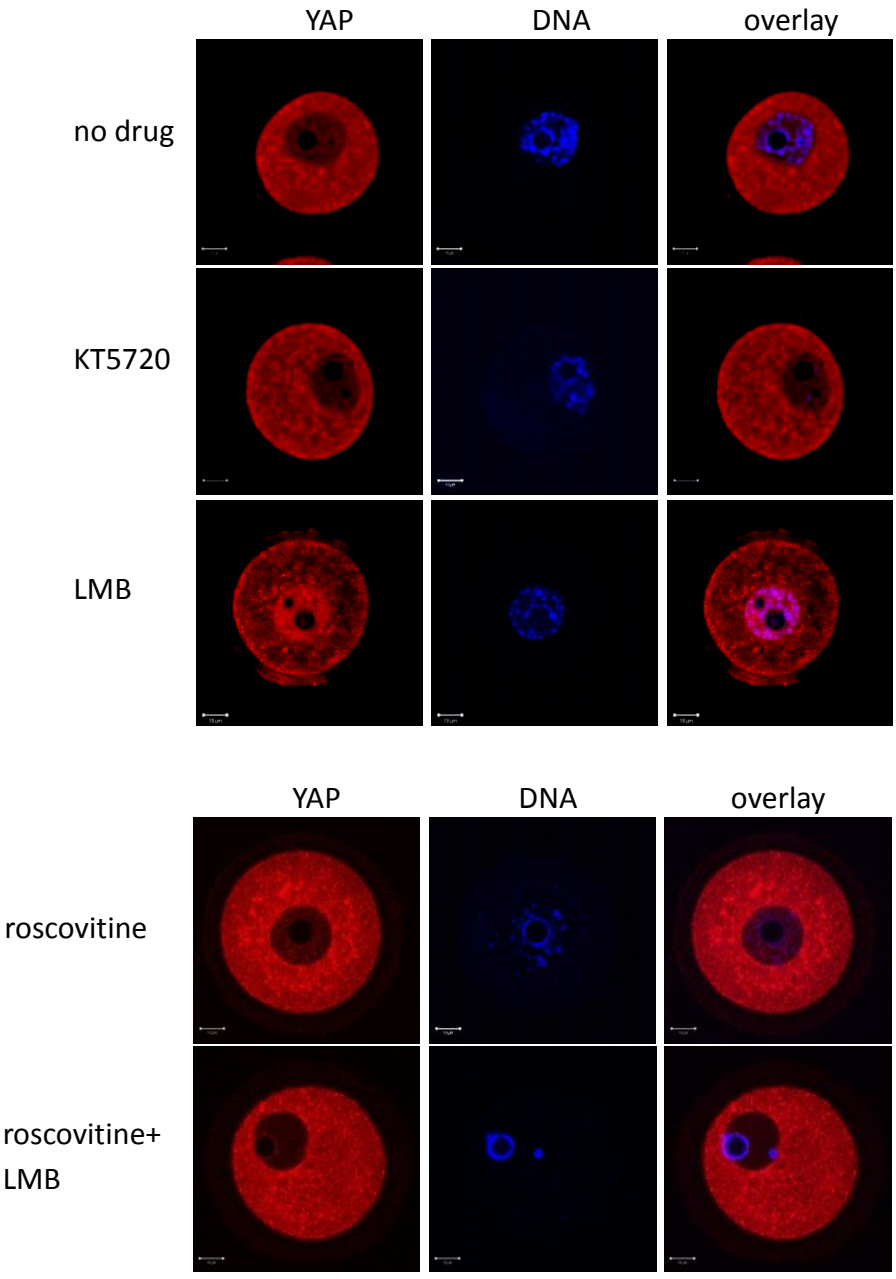


Figure 19: Transient nuclear localization of YAP in growing oocytes

A) Growing oocytes were incubated overnight under control conditions or in the presence of the protein kinase A inhibitor, KT5720, or the nuclear expost inhitor, leptomycn B (LMB), then stained using anti-YAP. LMB induces accumulation of nuclear YAP. B) Fully grown oocytes were incubated overnight in the presence of the CDK1 inhibitor, roscovitine, or roscovitine and LMB. No nuclear accumulation of YAP is detectable. Bar ¼ 10 µm.

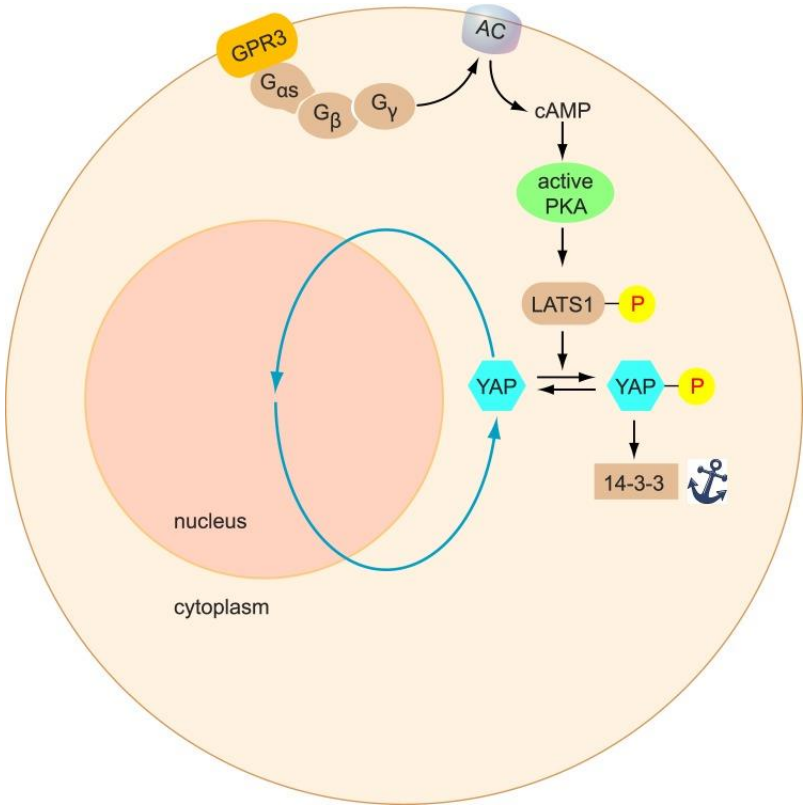


Figure 20: Control of YAP intracellular localization in oocytes

The cAMP synthesized by oocytes via G-protein receptor (GPR)-coupled adenyl cyclase (AC) maintains high activity of protein kinase A (PKA), which in turn directly or indirectly phosphorylates LATS1, thereby increasing its kinase activity toward YAP. S112 phosphorylated YAP associates with 14-3-3 proteins that anchor it in the cytoplasm. A portion of the YAP in growing oocytes remains nonphosphorylated and enters the nucleus but is rapidly exported back to the cytoplasm.

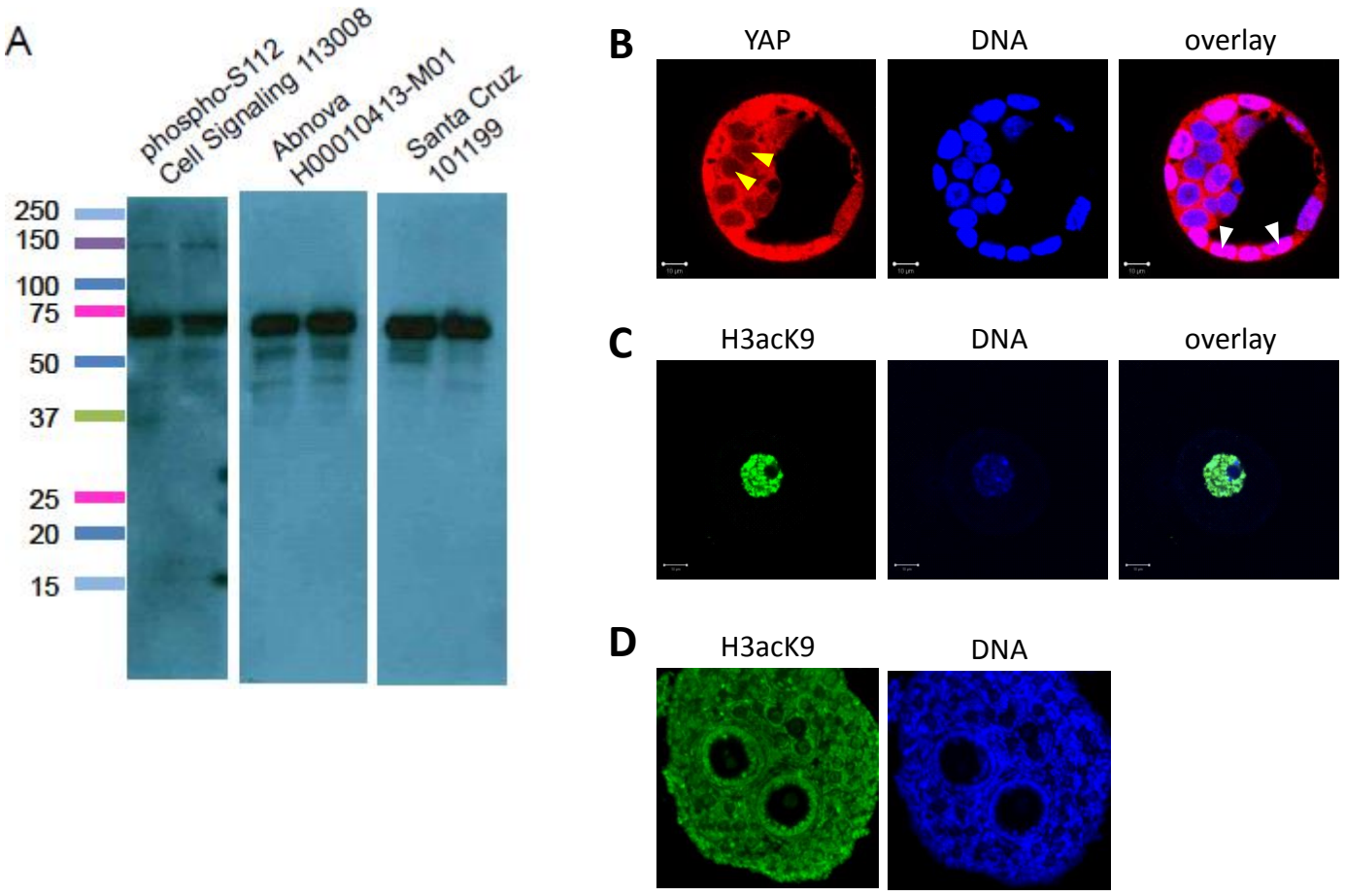


Figure 21: Fully grown oocytes were immunoblotted using the indicated antibodies (Supplemental data)

Each lane contains 75 oocytes. (B) Blastocysts were stained using anti-YAP from Abnova. Yellow arrowheads show unstained nuclei of the inner cell mass. White arrowheads show stained nuclei of trophectodermal cells. (C) Isolated oocytes were stained using anti-acetylated histone H3K9. (D) Ovarian paraffin sections were stained using anti-acetylated histone H3K9. Scale bar = 10 µm

References

Adhikari, D., N. Gorre, S. Risal, Z. Zhao, H. Zhang, Y. Shen and K. Liu (2012). "The safe use of a PTEN inhibitor for the activation of dormant mouse primordial follicles and generation of fertilizable eggs." PLoS one 7(6): e39034.

Allard, P., Q. Yang, W. F. Marzluff and H. J. Clarke (2005). "The stem-loop binding protein regulates translation of histone mRNA during mammalian oogenesis." Developmental biology 286(1): 195-206.

Basu, S., N. F. Totty, M. S. Irwin, M. Sudol and J. Downward (2003). "Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis." Molecular cell 11(1): 11-23.

Brito, I. R., I. M. Lima, M. Xu, L. D. Shea, T. K. Woodruff and J. R. Figueiredo (2014). "Three-dimensional systems for in vitro follicular culture: overview of alginate-based matrices." Reproduction, Fertility and Development 26(7): 915-930.

Chen, Q., N. Zhang, R. Xie, W. Wang, J. Cai, K.-S. Choi, K. K. David, B. Huang, N. Yabuta and H. Nojima (2015). "Homeostatic control of Hippo signaling activity revealed by an endogenous activating mutation in YAP." Genes & development 29(12): 1285-1297.

Cheng, Y., Y. Feng, L. Jansson, Y. Sato, M. Deguchi, K. Kawamura and A. J. Hsueh (2015). "Actin polymerization-enhancing drugs promote ovarian follicle growth mediated by the Hippo signaling effector YAP." The FASEB Journal 29(6): 2423-2430.

Clarke, H. J. (2018). "Regulation of germ cell development by intercellular signaling in the mammalian ovarian follicle." Wiley Interdisciplinary Reviews: Developmental Biology 7(1): e294.

Clarke, H. J., C. Oblin and M. Bustin (1992). "Developmental regulation of chromatin composition during mouse embryogenesis: somatic histone H1 is first detectable at the 4-cell stage." Development 115(3): 791-799.

Cockburn, K., S. Biechele, J. Garner and J. Rossant (2013). "The Hippo pathway member Nf2 is required for inner cell mass specification." Current biology 23(13): 1195-1201.

Conti, M., M. Hsieh, A. M. Zamah and J. S. Oh (2012). "Novel signaling mechanisms in the ovary during oocyte maturation and ovulation." Molecular and cellular endocrinology 356(1-2): 65-73.

De, S., J. L. Marcinkiewicz, S. Vijayaraghavan and D. Kline (2012). "Expression of 14-3-3 protein isoforms in mouse oocytes, eggs and ovarian follicular development." BMC research notes 5(1): 57.

de Vantéry Arrighi, C., A. Campana and S. Schorderet-Slatkine (2000). "A role for the MEK-MAPK pathway in okadaic acid-induced meiotic resumption of incompetent growing mouse oocytes." Biology of reproduction 63(2): 658-665.

Demeestere, I., A. K. Streiff, J. Suzuki, S. Al-Khabouri, E. Mahrous, S. L. Tan and H. J. Clarke (2012). "Follicle-stimulating hormone accelerates mouse oocyte development in vivo." Biology of reproduction 87(1): 3, 1-11.

Diepenbruck, M., L. Waldmeier, R. Ivanek, P. Berninger, P. Arnold, E. van Nimwegen and G. Christofori (2014). "Tead2 expression levels control Yap/Taz subcellular distribution, zyxin expression, and epithelial-mesenchymal transition." J Cell Sci: jcs. 139865.

Dong, J., G. Feldmann, J. Huang, S. Wu, N. Zhang, S. A. Comerford, M. F. Gayyed, R. A. Anders, A. Maitra and D. Pan (2007). "Elucidation of a universal size-control mechanism in Drosophila and mammals." Cell 130(6): 1120-1133.

Dupont, S., L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato and S. Bicciato (2011). "Role of YAP/TAZ in mechanotransduction." Nature 474(7350): 179.

El-Hayek, S. and H. J. Clarke (2015). "Follicle-stimulating hormone increases gap junctional communication between somatic and germ-line follicular compartments during murine oogenesis." Biology of reproduction 93(2).

El-Hayek, S., I. Demeestere and H. J. Clarke (2014). "Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle." Proceedings of the National Academy of Sciences 111(47): 16778-16783.

Eppig, J. J. and M. J. O'brien (1996). "Development in vitro of mouse oocytes from primordial follicles." Biology of reproduction 54(1): 197-207.

Finch, M. L., A. M. Passman, R. P. Strauss, G. C. Yeoh and B. A. Callus (2015). "Sub-cellular localisation studies may spuriously detect the Yes-associated protein, YAP, in nucleoli leading to potentially invalid conclusions of its function." PloS one 10(2): e0114813.

Freudzon, L., R. P. Norris, A. R. Hand, S. Tanaka, Y. Saeki, T. L. Jones, M. M. Rasenick, C. H. Berlot, L. M. Mehlmann and L. A. Jaffe (2005). "Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the Gs G protein." J Cell Biol 171(2): 255-265.

Handel, M. A., J. J. Eppig and J. C. Schimenti (2014). "Applying "gold standards" to in-vitro-derived germ cells." Cell 157(6): 1257-1261.

Hansen, C. G., T. Moroishi and K.-L. Guan (2015). "YAP and TAZ: a nexus for Hippo signaling and beyond." Trends in cell biology 25(9): 499-513.

Harrouk, W. and H. J. Clarke (1995). "Mitogen-activated protein (MAP) kinase during the acquisition of meiotic competence by growing oocytes of the mouse." Molecular reproduction and development 41(1): 29-36.

Harvey, K. F., X. Zhang and D. M. Thomas (2013). "The Hippo pathway and human cancer." Nature Reviews Cancer 13(4): 246.

Hayashi, K., S. Ogushi, K. Kurimoto, S. Shimamoto, H. Ohta and M. Saitou (2012). "Offspring from oocytes derived from in vitro primordial germ cell–like cells in mice." Science: 1222482.

Hirate, Y., K. Cockburn, J. Rossant and H. Sasaki (2012). "Tead4 is constitutively nuclear, while nuclear vs. cytoplasmic Yap distribution is regulated in preimplantation mouse embryos." Proceedings of the National Academy of Sciences 109(50): E3389-E3390.

Imajo, M., K. Miyatake, A. Iimura, A. Miyamoto and E. Nishida (2012). "A molecular mechanism that links Hippo signalling to the inhibition of Wnt/β-catenin signalling." The EMBO journal 31(5): 1109-1122.

Jagarlamudi, K., L. Liu, D. Adhikari, P. Reddy, A. Idahl, U. Ottander, E. Lundin and K. Liu (2009). "Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation." PloS one 4(7): e6186.

Kanai, F., P. A. Marignani, D. Sarbassova, R. Yagi, R. A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito and L. C. Cantley (2000). "TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins." The EMBO journal 19(24): 6778-6791.

Kaneko, K. J., E. B. Cullinan, K. E. Latham and M. L. DePamphilis (1997). "Transcription factor mTEAD-2 is selectively expressed at the beginning of zygotic gene expression in the mouse." Development 124(10): 1963-1973.

Kawamura, K., Y. Cheng, N. Suzuki, M. Deguchi, Y. Sato, S. Takae, C.-h. Ho, N. Kawamura, M. Tamura and S. Hashimoto (2013). "Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment." Proceedings of the National Academy of Sciences 110(43): 17474-17479.

Kim, M., M. Kim, S. Lee, S. Kuninaka, H. Saya, H. Lee, S. Lee and D. S. Lim (2013). "cAMP/PKA signalling reinforces the LATS–YAP pathway to fully suppress YAP in response to actin cytoskeletal changes." The EMBO journal 32(11): 1543-1555.

Kovo, M., M. Kandli-Cohen, M. Ben-Haim, D. Galiani, D. W. Carr and N. Dekel (2006). "An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes." Reproduction 132(1): 33-43.

Li, J., K. Kawamura, Y. Cheng, S. Liu, C. Klein, S. Liu, E.-K. Duan and A. J. Hsueh (2010). "Activation of dormant ovarian follicles to generate mature eggs." Proceedings of the National Academy of Sciences 107(22): 10280-10284.

Liu-Chittenden, Y., B. Huang, J. S. Shim, Q. Chen, S.-J. Lee, R. A. Anders, J. O. Liu and D. Pan (2012). "Genetic and pharmacological disruption of the TEAD–YAP complex suppresses the oncogenic activity of YAP." Genes & development 26(12): 1300-1305.

Mehlmann, L. M., T. L. Jones and L. A. Jaffe (2002). "Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte." Science 297(5585): 1343-1345.

Mehlmann, L. M., Y. Saeki, S. Tanaka, T. J. Brennan, A. V. Evsikov, F. L. Pendola, B. B. Knowles, J. J. Eppig and L. A. Jaffe (2004). "The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes." Science 306(5703): 1947-1950.

Mo, J.-S., Z. Meng, Y. C. Kim, H. W. Park, C. G. Hansen, S. Kim, D.-S. Lim and K.-L. Guan (2015). "Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway." Nature cell biology 17(4): 500.

Mo, J. S., H. W. Park and K. L. Guan (2014). "The Hippo signaling pathway in stem cell biology and cancer." EMBO reports 15(6): 642-656.

Monniaux, D., F. Clément, R. Dalbiès-Tran, A. Estienne, S. Fabre, C. Mansanet and P. Monget (2014). "The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link?" Biology of Reproduction 90(4): 85, 81-11.

Moroishi, T., C. G. Hansen and K.-L. Guan (2015). "The emerging roles of YAP and TAZ in cancer." Nature Reviews Cancer 15(2): 73.

Nishimura, T., W. Fujii, K. Sugiura and K. Naito (2014). "Cytoplasmic anchoring of cAMP-dependent protein kinase (PKA) by A-kinase anchor proteins (AKAPs) is required for meiotic arrest of porcine full-grown and growing oocytes." Biology of reproduction 90(3).

Nogueira, D., C. Albano, T. Adriaenssens, R. Cortvrindt, C. Bourgain, P. Devroey and J. Smitz (2003). "Human oocytes reversibly arrested in prophase I by phosphodiesterase type 3 inhibitor in vitro." Biology of reproduction 69(3): 1042-1052.

Norris, R. P., W. J. Ratzan, M. Freudzon, L. M. Mehlmann, J. Krall, M. A. Movsesian, H. Wang, H. Ke, V. O. Nikolaev and L. A. Jaffe (2009). "Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte." Development 136(11): 1869-1878.

Novella-Maestre, E., S. Herraiz, B. Rodríguez-Iglesias, C. Díaz-García and A. Pellicer (2015). "Short-term PTEN inhibition improves in vitro activation of primordial follicles, preserves follicular viability, and restores AMH levels in cryopreserved ovarian tissue from cancer patients." PLoS One 10(5): e0127786.

Oku, Y., N. Nishiya, T. Shito, R. Yamamoto, Y. Yamamoto, C. Oyama and Y. Uehara (2015). "Small molecules inhibiting the nuclear localization of YAP/TAZ for chemotherapeutics and chemosensitizers against breast cancers." FEBS Open Bio 5: 542-549.

Ota, M. and H. Sasaki (2008). "Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling." Development 135(24): 4059-4069.

Piccolo, S., S. Dupont and M. Cordenonsi (2014). "The biology of YAP/TAZ: hippo signaling and beyond." Physiological reviews 94(4): 1287-1312.

Picton, H., D. Briggs and R. Gosden (1998). "The molecular basis of oocyte growth and development." Molecular and cellular endocrinology 145(1-2): 27-37.

Reddy, P., L. Liu, D. Adhikari, K. Jagarlamudi, S. Rajareddy, Y. Shen, C. Du, W. Tang, T. Hämäläinen and S. L. Peng (2008). "Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool." Science 319(5863): 611-613.

Ren, F., L. Zhang and J. Jiang (2010). "Hippo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms." Developmental biology 337(2): 303-312.

Richard, S. and J. M. Baltz (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." Biology of reproduction 90(6): 137, 131-110.

Richardson, H. E. (2011). "Actin up for Hippo." The EMBO journal 30(12): 2307-2309.

Sánchez, F. and J. Smitz (2012). "Molecular control of oogenesis." Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1822(12): 1896-1912.

Shea, L. D., T. K. Woodruff and A. Shikanov (2014). "Bioengineering the ovarian follicle microenvironment." Annual review of biomedical engineering 16: 29-52.

Smitz, J., M.-M. Dolmans, J. Donnez, J. Fortune, O. Hovatta, K. Jewgenow, H. Picton, C. Plancha, L. Shea and R. Stouffer (2010). "Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation." Human reproduction update 16(4): 395-414.

Sun, T., M. E. Pepling and F. J. Diaz (2015). "Lats1 deletion causes increased germ cell apoptosis and follicular cysts in mouse ovaries." Biology of reproduction 93(1).

Sun, X., Y. Su, Y. He, J. Zhang, W. Liu, H. Zhang, Z. Hou, J. Liu and J. Li (2015). "New strategy for in vitro activation of primordial follicles with mTOR and PI3K stimulators." Cell Cycle 14(5): 721-731.

Telfer, E. E. and M. McLaughlin (2011). In vitro development of ovarian follicles. Seminars in reproductive medicine, © Thieme Medical Publishers.

Telfer, E. E. and M. B. Zelinski (2013). "Ovarian follicle culture: advances and challenges for human and nonhuman primates." Fertility and sterility 99(6): 1523-1533.

Varelas, X. (2014). "The Hippo pathway effectors TAZ and YAP in development, homeostasis and disease." Development 141(8): 1614-1626.

Wang, W., J. Huang and J. Chen (2011). "Angiomotin-like proteins associate with and negatively regulate YAP1." Journal of Biological Chemistry 286(6): 4364-4370.

Wang, Y., Z. Teng, G. Li, X. Mu, Z. Wang, L. Feng, W. Niu, K. Huang, X. Xiang and C. Wang (2015). "Cyclic AMP in oocytes controls meiotic prophase I and primordial folliculogenesis in the perinatal mouse ovary." Development 142(2): 343-351.

Woodruff, T. K. (2015). "Oncofertility: a grand collaboration between reproductive medicine and oncology." Reproduction: REP-15-0163.

Yang, Q., P. Allard, M. Huang, W. Zhang and H. J. Clarke (2010). "Proteasomal activity is required to initiate and to sustain translational activation of messenger RNA encoding the stem-

loop-binding protein during meiotic maturation in mice." Biology of reproduction 82(1): 123-131.

Yu, F.-X., Y. Zhang, H. W. Park, J. L. Jewell, Q. Chen, Y. Deng, D. Pan, S. S. Taylor, Z.-C. Lai and K.-L. Guan (2013). "Protein kinase A activates the Hippo pathway to modulate cell proliferation and differentiation." Genes & development 27(11): 1223-1232.

Yu, F.-X., B. Zhao, N. Panupinthu, J. L. Jewell, I. Lian, L. H. Wang, J. Zhao, H. Yuan, K. Tumaneng and H. Li (2012). "Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling." Cell 150(4): 780-791.

Zanconato, F., M. Forcato, G. Battilana, L. Azzolin, E. Quaranta, B. Bodega, A. Rosato, S. Bicciato, M. Cordenonsi and S. Piccolo (2015). "Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth." Nature cell biology 17(9): 1218.

Zeng, H.-t., Z. Ren, L. Guzman, X. Wang, M. L. Sutton-McDowall, L. J. Ritter, M. De Vos, J. Smitz, J. G. Thompson and R. B. Gilchrist (2013). "Heparin and cAMP modulators interact during pre-in vitro maturation to affect mouse and human oocyte meiosis and developmental competence." Human Reproduction 28(6): 1536-1545.

Zhang, M., Y.-Q. Su, K. Sugiura, G. Xia and J. J. Eppig (2010). "Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes." Science 330(6002): 366-369.

Zhao, B., L. Li, Q. Lu, L. H. Wang, C.-Y. Liu, Q. Lei and K.-L. Guan (2011). "Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein." Genes & development 25(1): 51-63.

Zhao, B., L. Li, K. Tumaneng, C.-Y. Wang and K.-L. Guan (2010). "A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCFβ-TRCP." Genes & development 24(1): 72-85.

Zhao, B., X. Wei, W. Li, R. S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu and L. Li (2007). "Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control." Genes & development 21(21): 2747-2761.

Zhao, B., X. Ye, J. Yu, L. Li, W. Li, S. Li, J. Yu, J. D. Lin, C.-Y. Wang and A. M. Chinnaiyan (2008). "TEAD mediates YAP-dependent gene induction and growth control." Genes & development 22(14): 000-000.

Transitional Statement

As previously mentioned in chapter 1, oocyte healthy development- which ensures future normal fertilization, embryogenesis, and live birth- is tightly relied on its appropriate growth and subsequent meiotic maturation. Noticebly, both oocyte growth and meiotic maturation are accurately controlled by the surruonding granulosa cell.

In Chapter 2 we focused on the first part of oocyte develoment, growth, through which the oocyte incereases in size dramatically aiming to provide suffucient supply for the future early embryo. Therefore, understanding molecular mechanisms which regulate oocyte proper growth are of high importance in the physiology of oocyte development. In chapter 2, we particularly investigated the role of a major regulator of growth and developmental processes, YAP, in the context of oocyte growth.

In the next chapter we will move on to the the second part of oocyte development, meiotic maturation, and will specifically concentrate on the concept of oocyte-granulosa cells communication throughout meiotic maturation. Since transzonal projections (TZPs) are the only means of physical contact between the oocyte and its surrounding granulosa cells, however are completely lost during meiotic maturation, we will explore the molecular mechanism controlling TZPs dynamics during meiotic maturation, in the course of upcoming chapter.

Chapter 3

MANUSCRIPT II

Depletion of cyclic GMP in the somatic compartment triggers retraction of filopodia mediating germline-soma communication in females

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Running title: cGMP controls TZP stability

Key words: follicle, filopodia, cyclic GMP, intercellular communication, oocyte

Preface

In the present chapter (Manuscript II) we target the second and final part of oogenesis, meiotic maturation, which commences immediately after the oocyte reaching its full size. Although the filamentous bridges, TZPs, whose mission is to provide germline-soma communication are carefully preserved during oocyte growth, they vanish during meiotic maturation to release the oocyte from the influence of the granulosa cells in preparation for meeting the sperm. However, neither the timing and dynamics of this loss, nor the molecular mechanisms behind it have been studied so far. Accordingly, we sought to clarify these issues by pursuing the following objectives:

IIa) Examine the dynamics of TZPs loss within the oocyte-cumulus complexes during *in vitro* meiotic maturation

IIb) Determine whether the factors involved in regulating meiotic maturation are also implicated in TZP loss.

IIc) Identify which of the two cell types, either oocyte or cumulus cells, governs the process of TZPs loss.

This work is discussed in Chapter 3 (Manuscript II) which is in preparation for submission.

Significance statement

Filopodia are bridges along which regulatory molecules can be transported between physically distant cells. Despite their importance in controlling intercellular communication under normal and pathological conditions, their delicate nature has made them difficult to study. During its growth, the mammalian oocyte is linked by thousands of easily visualized filopodia that extend from the somatic follicular cells that surround it. During the final stage of oocyte development, termed maturation, these filopodia rapidly disappear, freeing the oocyte from this external control in preparation for fertilization. Here, we identify the signaling pathways that control the filopodial loss and show that they are activated by the signals that trigger oocyte maturation. Our results identify the molecular basis of developmentally programmed loss of intercellular communication.

Abstract

Filopodia play essential roles regulating intercellular communication, serving as physical bridges that enable regulatory molecules to be exchanged between physically distant cells. However, mechanisms controlling filopodial dynamics remain incompletely understood. During oocyte growth in mammals, the surrounding somatic follicular granulosa cells elaborate actin-rich filopodia, termed transzonal projections (TZPs), that traverse the zona pellucida separating the two cell types and contact the oocyte plasma membrane, enabling transmission of signals from the granulosa cells to the oocyte. During meiotic maturation, the TZPs are lost, freeing the oocyte from direct maternal control in preparation for fertilization. The molecular mechanisms regulating TZP loss, however, are unknown. We show here that, when oocytes reach full-size, cyclic GMP (cGMP) produced by the granulosa cells prevents the TZPs from undergoing a spontaneous slow retraction. Activation of epidermal growth factor receptor (EGFR) signaling, which can initiate oocyte maturation, triggers a more rapid retraction of the TZPs. Unexpectedly, EGFR-triggered retraction is independent of oocyte maturation; rather, it is controlled exclusively by signaling within the granulosa cells. EGFR-triggered TZP retraction requires activity of extracellular signal regulated kinase and of cGMP-specific phosphodiesterase. cGMP is known to inhibit Rho-associated kinase (ROCK), which regulates actomyosin contractility. Strikingly, pharmacological inhibition of ROCK fully prevents TZP retraction. We propose that high cGMP within granulosa cells inhibits actomyosin contraction, thereby maintaining TZPs; conversely, when the hormonal signals that initiate maturation cause cGMP to decrease, activation of actomyosin contraction triggers TZP retraction. Our results provide a molecular mechanism underpinning a developmentally programmed loss of filopodia mediated intercellular communication.

Introduction

Intercellular signaling plays an indispensable role in the development and function of multicellular organisms. Signals are exchanged not only between adjacent cells but also between cells that are physically separated from each other. Such long-range signaling has until recently been attributed to the diffusion of secreted factors from their site of synthesis to their site of action. It is now increasingly recognized, however, that long cytoplasmic bridges can link distant cells and that these can serve as a means to directly transport signaling molecules from their source to their target cells. The best-known of these structures are filopodia, which play a wide-range of roles both in normal development and during disease (Fairchild and Barna 2014, Jacquemet, Hamidi et al. 2015, Stuelten, Parent et al. 2018). Related structures, such as tunneling nanotubes and cytonemes, also transmit signals over long intercellular distances (Marzo, Gousset et al. 2012, Inaba, Buszczak et al. 2015, Roy and Kornberg 2015). Thus, a better knowledge of the mechanisms that regulate the assembly and disassembly of these distinctive structures is needed to achieve a deeper understanding of cellular behaviors and interactions under both normal and pathogenic conditions. Oocytes of all species are surrounded by a thick extracellular coat that separates them from the adjacent somatic cells. Contact is maintained through filopodia-like structures that emanate from the follicle cells and penetrate through the protective coating to reach the oocyte plasma membrane (Clarke 2018). In mammals, the extracellular coat is termed the zona pellucida and the filopodia-like structures are termed transzonal projections (TZPs) (Chiquoine 1960, Albertini, Combelles et al. 2001, Li and Albertini 2013, Wassarman and Litscher 2013, Wassarman and Litscher 2013, Clarke 2018). Gap junctions that are assembled where the tips of the TZPs contact the oocyte membrane allow the granulosa cells to transfer to the oocytes nutrients and regulatory signals that are essential for its development

(Sánchez and Smitz 2012). The anatomically simple architecture of the mammalian ovarian follicle, the localization of the TZPs in a defined position, and the development of culture conditions that support follicular growth in vitro (O'Brien, Pendola et al. 2003) together make it an ideal model system in which to experimentally study the dynamics and function of intercellular bridges of communication. During growth of the oocyte, which requires about three weeks in the mouse, the adjacent follicular granulosa cells proliferate mitotically so that they continue to fully enclose the expanding surface area of the germ cell and continuously elaborate new TZPs, leading to a steady increase in the number of TZPs (El-Hayek, Yang et al. 2018). Factors secreted by the oocyte, including the transforming growth factor β super-family member, growth-differentiation factor 9 (Peng, Li et al. 2013), promote the elaboration of new TZPs. Thus, when the follicle has reached the preovulatory antral stage, a rich network of TZPs connects the full-grown oocyte to the cumulus granulosa cells that surround it (Wassarman and Litscher 2013, El-Hayek, Yang et al. 2018). In response to the ovulatory surge of luteinizing hormone, the oocyte undergoes the final stage of differentiation, termed meiotic maturation (Coticchio, Dal Canto et al. 2015, Jaffe and Egbert 2017). Coincident with maturation, the TZPs are lost, and the granulosa cells become separated both from the oocyte and from each other through a process known as cumulus layer expansion. Thus, in contrast to the relatively slow accumulation of TZPs linked to oocyte growth, their loss during maturation is relatively rapid. The loss of the TZPs frees the oocyte from the direct influence of the granulosa cells prior to fertilization and initiation of embryonic development. Loss of the TZPs, which are firmly embedded in the zona pellucida, may be necessary to fully disengage the tightly packed innermost layer of cumulus granulosa cells from the *zona pellucida* and thereby facilitate sperm access to the oocyte. Yet, the mechanism underlying this key developmental transition has

remained unknown. Here, we identify mechanisms that maintain TZPs within the cumulus granulosa cell-oocyte complexes (COCs) of antral follicles and trigger their loss during meiotic maturation. We show that the maintenance and subsequent disassembly of these intercellular bridges of communication are precisely orchestrated events. Our results may contribute to an understanding of some cases of human infertility and more generally demonstrate how the structures that permit intercellular communication between physically separated cells can be disassembled in a developmentally regulated manner.

Material and methods

Animals

Mice

All experiments were performed in compliance with the regulations and policies of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Research Institute of the McGill University Health Centre (RI-MUHC). CD-1 mice were obtained from Charles River (St-Constant, QC). mTmG (membrane-Tomato/membrane-Green) founder mice were obtained (Jackson Laboratory, Bar Harbor, ME; strain 007676) and a colony established at the RI-MUHC. These mice carry a transgene encoding a membrane-targeted fluorescent protein. Targeting is mediated via the first 8 amino acids of the plasma membrane associated protein, MARCKS.

Pig

Swine ovaries were obtained from pre-pubertal gilts collected from a local abattoir (Olymel, S.E.C./L.P, Saint Esprit, Quebec, Canada).

Collection and culture of cells

Mice

COCs containing fully grown oocytes and granulosa cell-oocyte complexes (GOCs) containing growing oocytes were collected from post-natal day (PD) 19-21 mice that had received an intraperitoneal injection of 5 IU of equine chorionic gonadotropin (Sigma, Windsor, ON) 44 hr previously and PD 17 mice, respectively, as described (O'Brien, Pendola et al. 2003, El-Hayek and Clarke 2016, El-Hayek, Yang et al. 2018). COCs were collected in MEMH containing cilostamide (20 μM, Sigma) to prevent meiotic resumption.

To permit meiotic resumption, COCs were incubated in cilostamide-free MEM in the absence of cilostamide. Depending on the experiment, the following supplements were added: CNP (100 nM, Sigma N8768), 8-pCPT-cGMP (2.5 mM, Sigma C5438), EGF (10 ng/ml, BD Biosciences 354052), U0126 (25 μM, Sigma U120), Sildenafil (100 μM, Tocris 3784), Y-27632 (250 μM, Sigma Y0503), cilostamide (15 μM, Sigma C7971), or Roscovitine (10 μM, Sigma R7772). GOCs were incubated in the presence or absence of EGF, VEGF (150 ng/mL, R&D 220-BB-010), and PDGF (50 ng/mL, R&D 293-VE-010).

Pig

Cumulus oocyte complexes (COC) were collected from 3–6 mm follicles and those having at least three layers of cumulus cells and homogeneous cytoplasm were selected for maturation. Groups of 30 COCs were matured as described (Bohrer, Coutinho et al. 2015).

Immunofluorescence

COCs, GOCs, and denuded oocytes were fixed using in freshly prepared 2% (w/v) paraformaldehyde (Fisher Scientific 04042) in phosphate-buffered saline (PBS, pH 7.2) containing 0.1% Triton X-100 (ACROS 9002-93-1) and processed for immunofluorescence as described ((Yang, Allard et al. 2010)). To mount the samples, a 9 x 0.12 mm spacer (GBL654008, Sigma) was attached to a glass microscope slide. A 2 µl drop of PBS containing 0.3% Polyvinylpyrrolidone (Sigma, PVP360) was placed in the center of the spacer and covered with 20 µl of mineral oil. Samples were then transferred into the drop of PBS and a cover slip was placed on top. Samples were imaged using a LSM 880 confocal microscope (Zeiss, Toronto, ON). The following reagents were used for fluorescent detection of specific proteins: Phalloidin-TRITC (1:100, P1951, Sigma), Phalloidin- Alexa 488 (1:100, A12379, Thermo Fisher), DAPI (1:100, Roche 10236276001), anti-E-Cadherin (1:100, BD Transduction Laboratories, 610181), anti-β-catenin (1:100, BD Transduction Laboratories, 610153), anti-RFP (1: 400, Cedarlane, 600-401-379), anti-rabbit IgG-Alexa 488 (1:100, Thermo Fisher, A11008). Tyramide SuperBoost™ Kit with Alexa Fluor™ Tyramide (Invitrogen) was used to amplify the β-catenin signal, following the manufacturer's directions.

Immunoblotting

Immunoblotting was performed as described (REF) except that proteins were separated using pre-cast 12% gels (456-8045, Bio Rad). Antibodies used were against phospho-p44/42 ERK (1:1000, Cell Signalling 9106) and TACC3 (1:1000, Abcam 134154).

Confocal image analysis

To quantify the number of actin-TZPs, a confocal optical section was obtained at the equatorial plane of the oocyte. Using Fiji software (National Institutes of Health, Bethesda, MD), segmented circle was created around the oocyte circumference, in the middle of the zona pellucida, and the fluorescence intensity at each point on the line was determined. Each point whose value was above the background value of the oocyte cytoplasm and higher than each of its immediately neighboring points was counted as a TZP. The total number of TZPs counted by this method, although not representing the total number of TZPs in the specimen, was considered as the TZP number associated with the oocyte.

Analysis of gap junctional coupling using microinjection of fluorescence Lucifer Yellow dye

The oocyte of each COC was injected with about 10 pl of a 100 mM solution of Lucifer Yellow (Thermo Fisher), using a Zeiss Axio Observer Z1 microscope and PLI-100 microinjector (Medical Systems, NY). The complexes were incubated for 30 minutes to allow dye transfer to the cumulus granulosa cells and then examined using fluorescence microscopy.

Statistical Analysis

All experiments were carried out at least three times. Quantitative data were analyzed using one-way ANOVA followed by Tukey HSD. Error bars indicate SEM. A p-value of less than 0.05 was considered significant, and is indicated by different letters above bars of the histograms.

Results

TZPs are lost spontaneously in a time-dependent manner following removal of COCs from the follicle.

Using the F-actin binding peptide, phalloidin, TZPs can be visualized as filopodia-like structures that originate from the innermost layer(s) of granulosa cells, pass through the zona pellucida, and eventually contact the oocyte plasma membrane (Figure. 22A) (Barrett, Shea et al. 2010, Li and Albertini 2013, Macaulay, Gilbert et al. 2014, Makita and Miyano 2014, McGinnis and Kinsey 2015, El-Hayek, Yang et al. 2018). To examine the process of TZP loss during oocyte maturation, we collected COCs and incubated them in vitro. At different intervals, COCs were removed from the culture, fixed, and stained using Alexa Fluor 488-conjugated phalloidin. Using confocal images, the number of TZPs within the equatorial circumference of the zona pellucida of each oocyte - still fully enclosed by cumulus cells – was quantified. As shown in Figure 22 (B, C), the number of TZPs progressively decreased in a time dependent manner, such that less than 5% of the TZP population present at 0 hr remained after 15 hr of incubation. Since the duration of meiotic maturation in mouse is relatively short (~ 12 hr) (El-Hayek and Clarke 2016), we also assessed TZP dynamics in a species whose duration is longer and thus closer to human. To this end, we examined pig COCs, whose meiotic maturation requires 42-46 hr (Edwards 1965). We observed the same gradual decrease in the number of TZPs as the oocytes matured (Fig. 22D, E). The loss of TZPs in both species was characterized by shortening and clustering of TZPs towards the cumulus cell as well as an accumulation of large foci of actin rooted at the basis of the cumulus granulosa cells (Fig. 22F). This phenotype suggests that the loss of TZPs is due to their active retraction into the cumulus cell of origin. Together, these results demonstrate that TZPs, which continuously grow out from the granulosa cells towards the oocyte during the prolonged

period of oocyte growth in the mouse (El-Hayek, Yang et al. 2018), retract within a relatively brief period of time when the COC is removed from the antral follicle and placed in culture. We next explored the molecular basis of these TZP dynamics.

NPPC-cGMP signalling pathway in the cumulus cells maintains TZPs

Because TZPs are preserved while the COC is in the follicle yet are lost when it is removed from the follicle, we speculated that a factor within the follicular environment might prevent TZP loss. C-type natriuretic peptide (CNP, also termed NPPC) produced by mural granulosa cells (Norris, Ratzan et al. 2009, Zhang, Su et al. 2010, Kawamura, Cheng et al. 2011, Zhang, Su et al. 2011, Franciosi, Coticchio et al. 2014) binds to and activates its receptor guanylyl cyclase, NPR2, on the surface of both mural and cumulus granulosa cells, which in turn induces synthesis of cyclic guanosine monophosphate (cGMP) within these cells. cGMP is then transferred from the cumulus granulosa cells to the oocyte through gap junctions (Norris, Ratzan et al. 2009, Zhang, Su et al. 2010) and suppresses phosphodiesterase 1 (PDE) 3A in the oocyte to maintain high cyclic guanosine monophosphate (cAMP)-dependent protein kinase A activity, which blocks meiotic resumption (Conti, Hsieh et al. 2012). We hypothesized that CNP-cGMP signalling maintains TZPs, as it maintains oocyte meiotic arrest. To test this hypothesis, we incubated COCs overnight in the presence and absence of either CNP or a membrane-permeable analog of cGMP (8-pCPT cGMP) and then quantified TZP8 number. We found that addition of either CNP or the cGMP analog fully preserved the TZPs within the COCs during overnight culture (Fig. 23 A, B), consistent with recent reports (Franciosi, Coticchio et al. 2014, Sánchez, Lolicato et al. 2017). As expected, the oocytes in both groups remained meiotically arrested, confirming that the drugs generated high cGMP within the COCs. Importantly, the ability of CNP to maintain

meiotic arrest demonstrates that the TZPs remained functional, as it indicates that cGMP produced in the cumulus granulosa cells was transferred to the oocyte. Both TZP-loss and maturation occurred when the COCs were subsequently incubated in drug-free medium, indicating that the inhibitory effects were reversible (data not shown). These results demonstrate that either inducing endogenous cGMP production within the cumulus cells by activating NPPC-NPR2 signalling or directly delivering a cGMP analog to the COCs by adding it to the culture medium efficiently maintains the TZPs. Because a portion of the cGMP produced in the cumulus cells is transferred to the oocyte, where it prevents meiotic maturation, we then asked in which cell compartment it acts to prevent TZP retraction. We incubated COCs overnight in the presence of CNP and carbenoxolone, which blocks gap junction activity. As expected, abrogating the flow of cGMP from the cumulus granulosa cells to the oocyte triggered meiotic resumption (Fig. 23C). Strikingly, however, the TZPs were preserved such that, at the end of the incubation period, we observed mature oocytes fully surrounded by TZPs (Fig. 23C, D). These results clearly show that cGMP acts within the cumulus granulosa cells to maintain TZPs.

Activation of EGFR signalling accelerates TZP loss

Although fully grown oocytes undergo meiotic maturation spontaneously when removed from the follicle and placed in culture, maturation under physiological conditions is initiated through a cascade of events triggered by the pre-ovulatory LH surge and mediated at least in part via trans-activation of the epidermal growth factor receptor (EGFR) (Conti, Hsieh et al. 2012, Jaffe and Egbert 2017). Therefore, we tested whether EGFR signaling also regulated TZP loss. When we cultured COCs in the presence of EGF, we observed a dramatically accelerated loss of TZPs such that fewer than 10% remained by 8 hr after maturation initiation, whereas almost 80%

remained in the untreated COCs (Fig. 24A, B). The rapid loss of TZPs led us to question whether EGFR activation triggered loss of the entire filopodial structure or only of its actin cytoskeleton. To distinguish between these possibilities, we used two approaches to visualize the TZP plasma membrane. We first used a genetically engineered mouse model, mTmG, which constitutively express membrane-targeted Tomato, a modified form of red fluorescent protein (RFP) that can be stained using anti-RFP antibodies (Boyd, Skove et al. 2015). Second, using non-transgenic mice, we defined the TZP 1 membrane using anti-β-catenin. We collected COCs and cultured them with EGF for 8 hr and then stained with phalloidin and anti-RFP or anti-β-catenin. Both approaches revealed that both the actin filaments and the plasma membrane of the TZPs had disappeared by 8 hr in EGF-treated COCs (Fig. 24C). These results confirm that EGF triggers rapid loss of the TZPs.

TZP loss is independent of oocyte meiotic maturation

Because TZP loss occurs coincident with oocyte maturation, we considered that it might be dependent on this process. TZPs are thought to stably attach to the oocyte via interactions between N-cadherin on the tips of the TZP and E-cadherin on the oocyte plasma membrane (Li and Albertini 2013), and we observed a striking loss of both E-cadherin and β-catenin from the oocyte plasma membrane during maturation (Fig. 25A), providing conceptual support for this hypothesis. To test it, we blocked oocyte maturation in EGF-treated COCs, using either cilostamide, an inhibitor of PDE3A, or roscovitine, an inhibitor of CDK1 activity. As expected, the oocytes remained in meiotic arrest. Moreover, E-cadherin remained localized at the plasma membrane (Fig. 25B). Unexpectedly, however, the TZPs were lost (Fig. 25C, D). Because

EGFR-activated TZP loss can occur independently of meiotic maturation, we conclude that it is controlled principally or solely by mechanisms operating within the granulosa cells.

TZP loss requires ERK-MAP kinase signaling

EGFR signalling within the granulosa cells is mediated through the ERK-MAP kinase (hereafter termed ERK) pathway (Panigone, Hsieh et al. 2008, Sánchez and Smitz 2012). To investigate whether the ERK pathway mediates EGFR-induced TZP loss, we co-cultured COCs with EGF and U0126, a highly selective inhibitor of this pathway. Strikingly, TZP loss was completely abrogated by U0126 (Fig. 25E, F). To test whether these TZPs remained functional, we treated COCs as above and, at the end of the 8-hr culture period, injected Lucifer Yellow, a fluorescent dye that can pass through gap junctions, into the oocyte. Following a 30-minute incubation to allow dye-transfer, we imaged the COCs. In COCs cultured in EGF alone, no fluorescence was observed in the cumulus cells, indicating that gap junctions no longer connected them to the oocyte. In contrast, extensive dye-transfer to the cumulus granulosa cells occurred in COCs cultured in EGF and U0126 (Fig. 25G), indicating that functional gap junctions remained. These results indicate that EGFR-mediated loss of TZPs depends on activation of the ERK pathway. Unexpectedly, in contrast to its activity to prevent TZP loss, U0126 did not prevent the loss of detectable E-cadherin from the oocyte membrane (Fig. 25B). This result suggests that the maintenance of functional TZPs does not require E-cadherin at the oocyte membrane.

PDE5 is the link between EGF and cGMP signalling in regulating TZPs dynamics

Because elevated cGMP in the cumulus granulosa cells maintains TZPs, whereas EGF triggers their loss, we then asked whether these opposing activities were mechanistically linked. The

amount of cGMP in the granulosa cells is determined by the balance between its synthesis, via NPR2, and its degradation, via cGMP-specific phosphodiesterase type 5 (PDE5), which is expressed in the granulosa cells of antral follicles and becomes activated upon LH-induced maturation (Egbert, Uliasz et al. 2016). To test whether the EGF-triggered loss of TZPs occurred via a decrease in cGMP, we co-cultured COCs with EGF and sildenafil, a highly selective PDE5 inhibitor. Strikingly, inhibiting PDE5 activity entirely prevented EGF-induced TZP loss (Fig. 26A, B). These results imply that activation of EGFR signaling triggers TZP loss via a reduction in cGMP within the cumulus granulosa cells.

We then sought to identify a mechanism linking the reduction in cGMP to the retraction of the TZPs. Rho-associated protein kinase (ROCK) regulates actin dynamics and remodelling (Kimura, Ito et al. 1996, Gallo 2004, Zaidel-Bar, Zhenhuan et al. 2015, Pandya, Orgaz et al. 2017). Strikingly, through its activation of protein kinase G, cGMP suppresses Rho-ROCK signalling (Kato, Blanton et al. 2012, Aburima, Walladbegi et al. 2017). This led us to hypothesize that TZP retraction may be mediated through activated ROCK. Indeed, we found that co-culture with Y-27632, a selective ROCK inhibitor, fully prevented the loss of TZPs in EGF-stimulated COCs (Fig. 26C, D). This inhibition was reversible, confirming that it was not due to a cell-toxic effect of Y-27632 (data not shown). Taken together, these data suggest that TZP loss is triggered by relief of cGMP-mediated inhibition of ROCK activity.

Growth factors do not trigger loss of TZPs in pre-antral follicles

To identify at which stage of follicular development granulosa cells acquire the ability to retract their TZPs, we collected GOCs from late pre-antral follicles and cultured them overnight with

EGF, as well as with VEGF or PDGF, which also activate the ERK pathway in target cells. Immunoblotting of the granulosa cells that were isolated from GOCs immediately following 1 hr exposure to the growth factors confirmed that each growth factor was able to activate ERK signaling, as indicated by phosphorylation of ERK (Fig. 27A). Following overnight culture, however, in no case did we observe a decrease in the number of TZPs (Fig. 27B). We conclude that the granulosa cells from pre-antral follicles do not possess the required machinery downstream of ERK phosphorylation to trigger TZP loss.

Discussion

Although it is well-established that TZPs are lost during meiotic maturation of the oocyte, the mechanism underlying this key developmental transition has remained unknown. Our results identify a mechanism that is responsible for maintaining TZPs within antral follicles prior to meiotic maturation and ovulation; establish that TZP loss, although triggered by the same signaling pathway that can initiate oocyte maturation, is regulated entirely independently of this germ-cell process; and provide evidence that activation of actomyosin contractility within the cumulus granulosa cells may represent the biophysical mechanism through which TZPs retract into their cell of origin.

cGMP produced by the granulosa cells is transferred to the oocyte where, by inhibiting the activity of PDE3A, it maintains meiotic arrest. Our results identify a new function for cGMP – to maintain the TZPs within COCs – and show that this function requires cGMP within the cumulus granulosa cells themselves. Thus, meiotic arrest of the oocyte requires cGMP function in both the somatic and germ cell compartments. cGMP within the granulosa cells maintains the TZPs,

and the TZPs in turn, via the gap junctions located at their points of contact with the oocyte, enable cGMP to be transferred to the oocyte where it maintains meiotic arrest (Fig. 28A). Our results thus reveal that mechanistically independent but developmentally coordinated functions of cGMP maintain the oocyte in a state where it is poised to initiate maturation in response to the ovulatory stimulus.

Activation of EGFR signaling, which promotes oocyte maturation, triggers a relatively rapid loss of the TZPs, and we suggest that this is the physiological pathway through which TZPs are lost. Because this loss is temporally coordinated with maturation and because E-cadherin, which is thought to mediate TZP attachment to the oocyte plasma membrane via heterotypic interactions with N-cadherin (Li and Albertini 2013), becomes undetectable on the oocyte membrane during maturation, we initially hypothesized that retraction was mechanistically linked to oocyte maturation. In contrast, however, we found that the two processes could be experimentally dissociated. Moreover, when ERK signalling was blocked, TZPs remained intact and functional, as assayed by dye-transfer, despite the loss of detectable E-cadherin from the oocyte plasma membrane. Thus, whereas the growth of TZPs is promoted by factors produced by the oocyte (El-Hayek, Yang et al. 2018), their retraction appears to be regulated solely by the cumulus granulosa cells.

Because EGFR-triggered loss of TZPs is inhibited when activity of the cGMP-specific PDE5 is blocked, we propose that an EGFR-triggered decrease in the intracellular level of cGMP is a key step in this process. Although the mechanism by which a decrease in cGMP triggers TZP loss remains to be established, it is intriguing that loss also requires ROCK activity. Through its

activity to promote phosphorylation of the regulatory light chain of myosin II, ROCK promote actomyosin contractility (Kimura, Ito et al. 1996, Ueda, Murata-Hori et al. 2002, Gallo 2006, Amano, Nakayama et al. 2010, Pandya, Orgaz et al. 2017). Strikingly, cGMP, by activating protein kinase G, suppresses ROCK activity and actomyosin contractility in a range of cell types (Sauzeau, Le Jeune et al. 2000, Murthy, Zhou et al. 2003, Sunico, González-Forero et al. 2010, Zhang, Peng et al. 2010, Huang, Gai et al. 2011, Mandal, Stanco et al. 2013, González-Forero and Moreno-López 2014). Based on this data, we propose that TZP retraction occurs via a two-step mechanism. First, the TZP detaches from the oocyte plasma membrane, possibly via disruption of the N-cadherin/E6 cadherin interaction. Subsequently, the decrease in cGMP within cumulus granulosa cells enables activation of ROCK, which in turn activates actomyosin contraction within the TZPs, thereby causing their retraction (Fig. 28B).

Although the TZPs are spontaneously lost when COCs are placed in culture, they remain stable when GOCs are placed in culture. Moreover, EGF and other growth factors are unable to trigger TZP loss in GOCs even though they activate their respective receptors, as indicated by phosphorylation of ERK. Thus, it appears that the establishment of the cumulus granulosa cell population in antral follicles is accompanied by an important shift in the physiology of these cells, such that maintenance of TZPs becomes sensitive to the exracellular milieu. One possibility is that the cGMP-specific PDE5 is not present or not functional in the granulosa cells prior to their differentiation into cumulus granulosa cells. Alternatively, the cGMP-inhibited mechanism that effects the loss of TZPs may not yet be in place. The inability of granulosa cells within GOCs to activate TZP retraction may represent a failsafe mechanism to ensure that contact and gap junctional communication between the granulosa cells and oocyte is not

precociously lost, which could trigger oocyte maturation prior to the ovulatory signal and compromise embryonic development.

Acknowledgements

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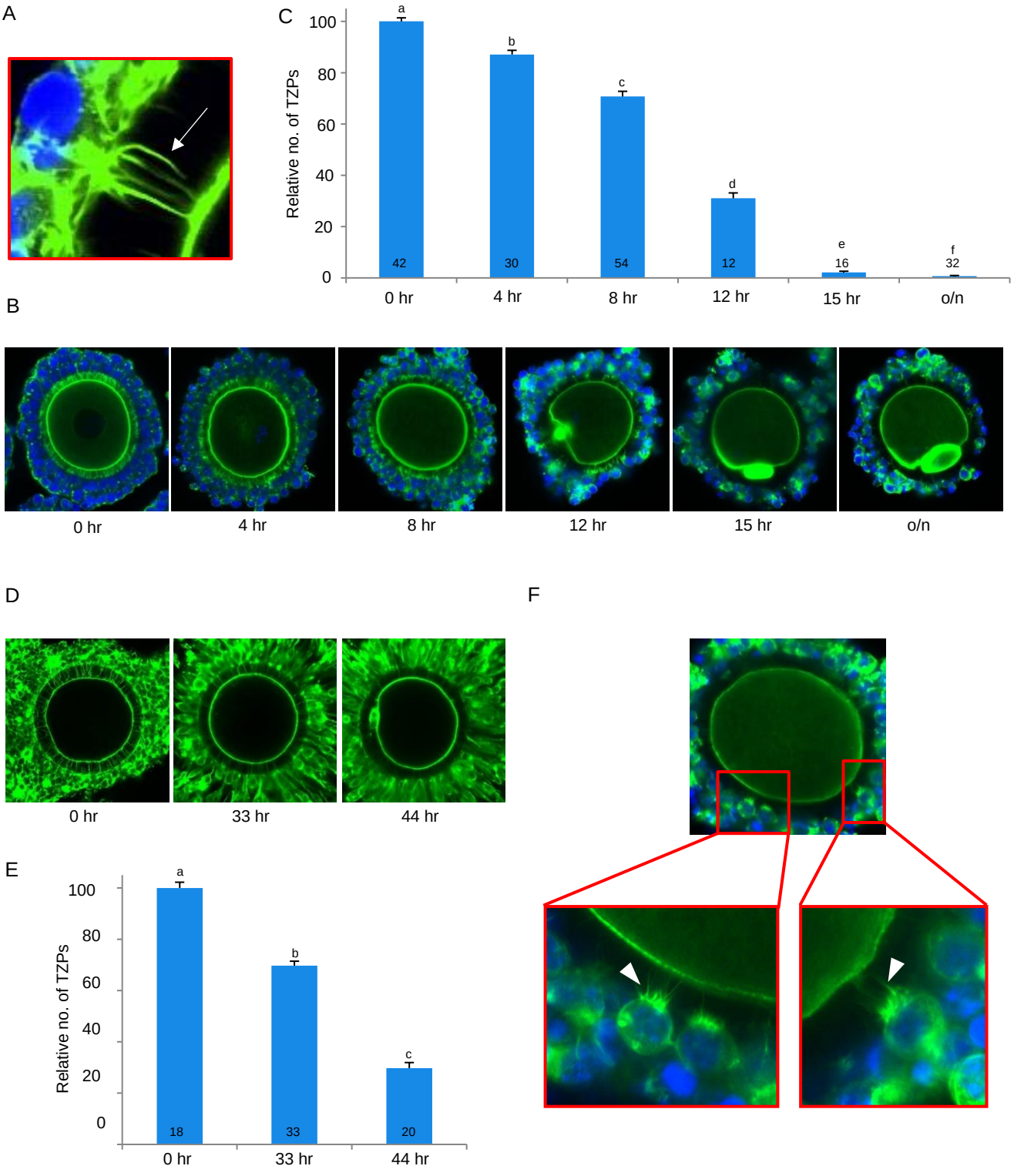

A
C
Relative no. of TZPs
100
80
60
40
20
0
a
b
c
d
e
f
42
30
54
12
16
32
0 hr
4 hr
8 hr
12 hr
15 hr
o/n
B
0 hr
4 hr
8 hr
12 hr
15 hr
o/n
D
0 hr
33 hr
44 hr
E
Relative no. of TZPs
100
80
60
40
20
0
a
b
c
18
33
20
0 hr
33 hr
44 hr
F

Figure 22: TZPs are lost spontaneously and time-dependently after COCs are removed from the antral follicles and placed in culture.

(A) Confocal micrograph showing TZPs extending from a cumulus granulosa cell (left) to the oocyte (right).

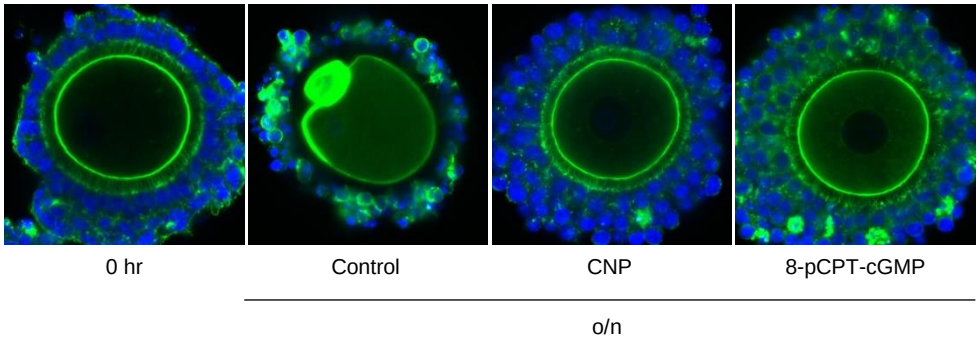
(B) COCs were incubated for the indicated periods of time, then fixed and stained using Alexa 488-phalloidin to visualize actin-rich TZPs and DAPI to visualize cell nuclei. The large cell in the middle is the oocyte and the bright ring at its circumference is the cortex. Small cells surrounding the oocyte are the granulosa cells. Hair-like TZPs penetrate the zona pellucida which separates the two cell types. Brightly stained structure visible at 12 hr, 15 hr and o/n is the first polar body.

(C) Mean number of TZPs detected in an equatorial confocal section at the indicated time-points, normalized to the number in the 0 hr group. Number of COCs examined is shown at the bottom of each bar.

(D) Porcine COCs were incubated for the indicated periods of time, then fixed and stained using Alexa 488-phalloidin. (E) Porcine COC images were analyzed as in (C).

(F) A murine COC after overnight culture. Lower panels show enlargement of the boxed areas. Arrowheads point to TZPs apparently retracted towards the cumulus cell bodies.

A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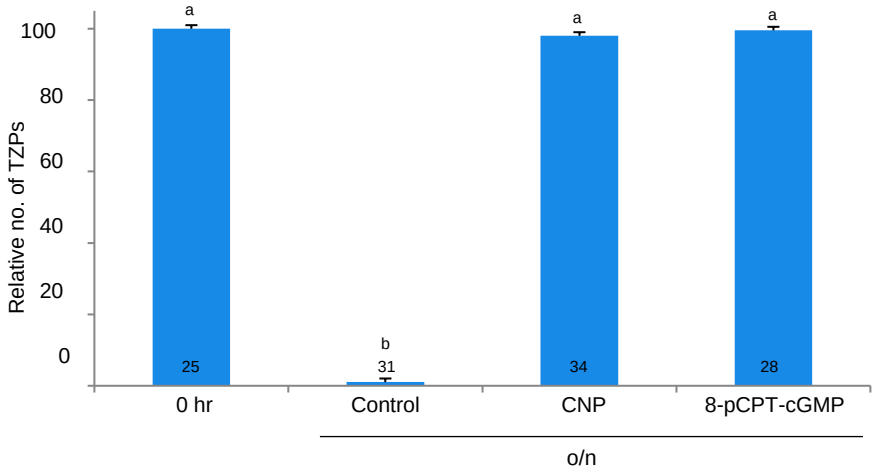
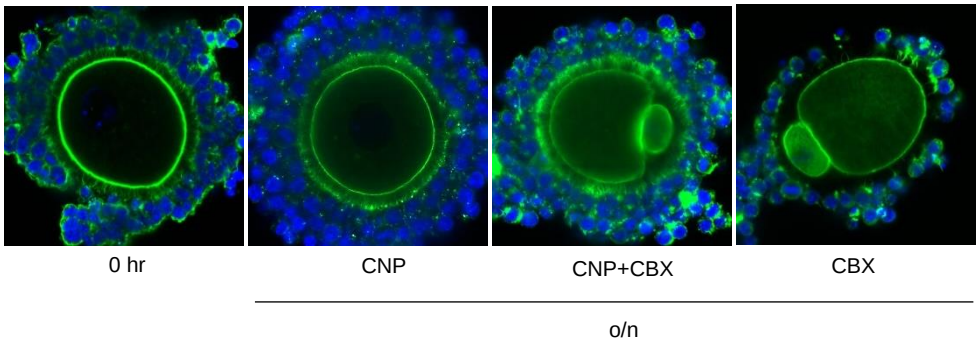
Control
CNP
8-pCPT-cGMP
o/n

B


Relative no. of TZPs
100
80
60
40
20
0
a
b
a
a
25
31
34
28
0 hr
Control
CNP
8-pCPT-cGMP
o/n

C


0 hr
CNP
CNP+CBX
CBX
o/n

D

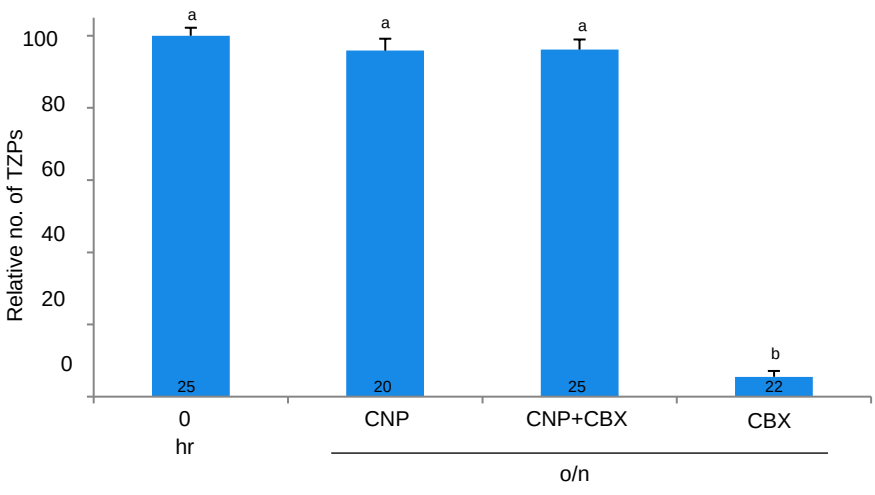
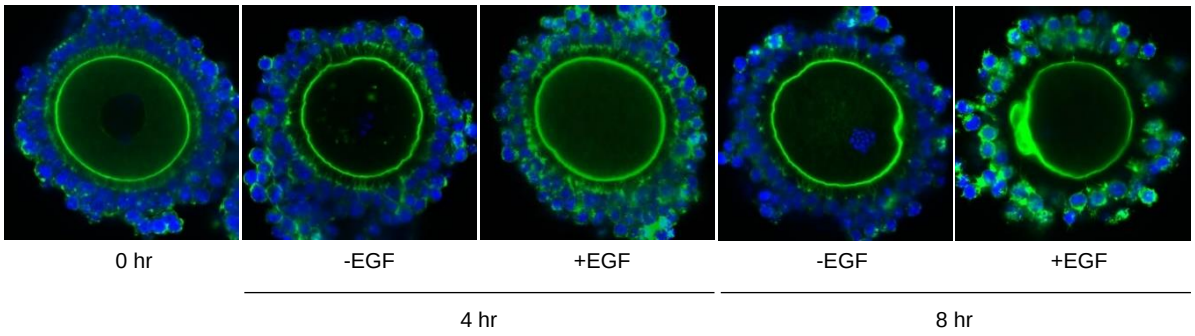

Relative no. of TZPs
100
80
60
40
20
0
a
a
a
b
25
20
25
22
0 hr
CNP
CNP+CBX
CBX
o/n

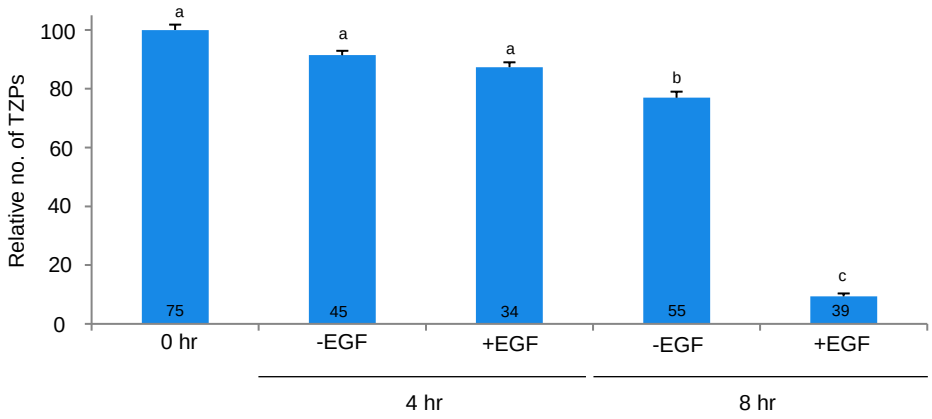
Figure 23: NPPC-cGMP signalling maintains TZP

(A, C) Murine COCs were fixed immediately (0 hr) or incubated overnight under control conditions or in the presence of the indicated drugs and then fixed. Phalloidin-stained actin is green; DNA is blue. In (A), only the control-overnight oocyte has undergone maturation and produced a polar body. (B, D) Mean number of TZPs detected in an equatorial confocal section at the indicated time-points for A and C, respectively, normalized to the number in the 0 hr group. Number of COCs examined is shown at the bottom of each bar.

A


0 hr
-EGF
+EGF
-EGF
+EGF
4 hr
8 hr

B


Relative no. of TZPs
100
80
60
40
20
0
a
a
a
b
c
75
45
34
55
39
0 hr
-EGF
+EGF
-EGF
+EGF
4 hr
8 hr

C

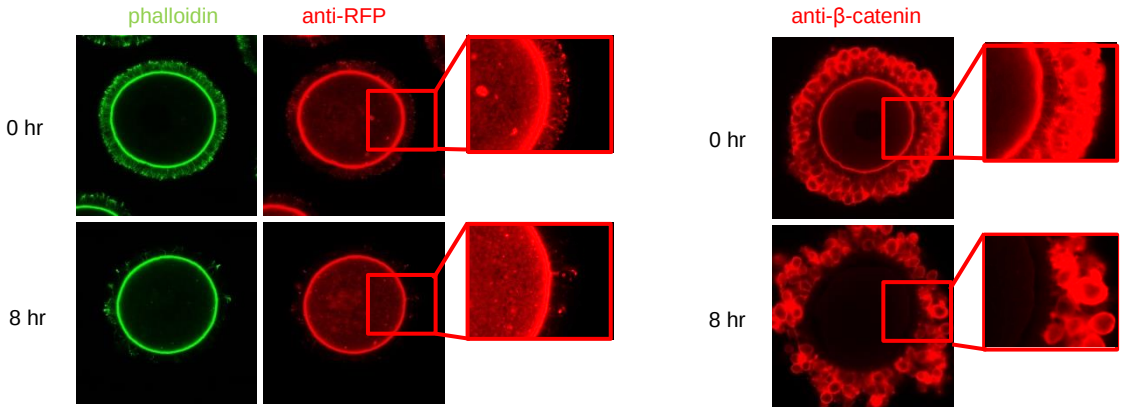

phalloidin
anti-RFP
anti-β-catenin
0 hr
8 hr
0 hr
8 hr

Figure 24: EGFR signalling promotes loss of TZPs

(A) COCs were incubated in the absence or presence of EGF for either 4 or 8 hr, then stained as described.

(B) Mean number of TZPs detected in an equatorial confocal section at the indicated time-points, normalized to the number in the 0 hr group. Number of COCs examined is shown at the bottom of each bar. (C) COCs obtained from transgenic 1 mTmG (left) or wild-type (right) mice were isolated from antral follicles and either fixed immediately (0 hr) or incubated in the presence of EGF for 8 hr and then fixed. Phalloidin-stained actin is green; immunofluorescence using indicated antibodies is red. Boxed area is enlarged and shown with increased brightness. All image-pairs were processed identically. Phalloidin and anti-RFP signals were collected in TRITC and Alexa-488 channels, respectively. To maintain consistency with green phalloidin in the other figures, after acquiring confocal images, the colours were exchanged using Fiji software. Note also loss of β-catenin staining from oocyte membrane at 8 hr.

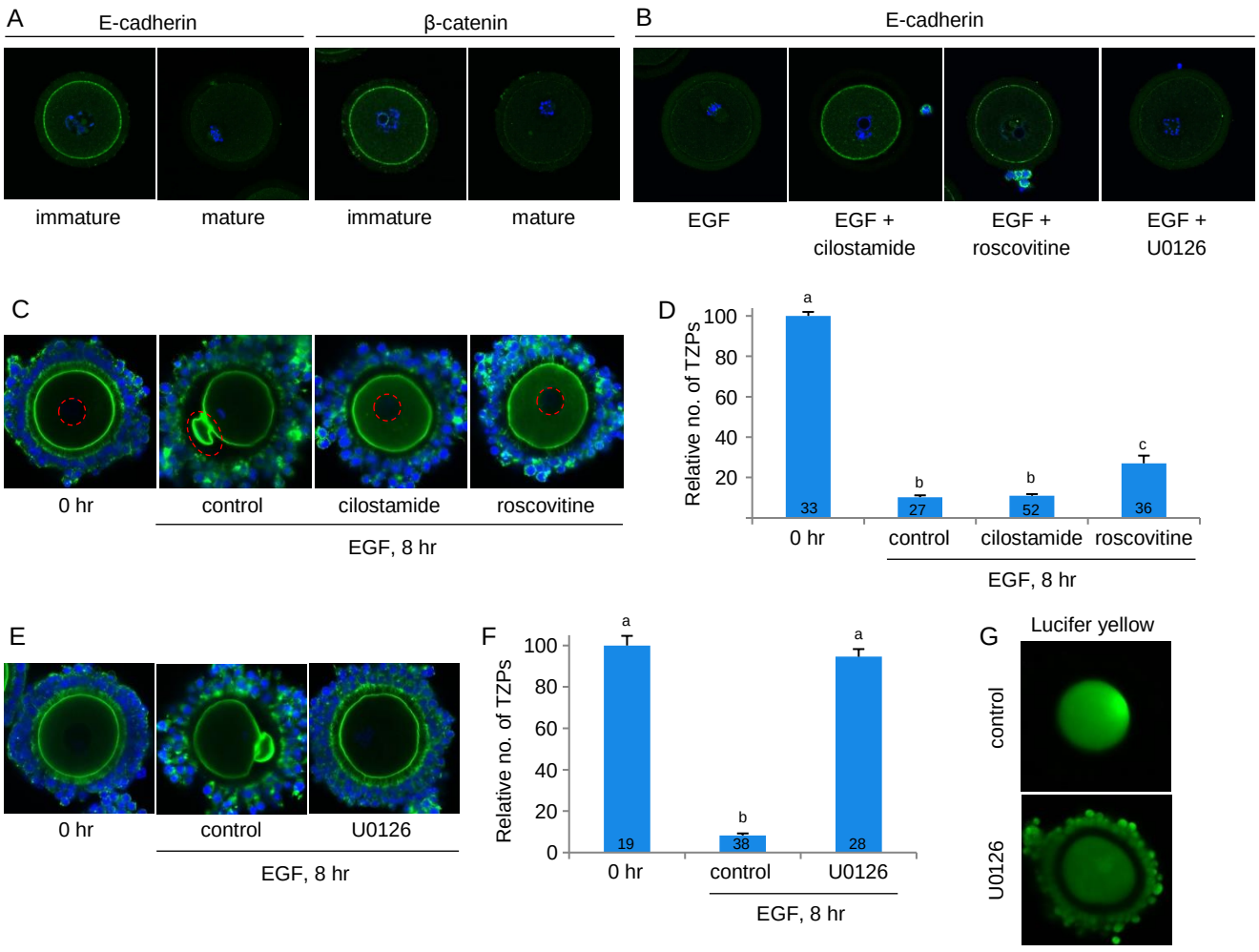


Figure 25: TZP loss is independent of the oocyte meiotic maturation but requires ERK signaling in the cumulus granulosa cells.

(A) Immature and mature oocytes were fixed and stained using anti-E-cadherin or anti-β-catenin. DNA was stained using DAPI. The cumulus granulosa cells were removed so that the immunofluorescent signal could be detected.

(B) COCs were incubated for 8 hr under the indicated conditions, then fixed and stained using anti-E-cadherin and DAPI.

(C) COCs were incubated under the indicated conditions. Cilostamide and roscovitine prevent germinal vesicle breakdown (nucleus indicated by dashed circle), but not TZP loss.

(D) Images were quantified as described.

(E, F) COCs were incubated as indicated and TZPs were quantified as described.

(G) COCs were incubated for 8 hr with EGF in the presence or absence of U0126. A solution of Lucifer Yellow was then injected into the oocytes and 30 minutes later the complexes were examined using fluorescence microscopy.

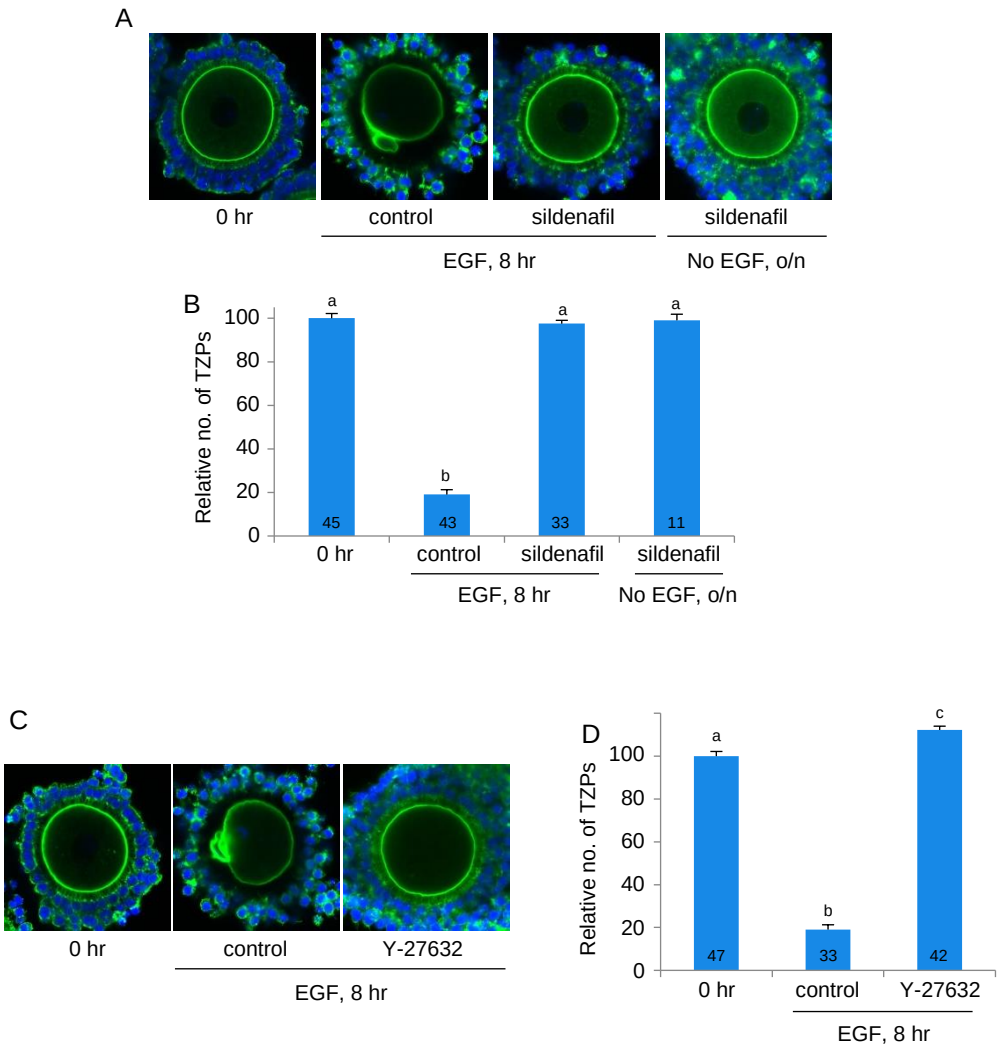


Figure 26: cGMP-regulated activation of ROCK in cumulus granulosa cells triggers TZP loss.

(A, B) COCs were incubated for 8 hr with EGF in the presence or absence of PDE5 inhibitor, sildenafil or overnight with sildenafil, then processed as described. Data was analyzed as previously described.

(C, D) COCs were incubated for 8 hr with EGF in the presence or absence of ROCK inhibitor, Y-27632, then processed as described. Data was analyzed as previously described.

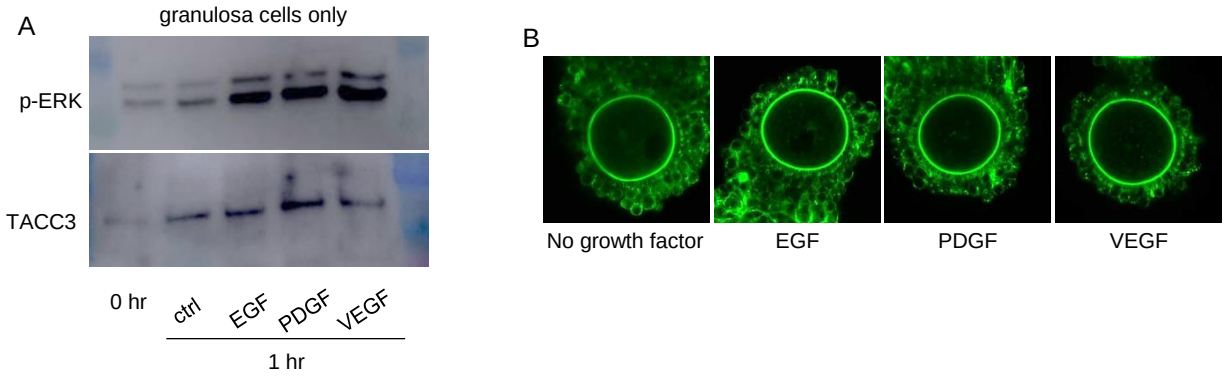


Figure 27: Growth factors do not influence the TZPs of pre-antral follicles.

(A) GOCs from large pre-antral follicles were incubated overnight in the presence or absence of the indicated growth factors. The granulosa cells were then separated from the oocyte and subjected to immunoblotting to detect phospho-ERK and a loading control, TACC3.

(B) GOCs were collected and treated as in (A), then fixed and stained using Alexa 488-phalloidin.

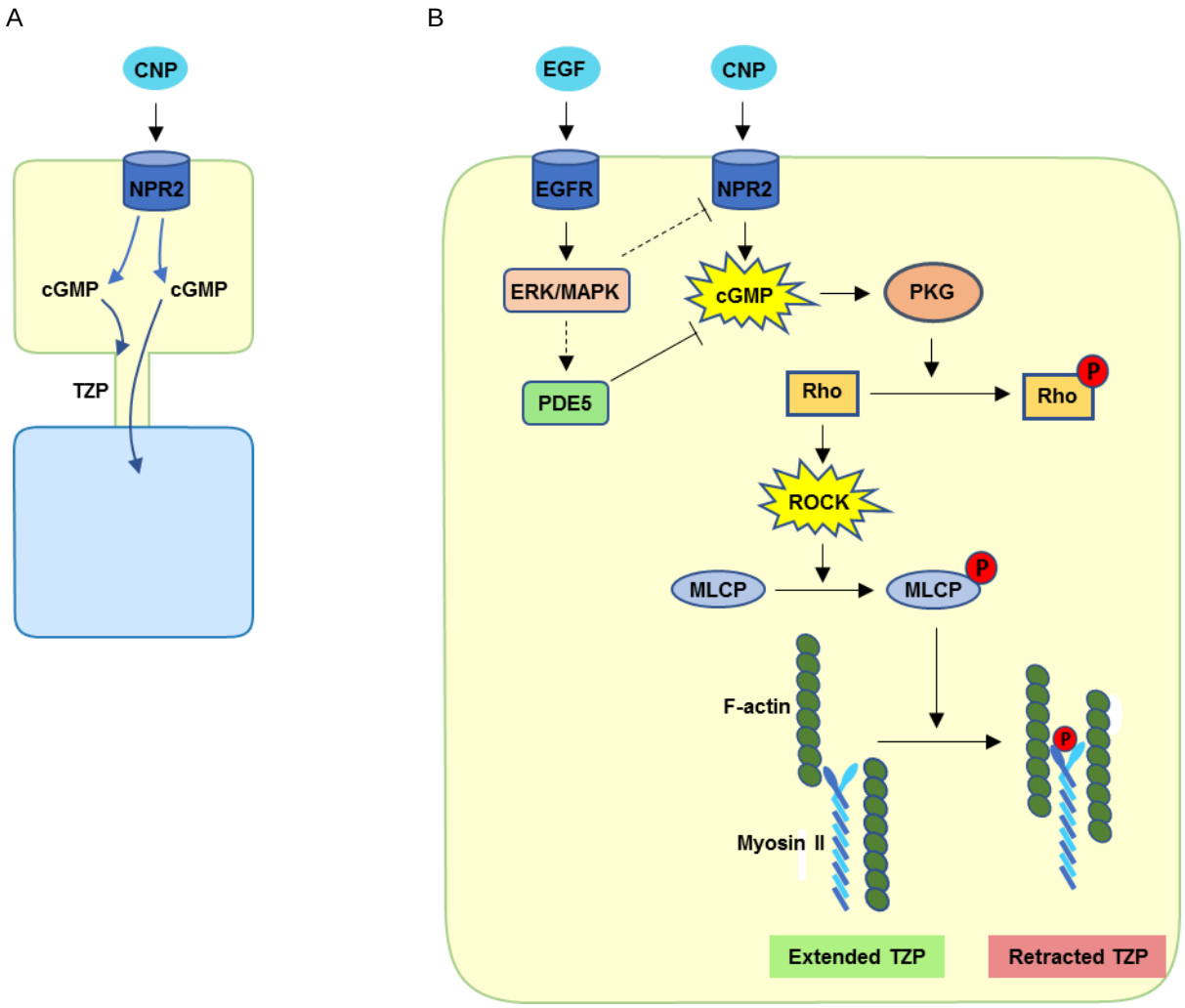


Figure 28: Proposed molecular basis of TZP maintenance and retraction.

(A) In COCs, cGMP generated by C-type natriuretic peptide (CNP)-activated NPR2 serves two functions. Within the cumulus granulosa cells, it maintains the TZPs. This permits additional cGMP to be transferred to the oocyte via gap junctions at the TZP tips. Within the oocyte, cGMP prevents meiotic maturation by inhibiting PDE3A.

(B) Activation of EGFR triggers phosphorylation and activation of the ERK MAP kinase, leading to a reduction in cyclic GMP. Activated ERK may reduce cGMP synthesis or increase its hydrolysis via PDE5 or both. Reduced cGMP in turn impairs the activity of protein kinase G (PKG), which enables dephosphorylation and activation of Rho. Newly active Rho-associated kinase (ROCK) phosphorylates and inactivates myosin light chain phosphatase (MLCP). This leads to an increase in the phosphorylation of myosin light chain, which activates actomyosin contractility, shortening actin fibres.

References

Aburima, A., K. Walladbegi, J. Wake and K. Naseem (2017). "cGMP signaling inhibits platelet shape change through regulation of the RhoA-Rho Kinase-MLC phosphatase signaling pathway." Journal of Thrombosis and Haemostasis 15(8): 1668-1678.

Albertini, D. F., C. Combelles, E. Benecchi and M. J. Carabatsos (2001). "Cellular basis for paracrine regulation of ovarian follicle development." Reproduction 121(5): 647-653.

Amano, M., M. Nakayama and K. Kaibuchi (2010). "Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity." Cytoskeleton 67(9): 545-554.

Barrett, S. L., L. D. Shea and T. K. Woodruff (2010). "Noninvasive index of cryorecovery and growth potential for human follicles in vitro." Biology of reproduction 82(6): 1180-1189.

Bohrer, R. C., A. R. S. Coutinho, R. Duggavathi and V. Bordignon (2015). "The incidence of DNA double-strand breaks is higher in late-cleaving and less developmentally competent porcine embryos." Biology of reproduction 93(3): 59, 51-58.

Boyd, J. L., S. L. Skove, J. P. Rouanet, L.-J. Pilaz, T. Bepler, R. Gordân, G. A. Wray and D. L. Silver (2015). "Human-chimpanzee differences in a FZD8 enhancer alter cell-cycle dynamics in the developing neocortex." Current Biology 25(6): 772-779.

Chiquoine, A. D. (1960). "The development of the zona pellucida of the mammalian ovum." American Journal of Anatomy 106(2): 149-169.

Clarke, H. J. (2018). "Regulation of germ cell development by intercellular signaling in the mammalian ovarian follicle." Wiley Interdisciplinary Reviews: Developmental Biology 7(1): e294.

Conti, M., M. Hsieh, A. M. Zamah and J. S. Oh (2012). "Novel signaling mechanisms in the ovary during oocyte maturation and ovulation." Molecular and cellular endocrinology 356(1-2): 65-73.

Coticchio, G., M. Dal Canto, M. Mignini Renzini, M. C. Guglielmo, F. Brambillasca, D. Turchi, P. V. Novara and R. Fadini (2015). "Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization." Human reproduction update 21(4): 427-454.

Edwards, R. (1965). "Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes." Nature 208(5008): 349.

Egbert, J. R., T. F. Uliasz, L. C. Shuhaibar, A. Geerts, F. Wunder, R. J. Kleiman, J. M. Humphrey, P. D. Lampe, N. O. Artemyev and S. D. Rybalkin (2016). "Luteinizing hormone causes phosphorylation and activation of the cGMP phosphodiesterase PDE5 in rat ovarian follicles, contributing, together with PDE1 activity, to the resumption of meiosis." Biology of reproduction 94(5): 110, 111-111.

El-Hayek, S. and H. J. Clarke (2016). Control of oocyte growth and development by intercellular communication within the follicular niche. Molecular Mechanisms of Cell Differentiation in Gonad Development, Springer: 191-224.

El-Hayek, S., Q. Yang, L. Abbassi, G. FitzHarris and H. J. Clarke (2018). "Mammalian Oocytes Locally Remodel Follicular Architecture to Provide the Foundation for Germline-Soma Communication." Current Biology 28(7): 1124-1131. e1123.

El-Hayek, S., Q. Yang, L. Abbassi, G. FitzHarris and H. J. Clarke (2018). "Mammalian Oocytes Locally Remodel Follicular Architecture to Provide the Foundation for Germline-Soma Communication." Current Biology 28(7): 1124-1131.e1123.

Fairchild, C. L. and M. Barna (2014). "Specialized filopodia: at the 'tip'of morphogen transport and vertebrate tissue patterning." Current opinion in genetics & development 27: 67-73.

Franciosi, F., G. Coticchio, V. Lodde, I. Tessaro, S. C. Modina, R. Fadini, M. Dal Canto, M. M. Renzini, D. F. Albertini and A. M. Luciano (2014). "Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes." Biology of reproduction 91(3).

Gallo, G. (2004). "Myosin II activity is required for severing-induced axon retraction in vitro." Experimental neurology 189(1): 112-121.

Gallo, G. (2006). "RhoA-kinase coordinates F-actin organization and myosin II activity during semaphorin-3A-induced axon retraction." Journal of cell science 119(16): 3413-3423.

González-Forero, D. and B. Moreno-López (2014). "Retrograde response in axotomized motoneurons: nitric oxide as a key player in triggering reversion toward a dedifferentiated phenotype." Neuroscience 283: 138-165.

Huang, X., Y. Gai, N. Yang, B. Lu, C. S. Samuel, V. J. Thannickal and Y. Zhou (2011). "Relaxin regulates myofibroblast contractility and protects against lung fibrosis." The American journal of pathology 179(6): 2751-2765.

Inaba, M., M. Buszczak and Y. M. Yamashita (2015). "Nanotubes mediate niche–stem-cell signalling in the Drosophila testis." Nature 523(7560): 329.

Jacquemet, G., H. Hamidi and J. Ivaska (2015). "Filopodia in cell adhesion, 3D migration and cancer cell invasion." Current opinion in cell biology 36: 23-31.

Jaffe, L. A. and J. R. Egbert (2017). "Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle." Annual review of physiology 79: 237-260.

Kato, M., R. Blanton, G.-R. Wang, T. J. Judson, Y. Abe, M. Myoishi, R. H. Karas and M. E. Mendelsohn (2012). "Direct binding and regulation of RhoA by cyclic GMP-dependent protein kinase Iα." Journal of Biological Chemistry: jbc. M112. 421040.

Kawamura, K., Y. Cheng, N. Kawamura, S. Takae, A. Okada, Y. Kawagoe, S. Mulders, Y. Terada and A. J. Hsueh (2011). "Pre-ovulatory LH/hCG surge decreases C-type natriuretic

peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes." Human reproduction 26(11): 3094-3101.

Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano and K. Okawa (1996). "Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)." Science 273(5272): 245-248.

Li, R. and D. F. Albertini (2013). "The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte." Nature reviews Molecular cell biology 14(3): 141.

Macaulay, A. D., I. Gilbert, J. Caballero, R. Barreto, E. Fournier, P. Tossou, M.-A. Sirard, H. J. Clarke, É. W. Khandjian and F. J. Richard (2014). "The gametic synapse: RNA transfer to the bovine oocyte." Biology of reproduction 91(4): 90, 91-12.

Makita, M. and T. Miyano (2014). "Steroid hormones promote bovine oocyte growth and connection with granulosa cells." Theriogenology 82(4): 605-612.

Mandal, S., A. Stanco, E. S. Buys, G. Enikolopov and J. L. Rubenstein (2013). "Soluble guanylate cyclase generation of cGMP regulates migration of MGE neurons." Journal of Neuroscience 33(43): 16897-16914.

Marzo, L., K. Gousset and C. Zurzolo (2012). "Multifaceted roles of tunneling nanotubes in intercellular communication." Frontiers in physiology 3: 72.

McGinnis, L. K. and W. H. Kinsey (2015). "Role of focal adhesion kinase in oocyte-follicle communication." Molecular reproduction and development 82(2): 90-102.

Murthy, K. S., H. Zhou, J. R. Grider and G. M. Makhlouf (2003). "Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA." American Journal of Physiology-Gastrointestinal and Liver Physiology 284(6): G1006-G1016.

Norris, R. P., W. J. Ratzan, M. Freudzon, L. M. Mehlmann, J. Krall, M. A. Movsesian, H. Wang, H. Ke, V. O. Nikolaev and L. A. Jaffe (2009). "Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte." Development 136(11): 1869-1878.

O'Brien, M. J., J. K. Pendola and J. J. Eppig (2003). "A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence." Biology of reproduction 68(5): 1682-1686.

Pandya, P., J. L. Orgaz and V. Sanz-Moreno (2017). "Actomyosin contractility and collective migration: may the force be with you." Current opinion in cell biology 48: 87-96.

Panigone, S., M. Hsieh, M. Fu, L. Persani and M. Conti (2008). "Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway." Molecular endocrinology 22(4): 924-936.

Peng, J., Q. Li, K. Wigglesworth, A. Rangarajan, C. Kattamuri, R. T. Peterson, J. J. Eppig, T. B. Thompson and M. M. Matzuk (2013). "Growth differentiation factor 9: bone morphogenetic

protein 15 heterodimers are potent regulators of ovarian functions." Proceedings of the National Academy of Sciences 110(8): E776-E785.

Roy, S. and T. B. Kornberg (2015). "Paracrine signaling mediated at cell–cell contacts." Bioessays 37(1): 25-33.

Sánchez, F., F. Lolicato, S. Romero, M. De Vos, H. Van Ranst, G. Verheyen, E. Anckaert and J. Smitz (2017). "An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield." Human Reproduction 32(10): 2056-2068.

Sánchez, F. and J. Smitz (2012). "Molecular control of oogenesis." Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease 1822(12): 1896-1912.

Sauzeau, V., H. Le Jeune, C. Cario-Toumaniantz, A. Smolenski, S. M. Lohmann, J. Bertoglio, P. Chardin, P. Pacaud and G. Loirand (2000). "Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle." Journal of Biological Chemistry.

Stuelten, C. H., C. A. Parent and D. J. Montell (2018). "Cell motility in cancer invasion and metastasis: insights from simple model organisms." Nature Reviews Cancer.

Sunico, C. R., D. González-Forero, G. Domínguez, J. M. García-Verdugo and B. Moreno-López (2010). "Nitric oxide induces pathological synapse loss by a protein kinase G-, Rho kinase-dependent mechanism preceded by myosin light chain phosphorylation." Journal of Neuroscience 30(3): 973-984.

Ueda, K., M. Murata-Hori, M. Tatsuka and H. Hosoya (2002). "Rho-kinase contributes to diphosphorylation of myosin II regulatory light chain in nonmuscle cells." Oncogene 21(38): 5852.

Wassarman, P. M. and E. S. Litscher (2013). Biogenesis of the mouse egg's extracellular coat, the zona pellucida. Current topics in developmental biology, Elsevier. 102: 243-266.

Wassarman, P. M. and E. S. Litscher (2013). "Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility." International Journal of Developmental Biology 56(10-11-12): 833-839.

Yang, Q., P. Allard, M. Huang, W. Zhang and H. J. Clarke (2010). "Proteasomal activity is required to initiate and to sustain translational activation of messenger RNA encoding the stem-loop-binding protein during meiotic maturation in mice." Biology of reproduction 82(1): 123-131.

Zaidel-Bar, R., G. Zhenhuan and C. Luxenburg (2015). "The contractome–a systems view of actomyosin contractility in non-muscle cells." J Cell Sci: jcs. 170068.

Zhang, M., Y.-Q. Su, K. Sugiura, K. Wigglesworth, G. Xia and J. J. Eppig (2011). "Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro." Endocrinology 152(11): 4377-4385.

Zhang, M., Y.-Q. Su, K. Sugiura, G. Xia and J. J. Eppig (2010). "Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes." Science 330(6002): 366-369.

Zhang, W.-C., Y.-J. Peng, G.-S. Zhang, W.-Q. He, Y.-N. Qiao, Y.-Y. Dong, Y.-Q. Gao, C. Chen, C.-H. Zhang and W. Li (2010). "Myosin light chain kinase is necessary for tonic airway smooth muscle contraction." Journal of Biological Chemistry 285(8): 5522-5531.

Chapter 4

DISCUSSION and CONCLUSION

Discussion

An oocyte is not able to be fertilized and produce a healthy viable embryo unless it becomes developmentally competent. The journey of oocyte developmental competence consists of two crucial phases: growth and meiotic maturation, which both must be carefully regulated (Sánchez and Smitz 2012, Clarke 2018). Growth comes first through which the oocyte increases in size more than 100 times as it accumulates an adequate supply of nutrients, mRNA and organelles for the utilization of the future early embryo (Moore and Lintern-Moore 1974, Moore, Lintern-Moore et al. 1974, Moore and Lintern-Moore 1978, Sternlicht and Schultz 1981, De Leon, Johnson et al. 1983, Bachvarova, De Leon et al. 1985, Eichenlaub-Ritter and Peschke 2002, Mahrous, Yang et al. 2012, Sánchez and Smitz 2012, Svoboda, Franke et al. 2015). Meiotic maturation comes next, only after the oocyte reaches its maximum size (Sánchez and Smitz 2012). Because these two stages of oocyte development play a critical role in assuring healthy reproduction, molecular mechanisms regulating them under the physiological conditions are important to discover. This thesis targeted oocyte development from both angles of growth and meiotic maturation and investigated the role of potential molecular mechanisms in regulating each of these two processes.

Hippo is a canonical and highly conserved signalling pathway which is widely known for its key role in regulating cell growth (Harvey, Zhang et al. 2013, Piccolo, Dupont et al. 2014, Varelas 2014, Moroishi, Hansen et al. 2015), in terms of both cell size and cell number (Wang, Mao et al. 2014, Goodman, Dietz et al. 2015, Zanconato, Forcato et al. 2015, Mugahid, Kalocsay et al. 2018, Plouffe, Lin et al. 2018). Consequently, it is well established that the Hippo pathway is actively involved in the control of tissue and organ size, as well as tumorigenesis. Thus, due to

its extensive implication in developmental biology and oncology, numerous studies have recently focused on the role of this pathway in the physiological or pathological growth of a broad range of somatic cell types (Piccolo, Dupont et al. 2014). In this thesis, we particularly investigated whether YAP, as the key effector of the Hippo pathway, is associated with the control of oocyte growth and our findings are reported in chapter II (manuscript I).

The activity of YAP as a transcription co-factor and the chief downstream effector of Hippo pathway is directly dependent on its nuclear accumulation. YAP subcellular localization is defined by its phosphorylation state on the specific residue of S127 in human and S112 in mouse, which is under the regulation of LATS1/2 as other core components of the pathway. In fact, when the Hippo pathway is on, LATS 1/2 phosphorylate YAP causing it to bind 14-3-3 protein, become sequestered in the cytoplasm, and consequently remain transcriptionally inactive. In contrast, when Hippo pathway is off, LATS 1/2 are inactive and thus incapable of phosphorylating YAP which in turn allows it to translocate into the nucleus, reach the genome, and induce the transcription of its target genes which promote cell proliferation (Basu, Totty et al. 2003, Dong, Feldmann et al. 2007, Zhao, Wei et al. 2007, Piccolo, Dupont et al. 2014, Oku, Nishiya et al. 2015, Zanconato, Forcato et al. 2015).

Our study, for the first time, systematically assessed the pattern of YAP expression and subcellular localization throughout prenatal and the entire postnatal stages of oocyte development. We found that YAP is expressed in the oocyte beginning during embryonic life, and this expression robustly continues during all the stages of oocyte postnatal development including meiotic maturation. However, we showed that YAP is constitutively phosphorylated at

S112, excluded from the nucleus, and localized in the cytoplasm during the entire period of oocyte growth but dephosphorylated at this site as the oocyte re-enters the cell cycle by resuming meiotic maturation. These results strongly suggest that, even though nuclear YAP plays a crucial role in the control of cell growth in various somatic cell types (Harvey, Zhang et al. 2013, Piccolo, Dupont et al. 2014, Varelas 2014, Wang, Mao et al. 2014, Goodman, Dietz et al. 2015, Moroishi, Hansen et al. 2015, Mugahid, Kalocsay et al. 2018, Plouffe, Lin et al. 2018), it does not regulate oocyte growth under physiological conditions. We then identified three independent molecular mechanisms which cooperatively prevent YAP from nuclear accumulation.

It is known that i) cAMP-dependent protein kinase A is highly active in the growing and fully grown oocytes (Nogueira, Albano et al. 2003, Kovo, Kandli-Cohen et al. 2006, Conti, Hsieh et al. 2012, Zeng, Ren et al. 2013, Nishimura, Fujii et al. 2014); ii) cAMP-dependent protein kinase A, via activating LATS1/2, induces YAP phosphorylation at S112 in some somatic cell types (Yu, Zhao et al. 2012, Kim, Kim et al. 2013, Yu, Zhang et al. 2013); and iii) S112 phosphorylation of YAP is a powerful lever for keeping it out of the nucleus in numerous somatic cell types (Dupont, Morsut et al. 2011, Chen, Zhang et al. 2015, Hansen, Moroishi et al. 2015). Based on the above facts we hypothesized and subsequently showed that in both growing and fully grown oocytes cAMP-dependent protein kinase A phosphorylates YAP at S112 which likely causes its cytoplasmic retention.

Next, by measuring the protein levels, we observed that a portion of YAP in the growing oocytes is not phosphorylated at S112. However, to our surprise, this portion was not detectable in the nuclei of the growing oocytes. Strikingly, when we used the inhibitor of nuclear export inhibitor,

leptomycin B, a robust accumulation of YAP occurred in the nuclei of the growing oocytes. This suggests that although a subset of YAP molecules within the growing oocytes is dephosphorylated at S112 and thus capable of "entering" the nucleus, lack or weakness of the required machinery for keeping it inside the nucleus causes its instant return to the cytoplasm.

Furthermore, we found no trace of YAP in the nucleus of fully grown oocyte even when we pharmacologically inhibited both S122 phosphorylation of YAP and the nuclear export signal. This suggest that in contrast to growing oocytes, in fully grown oocytes even the dephosphorylated form of YAP is incapable of entering the nucleus.

Altogether we conclude that multiple mechanisms, which act independent of the canonical Hippo pathway, cooperate to prevent YAP from nuclear localization. However, what crucial effect is associated with YAP nuclear exclusion and what function is contributed to the cytoplasmic YAP still remain to be elucidated as the future directions. Considering the fact that nuclear YAP potently promotes the cell cycle (Varelas 2014, Moroishi, Hansen et al. 2015), it is possible that nuclear exclusion of YAP is essential to prevent precocious meiotic resumption, before the oocyte fulfills its growth period. It is also possible that cytoplasmic YAP plays certain roles in regulation of oocyte normal development, perhaps by sequestering molecules away from the nucleus (Imajo, Miyatake et al. 2012). This possibility could be investigated by the conditional deletion of *Yap1* in the oocyte and detecting any changes in its gene expression profile, developmental competence, fertilization rate, and embryo development.

In the third chapter (Manuscript II), we moved beyond the oocyte growth and focused on the next and final stage of oocyte devlopment, meiotic maturation, and particularly studied the dynamics of germline-soma communication during this period.

As discussed earlier in the thesis introduction, within a primordial follicle and before the oocyte initiates its growth, the oocyte and the adjacent granulosa cells are immediately apposed to each other without the presence of any physical mediator, and are directly coupled through adherens and gap junctions. However, as the oocyte enters the growth phase and the newly formed zona pellucida pushes the granulosa cells away from the oocyte, TZPs germinate from the granulosa cells bodies and extend towards the oocyte to maintain the contact between the two cell types. However, the number of TZPs is not constant during different stages of oocyte development. We have shown recently that TZPs increase in number in parallel with the gradual increase in the oocyte circumference due to its growth. (El-Hayek, Yang et al. 2018). Here we show that TZPs are not permanent structures and their growth is not an endless process. In fact, we show that when the fully grown oocyte resume meiosis, TZPs decrease in number in parallel with the progress of meiotic maturation such that when the oocyte reaches the metaphase of the second meiotic division, very few or no TZP remain.

How do TZPs disappear? The accumulation of large aggregates of actin at the base of the inner most granulosa cells, at the same spots where TZPs had initially originated and extend from, suggests that TZPs loss is a manifestation of their active pulling pack towards the granulosa cells bodies, a behaviour which we define as TZP retraction and is phenotypically very similar to the

neurite retraction occurring in developing nervous system or in response to adult neural disorders (Luo and O'Leary 2005).

We have previously shown that oocyte, via GDF9 secretion, signals to its neighboring granulosa cells (El-Hayek, Yang et al. 2018) to generate new TZPs. In this study, we elucidated the cascade of molecular mechanisms which initiate TZP loss. Our observation that there is a robust coincidence between TZPs presence and meiotic arrest as well as between TZPs loss and meiotic resumption suggests that there might be a correlation between TZPs numbers and molecular events related to meiotic maturation. During the follicular growth there is a high concentration of NPPC in the granulosa cells which produces a high level of cGMP that plays the key role in meiotic arrest. When the follicle is fully grown and reaches the pre-ovulatory stage, LH surge triggers meiotic resumption by decreasing cGMP level via two distinct pathways: first, through reduction in NPPC level and NPR2 activity which result in a decrease in NPPC-dependent cGMP production, and secondly through activation of EGF network which also causes a decrease in follicle cGMP level; however, the molecular involvement of EGF in mediating the LH-induced decrease in cGMP content is still unknown (Robinson, Zhang et al. 2012, Liu, Xie et al. 2014, Richards and Ascoli 2018). PDE5 is a highly specific hydrolyzer of cGMP which is expressed in the cumulus cells of pre-ovulatory follicles. Hence, PDE5 inhibitor treatment of these follicles elevates cGMP 5-fold above basal levels and reversibly blocks meiotic maturation (Wang, Ning et al. 2008, Vaccari, Weeks et al. 2009). Therefore, it is strongly probable that PDE5 activity mediates LH-induced EGF effect on reducing cGMP levels and promoting meiotic maturation.

We show that TZPs are entirely lost during COCs overnight *in vitro* maturation. But addition of either cGMP membrane permeable analog, exogenous NPPC to continuously produce cGMP, or PDE5 inhibitor to prevent cGMP degradation, fully prevent TZP loss during overnight culture, proving that cGMP plays a central role in regulating TZPs, as it does in regulating meiotic maturation (Norris, Ratzan et al. 2009).

We then identify the upstream and downstream effectors of cGMP as the other components of the cascade regulating TZPs numbers. We indicate while TZPs are gradually lost during overnight COCs culture, EGF accelerates this process. However, EGF in the presence of PDE5 inhibitor is completely incapable of promoting TZPs loss. These results suggest while gradual degradation of cGMP during COCs overnight incubation causes gradual TZPs loss, EGF via activating cGMP hydrolyzer, PDE5, speeds up cGMP degradation and TZP loss. We also revealed that deactivation of ERK/MAPK, the downstream effector of EGF in the granulosa cells (Sánchez and Smitz 2012), fully prevents EGF-induced TZPs loss, demonstrating that EGF acts also through ERK/MAPK signalling to exert its effect on TZP loss. Since PDE5 is known to be directly phosphorylated and activated by PKA (Conti and Beavo 2007, Azevedo, Faucz et al. 2013) and on the other hand ERK/MAPK in response to EGF increases PKA activity in the cumulus cells (Heiligentag and Eichenlaub-Ritter 2018), we propose that ERK/MAPK acts as the upstream effector of PDE5 mediating EGF signal to PDE5.

We next discover that ROCK is the molecular target of cGMP to regulate the dynamic of TZPs skeleton. ROCK, initially defined as a downstream effector of GTPase (Ras homolog family member A) plays a critical role in rearrangements of actomyosin cytoskeleton (Amano,

Nakayama et al. 2010, Pandya, Orgaz et al. 2017). In fact, Rho-ROCK signalling mediates activating phosphorylation of the regulatory light chain of myosin II (MLC2) (Ueda, Murata-Hori et al. 2002, Amano, Nakayama et al. 2010) and phosphorylated myosin II removes actin stress fibres from relaxation state and promotes actomysin-based contractility of the cell processes (Kimura, Ito et al. 1996, Gallo 2004, Zaidel-Bar, Zhenhuan et al. 2015, Pandya, Orgaz et al. 2017). Interestingly, it is also shown that cGMP, through PKG (cGMP-dependent protein Kinase) activity, suppresses Rho-ROCK signalling and therefore prevents actomyosin contraction in vascular (Sauzeau, Le Jeune et al. 2000, Mills, Lewis et al. 2003, Surks 2007), gastric (Murthy, Zhou et al. 2003), or airway smooth muscle cells (Zhang, Peng et al. 2010), as well as in neurites (Sunico, González-Forero et al. 2010, Mandal, Stanco et al. 2013, González-Forero and Moreno-López 2014), and lung myofibroblasts (Huang, Gai et al. 2011). According to this evidence, we suggest that cGMP is likely linked to ROCK through PKG activity in the cumulus cells.

Overall, we propose a model which holds that before maturation, high cGMP levels in cumulus cells keeps ROCK inactive and thus TZPs actin stress fibres relaxed and extended. In contrast, at the time of maturation, high EGF levels through EGF/MAPK activity stimulates PDE5 and thus results in cGMP degradation. Loss of cGMP in the cumulus cells subsequently allows ROCK to become active and cause TZPs actin stress fibers contraction resulting in TZPs shortening towards granulosa cells bodies which we refer to as “TZPs retraction”.

We next prove blocking oocyte meiotic maturation, or even eliminating all oocyte-dependent effects by killing the oocyte, does not interfere with EGF-induced TZP loss. This suggests that

TZP loss does not require oocyte meiotic maturation. Therefore, although TZP maintenance coincidences with oocyte meiotic arrest, and TZP loss coincidences with oocyte meiotic maturation, we show that TZPs dynamics and meiotic maturation are two independent processes which are both driven by cGMP level – thus, this co-dependence serves to synchronize these two events. It is thus not surprising that we observed EGF-treatment not only accelerated TZPs loss, but also accelerated in vitro meiotic maturation such that most of the oocytes reached MII stage only after 8 hr of incubation. Finally, we observe that granulosa cells will acquire the competency for TZP loss only when they differentiate into cumulus cells at the antral phase. This could be due to the lack of PDE5 in the pre-antral granulosa cells (Wang, Ning et al. 2008) which interrupts the regulatory pathway downstream of EGF-ERK/MAPK. Overall, our study shows for the first time that cGMP plays a key functional role not only in the oocyte to regulate meiotic maturation (Norris, Ratzan et al. 2009), but also in the granulosa cells to regulate TZP dynamics. We also indicate while the oocyte manages TZP generation during oocyte growth (El-Hayek, Yang et al. 2018), granulosa cells manage TZP loss during oocyte meiotic maturation.

Inappropriate activation of this signalling pathway in the granulosa cells surrounding the growing oocytes may trigger precocious uncoupling between the two cell types prior to the oocyte completing pre-ovulatory development, resulting in oocytes incapable of being fertilized and giving rise to a healthy embryo. In contrast, a delay in the activation of EGF-ERK/MAPK-PDE5-cGMP-ROCK signalling pathway in the cumulus cells may delay fully grown oocyte release from the surrounding cumulus cells and therefore block sperm penetration and fertilization. Therefore, we propose that programmed activation of TZP regulatory signals is essential to ensure oocyte normal growth and fertilization.

Conclusion

From the female side, the majority of the success rate in fertilization and early embryogenesis is a reflection of oocyte quality, which itself is deeply dependent on two stages of development: increase in size followed by meiotic resumption. This thesis is focused on these two concepts and investigates key molecular mechanisms in regulating each of them.

The results of the first manuscript rule out the *in vivo* involvment of nuclear YAP in the oocyte growth and likely follicle development, while its role as a key regulator of organ size and organ development is well-established (Piccolo, Dupont et al. 2014, Varelas 2014, Hansen, Moroishi et al. 2015). Our finding also highlights the necessity of reconsidering previous *in vitro* works suggesting that enforced nuclear inclusion of YAP in the oocytes and granulosa cells, via disruption of the Hippo pathway, triggers oocyte and follicle growth which could consequently be applied as a tool for the treatment of infertility in patients with premature ovarian insufficiency (POI) (Kawamura, Cheng et al. 2013, Chen, Zhang et al. 2015).

In the second manuscript, we proceeded to further study oocyte development, but this time in the window of meiotic maturation. Oocyte appropriate development is necessarily dependent on its communication with the neighboring granulosa cells (Vanderhyden 2002, Kidder and Vanderhyden 2010); and TZPs, as filopodia-like structures, are the only means of physical contact between the two cell types (Clarke 2018). Even though TZPs are constantly present throughout oocyte growth and even increase in number as the oocyte gets bigger (El-Hayek, Yang et al. 2018), they disappear during meiotic maturation . We showed that loss of TZPs during oocyte meiotic maturation is an active and programmed event and subsequently

discovered the robust molecular cascade within the granulosa cells which tightly regulates this loss. Considering the fact that filopodia serve as the environment sensors and migrating feet of certain cell types including metastatic cancer cells, the pathway we identified as the powerful regulator of TZPs loss could be investigated and targeted in the metastatic cancer cells aiming to disable the migrating potential of these cells and thus prevent their metastasis.

References

Abe, K.-i., A. Inoue, M. G. Suzuki and F. Aoki (2010). "Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes." Journal of Reproduction and Development 56(5): 502-507.

Aburima, A., K. Walladbegi, J. Wake and K. Naseem (2017). "cGMP signaling inhibits platelet shape change through regulation of the RhoA-Rho Kinase-MLC phosphatase signaling pathway." Journal of Thrombosis and Haemostasis **15**(8): 1668-1678.

Ackert, C. L., J. E. Gittens, M. J. O'Brien, J. J. Eppig and G. M. Kidder (2001). "Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse." Developmental biology **233**(2): 258-270.

Adhikari, D., N. Gorre, S. Risal, Z. Zhao, H. Zhang, Y. Shen and K. Liu (2012). "The safe use of a PTEN inhibitor for the activation of dormant mouse primordial follicles and generation of fertilizable eggs." PLoS one **7**(6): e39034.

Adhikari, D. and K. Liu (2010). mTOR signaling in the control of activation of primordial follicles, Taylor & Francis.

Adhikari, D. and K. Liu (2014). "The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes." Molecular and cellular endocrinology **382**(1): 480-487.

Albertini, D. F. (1987). "Cytoplasmic reorganization during the resumption of meiosis in cultured preovulatory rat oocytes." Developmental biology **120**(1): 121-131.

Albertini, D. F., C. Combelles, E. Benecchi and M. J. Carabatsos (2001). "Cellular basis for paracrine regulation of ovarian follicle development." Reproduction **121**(5): 647-653.

Albertini, D. F. and V. Rider (1994). "Patterns of intercellular connectivity in the mammalian cumulus-oocyte complex." Microscopy research and technique **27**(2): 125-133.

Albrecht, K. H. and E. M. Eicher (2001). "Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor." Developmental biology **240**(1): 92-107.

Allard, P., Q. Yang, W. F. Marzluff and H. J. Clarke (2005). "The stem-loop binding protein regulates translation of histone mRNA during mammalian oogenesis." Developmental biology **286**(1): 195-206.

Amano, M., M. Nakayama and K. Kaibuchi (2010). "Rho-kinase/ROCK: a key regulator of the cytoskeleton and cell polarity." Cytoskeleton **67**(9): 545-554.

Amsterdam, A., S. Rotmensch, A. Furman, E. A. Venter and I. Vlodavsky (1989). "Synergistic effect of human chorionic gonadotropin and extracellular matrix on in vitro differentiation of human granulosa cells: progesterone production and gap junction formation." Endocrinology **124**(4): 1956-1964.

Andreu-Vieyra, C. V., R. Chen, J. E. Agno, S. Glaser, K. Anastassiadis, A. F. Stewart and M. M. Matzuk (2010). "MLL2 is required in oocytes for bulk histone 3 lysine 4 trimethylation and transcriptional silencing." PLoS biology **8**(8): e1000453.

Arango, N. A., R. Lovell-Badge and R. R. Behringer (1999). "Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development." Cell **99**(4): 409-419.

Ashkenazi, H., X. Cao, S. Motola, M. Popliker, M. Conti and A. Tsafriri (2005). "Epidermal growth factor family members: endogenous mediators of the ovulatory response." Endocrinology **146**(1): 77-84.

Avella, M. A., B. Xiong and J. Dean (2013). "The molecular basis of gamete recognition in mice and humans." MHR: Basic science of reproductive medicine **19**(5): 279-289.

Azevedo, M. F., F. R. Faucz, E. Bimpaki, A. Horvath, I. Levy, R. B. de Alexandre, F. Ahmad, V. Manganiello and C. A. Stratakis (2013). "Clinical and molecular genetics of the phosphodiesterases (PDEs)." Endocrine reviews **35**(2): 195-233.

Bacha Jr, W. J. and L. M. Bacha (2012). Color atlas of veterinary histology, John Wiley & Sons.

Bachvarova, R., V. De Leon, A. Johnson, G. Kaplan and B. V. Paynton (1985). "Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes." Developmental biology **108**(2): 325-331.

Barnes, R. B., A. B. Namnoum, R. L. Rosenfield and L. C. Layman (2002). "The role of LH and FSH in ovarian androgen secretion and ovarian follicular development: clinical studies in a patient with isolated FSH deficiency and multicystic ovaries: case report." Human Reproduction **17**(1): 88-91.

Barnett, K., C. Schilling, C. Greenfeld, D. Tomic and J. Flaws (2006). "Ovarian follicle development and transgenic mouse models." Human reproduction update **12**(5): 537-555.

Barrett, S. L., L. D. Shea and T. K. Woodruff (2010). "Noninvasive index of cryorecovery and growth potential for human follicles in vitro." Biology of reproduction **82**(6): 1180-1189.

Bartol, F., A. Wiley, J. Floyd, T. Ott, F. Bazer, C. Gray and T. Spencer (1999). "Uterine differentiation as a foundation for subsequent fertility." Journal of reproduction and fertility. Supplement **54**: 287-302.

Bassett, D. L. (1943). "The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle." American Journal of Anatomy **73**(2): 251-291.

Basu, S., N. F. Totty, M. S. Irwin, M. Sudol and J. Downward (2003). "Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis." Molecular cell **11**(1): 11-23.

Bedell, M. A., C. I. Brannan, E. P. Evans, N. G. Copeland, N. A. Jenkins and P. J. Donovan (1995). "DNA rearrangements located over 100 kb 5'of the Steel (Sl)-coding region in Steel-

panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development." Genes & development **9**(4): 455-470.

Ben-Ami, I., S. Freimann, L. Armon, A. Dantes, D. Strassburger, S. Friedler, A. Raziel, R. Seger, R. Ron-El and A. Amsterdam (2006). "PGE2 up-regulates EGF-like growth factor biosynthesis in human granulosa cells: new insights into the coordination between PGE2 and LH in ovulation." Molecular human reproduction **12**(10): 593-599.

Biggers, J., D. Whittingham and R. Donahue (1967). "The pattern of energy metabolism in the mouse oöcyte and zygote." Proceedings of the National Academy of Sciences **58**(2): 560-567.

Birk, O. S., D. E. Casiano, C. A. Wassif, T. Cogliati, L. Zhao, Y. Zhao, A. Grinberg, S. Huang, J. A. Kreidberg and K. L. Parker (2000). "The LIM homeobox gene Lhx9 is essential for mouse gonad formation." Nature **403**(6772): 909.

Bitgood, M. J., L. Shen and A. P. McMahon (1996). "Sertoli cell signaling by Desert hedgehog regulates the male germline." Current Biology **6**(3): 298-304.

Blaustein, A. (1978). "Pathology of the Female Genital Tract." The American Journal of Surgical Pathology **2**(1): 111-112.

Bohrer, R. C., A. R. S. Coutinho, R. Duggavathi and V. Bordignon (2015). "The incidence of DNA double-strand breaks is higher in late-cleaving and less developmentally competent porcine embryos." Biology of reproduction **93**(3): 59, 51-58.

Boone, D. L. and B. K. Tsang (1997). "Identification and localization of deoxyribonuclease I in the rat ovary." Biology of reproduction **57**(4): 813-821.

Boone, D. L., W. Yan and B. K. Tsang (1995). "Identification of a deoxyribonuclease I-like endonuclease in rat granulosa and luteal cell nuclei." Biology of reproduction **53**(5): 1057-1065.

Bornslaeger, E. A., P. Mattei and R. M. Schultz (1986). "Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation." Developmental biology **114**(2): 453-462.

Bouniol-Baly, C., L. Hamraoui, J. Guibert, N. Beaujean, M. S. Szöllösi and P. Debey (1999). "Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes." Biology of Reproduction **60**(3): 580-587.

Bowles, J. and P. Koopman (2010). "Sex determination in mammalian germ cells: extrinsic versus intrinsic factors." Reproduction **139**(6): 943-958.

Boyd, J. L., S. L. Skove, J. P. Rouanet, L.-J. Pilaz, T. Bepler, R. Gordân, G. A. Wray and D. L. Silver (2015). "Human-chimpanzee differences in a FZD8 enhancer alter cell-cycle dynamics in the developing neocortex." Current Biology **25**(6): 772-779.

Brennan, J. and B. Capel (2004). "One tissue, two fates: molecular genetic events that underlie testis versus ovary development." Nature Reviews Genetics **5**(7): 509.

Brennan, J., C. Tilmann and B. Capel (2003). "Pdgfr-α mediates testis cord organization and fetal Leydig cell development in the XY gonad." Genes & development **17**(6): 800-810.

Brinster, R. (1971). "Oxidation of pyruvate and glucose by oocytes of the mouse and rhesus monkey." Journal of reproduction and fertility **24**(2): 187-191.

Bristol-Gould, S. K., P. K. Kreeger, C. G. Selkirk, S. M. Kilen, R. W. Cook, J. L. Kipp, L. D. Shea, K. E. Mayo and T. K. Woodruff (2006). "Postnatal regulation of germ cells by activin: the establishment of the initial follicle pool." Developmental biology **298**(1): 132-148.

Brito, I. R., I. M. Lima, M. Xu, L. D. Shea, T. K. Woodruff and J. R. Figueiredo (2014). "Three-dimensional systems for in vitro follicular culture: overview of alginate-based matrices." Reproduction, Fertility and Development **26**(7): 915-930.

Brower, P. T. and R. M. Schultz (1982). "Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth." Developmental biology **90**(1): 144-153.

Browne, C. L. and W. Werner (1984). "Intercellular junctions between the follicle cells and oocytes of Xenopus laevis." Journal of Experimental Zoology **230**(1): 105-113.

Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis and M. E. Greenberg (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." cell **96**(6): 857-868.

Brunet, S. and B. Maro (2007). "Germinal vesicle position and meiotic maturation in mouse oocyte." Reproduction **133**(6): 1069-1072.

Buccione, R., S. Cecconi, C. Tatone, F. Mangia and R. Colonna (1987). "Follicle cell regulation of mammalian oocyte growth." Journal of Experimental Zoology **242**(3): 351-354.

Bullejos, M. and P. Koopman (2001). "Spatially dynamic expression of Sry in mouse genital ridges." Developmental Dynamics **221**(2): 201-205.

Burns, K. H., C. Yan, T. R. Kumar and M. M. Matzuk (2001). "Analysis of ovarian gene expression in follicle-stimulating hormone β knockout mice." Endocrinology **142**(7): 2742-2751.

Callus, B. A., A. M. Verhagen and D. L. Vaux (2006). "Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation." The FEBS journal **273**(18): 4264-4276.

Camargo, F. D., S. Gokhale, J. B. Johnnidis, D. Fu, G. W. Bell, R. Jaenisch and T. R. Brummelkamp (2007). "YAP1 increases organ size and expands undifferentiated progenitor cells." Current biology **17**(23): 2054-2060.

Cameron, M. R., J. S. Foster, A. Bukovsky and J. Wimalasena (1996). "Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5'-monophosphates in porcine granulosa cells." Biology of reproduction **55**(1): 111-119.

Camp, T. A., J. O. Rahal and K. E. Mayo (1991). "Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary." Molecular endocrinology **5**(10): 1405-1417.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-1657.

Cantley, L. C. and B. G. Neel (1999). "New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway." Proceedings of the National Academy of Sciences **96**(8): 4240-4245.

Carabatsos, M. J., J. Elvin, M. M. Matzuk and D. F. Albertini (1998). "Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice." Developmental biology **204**(2): 373-384.

Castrillon, D. H., L. Miao, R. Kollipara, J. W. Horner and R. A. DePinho (2003). "Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a." Science **301**(5630): 215-218.

Cattanach, B., C. A. Iddon, H. Charlton, S. A. CHIAPPA and G. Fink (1977). "Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism." Nature **269**(5626): 338.

Chabot, J.-G., R. St-Arnaud, P. Walker and G. Pelletier (1986). "Distribution of epidermal growth factor receptors in the rat ovary." Molecular and cellular endocrinology **44**(2): 99-108.

Chaigne, A., C. Campillo, N. Gov, R. Voituriez, C. Sykes, M. Verlhac and M. Terret (2015). "A narrow window of cortical tension guides asymmetric spindle positioning in the mouse oocyte." Nature communications **6**: 6027.

Chalhoub, N. and S. J. Baker (2009). "PTEN and the PI3-kinase pathway in cancer." Annual Review of Pathological Mechanical Disease **4**: 127-150.

Chan, E. H., M. Nousiainen, R. B. Chalamalasetty, A. Schäfer, E. A. Nigg and H. H. Sillje (2005). "The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1." Oncogene **24**(12): 2076.

Chang, H.-M., J. Qiao and P. C. Leung (2016). "Oocyte–somatic cell interactions in the human ovary—novel role of bone morphogenetic proteins and growth differentiation factors." Human reproduction update **23**(1): 1-18.

Chen, J., C. Melton, N. Suh, J. S. Oh, K. Horner, F. Xie, C. Sette, R. Blelloch and M. Conti (2011). "Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition." Genes & development **25**(7): 755-766.

Chen, L., P. Russell and W. Larsen (1993). "Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass." Molecular reproduction and development **34**(1): 87-93.

Chen, Q., N. Zhang, R. Xie, W. Wang, J. Cai, K.-S. Choi, K. K. David, B. Huang, N. Yabuta and H. Nojima (2015). "Homeostatic control of Hippo signaling activity revealed by an endogenous activating mutation in YAP." Genes & development **29**(12): 1285-1297.

Chen, X., B. Zhou, J. Yan, B. Xu, P. Tai, J. Li, S. Peng, M. Zhang and G. Xia (2008). "Epidermal growth factor receptor activation by protein kinase C is necessary for FSH-induced meiotic resumption in porcine cumulus–oocyte complexes." Journal of Endocrinology **197**(2): 409-419.

Cheng, Y., Y. Feng, L. Jansson, Y. Sato, M. Deguchi, K. Kawamura and A. J. Hsueh (2015). "Actin polymerization-enhancing drugs promote ovarian follicle growth mediated by the Hippo signaling effector YAP." The FASEB Journal **29**(6): 2423-2430.

Chiquoine, A. D. (1960). "The development of the zona pellucida of the mammalian ovum." American Journal of Anatomy **106**(2): 149-169.

Cho, W. K., S. Stern and J. D. Biggers (1974). "Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro." Journal of Experimental Zoology **187**(3): 383-386.

Choi, Y., D. Yuan and A. Rajkovic (2008). "Germ cell-specific transcriptional regulator sohlh2 is essential for early mouse folliculogenesis and oocyte-specific gene expression." Biology of reproduction **79**(6): 1176-1182.

Christians, E., M. Boiani, S. Garagna, C. Dessy, C. A. Redi, J. P. Renard and M. Zuccotti (1999). "Gene expression and chromatin organization during mouse oocyte growth." Developmental biology **207**(1): 76-85.

Clark, A. M., K. K. Garland and L. D. Russell (2000). "Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules." Biology of reproduction **63**(6): 1825-1838.

Clarke, H. J. (2018). "Regulation of germ cell development by intercellular signaling in the mammalian ovarian follicle." Wiley Interdisciplinary Reviews: Developmental Biology **7**(1): e294.

Clarke, H. J., C. Oblin and M. Bustin (1992). "Developmental regulation of chromatin composition during mouse embryogenesis: somatic histone H1 is first detectable at the 4-cell stage." Development **115**(3): 791-799.

Clement, P. B. (1987). "Histology of the ovary." The American journal of surgical pathology **11**(4): 277-303.

Cockburn, K., S. Biechele, J. Garner and J. Rossant (2013). "The Hippo pathway member Nf2 is required for inner cell mass specification." Current biology **23**(13): 1195-1201.

Collado-Fernandez, E., H. M. Picton and R. Dumollard (2013). "Metabolism throughout follicle and oocyte development in mammals." International Journal of Developmental Biology **56**(10-11-12): 799-808.

Colonna, R. and F. Mangia (1983). "Mechanisms of amino acid uptake in cumulus-enclosed mouse oocytes." Biology of reproduction **28**(4): 797-803.

Colvin, J. S., R. P. Green, J. Schmahl, B. Capel and D. M. Ornitz (2001). "Male-to-female sex reversal in mice lacking fibroblast growth factor 9." Cell **104**(6): 875-889.

Comiskey, M. and C. M. Warner (2007). "Spatio-temporal localization of membrane lipid rafts in mouse oocytes and cleaving preimplantation embryos." Developmental biology **303**(2): 727-739.

Comizzoli, P., B. S. Pukazhenthi and D. E. Wildt (2011). "The competence of germinal vesicle oocytes is unrelated to nuclear chromatin configuration and strictly depends on cytoplasmic quantity and quality in the cat model." Human reproduction **26**(8): 2165-2177.

Conti, M., C. B. Andersen, F. Richard, C. Mehats, S.-Y. Chun, K. Horner, C. Jin and A. Tsafriri (2002). "Role of cyclic nucleotide signaling in oocyte maturation." Molecular and cellular endocrinology **187**(1-2): 153-159.

Conti, M. and J. Beavo (2007). "Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling." Annu. Rev. Biochem. **76**: 481-511.

Conti, M., M. Hsieh, A. M. Zamah and J. S. Oh (2012). "Novel signaling mechanisms in the ovary during oocyte maturation and ovulation." Molecular and cellular endocrinology **356**(1-2): 65-73.

Cortvrindt, R., J. Smitz and A. Van Steirteghem (1996). "Ovary and ovulation: In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system." Human Reproduction **11**(12): 2656-2666.

Cortvrindt, R., J. Smitz and A. Van Steirteghem (1997). "Assessment of the need for follicle stimulating hormone in early preantral mouse follicle culture in vitro." Human Reproduction (Oxford, England) **12**(4): 759-768.

Coticchio, G., M. Dal Canto, M. Mignini Renzini, M. C. Guglielmo, F. Brambillasca, D. Turchi, P. V. Novara and R. Fadini (2015). "Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization." Human reproduction update **21**(4): 427-454.

Couse, J. F., M. M. Yates, B. J. Deroo and K. S. Korach (2005). "Estrogen receptor-β is critical to granulosa cell differentiation and the ovulatory response to gonadotropins." Endocrinology **146**(8): 3247-3262.

Cui, X.-S., X.-Y. Li, X.-J. Yin, J.-J. KANG and N.-H. KIM (2007). "Maternal gene transcription in mouse oocytes: genes implicated in oocyte maturation and fertilization." Journal of Reproduction and Development **53**(2): 405-418.

Daneau, I., N. Pilon, A. Boyer, R. Behdjani, P. A. Overbeek, R. Viger, J. Lussier and D. W. Silversides (2002). "The porcine SRY promoter is transactivated within a male genital ridge environment." Genesis **33**(4): 170-180.

Danilovich, N., P. S. Babu, W. Xing, M. Gerdes, H. Krishnamurthy and M. R. Sairam (2000). "Estrogen deficiency, obesity, and skeletal abnormalities in follicle-stimulating hormone receptor knockout (FORKO) female mice." Endocrinology **141**(11): 4295-4308.

Davis, B. J., D. E. Lennard, C. A. Lee, H. F. Tiano, S. G. Morham, W. C. Wetsel and R. Langenbach (1999). "Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1β." Endocrinology **140**(6): 2685-2695.

De La Fuente, R. (2006). "Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes." Developmental biology **292**(1): 1-12.

De La Fuente, R. and J. J. Eppig (2001). "Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling." Developmental biology **229**(1): 224-236.

De La Fuente, R., M. J. O'Brien and J. J. Eppig (1999). "Epidermal growth factor enhances preimplantation developmental competence of maturing mouse oocytes." Human Reproduction **14**(12): 3060-3068.

De La Fuente, R., M. M. Viveiros, K. H. Burns, E. Y. Adashi, M. M. Matzuk and J. J. Eppig (2004). "Major chromatin remodeling in the germinal vesicle (GV) of mammalian oocytes is dispensable for global transcriptional silencing but required for centromeric heterochromatin function." Developmental biology **275**(2): 447-458.

De Leon, V., A. Johnson and R. Bachvarova (1983). "Half-lives and relative amounts of stored and polysomal ribosomes and poly (A)+ RNA in mouse oocytes." Developmental biology **98**(2): 400-408.

de Lesegno, C. V., K. Reynaud, C. Pechoux, S. Thoumire and S. Chastant-Maillard (2008). "Ultrastructure of canine oocytes during in vivo maturation." Molecular Reproduction and Development: Incorporating Gamete Research **75**(1): 115-125.

De, S., J. L. Marcinkiewicz, S. Vijayaraghavan and D. Kline (2012). "Expression of 14-3-3 protein isoforms in mouse oocytes, eggs and ovarian follicular development." BMC research notes **5**(1): 57.

De Santa Barbara, P., N. Bonneaud, B. Boizet, M. Desclozeaux, B. Moniot, P. Sudbeck, G. Scherer, F. Poulat and P. Berta (1998). "Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene." Molecular and cellular biology **18**(11): 6653-6665.

De Smedt, V. and D. Szöllösi (1991). "Cytochalasin D treatment induces meiotic resumption in follicular sheep oocytes." Molecular reproduction and development **29**(2): 163-171.

de Vantéry Arrighi, C., A. Campana and S. Schorderet-Slatkine (2000). "A role for the MEK-MAPK pathway in okadaic acid-induced meiotic resumption of incompetent growing mouse oocytes." Biology of reproduction **63**(2): 658-665.

Dean, J. (1992). "Biology of mammalian fertilization: role of the zona pellucida." The Journal of clinical investigation **89**(4): 1055-1059.

Debey, P., M. S. Szöllösi, D. Szöllösi, D. Vautier, A. Girousse and D. Besombes (1993). "Competent mouse oocytes isolated from antral follicles exhibit different chromatin organization

and follow different maturation dynamics." Molecular reproduction and development **36**(1): 59-74.

Dekel, N. and W. H. Beers (1978). "Rat oocyte maturation in vitro: relief of cyclic AMP inhibition by gonadotropins." Proceedings of the National Academy of Sciences **75**(9): 4369-4373.

Dekel, N. and I. Sherizly (1985). "Epidermal growth factor induces maturation of rat follicle-enclosed oocytes." Endocrinology **116**(1): 406-409.

Demeestere, I., A. K. Streiff, J. Suzuki, S. Al-Khabouri, E. Mahrous, S. L. Tan and H. J. Clarke (2012). "Follicle-stimulating hormone accelerates mouse oocyte development in vivo." Biology of reproduction **87**(1): 3, 1-11.

Diaz, F. J., K. Wigglesworth and J. J. Eppig (2007). "Oocytes determine cumulus cell lineage in mouse ovarian follicles." Journal of cell science **120**(8): 1330-1340.

Diepenbruck, M., L. Waldmeier, R. Ivanek, P. Berninger, P. Arnold, E. van Nimwegen and G. Christofori (2014). "Tead2 expression levels control Yap/Taz subcellular distribution, zyxin expression, and epithelial-mesenchymal transition." J Cell Sci: jcs. 139865.

Dierich, A., M. R. Sairam, L. Monaco, G. M. Fimia, A. Gansmuller, M. LeMeur and P. Sassone-Corsi (1998). "Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance." Proceedings of the National Academy of Sciences **95**(23): 13612-13617.

Dintzis, S. and P. Treuting (2011). Comparative Anatomy and Histology: A Mouse and Human Atlas, Academic.

Dissen, G. A., C. Garcia-Rudaz and S. R. Ojeda (2009). Role of neurotrophic factors in early ovarian development. Seminars in reproductive medicine, NIH Public Access.

Dissen, G. A., D. F. Hill, M. E. Costa, C. Les Dees, H. E. Lara and S. R. Ojeda (1996). "A role for trkA nerve growth factor receptors in mammalian ovulation." Endocrinology **137**(1): 198-209.

Dissen, G. A., C. Romero, A. N. Hirshfield and S. R. Ojeda (2001). "Nerve growth factor is required for early follicular development in the mammalian ovary." Endocrinology **142**(5): 2078-2086.

Donahue, R. and S. Stern (1968). "Follicular cell support of oocyte maturation: production of pyruvate in vitro." Journal of reproduction and fertility **17**(2): 395-398.

Dong, J., D. F. Albertini, K. Nishimori, T. R. Kumar, N. Lu and M. M. Matzuk (1996). "Growth differentiation factor-9 is required during early ovarian folliculogenesis." Nature **383**(6600): 531.

Dong, J., G. Feldmann, J. Huang, S. Wu, N. Zhang, S. A. Comerford, M. F. Gayyed, R. A. Anders, A. Maitra and D. Pan (2007). "Elucidation of a universal size-control mechanism in Drosophila and mammals." Cell **130**(6): 1120-1133.

Dorrington, J. H. and D. Armstrong (1979). Effects of FSH on gonadal functions. Proceedings of the 1978 Laurentian Hormone Conference, Elsevier.

Dorrington, J. H., Y. S. Moon and D. T. Armstrong (1975). "Estradiol-17β biosynthesis in cultured granulosa cells from hypophysectomized immature rats; stimulation by follicle-stimulating hormone1." Endocrinology **97**(5): 1328-1331.

Downs, S. M., S. A. Daniel, E. A. Bornslaeger, P. C. Hoppe and J. J. Eppig (1989). "Maintenance of meiotic arrest in mouse oocytes by purines: modulation of cAMP levels and cAMP phosphodiesterase activity." Gamete research **23**(3): 323-334.

Downs, S. M. and A. M. Mastropolo (1994). "The participation of energy substrates in the control of meiotic maturation in murine oocytes." Developmental biology **162**(1): 154-168.

Dragovic, R. A., L. J. Ritter, S. J. Schulz, F. Amato, D. T. Armstrong and R. B. Gilchrist (2005). "Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion." Endocrinology **146**(6): 2798-2806.

Dube, J. L., P. Wang, J. Elvin, K. M. Lyons, A. J. Celeste and M. M. Matzuk (1998). "The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes." Molecular endocrinology **12**(12): 1809-1817.

Dupont, S., L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato and S. Bicciato (2011). "Role of YAP/TAZ in mechanotransduction." Nature **474**(7350): 179.

Durinzi, K. L., E. M. Saniga and S. E. Lanzendorf (1995). "The relationship between size and maturation in vitro in the unstimulated human oocyte." Fertility and sterility **63**(2): 404-406.

Durlinger, A. L., M. J. Gruijters, P. Kramer, B. Karels, H. A. Ingraham, M. W. Nachtigal, J. T. J. Uilenbroek, J. A. Grootegoed and A. P. Themmen (2002). "Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary." Endocrinology **143**(3): 1076-1084.

Durlinger, A. L., P. Kramer, B. Karels, F. H. de Jong, J. T. J. Uilenbroek, J. A. Grootegoed and A. P. Themmen (1999). "Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary." Endocrinology **140**(12): 5789-5796.

Edwards, R. (1965). "Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes." Nature **208**(5008): 349.

Egbert, J. R., L. C. Shuhaibar, A. B. Edmund, D. A. Van Helden, J. W. Robinson, T. F. Uliasz, V. Baena, A. Geerts, F. Wunder and L. R. Potter (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.

Egbert, J. R., T. F. Uliasz, L. C. Shuhaibar, A. Geerts, F. Wunder, R. J. Kleiman, J. M. Humphrey, P. D. Lampe, N. O. Artemyev and S. D. Rybalkin (2016). "Luteinizing hormone causes phosphorylation and activation of the cGMP phosphodiesterase PDE5 in rat ovarian follicles, contributing, together with PDE1 activity, to the resumption of meiosis." Biology of reproduction **94**(5): 110, 111-111.

Eichenlaub-Ritter, U. and M. Peschke (2002). "Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level." Human Reproduction Update **8**(1): 21-41.

Eicher, E. M. and L. L. Washburn (1986). "Genetic control of primary sex determination in mice." Annual review of genetics **20**(1): 327-360.

El-Fouly, M., B. Cook, M. Nekola and A. Nalbandov (1970). "Role of the ovum in follicular luteinization." Endocrinology **87**(2): 288-293.

El-Hayek, S. and H. J. Clarke (2015). "Follicle-stimulating hormone increases gap junctional communication between somatic and germ-line follicular compartments during murine oogenesis." Biology of reproduction **93**(2).

El-Hayek, S. and H. J. Clarke (2016). Control of oocyte growth and development by intercellular communication within the follicular niche. Molecular Mechanisms of Cell Differentiation in Gonad Development, Springer**:** 191-224.

El-Hayek, S., I. Demeestere and H. J. Clarke (2014). "Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle." Proceedings of the National Academy of Sciences **111**(47): 16778-16783.

El-Hayek, S., Q. Yang, L. Abbassi, G. FitzHarris and H. J. Clarke (2018). "Mammalian Oocytes Locally Remodel Follicular Architecture to Provide the Foundation for Germline-Soma Communication." Current Biology **28**(7): 1124-1131. e1123.

El-Hayek, S., Q. Yang, L. Abbassi, G. FitzHarris and H. J. Clarke (2018). "Mammalian Oocytes Locally Remodel Follicular Architecture to Provide the Foundation for Germline-Soma Communication." Current Biology **28**(7): 1124-1131.e1123.

El-Danasouri, I., A. Frances and L. Westphal (1993). "Immunocytochemical localization of transforming growth factor-α and epidermal growth factor receptor in human fallopian tubes and cumulus cells." American Journal of Reproductive Immunology **30**(2-3): 82-87.

Elvin, J. A., A. T. Clark, P. Wang, N. M. Wolfman and M. M. Matzuk (1999). "Paracrine actions of growth differentiation factor-9 in the mammalian ovary." Molecular endocrinology **13**(6): 1035-1048.

Elvin, J. A., C. Yan, P. Wang, K. Nishimori and M. M. Matzuk (1999). "Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary." Molecular endocrinology **13**(6): 1018-1034.

Emmen, J. and K. Korach (2003). "Estrogen receptor knockout mice: phenotypes in the female reproductive tract." Gynecological endocrinology **17**(2): 169-176.

Eppig, J., F. Chesnel, Y. Hirao, M. O'brien, F. Pendola, S. Watanabe and K. Wigglesworth (1997). "Oocyte control of granulosa cell development: how and why." Human reproduction (Oxford, England) **12**(11 Suppl): 127-132.

Eppig, J. J. (1981). "Prostaglandin E2 stimulates cumulus expansion and hyaluronic acid synthesis by cumuli oophori isolated from mice." Biology of Reproduction **25**(1): 191-195.

Eppig, J. J. (1991). "Intercommunication between mammalian oocytes and companion somatic cells." Bioessays **13**(11): 569-574.

Eppig, J. J. (2001). "Oocyte control of ovarian follicular development and function in mammals." Reproduction **122**(6): 829-838.

Eppig, J. J. and M. J. O'brien (1996). "Development in vitro of mouse oocytes from primordial follicles." Biology of reproduction **54**(1): 197-207.

Eppig, J. J. and M. L. Steckman (1976). "Comparison of exogenous energy sources for in vitro maintenance of follicle cell-freeXenopus laevis oocytes." In vitro **12**(3): 173-179.

Eppig, J. J., K. Wigglesworth, F. Pendola and Y. Hirao (1997). "Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells." Biology of reproduction **56**(4): 976-984.

Eppig, J. J., K. Wigglesworth and F. L. Pendola (2002). "The mammalian oocyte orchestrates the rate of ovarian follicular development." Proceedings of the National Academy of Sciences **99**(5): 2890-2894.

Erickson, G., C. Wang and A. Hsueh (1979). "FSH induction of functional LH receptors in granulosa cells cultured in a chemically defined medium." Nature **279**(5711): 336.

Erickson, G. F. and S. Shimasaki (2001). "The physiology of folliculogenesis: the role of novel growth factors." Fertility and Sterility **76**(5): 943-949.

Evans, W. H. (2015). Cell communication across gap junctions: a historical perspective and current developments, Portland Press Limited.

Fagbohun, C. and S. Downs (1992). "Requirement for glucose in ligand-stimulated meiotic maturation of cumulus cell-enclosed mouse oocytes." Journal of reproduction and fertility **96**(2): 681-697.

Fairchild, C. L. and M. Barna (2014). "Specialized filopodia: at the 'tip'of morphogen transport and vertebrate tissue patterning." Current opinion in genetics & development **27**: 67-73.

Faix, J., D. Breitsprecher, T. E. Stradal and K. Rottner (2009). "Filopodia: Complex models for simple rods." The international journal of biochemistry & cell biology **41**(8-9): 1656-1664.

Finch, M. L., A. M. Passman, R. P. Strauss, G. C. Yeoh and B. A. Callus (2015). "Sub-cellular localisation studies may spuriously detect the Yes-associated protein, YAP, in nucleoli leading to potentially invalid conclusions of its function." PloS one **10**(2): e0114813.

Fitzpatrick, S. L. and J. S. Richards (1991). "Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells." Endocrinology **129**(3): 1452-1462.

Flaws, J. A., R. Abbud, R. J. Mann, J. H. Nilson and A. N. Hirshfield (1997). "Chronically elevated luteinizing hormone depletes primordial follicles in the mouse ovary." Biology of Reproduction **57**(5): 1233-1237.

Fortune, J. (2003). "The early stages of follicular development: activation of primordial follicles and growth of preantral follicles." Animal reproduction science **78**(3-4): 135-163.

Fortune, J. and D. Armstrong (1977). "Androgen production by theca and granulosa isolated from proestrous rat follicles." Endocrinology **100**(5): 1341-1347.

Fortune, J. and J. Eppig (1979). "Effects of gonadotropins on steroid secretion by infantile and juvenile mouse ovaries in vitro." Endocrinology **105**(3): 760-768.

Franciosi, F., G. Coticchio, V. Lodde, I. Tessaro, S. C. Modina, R. Fadini, M. Dal Canto, M. M. Renzini, D. F. Albertini and A. M. Luciano (2014). "Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes." Biology of reproduction **91**(3).

Freudzon, L., R. P. Norris, A. R. Hand, S. Tanaka, Y. Saeki, T. L. Jones, M. M. Rasenick, C. H. Berlot, L. M. Mehlmann and L. A. Jaffe (2005). "Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the Gs G protein." J Cell Biol **171**(2): 255-265.

Fru, K., M. Cherian-Shaw, M. Puttabyatappa, C. VandeVoort and C. Chaffin (2007). "Regulation of granulosa cell proliferation and EGF-like ligands during the periovulatory interval in monkeys." Human reproduction **22**(5): 1247-1252.

Führer, F., B. Mayr, K. Schellander, M. Kalat and W. Schleger (1989). "Maturation competence and chromatin behaviour in growing and fully grown cattle oocytes." Journal of Veterinary Medicine Series A **36**(1-10): 285-291.

Fülöp, C., A. Salustri and V. C. Hascall (1997). "Coding sequence of a hyaluronan synthase homologue expressed during expansion of the mouse cumulus–oocyte complex." Archives of Biochemistry and Biophysics **337**(2): 261-266.

Gall, L., N. Chene, M. Dahirel, S. Ruffini and C. Boulesteix (2004). "Expression of epidermal growth factor receptor in the goat cumulus–oocyte complex." Molecular Reproduction and Development: Incorporating Gamete Research **67**(4): 439-445.

Gallo, G. (2004). "Myosin II activity is required for severing-induced axon retraction in vitro." Experimental neurology **189**(1): 112-121.

Gallo, G. (2006). "RhoA-kinase coordinates F-actin organization and myosin II activity during semaphorin-3A-induced axon retraction." Journal of cell science **119**(16): 3413-3423.

Galloway, S. M., K. P. McNatty, L. M. Cambridge, M. P. Laitinen, J. L. Juengel, T. S. Jokiranta, R. J. McLaren, K. Luiro, K. G. Dodds and G. W. Montgomery (2000). "Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner." Nature genetics **25**(3): 279.

Gao, T., D. Zhou, C. Yang, T. Singh, A. Penzo–Méndez, R. Maddipati, A. Tzatsos, N. Bardeesy, J. Avruch and B. Z. Stanger (2013). "Hippo signaling regulates differentiation and maintenance in the exocrine pancreas." Gastroenterology **144**(7): 1543-1553. e1541.

George, N. M., C. E. Day, B. P. Boerner, R. L. Johnson and N. E. Sarvetnick (2012). "Hippo signaling regulates pancreas development through inactivation of Yap." Molecular and cellular biology: MCB. 01034-01012.

Gilchrist, R. B., M. Lane and J. G. Thompson (2008). "Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality." Human reproduction update **14**(2): 159-177.

Gilula, N. B., O. R. Reeves and A. Steinbach (1972). "Metabolic coupling, ionic coupling and cell contacts." Nature **235**(5336): 262.

Ginsburg, M., M. Snow and A. McLAREN (1990). "Primordial germ cells in the mouse embryo during gastrulation." Development **110**(2): 521-528.

Ginther, O., M. Beg, D. Bergfelt, F. Donadeu and K. Kot (2001). "Follicle selection in monovular species." Biology of reproduction **65**(3): 638-647.

Gittens, J. E., K. J. Barr, B. C. Vanderhyden and G. M. Kidder (2005). "Interplay between paracrine signaling and gap junctional communication in ovarian follicles." J Cell Sci **118**(1): 113-122.

Gittens, J. E., A. A. Mhawi, D. Lidington, Y. Ouellette and G. M. Kidder (2003). "Functional analysis of gap junctions in ovarian granulosa cells: distinct role for connexin43 in early stages of folliculogenesis." American Journal of Physiology-Cell Physiology **284**(4): C880-C887.

Glister, C., N. P. Groome and P. G. Knight (2003). "Oocyte-mediated suppression of follicle-stimulating hormone-and insulin-like growth factor-induced secretion of steroids and inhibin-related proteins by bovine granulosa cells in vitro: possible role of transforming growth factor α." Biology of reproduction **68**(3): 758-765.

Goldenberg, R., J. Vaitukaitis and G. T. ROSS (1972). "Estrogen and follicle stimulating hormone interactions on follicle growth in rats." Endocrinology **90**(6): 1492-1498.

González-Forero, D. and B. Moreno-López (2014). "Retrograde response in axotomized motoneurons: nitric oxide as a key player in triggering reversion toward a dedifferentiated phenotype." Neuroscience **283**: 138-165.

Goodfellow, P. N. and R. Lovell-Badge (1993). "SRY and sex determination in mammals." Annual review of genetics **27**(1): 71-92.

Goodman, C. A., J. M. Dietz, B. L. Jacobs, R. M. McNally, J.-S. You and T. A. Hornberger (2015). "Yes-Associated Protein is up-regulated by mechanical overload and is sufficient to induce skeletal muscle hypertrophy." FEBS letters **589**(13): 1491-1497.

Göritz, F., K. Jewgenow and H. Meyer (1996). "Epidermal growth factor and epidermal growth factor receptor in the ovary of the domestic cat (Felis catus)." Journal of reproduction and fertility **106**(1): 117-124.

Gougeon, A. Dynamics of human follicular growth: morphologic, dynamic, and functional aspects. Ovary (2004) 2: 25–43. doi: 10.1016, B978-012444562-8/50003-3.[Cross Ref].

Gougeon, A. (1996). "Regulation of ovarian follicular development in primates: facts and hypotheses." Endocrine reviews **17**(2): 121-155.

Gougeon, A. (1998). "Ovarian follicular growth in humans: ovarian ageing and population of growing follicles." Maturitas **30**(2): 137-142.

Gougeon, A. (2003). "Dynamics for Human Growth: Morphologic, Dynamic, and Functional Aspects." The ovary **2**: 25-43.

Gougeon, A. (2010). Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. Annales d'endocrinologie, Elsevier.

Gougeon, A. and D. Busso (2000). "Morphologic and functional determinants of primordial and primary follicles in the monkey ovary." Molecular and cellular endocrinology **163**(1-2): 33-42.

Gougeon, A., R. Ecochard and J. C. Thalabard (1994). "Age-related changes of the population of human ovarian follicles: increase in the disappearance rate of non-growing and early-growing follicles in aging women." Biology of reproduction **50**(3): 653-663.

Grainger, D. (1999). "Uterus, human." Encyclopedia of reproduction **4**: 942-950.

Green, D. (1997). "Three-dimensional structure of the zona pellucida." Reviews of Reproduction **2**(3): 147-156.

Greenwald, G. S. (1972). "Of eggs and follicles." American Journal of Anatomy **135**(1): 1-3.

Gubbay, J., J. Collignon, P. Koopman, B. Capel, A. Economou, A. Münsterberg, N. Vivian, P. Goodfellow and R. Lovell-Badge (1990). "A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes." Nature **346**(6281): 245.

Gui, L.-M. and I. M. Joyce (2005). "RNA interference evidence that growth differentiation factor-9 mediates oocyte regulation of cumulus expansion in mice." Biology of reproduction **72**(1): 195-199.

Hacker, A., B. Capel, P. Goodfellow and R. Lovell-Badge (1995). "Expression of Sry, the mouse sex determining gene." Development **121**(6): 1603-1614.

Hadek, R. (1965). "The structure of the mammalian egg." Int. Rev. Cytol **18**: 29-68.

Haghighat, N. and L. J. Van Winkle (1990). "Developmental change in follicular cell-enhanced amino acid uptake into mouse oocytes that depends on intact gap junctions and transport system Gly." Journal of Experimental Zoology **253**(1): 71-82.

Halpin, D. and H. Charlton (1988). "Effects of short-term injection of gonadotrophins on ovarian follicle development in hypogonadal (hpg) mice." Journal of reproduction and fertility **82**(1): 393-400.

Hammes, A., J.-K. Guo, G. Lutsch, J.-R. Leheste, D. Landrock, U. Ziegler, M.-C. Gubler and A. Schedl (2001). "Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation." Cell **106**(3): 319-329.

Han, S. J., R. Chen, M. P. Paronetto and M. Conti (2005). "Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse." Current Biology **15**(18): 1670-1676.

Handel, M. A., J. J. Eppig and J. C. Schimenti (2014). "Applying "gold standards" to in-vitro-derived germ cells." Cell **157**(6): 1257-1261.

Hansen, C. G., T. Moroishi and K.-L. Guan (2015). "YAP and TAZ: a nexus for Hippo signaling and beyond." Trends in cell biology **25**(9): 499-513.

Harris, S. E., H. J. Leese, R. G. Gosden and H. M. Picton (2009). "Pyruvate and oxygen consumption throughout the growth and development of murine oocytes." Molecular Reproduction and Development: Incorporating Gamete Research **76**(3): 231-238.

Harrouk, W. and H. J. Clarke (1995). "Mitogen-activated protein (MAP) kinase during the acquisition of meiotic competence by growing oocytes of the mouse." Molecular reproduction and development **41**(1): 29-36.

Harvey, K. F., X. Zhang and D. M. Thomas (2013). "The Hippo pathway and human cancer." Nature Reviews Cancer **13**(4): 246.

Hawkins, J., A. Taylor, P. Berta, J. Levilliers, B. Van der Auwera and P. Goodfellow (1992). "Mutational analysis of SRY: nonsense and missense mutations in XY sex reversal." Human genetics **88**(4): 471-474.

Hayashi, K., S. Ogushi, K. Kurimoto, S. Shimamoto, H. Ohta and M. Saitou (2012). "Offspring from oocytes derived from in vitro primordial germ cell–like cells in mice." Science: 1222482.

Hayashi, M., E. McGee, G. Min, C. Klein, U. M. Rose, M. v. Duin and A. J. Hsueh (1999). "Recombinant growth differentiation factor-9 (GDF-9) enhances growth and differentiation of cultured early ovarian follicles." Endocrinology **140**(3): 1236-1244.

Heallen, T., M. Zhang, J. Wang, M. Bonilla-Claudio, E. Klysik, R. L. Johnson and J. F. Martin (2011). "Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size." Science **332**(6028): 458-461.

Heffner, L. J. and D. J. Schust (2010). The reproductive system at a glance, John Wiley & Sons.

Heiligentag, M. and U. Eichenlaub-Ritter (2018). "Preantral follicle culture and oocyte quality." Reproduction, Fertility and Development **30**(1): 18-43.

Heller, D. T. and R. M. Schultz (1980). "Ribonucleoside metabolism by mouse oocytes: metabolic cooperativity between the fully grown oocyte and cumulus cells." Journal of Experimental Zoology **214**(3): 355-364.

Hérubel, F., S. El Mouatassim, P. Guérin, R. Frydman and Y. Ménézo (2002). "Genetic expression of monocarboxylate transporters during human and murine oocyte maturation and early embryonic development." Zygote **10**(2): 175-181.

Hickey, G. J., S. Chen, M. J. Besman, J. E. Shively, P. F. Hall, D. Gaddy-Kurten and J. S. Richards (1988). "Hormonal regulation, tissue distribution, and content of aromatase cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis." Endocrinology **122**(4): 1426-1436.

Hill, J., K. Hammar, P. Smith and D. Gross (1999). "Stage-dependent effects of epidermal growth factor on Ca2+ efflux in mouse oocytes." Molecular Reproduction and Development: Incorporating Gamete Research **53**(2): 244-253.

Hillier, S. G., P. F. Whitelaw and C. D. Smyth (1994). "Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin'model revisited." Molecular and cellular endocrinology **100**(1-2): 51-54.

Hinrichs, K., A. Schmidt, P. Friedman, J. Selgrath and M. Martin (1993). "In vitro maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy." Biology of Reproduction **48**(2): 363-370.

Hirate, Y., K. Cockburn, J. Rossant and H. Sasaki (2012). "Tead4 is constitutively nuclear, while nuclear vs. cytoplasmic Yap distribution is regulated in preimplantation mouse embryos." Proceedings of the National Academy of Sciences **109**(50): E3389-E3390.

Hirshfield, A. N. (1989). "Granulosa cell proliferation in very small follicles of cycling rats studied by long-term continuous tritiated-thymidine infusion." Biology of reproduction **41**(2): 309-316.

Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. International review of cytology, Elsevier. **124:** 43-101.

Hirshfield, A. N. (1994). "Relationship between the supply of primordial follicles and the onset of follicular growth in rats." Biology of reproduction **50**(2): 421-428.

Holt, J. E., S. I. Lane and K. T. Jones (2013). The control of meiotic maturation in mammalian oocytes. Current topics in developmental biology, Elsevier. **102:** 207-226.

Holt, J. E., S. M.-T. Tran, J. L. Stewart, K. Minahan, I. García-Higuera, S. Moreno and K. T. Jones (2011). "The APC/C activator FZR1 coordinates the timing of meiotic resumption during prophase I arrest in mammalian oocytes." Development: dev. 059022.

Holt, J. E., J. Weaver and K. T. Jones (2010). "Spatial regulation of APCCdh1-induced cyclin B1 degradation maintains G2 arrest in mouse oocytes." Development: dev. 047555.

Hreinsson, J. G., J. E. Scott, C. Rasmussen, M. L. Swahn, A. J. Hsueh and O. Hovatta (2002). "Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture." The Journal of Clinical Endocrinology & Metabolism **87**(1): 316-321.

Hsieh, M., A. M. Zamah and M. Conti (2009). Epidermal growth factor-like growth factors in the follicular fluid: role in oocyte development and maturation. Seminars in reproductive medicine, NIH Public Access.

Hu, Y.-C., P.-H. Wang, S. Yeh, R.-S. Wang, C. Xie, Q. Xu, X. Zhou, H.-T. Chao, M.-Y. Tsai and C. Chang (2004). "Subfertility and defective folliculogenesis in female mice lacking androgen receptor." Proceedings of the National Academy of Sciences **101**(31): 11209-11214.

Huang, E. J., K. Manova, A. I. Packer, S. Sanchez, R. F. Bachvarova and P. Besmer (1993). "The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles." Developmental biology **157**(1): 100-109.

Huang, X., Y. Gai, N. Yang, B. Lu, C. S. Samuel, V. J. Thannickal and Y. Zhou (2011). "Relaxin regulates myofibroblast contractility and protects against lung fibrosis." The American journal of pathology **179**(6): 2751-2765.

Hubbard, G. M. and G. F. Erickson (1988). "Luteinizing hormone-independent luteinization and ovulation in the hypophysectomized rat: a possible role for the oocyte?" Biology of reproduction **39**(1): 183-194.

Hunt, P. A. and T. J. Hassold (2008). "Human female meiosis: what makes a good egg go bad?" Trends in Genetics **24**(2): 86-93.

Hussein, M. R. (2005). "Apoptosis in the ovary: molecular mechanisms." Human reproduction update **11**(2): 162-178.

Hutt, K., E. McLaughlin and M. Holland (2006). "Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis." Molecular human reproduction **12**(2): 61-69.

Igarashi, H., T. Takahashi, E. Takahashi, N. Tezuka, K. Nakahara, K. Takahashi and H. Kurachi (2005). "Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization." Biology of reproduction **72**(5): 1256-1261.

Imajo, M., K. Miyatake, A. Iimura, A. Miyamoto and E. Nishida (2012). "A molecular mechanism that links Hippo signalling to the inhibition of Wnt/β-catenin signalling." The EMBO journal **31**(5): 1109-1122.

Inaba, M., M. Buszczak and Y. M. Yamashita (2015). "Nanotubes mediate niche–stem-cell signalling in the Drosophila testis." Nature **523**(7560): 329.

Inoue, Y., S. Miyamoto, T. Fukami, K. Shirota, F. Yotsumoto and T. Kawarabayashi (2009). "Amphiregulin is much more abundantly expressed than transforming growth factor-alpha and epidermal growth factor in human follicular fluid obtained from patients undergoing in vitro fertilization–embryo transfer." Fertility and sterility **91**(4): 1035-1041.

Irving-Rodgers, H. F. and R. Rodgers (2000). "Ultrastructure of the basal lamina of bovine ovarian follicles and its relationship to the membrana granulosa." Journal of reproduction and fertility **118**(2): 221-228.

Jacquemet, G., H. Hamidi and J. Ivaska (2015). "Filopodia in cell adhesion, 3D migration and cancer cell invasion." Current opinion in cell biology **36**: 23-31.

Jaffe, L. A. and J. R. Egbert (2017). "Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle." Annual review of physiology **79**: 237-260.

Jagarlamudi, K., L. Liu, D. Adhikari, P. Reddy, A. Idahl, U. Ottander, E. Lundin and K. Liu (2009). "Oocyte-specific deletion of Pten in mice reveals a stage-specific function of PTEN/PI3K signaling in oocytes in controlling follicular activation." PloS one **4**(7): e6186.

Jiménez, R., A. Sánchez, M. Burgos and R. D. de la Guardia (1996). "Puzzling out the genetics of mammalian sex determination." Trends in Genetics **12**(5): 164-166.

Jin, X., C. S. Han, F. Q. Yu, P. Wei, Z. Y. Hu and Y. X. Liu (2005). "Anti-apoptotic action of stem cell factor on oocytes in primordial follicles and its signal transduction." Molecular Reproduction and Development: Incorporating Gamete Research **70**(1): 82-90.

Jin, Y.-X., H.-S. Lee, X.-J. Yin, X.-S. Cui, I.-K. Kong and N.-H. Kim (2006). "Chromatin, microtubule and microfilament configurations in the canine oocyte." Reproduction, Fertility and Development **18**(8): 849-856.

John, G. B., T. D. Gallardo, L. J. Shirley and D. H. Castrillon (2008). "Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth." Developmental biology **321**(1): 197-204.

Johnson, M. T., E. A. Freeman, D. K. Gardner and P. A. Hunt (2007). "Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo." Biology of reproduction **77**(1): 2-8.

Jones, R. E. and K. H. Lopez (2013). Human reproductive biology, Academic Press.

Joyce, I. M., A. T. Clark, F. L. Pendola and J. J. Eppig (2000). "Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles." Biology of reproduction **63**(6): 1669-1675.

Joyce, I. M., F. L. Pendola, K. Wigglesworth and J. J. Eppig (1999). "Oocyte regulation of kit ligand expression in mouse ovarian follicles." Developmental biology **214**(2): 342-353.

Juan, W. C. and W. Hong (2016). "Targeting the Hippo signaling pathway for tissue regeneration and cancer therapy." Genes **7**(9): 55.

Juneja, S. C., K. J. Barr, G. C. Enders and G. M. Kidder (1999). "Defects in the germ line and gonads of mice lacking connexin43." Biology of Reproduction **60**(5): 1263-1270.

Kaivo-oja, N., L. A. Jeffery, O. Ritvos and D. G. Mottershead (2006). "Smad signalling in the ovary." Reproductive Biology and Endocrinology **4**(1): 21.

Kanai, F., P. A. Marignani, D. Sarbassova, R. Yagi, R. A. Hall, M. Donowitz, A. Hisaminato, T. Fujiwara, Y. Ito and L. C. Cantley (2000). "TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins." The EMBO journal **19**(24): 6778-6791.

Kaneko, K. J., E. B. Cullinan, K. E. Latham and M. L. DePamphilis (1997). "Transcription factor mTEAD-2 is selectively expressed at the beginning of zygotic gene expression in the mouse." Development **124**(10): 1963-1973.

Kato, M., R. Blanton, G.-R. Wang, T. J. Judson, Y. Abe, M. Myoishi, R. H. Karas and M. E. Mendelsohn (2012). "Direct binding and regulation of RhoA by cyclic GMP-dependent protein kinase Iα." Journal of Biological Chemistry: jbc. M112. 421040.

Katoh-Fukui, Y., R. Tsuchiya, T. Shiroishi, Y. Nakahara, N. Hashimoto, K. Noguchi and T. Higashinakagawa (1998). "Male-to-female sex reversal in M33 mutant mice." Nature **393**(6686): 688.

Kawamura, K., Y. Cheng, N. Kawamura, S. Takae, A. Okada, Y. Kawagoe, S. Mulders, Y. Terada and A. J. Hsueh (2011). "Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes." Human reproduction **26**(11): 3094-3101.

Kawamura, K., Y. Cheng, N. Suzuki, M. Deguchi, Y. Sato, S. Takae, C.-h. Ho, N. Kawamura, M. Tamura and S. Hashimoto (2013). "Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment." Proceedings of the National Academy of Sciences **110**(43): 17474-17479.

Keel, B. A., J. M. Hildebrandt, J. V. May and J. S. Davis (1995). "Effects of epidermal growth factor on the tyrosine phosphorylation of mitogen-activated protein kinases in monolayer cultures of porcine granulosa cells." Endocrinology **136**(3): 1197-1204.

Kezele, P., E. E. Nilsson and M. K. Skinner (2005). "Keratinocyte growth factor acts as a mesenchymal factor that promotes ovarian primordial to primary follicle transition." Biology of reproduction **73**(5): 967-973.

Kidder, G. M. and B. C. Vanderhyden (2010). "Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence." Canadian journal of physiology and pharmacology **88**(4): 399-413.

Kim, M., M. Kim, S. Lee, S. Kuninaka, H. Saya, H. Lee, S. Lee and D. S. Lim (2013). "cAMP/PKA signalling reinforces the LATS–YAP pathway to fully suppress YAP in response to actin cytoskeletal changes." The EMBO journal **32**(11): 1543-1555.

Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano and K. Okawa (1996). "Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase)." Science **273**(5272): 245-248.

Kitamura, K., M. Yanazawa, N. Sugiyama, H. Miura, A. Iizuka-Kogo, M. Kusaka, K. Omichi, R. Suzuki, Y. Kato-Fukui and K. Kamiirisa (2002). "Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans." Nature genetics **32**(3): 359.

Kol, S. and E. Y. Adashi (1995). "Intraovarian factors regulating ovarian function." Current Opinion in Obstetrics and Gynecology **7**(3): 209-213.

Koopman, P., J. Gubbay, N. Vivian, P. Goodfellow and R. Lovell-Badge (1991). "Male development of chromosomally female mice transgenic for Sry." Nature **351**(6322): 117.

Koval, M., S. A. Molina and J. M. Burt (2014). "Mix and match: investigating heteromeric and heterotypic gap junction channels in model systems and native tissues." FEBS letters **588**(8): 1193-1204.

Kovo, M., M. Kandli-Cohen, M. Ben-Haim, D. Galiani, D. W. Carr and N. Dekel (2006). "An active protein kinase A (PKA) is involved in meiotic arrest of rat growing oocytes." Reproduction **132**(1): 33-43.

Kreidberg, J. A., H. Sariola, J. M. Loring, M. Maeda, J. Pelletier, D. Housman and R. Jaenisch (1993). "WT-1 is required for early kidney development." Cell **74**(4): 679-691.

Kudo, N. R., K. Wassmann, M. Anger, M. Schuh, K. G. Wirth, H. Xu, W. Helmhart, H. Kudo, M. Mckay and B. Maro (2006). "Resolution of chiasmata in oocytes requires separase-mediated proteolysis." Cell **126**(1): 135-146.

Kumar, T. R., J. Agno, J. A. Janovick, P. M. Conn and M. M. Matzuk (2003). "Regulation of FSHβ and GnRH receptor gene expression in activin receptor II knockout male mice." Molecular and cellular endocrinology **212**(1-2): 19-27.

Kumar, T. R., Y. Wang, N. Lu and M. M. Matzuk (1997). "Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility." Nature genetics **15**(2): 201.

Lam, X., C. Gieseke, M. Knoll and P. Talbot (2000). "Assay and importance of adhesive interaction between hamster (Mesocricetus auratus) oocyte-cumulus complexes and the oviductal epithelium." Biology of reproduction **62**(3): 579-588.

Lawson, K. and W. Hage (1994). "Clonal analysis of the origin of primordial germ cells in the mouse." Germline development **165**: 68-84.

Layman, L. C. and P. G. McDonough (2000). "Mutations of follicle stimulating hormone-β and its receptor in human and mouse: genotype/phenotype." Molecular and cellular endocrinology **161**(1-2): 9-17.

Lee, K.-B., M. Zhang, K. Sugiura, K. Wigglesworth, T. Uliasz, L. A. Jaffe and J. J. Eppig (2013). "Hormonal coordination of natriuretic peptide type C and natriuretic peptide receptor 3 expression in mouse granulosa cells." Biology of reproduction **88**(2): 42, 41-49.

Lee, K.-P., J.-H. Lee, T.-S. Kim, T.-H. Kim, H.-D. Park, J.-S. Byun, M.-C. Kim, W.-I. Jeong, D. F. Calvisi and J.-M. Kim (2010). "The Hippo–Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis." Proceedings of the National Academy of Sciences **107**(18): 8248-8253.

Lee, S., E. Lenton, L. Sexton and I. Cooke (1988). "The effect of age on the cyclical patterns of plasma LH, FSH, oestradiol and progesterone in women with regular menstrual cycles." Human reproduction **3**(7): 851-855.

Lee, W.-S., F. Otsuka, R. K. Moore and S. Shimasaki (2001). "Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat." Biology of reproduction **65**(4): 994-999.

Leese, H. J. and A. M. Barton (1985). "Production of pyruvate by isolated mouse cumulus cells." Journal of Experimental Zoology **234**(2): 231-236.

Lefevre, B., A. Gougeon, F. Nome and J. Testart (1989). "In vivo changes in oocyte germinal vesicle related to follicular quality and size at mid-follicular phase during stimulated cycles in the cynomolgus monkey." Reproduction Nutrition Development **29**(5): 523-532.

Lei, Q.-Y., H. Zhang, B. Zhao, Z.-Y. Zha, F. Bai, X.-H. Pei, S. Zhao, Y. Xiong and K.-L. Guan (2008). "TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway." Molecular and cellular biology **28**(7): 2426-2436.

Lew, D. J. and S. Kornbluth (1996). "Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control." Current opinion in cell biology **8**(6): 795-804.

Li, H. and R.-C. Chian (2017). Follicular Development and Oocyte Growth. Development of In Vitro Maturation for Human Oocytes, Springer**:** 37-57.

Li, J., K. Kawamura, Y. Cheng, S. Liu, C. Klein, S. Liu, E.-K. Duan and A. J. Hsueh (2010). "Activation of dormant ovarian follicles to generate mature eggs." Proceedings of the National Academy of Sciences **107**(22): 10280-10284.

Li, L., P. Zheng and J. Dean (2010). "Maternal control of early mouse development." Development **137**(6): 859-870.

Li, M., C.-G. Liang, B. Xiong, B.-Z. Xu, S.-L. Lin, Y. Hou, D.-Y. Chen, H. Schatten and Q.-Y. Sun (2008). "PI3-kinase and mitogen-activated protein kinase in cumulus cells mediate EGF-induced meiotic resumption of porcine oocyte." Domestic animal endocrinology **34**(4): 360-371.

Li, R. and D. F. Albertini (2013). "The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte." Nature reviews Molecular cell biology **14**(3): 141.

Lincoln, A. J., D. Wickramasinghe, P. Stein, R. M. Schultz, M. E. Palko, P. Maria, L. Tessarollo and P. J. Donovan (2002). "Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation." Nature genetics **30**(4): 446.

Lindbloom, S., T. Farmerie, C. Clay, G. Seidel Jr and E. Carnevale (2008). "Potential involvement of EGF-like growth factors and phosphodiesterases in initiation of equine oocyte maturation." Animal reproduction science **103**(1-2): 187-192.

Lintern-Moore, S. and G. Moore (1979). "The initiation of follicle and oocyte growth in the mouse ovary." Biology of Reproduction **20**(4): 773-778.

Liu-Chittenden, Y., B. Huang, J. S. Shim, Q. Chen, S.-J. Lee, R. A. Anders, J. O. Liu and D. Pan (2012). "Genetic and pharmacological disruption of the TEAD–YAP complex suppresses the oncogenic activity of YAP." Genes & development **26**(12): 1300-1305.

Liu, K., S. Rajareddy, L. Liu, K. Jagarlamudi, K. Boman, G. Selstam and P. Reddy (2006). "Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer." Developmental biology **299**(1): 1-11.

Liu, X., F. Xie, A. M. Zamah, B. Cao and M. Conti (2014). "Multiple pathways mediate luteinizing hormone regulation of cGMP signaling in the mouse ovarian follicle." Biology of reproduction **91**(1): 9, 1-11.

Liu, Y.-X. and A. J. Hsueh (1986). "Synergism between granulosa and theca-interstitial cells in estrogen biosynthesis by gonadotropin-treated rat ovaries: studies on the two-cell, two-gonadotropin hypothesis using steroid antisera." Biology of reproduction **35**(1): 27-36.

Lovell-Badge, R., C. Canning and R. Sekido (2002). Sex-determining genes in mice: building pathways. Novartis Foundation symposium, Chichester; New York; John Wiley; 1999.

Lovell-Badge, R. and E. Robertson (1990). "XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy." Development **109**(3): 635-646.

Lu, L., Y. Li, S. M. Kim, W. Bossuyt, P. Liu, Q. Qiu, Y. Wang, G. Halder, M. J. Finegold and J.-S. Lee (2010). "Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver." Proceedings of the National Academy of Sciences **107**(4): 1437-1442.

Lubahn, D. B., J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach and O. Smithies (1993). "Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene." Proceedings of the National Academy of Sciences **90**(23): 11162-11166.

Lucifero, D., M. R. Mann, M. S. Bartolomei and J. M. Trasler (2004). "Gene-specific timing and epigenetic memory in oocyte imprinting." Human molecular genetics **13**(8): 839-849.

Luck, M. (1994). "The gonadal extracellular matrix." Oxford Reviews of Reproductive Biology.

Luo, L. and D. D. O'Leary (2005). "Axon retraction and degeneration in development and disease." Annu. Rev. Neurosci. **28**: 127-156.

Luo, X., Y. Ikeda and K. L. Parker (1994). "A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation." Cell **77**(4): 481-490.

Macaulay, A. D., I. Gilbert, J. Caballero, R. Barreto, E. Fournier, P. Tossou, M.-A. Sirard, H. J. Clarke, É. W. Khandjian and F. J. Richard (2014). "The gametic synapse: RNA transfer to the bovine oocyte." Biology of reproduction **91**(4): 90, 91-12.

Maehama, T. and J. E. Dixon (1998). "The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate." Journal of Biological Chemistry **273**(22): 13375-13378.

Mahrous, E., Q. Yang and H. J. Clarke (2012). "Regulation of mitochondrial DNA accumulation during oocyte growth and meiotic maturation in the mouse." Reproduction: REP-12-0113.

Makita, M. and T. Miyano (2014). "Steroid hormones promote bovine oocyte growth and connection with granulosa cells." Theriogenology **82**(4): 605-612.

Maller, J. and E. Krebs (1977). "Progesterone-stimulated meiotic cell division in Xenopus oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3': 5'-monophosphate-dependent protein kinase." Journal of Biological Chemistry **252**(5): 1712-1718.

Maller, J. L. and E. G. Krebs (1980). Regulation of oocyte maturation. Current topics in cellular regulation, Elsevier. **16:** 271-311.

Mandal, S., A. Stanco, E. S. Buys, G. Enikolopov and J. L. Rubenstein (2013). "Soluble guanylate cyclase generation of cGMP regulates migration of MGE neurons." Journal of Neuroscience **33**(43): 16897-16914.

Manova, K., K. Nocka, P. Besmer and R. F. Bachvarova (1990). "Gonadal expression of c-kit encoded at the W locus of the mouse." Development **110**(4): 1057-1069.

Marzo, L., K. Gousset and C. Zurzolo (2012). "Multifaceted roles of tunneling nanotubes in intercellular communication." Frontiers in physiology **3**: 72.

Mason, A. J., J. S. Hayflick, R. T. Zoeller, W. S. Young, H. S. Phillips, K. Nikolics and P. H. Seeburg (1986). "A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse." Science **234**(4782): 1366-1371.

Matsuda-Minehata, F., N. Inoue, Y. Goto and N. Manabe (2006). "The regulation of ovarian granulosa cell death by pro-and anti-apoptotic molecules." Journal of Reproduction and Development **52**(6): 695-705.

Mattson, B. A. and D. F. Albertini (1990). "Oogenesis: chromatin and microtubule dynamics during meiotic prophase." Molecular reproduction and development **25**(4): 374-383.

McElreavey, K., E. Vilain, N. Abbas, I. Herskowitz and M. Fellous (1993). "A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development." Proceedings of the National Academy of Sciences **90**(8): 3368-3372.

McFarland, K., R. Sprengel, H. S. Phillips, M. Kohler, N. Rosemblit, K. Nikolics, D. L. Segaloff and P. H. Seeburg (1989). "Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family." Science **245**(4917): 494-499.

McGaughey, R. W., D. H. Montgomery and J. D. Richter (1979). "Germinal vesicle configurations and patterns of polypeptide synthesis of porcine oocytes from antral follicles of different size, as related to their competency for spontaneous maturation." Journal of Experimental Zoology **209**(2): 239-253.

McGee, E. A. and A. J. Hsueh (2000). "Initial and cyclic recruitment of ovarian follicles." Endocrine reviews **21**(2): 200-214.

McGinnis, L. K. and W. H. Kinsey (2015). "Role of focal adhesion kinase in oocyte-follicle communication." Molecular reproduction and development **82**(2): 90-102.

McGrath, S. A., A. F. Esquela and S.-J. Lee (1995). "Oocyte-specific expression of growth/differentiation factor-9." Molecular Endocrinology **9**(1): 131-136.

Mehlmann, L. M. (2005). "Oocyte-specific expression of Gpr3 is required for the maintenance of meiotic arrest in mouse oocytes." Developmental biology **288**(2): 397-404.

Mehlmann, L. M., T. L. Jones and L. A. Jaffe (2002). "Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte." Science **297**(5585): 1343-1345.

Mehlmann, L. M., Y. Saeki, S. Tanaka, T. J. Brennan, A. V. Evsikov, F. L. Pendola, B. B. Knowles, J. J. Eppig and L. A. Jaffe (2004). "The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes." Science **306**(5703): 1947-1950.

Menke, D. B. and D. C. Page (2002). "Sexually dimorphic gene expression in the developing mouse gonad." Gene Expression Patterns **2**(3): 359-367.

Meredith, S., G. Dudenhoeffer, R. Butcher, S. Lerner and T. Walls (1992). "Unilateral ovariectomy increases loss of primordial follicles and is associated with increased metestrous concentration of follicle-stimulating hormone in old rats." Biology of reproduction **47**(2): 162-168.

Meredith, S., D. Kirkpatrick-Keller and R. L. Butcher (1986). "The effects of food restriction and hypophysectomy on numbers of primordial follicles and concentrations of hormones in rats." Biology of reproduction **35**(1): 68-73.

Mihm, M. and A. Evans (2008). "Mechanisms for dominant follicle selection in monovulatory species: a comparison of morphological, endocrine and intraovarian events in cows, mares and women." Reproduction in Domestic Animals **43**: 48-56.

Mills, T., R. W. Lewis, C. Wingard, A. Linder, L. Jin and R. C. Webb (2003). "Vasoconstriction, RhoA/Rho-kinase and the erectile response." International journal of impotence research **15**(S5): S20.

Mitra, J. and R. M. Schultz (1996). "Regulation of the acquisition of meiotic competence in the mouse: changes in the subcellular localization of cdc2, cyclin B1, cdc25C and wee1, and in the concentration of these proteins and their transcripts." Journal of Cell Science **109**(9): 2407-2415.

Miyamoto, N., M. Yoshida, S. Kuratani, I. Matsuo and S. Aizawa (1997). "Defects of urogenital development in mice lacking Emx2." Development **124**(9): 1653-1664.

Mo, J.-S., Z. Meng, Y. C. Kim, H. W. Park, C. G. Hansen, S. Kim, D.-S. Lim and K.-L. Guan (2015). "Cellular energy stress induces AMPK-mediated regulation of YAP and the Hippo pathway." Nature cell biology **17**(4): 500.

Mo, J. S., H. W. Park and K. L. Guan (2014). "The Hippo signaling pathway in stem cell biology and cancer." EMBO reports **15**(6): 642-656.

Monniaux, D., F. Clément, R. Dalbiès-Tran, A. Estienne, S. Fabre, C. Mansanet and P. Monget (2014). "The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link?" Biology of Reproduction **90**(4): 85, 81-11.

Monti, M. and C. Redi (2009). "Oogenesis specific genes (Nobox, Oct4, Bmp15, Gdf9, Oogenesin1 and Oogenesin2) are differentially expressed during natural and gonadotropin-induced mouse follicular development." Molecular Reproduction and Development: Incorporating Gamete Research **76**(10): 994-1003.

Moore, G. and S. Lintern-Moore (1974). "A correlation between growth and RNA synthesis in the mouse oocyte." Journal of reproduction and fertility **39**(1): 163-166.

Moore, G. and S. Lintern-Moore (1978). "Transcription of the mouse oocyte genome." Biology of reproduction **18**(5): 865-870.

Moore, G., S. Lintern-Moore, H. Peters and M. Faber (1974). "RNA synthesis in the mouse oocyte." The Journal of cell biology **60**(2): 416-422.

Mora, J. M., M. A. Fenwick, L. Castle, M. Baithun, T. A. Ryder, M. Mobberley, R. Carzaniga, S. Franks and K. Hardy (2012). "Characterization and significance of adhesion and junction-related proteins in mouse ovarian follicles." Biology of reproduction **86**(5): 153, 151-114.

Moroishi, T., C. G. Hansen and K.-L. Guan (2015). "The emerging roles of YAP and TAZ in cancer." Nature Reviews Cancer **15**(2): 73.

Mossman, H. W. and K. L. Duke (1973). Comparative morphology of the mammalian ovary, Wisconsin University Press.

Motta, P. M., S. Makabe, T. NAGURO and S. CORRER (1994). "Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy." Archives of histology and cytology **57**(4): 369-394.

Mottershead, D. G., S. Sugimura, S. L. Al-Musawi, J.-J. Li, D. Richani, M. A. White, G. A. Martin, A. P. Trotta, L. J. Ritter and J. Shi (2015). "Cumulin, an oocyte-secreted heterodimer of the transforming growth factor-β family, is a potent activator of granulosa cells and improves oocyte quality." Journal of Biological Chemistry: jbc. M115. 671487.

Mugahid, D., M. Kalocsay, S. Gruver, L. Peshkin and M. W. Kirschner (2018). "YAP independently regulates cell size and population growth dynamics via non-cell autonomous mediators." bioRxiv: 482836.

Murthy, K. S., H. Zhou, J. R. Grider and G. M. Makhlouf (2003). "Inhibition of sustained smooth muscle contraction by PKA and PKG preferentially mediated by phosphorylation of RhoA." American Journal of Physiology-Gastrointestinal and Liver Physiology **284**(6): G1006-G1016.

Muscatelli, F., T. M. Strom, A. P. Walker, E. Zanaria, D. Récan, A. Meindl, B. Bardoni, S. Guioli, G. Zehetner and W. Rabl (1994). "Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism." Nature **372**(6507): 672.

Nayudu, P. and S. Osborn (1992). "Factors influencing the rate of preantral and antral growth of mouse ovarian follicles in vitro." Journal of Reproduction and Fertility **95**(2): 349-362.

Nekola, M. and A. Nalbandov (1971). "Morphological changes of rat follicular cells as influenced by oocytes." Biology of reproduction **4**(2): 154-160.

Nilsson, E. E. and M. K. Skinner (2003). "Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development." Biology of reproduction **69**(4): 1265-1272.

Nilsson, E. E. and M. K. Skinner (2004). "Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition." Molecular and cellular endocrinology **214**(1-2): 19-25.

Nishimura, T., W. Fujii, K. Sugiura and K. Naito (2014). "Cytoplasmic anchoring of cAMP-dependent protein kinase (PKA) by A-kinase anchor proteins (AKAPs) is required for meiotic arrest of porcine full-grown and growing oocytes." Biology of reproduction **90**(3).

Nogueira, D., C. Albano, T. Adriaenssens, R. Cortvrindt, C. Bourgain, P. Devroey and J. Smitz (2003). "Human oocytes reversibly arrested in prophase I by phosphodiesterase type 3 inhibitor in vitro." Biology of reproduction **69**(3): 1042-1052.

Norris, R. P., M. Freudzon, L. M. Mehlmann, A. E. Cowan, A. M. Simon, D. L. Paul, P. D. Lampe and L. A. Jaffe (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.

Norris, R. P., W. J. Ratzan, M. Freudzon, L. M. Mehlmann, J. Krall, M. A. Movsesian, H. Wang, H. Ke, V. O. Nikolaev and L. A. Jaffe (2009). "Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte." Development **136**(11): 1869-1878.

Novella-Maestre, E., S. Herraiz, B. Rodríguez-Iglesias, C. Díaz-García and A. Pellicer (2015). "Short-term PTEN inhibition improves in vitro activation of primordial follicles, preserves follicular viability, and restores AMH levels in cryopreserved ovarian tissue from cancer patients." PLoS One **10**(5): e0127786.

O'Brien, M. J., J. K. Pendola and J. J. Eppig (2003). "A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence." Biology of reproduction **68**(5): 1682-1686.

O'shaughnessy, P., K. Dudley and W. Rajapaksha (1996). "Expression of follicle stimulating hormone-receptor mRNA during gonadal development." Molecular and cellular endocrinology **125**(1-2): 169-175.

Ochsner, S. A., A. J. Day, M. S. Rugg, R. M. Breyer, R. H. Gomer and J. S. Richards (2003). "Disrupted function of tumor necrosis factor-α-stimulated gene 6 blocks cumulus cell-oocyte complex expansion." Endocrinology **144**(10): 4376-4384.

Odor, D. L. (1960). "Electron microscopic studies on ovarian oocytes and unfertilized tubal ova in the rat." The Journal of Cell Biology **7**(3): 567-574.

Oh, J. S., S. J. Han and M. Conti (2010). "Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption." The Journal of cell biology **188**(2): 199-207.

Oktay, K., D. Briggs and R. G. Gosden (1997). "Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles." The Journal of Clinical Endocrinology & Metabolism **82**(11): 3748-3751.

Oktay, K., H. Newton, J. Mullan and R. Gosden (1998). "Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone." Human reproduction (Oxford, England) **13**(5): 1133-1138.

Oku, Y., N. Nishiya, T. Shito, R. Yamamoto, Y. Yamamoto, C. Oyama and Y. Uehara (2015). "Small molecules inhibiting the nuclear localization of YAP/TAZ for chemotherapeutics and chemosensitizers against breast cancers." FEBS Open Bio **5**: 542-549.

Ota, M. and H. Sasaki (2008). "Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling." Development **135**(24): 4059-4069.

Otsuka, F., S. Yamamoto, G. F. Erickson and S. Shimasaki (2001). "Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression." Journal of Biological Chemistry.

Otsuka, F., Z. Yao, T.-h. Lee, S. Yamamoto, G. F. Erickson and S. Shimasaki (2000). "Bone morphogenetic protein-15: identification of target cells and biological functions." Journal of Biological Chemistry.

Packer, A. I., Y. C. Hsu, P. Besmer and R. F. Bachvarova (1994). "The ligand of the c-kit receptor promotes oocyte growth." Developmental biology **161**(1): 194-205.

Pakarainen, T., F.-P. Zhang, L. Nurmi, M. Poutanen and I. Huhtaniemi (2005). "Knockout of luteinizing hormone receptor abolishes the effects of follicle-stimulating hormone on preovulatory maturation and ovulation of mouse graafian follicles." Molecular Endocrinology **19**(10): 2591-2602.

Pandya, P., J. L. Orgaz and V. Sanz-Moreno (2017). "Actomyosin contractility and collective migration: may the force be with you." Current opinion in cell biology **48**: 87-96.

Pangas, S. A., Y. Choi, D. J. Ballow, Y. Zhao, H. Westphal, M. M. Matzuk and A. Rajkovic (2006). "Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8." Proceedings of the National Academy of Sciences **103**(21): 8090-8095.

Panigone, S., M. Hsieh, M. Fu, L. Persani and M. Conti (2008). "Luteinizing hormone signaling in preovulatory follicles involves early activation of the epidermal growth factor receptor pathway." Molecular endocrinology **22**(4): 924-936.

Parfenov, V., G. Potchukalina, L. Dudina, D. Kostyuchek and M. Gruzova (1989). "Human antral follicles: oocyte nucleus and the karyosphere formation (electron microscopic and autoradiographic data)." Gamete research **22**(2): 219-231.

Park, J.-Y., Y.-Q. Su, M. Ariga, E. Law, S.-L. C. Jin and M. Conti (2004). "EGF-like growth factors as mediators of LH action in the ovulatory follicle." Science **303**(5658): 682-684.

Parrott, J. A. and M. K. Skinner (1999). "Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis." Endocrinology **140**(9): 4262-4271.

Pelosi, E., A. Forabosco and D. Schlessinger (2015). "Genetics of the ovarian reserve." Frontiers in genetics **6**: 308.

Pencharz, R. I. (1940). "Effect of estrogens and androgens alone and in combination with chorionic gonadotropin on the ovary of the hypophysectomized rat." Science **91**(2371): 554-555.

Peng, J., Q. Li, K. Wigglesworth, A. Rangarajan, C. Kattamuri, R. T. Peterson, J. J. Eppig, T. B. Thompson and M. M. Matzuk (2013). "Growth differentiation factor 9: bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions." Proceedings of the National Academy of Sciences **110**(8): E776-E785.

Peng, X.-R., A. J. Hsueh, P. S. Lapolt, L. Bjersing and T. Ny (1991). "Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation." Endocrinology **129**(6): 3200-3207.

Pepling, M. E. (2006). "From primordial germ cell to primordial follicle: mammalian female germ cell development." genesis **44**(12): 622-632.

Pepling, M. E. and A. C. Spradling (1998). "Female mouse germ cells form synchronously dividing cysts." Development **125**(17): 3323-3328.

Pepling, M. E. and A. C. Spradling (2001). "Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles." Developmental biology **234**(2): 339-351.

Pernasetti, F., V. V. Vasilyev, S. B. Rosenberg, J. S. Bailey, H.-J. Huang, W. L. Miller and P. L. Mellon (2001). "Cell-specific transcriptional regulation of follicle-stimulating hormone-β by activin and gonadotropin-releasing hormone in the LβT2 pituitary gonadotrope cell model." Endocrinology **142**(6): 2284-2295.

Perry, M., A. Gilbert and A. Evans (1978). "Electron microscope observations on the ovarian follicle of the domestic fowl during the rapid growth phase." Journal of anatomy **125**(Pt 3): 481.

Pesce, M., F. G. Klinger and M. De Felici (2002). "Derivation in culture of primordial germ cells from cells of the mouse epiblast: phenotypic induction and growth control by Bmp4 signalling." Mechanisms of development **112**(1-2): 15-24.

Pesty, A., F. Miyara, P. Debey, B. Lefevre and C. Poirot (2006). "Multiparameter assessment of mouse oogenesis during follicular growth in vitro." Molecular human reproduction **13**(1): 3-9.

Piccolo, S., S. Dupont and M. Cordenonsi (2014). "The biology of YAP/TAZ: hippo signaling and beyond." Physiological reviews **94**(4): 1287-1312.

Picton, H., D. Briggs and R. Gosden (1998). "The molecular basis of oocyte growth and development." Molecular and cellular endocrinology **145**(1-2): 27-37.

Pierce, J. G. and T. F. Parsons (1981). "Glycoprotein hormones: structure and function." Annual review of biochemistry **50**(1): 465-495.

Pincus, G. and E. V. Enzmann (1935). "The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs." Journal of Experimental Medicine **62**(5): 665-675.

Piquette, G. N., P. S. Lapolt, M. Oikawa and A. J. Hsueh (1991). "Regulation of luteinizing hormone receptor messenger ribonucleic acid levels by gonadotropins, growth factors, and gonadotropin-releasing hormone in cultured rat granulosa cells." Endocrinology **128**(5): 2449-2456.

Pirino, G., M. P. Wescott and P. J. Donovan (2009). "Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes." Cell cycle **8**(4): 665-670.

Plouffe, S. W., K. C. Lin, J. L. Moore, F. E. Tan, S. Ma, Z. Ye, Y. Qiu, B. Ren and K.-L. Guan (2018). "The Hippo pathway effector proteins YAP and TAZ have both distinct and overlapping functions in the cell." Journal of Biological Chemistry: jbc. RA118. 002715.

Praskova, M., F. Xia and J. Avruch (2008). "MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation." Current Biology **18**(5): 311-321.

Pratt, H. P. (1982). "Preimplantation mouse embryos synthesize membrane sterols." Developmental biology **89**(1): 101-110.

Pratt, H. P., J. Keith and J. Chakraborty (1980). "Membrane sterols and the development of the preimplantation mouse embryo." Development **60**(1): 303-319.

Rajah, R., E. M. Glaser and A. N. Hirshfield (1992). "The changing architecture of the neonatal rat ovary during histogenesis." Developmental dynamics **194**(3): 177-192.

Rajkovic, A., S. A. Pangas, D. Ballow, N. Suzumori and M. M. Matzuk (2004). "NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression." Science **305**(5687): 1157-1159.

Redding, G., J. Bronlund and A. Hart (2007). "Mathematical modelling of oxygen transport-limited follicle growth." Reproduction **133**(6): 1095-1106.

Reddy, P., L. Liu, D. Adhikari, K. Jagarlamudi, S. Rajareddy, Y. Shen, C. Du, W. Tang, T. Hämäläinen and S. L. Peng (2008). "Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool." Science **319**(5863): 611-613.

Reddy, P., L. Shen, C. Ren, K. Boman, E. Lundin, U. Ottander, P. Lindgren, Y.-x. Liu, Q.-y. Sun and K. Liu (2005). "Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development." Developmental biology **281**(2): 160-170.

Reeka, N., F. Berg and C. Brucker (1998). "Presence of transforming growth factor alpha and epidermal growth factor in human ovarian tissue and follicular fluid." Human reproduction (Oxford, England) **13**(8): 2199-2205.

Reis, A., H.-Y. Chang, M. Levasseur and K. T. Jones (2006). "APC cdh1 activity in mouse oocytes prevents entry into the first meiotic division." Nature cell biology **8**(5): 539.

Reis, A., S. Madgwick, H.-Y. Chang, I. Nabti, M. Levasseur and K. T. Jones (2007). "Prometaphase APC cdh1 activity prevents non-disjunction in mammalian oocytes." nature cell biology **9**(10): 1192.

Ren, F., L. Zhang and J. Jiang (2010). "Hippo signaling regulates Yorkie nuclear localization and activity through 14-3-3 dependent and independent mechanisms." Developmental biology **337**(2): 303-312.

Reynaud, K., R. Cortvrindt, J. Smitz and M. A. Driancourt (2000). "Effects of kit ligand and anti-kit antibody on growth of cultured mouse preantral follicles." Molecular Reproduction and Development: Incorporating Gamete Research **56**(4): 483-494.

Richard, F. J., A. Tsafriri and M. Conti (2001). "Role of phosphodiesterase type 3A in rat oocyte maturation." Biology of reproduction **65**(5): 1444-1451.

Richard, S. and J. M. Baltz (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." Biology of reproduction **90**(6): 137, 131-110.

RICHARDS, J. S. (1975). "Estradiol receptor content in rat granulosa cells during follicular development: modification by estradiol and gonadotropins." Endocrinology **97**(5): 1174-1184.

Richards, J. S. (1994). "Hormonal control of gene expression in the ovary." Endocrine reviews **15**(6): 725-751.

Richards, J. S. and M. Ascoli (2018). "Endocrine, Paracrine, and Autocrine Signaling Pathways That Regulate Ovulation." Trends in Endocrinology & Metabolism.

Richards, J. S., J. J. Ireland, M. C. RAO, G. A. Bernath, A. R. Midgley JR and L. E. Reichert (1976). "Ovarian follicular development in the rat: hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone." Endocrinology **99**(6): 1562-1570.

Richards, J. S., T. Jahnsen, L. Hedin, J. Lifka, S. Ratoosh, J. M. Durica and N. B. Goldring (1987). Ovarian follicular development: from physiology to molecular biology. Proceedings of the 1986 Laurentian Hormone Conference, Elsevier.

Richards, J. S., J. A. Jonassen, A. I. Rolfes, K. Kersey and L. E. Reichert JR (1979). "Adenosine 3', 5'-monophosphate, luteinizing hormone receptor, and progesterone during granulosa cell differentiation: effects of estradiol and follicle-stimulating hormone." Endocrinology **104**(3): 765-773.

Richards, J. S., Y. A. Ren, N. Candelaria, J. E. Adams and A. Rajkovic (2017). "Ovarian Follicular Theca Cell Recruitment, Differentiation, and Impact on Fertility: 2017 Update." Endocrine reviews **39**(1): 1-20.

Richardson, H. E. (2011). "Actin up for Hippo." The EMBO journal **30**(12): 2307-2309.

Richardson, M., D. Davies, R. Watson, M. Dunsford, C. Inman and G. Masson (1992). "Cultured human granulosa cells as a model for corpus luteum function: relative roles of gonadotrophin and low density lipoprotein studied under defined culture conditions." Human Reproduction **7**(1): 12-18.

Rieger, D. and N. Loskutoff (1994). "Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro." Journal of reproduction and fertility **100**(1): 257-262.

Robinson, J. W., M. Zhang, L. C. Shuhaibar, R. P. Norris, A. Geerts, F. Wunder, J. J. Eppig, L. R. Potter and L. A. Jaffe (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." Developmental biology **366**(2): 308-316.

Roy, S. and T. B. Kornberg (2015). "Paracrine signaling mediated at cell–cell contacts." Bioessays **37**(1): 25-33.

Rushmer, R. and R. Brinster (1973). "Carbon dioxide production from pyruvate and glucose by bovine oocytes." Experimental cell research **82**(2): 252-254.

Russo, V., A. Martelli, P. Berardinelli, O. Di Giacinto, N. Bernabò, D. Fantasia, M. Mattioli and B. Barboni (2007). "Modifications in chromatin morphology and organization during sheep oogenesis." Microscopy research and technique **70**(8): 733-744.

Saiduddin, S. and H. Zassenhaus (1977). "Estradiol-17β receptors in the immature rat ovary." Steroids **29**(2): 197-213.

Sales, A., C. Lobo, A. Carvalho, A. Moura and A. Rodrigues (2013). "Structure, function, and localization of aquaporins: their possible implications on gamete cryopreservation." Genet Mol Res **12**(4): 6718-6732.

Salustri, A., A. Camaioni, M. Di Giacomo, C. Fulop and V. C. Hascall (1999). "Hyaluronan and proteoglycans in ovarian follicles." Human reproduction update **5**(4): 293-301.

Salustri, A., M. Yanagishita and V. C. Hascall (1989). "Synthesis and accumulation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification." Journal of Biological Chemistry **264**(23): 13840-13847.

Sánchez, F., T. Adriaenssens, S. Romero and J. Smitz (2010). "Different follicle-stimulating hormone exposure regimens during antral follicle growth alter gene expression in the cumulus-oocyte complex in mice." Biology of reproduction **83**(4): 514-524.

Sánchez, F., F. Lolicato, S. Romero, M. De Vos, H. Van Ranst, G. Verheyen, E. Anckaert and J. Smitz (2017). "An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield." Human Reproduction **32**(10): 2056-2068.

Sánchez, F., S. Romero, F. K. Albuz and J. Smitz (2012). "In vitro follicle growth under non-attachment conditions and decreased FSH levels reduces Lhcgr expression in cumulus cells and

promotes oocyte developmental competence." Journal of assisted reproduction and genetics **29**(2): 141-152.

Sánchez, F. and J. Smitz (2012). "Molecular control of oogenesis." Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease **1822**(12): 1896-1912.

Sato, E. and S. Koide (1984). "Forskolin and mouse oocyte maturation in vitro." Journal of Experimental Zoology **230**(1): 125-129.

Sato, N., K. Kawamura, J. Fukuda, Y. Honda, T. Sato, H. Tanikawa, H. Kodama and T. Tanaka (2003). "Expression of LDL receptor and uptake of LDL in mouse preimplantation embryos." Molecular and cellular endocrinology **202**(1-2): 191-194.

Sauzeau, V., H. Le Jeune, C. Cario-Toumaniantz, A. Smolenski, S. M. Lohmann, J. Bertoglio, P. Chardin, P. Pacaud and G. Loirand (2000). "Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle." Journal of Biological Chemistry.

Schedl, A. and N. Hastie (1998). "Multiple roles for the Wilms' tumour suppressor gene, WT1 in genitourinary development." Molecular and cellular endocrinology **140**(1-2): 65-69.

Schmahl, J., Y. Kim, J. S. Colvin, D. M. Ornitz and B. Capel (2004). "Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination." Development **131**(15): 3627-3636.

Schramm, R. D., M. T. Tennier, D. E. Boatman and B. D. Bavister (1993). "Chromatin configurations and meiotic competence of oocytes are related to follicular diameter in nonstimulated rhesus monkeys." Biology of reproduction **48**(2): 349-356.

Schroeder, T. E. (1981). "Microfilament-mediated surface change in starfish oocytes in response to 1-methyladenine: implications for identifying the pathway and receptor sites for maturation-inducing hormones." The Journal of cell biology **90**(2): 362-371.

Schultz, R. M., G. E. Letourneau and P. M. Wassarman (1979). "Program of early development in the mammal: Changes in patterns and absolute rates of tubulin and total protein synthesis during oogenesis and early embryogenesis in the mouse." Developmental biology **68**(2): 341-359.

Schultz, R. M., R. R. Montgomery and J. R. Belanoff (1983). "Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis." Developmental biology **97**(2): 264-273.

Schultz, R. M. and P. M. Wassarman (1977). "Biochemical studies of mammalian oogenesis: Protein synthesis during oocyte growth and meiotic maturation in the mouse." Journal of Cell Science **24**(1): 167-194.

Segaloff, D. L., H. Wang and J. S. Richards (1990). "Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization." Molecular endocrinology **4**(12): 1856-1865.

Sekiguchi, T., T. Mizutani, K. Yamada, T. Kajitani, T. Yazawa, M. Yoshino and K. Miyamoto (2004). "Expression of epiregulin and amphiregulin in the rat ovary." Journal of molecular endocrinology **33**(1): 281-291.

Sela-Abramovich, S., E. Chorev, D. Galiani and N. Dekel (2005). "Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles." Endocrinology **146**(3): 1236-1244.

Sela-Abramovich, S., I. Edry, D. Galiani, N. Nevo and N. Dekel (2006). "Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation." Endocrinology **147**(5): 2280-2286.

Sen, A. and S. R. Hammes (2010). "Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function." Molecular Endocrinology **24**(7): 1393-1403.

Shalgi, R., P. Kraicer, A. Rimon, M. Pinto and N. Soferman (1973). "Proteins of human follicular fluid: the blood-follicle barrier." Fertility and sterility **24**(6): 429-434.

Shea, L. D., T. K. Woodruff and A. Shikanov (2014). "Bioengineering the ovarian follicle microenvironment." Annual review of biomedical engineering **16**: 29-52.

Shiina, H., T. Matsumoto, T. Sato, K. Igarashi, J. Miyamoto, S. Takemasa, M. Sakari, I. Takada, T. Nakamura and D. Metzger (2006). "Premature ovarian failure in androgen receptor-deficient mice." Proceedings of the National Academy of Sciences **103**(1): 224-229.

Shuhaibar, L. C., J. R. Egbert, A. B. Edmund, T. F. Uliasz, D. M. Dickey, S.-P. Yee, L. R. Potter and L. A. Jaffe (2016). "Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase is required for rapid resumption of oocyte meiosis in response to luteinizing hormone." Developmental biology **409**(1): 194-201.

Shuhaibar, L. C., J. R. Egbert, R. P. Norris, P. D. Lampe, V. O. Nikolaev, M. Thunemann, L. Wen, R. Feil and L. A. Jaffe (2015). "Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles." Proceedings of the National Academy of Sciences **112**(17): 5527-5532.

Simon, A. M., D. A. Goodenough, E. Li and D. L. Paul (1997). "Female infertility in mice lacking connexin 37." Nature **385**(6616): 525.

Singh, B., J. M. Rutledge and D. T. Armstrong (1995). "Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles." Molecular reproduction and development **40**(4): 391-399.

Sirois, J., D. Simmons and J. Richards (1992). "Hormonal regulation of messenger ribonucleic acid encoding a novel isoform of prostaglandin endoperoxide H synthase in rat preovulatory follicles. Induction in vivo and in vitro." Journal of Biological Chemistry **267**(16): 11586-11592.

Skinner, M. K. (2005). "Regulation of primordial follicle assembly and development." Human Reproduction Update **11**(5): 461-471.

Smallwood, S. A., S.-i. Tomizawa, F. Krueger, N. Ruf, N. Carli, A. Segonds-Pichon, S. Sato, K. Hata, S. R. Andrews and G. Kelsey (2011). "Dynamic CpG island methylation landscape in oocytes and preimplantation embryos." Nature genetics **43**(8): 811.

Smitz, J., M.-M. Dolmans, J. Donnez, J. Fortune, O. Hovatta, K. Jewgenow, H. Picton, C. Plancha, L. Shea and R. Stouffer (2010). "Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation." Human reproduction update **16**(4): 395-414.

Smyth, C., R. Gosden, A. McNeilly and S. Hillier (1994). "Effect of inhibin immunoneutralization on steroidogenesis in rat ovarian follicles in vitro." Journal of endocrinology **140**(3): 437-443.

Solc, P., A. Saskova, V. Baran, M. Kubelka, R. M. Schultz and J. Motlik (2008). "CDC25A phosphatase controls meiosis I progression in mouse oocytes." Developmental biology **317**(1): 260-269.

Song, H., K. K. Mak, L. Topol, K. Yun, J. Hu, L. Garrett, Y. Chen, O. Park, J. Chang and R. M. Simpson (2010). "Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression." Proceedings of the National Academy of Sciences **107**(4): 1431-1436.

Spoerke, J. M., C. O'Brien, L. Huw, H. Koeppen, J. Fridlyand, R. Brachmann, P. M. Haverty, A. Pandita, S. Mohan and D. Sampath (2012). "Phosphoinositide 3-kinase (PI3K) pathway alterations are associated with histologic subtypes and are predictive of sensitivity to PI3K inhibitors in lung cancer preclinical models." Clinical Cancer Research: clincanres. 2347.2012.

Sprengel, R., T. Braun, K. Nikolics, D. L. Segaloff and P. H. Seeburg (1990). "The testicular receptor for follicle stimulating hormone: structure and functional expression of cloned cDNA." Molecular Endocrinology **4**(4): 525-530.

Sternlicht, A. L. and R. M. Schultz (1981). "Biochemical studies of mammalian oogenesis: Kinetics of accumulation of total and poly (A)-containing RNA during growth of the mouse oocyte." Journal of Experimental Zoology **215**(2): 191-200.

Stewart, C. A. and R. R. Behringer (2012). Mouse oviduct development. Mouse Development, Springer**:** 247-262.

Stewart, K. R., L. Veselovska, J. Kim, J. Huang, H. Saadeh, S.-i. Tomizawa, S. A. Smallwood, T. Chen and G. Kelsey (2015). "Dynamic changes in histone modifications precede de novo DNA methylation in oocytes." Genes & development.

Stuelten, C. H., C. A. Parent and D. J. Montell (2018). "Cell motility in cancer invasion and metastasis: insights from simple model organisms." Nature Reviews Cancer.

Su, Y.-Q., J. M. Denegre, K. Wigglesworth, F. L. Pendola, M. J. O'Brien and J. J. Eppig (2003). "Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte–cumulus cell complex." Developmental biology **263**(1): 126-138.

Su, Y.-Q., K. Sugiura, K. Wigglesworth, M. J. O'Brien, J. P. Affourtit, S. A. Pangas, M. M. Matzuk and J. J. Eppig (2008). "Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells." Development **135**(1): 111-121.

Su, Y.-Q., K. Sugiura, Y. Woo, K. Wigglesworth, S. Kamdar, J. Affourtit and J. J. Eppig (2007). "Selective degradation of transcripts during meiotic maturation of mouse oocytes." Developmental biology **302**(1): 104-117.

Su, Y.-Q., K. Wigglesworth, F. L. Pendola, M. J. O'brien and J. J. Eppig (2002). "Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse." Endocrinology **143**(6): 2221-2232.

Sugiura, K., F. L. Pendola and J. J. Eppig (2005). "Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism." Developmental biology **279**(1): 20-30.

Sugiura, K., Y. Q. Su and J. J. Eppig (2009). "Targeted suppression of Has2 mRNA in mouse cumulus cell–oocyte complexes by adenovirus-mediated short-hairpin RNA expression." Molecular Reproduction and Development: Incorporating Gamete Research **76**(6): 537-547.

Sui, H. S., Y. Liu, D. Q. Miao, J. H. Yuan, T. W. Qiao, M. J. Luo and J. H. Tan (2005). "Configurations of germinal vesicle (GV) chromatin in the goat differ from those of other species." Molecular Reproduction and Development: Incorporating Gamete Research **71**(2): 227-236.

Sun, T., M. E. Pepling and F. J. Diaz (2015). "Lats1 deletion causes increased germ cell apoptosis and follicular cysts in mouse ovaries." Biology of reproduction **93**(1).

Sun, X., Z. Li, Y. Yi, W. Ding, J. Chen, J. Engelhardt and G. Leno (2009). "Chromatin configurations in the ferret germinal vesicle that reflect developmental competence for in vitro maturation." Reproduction in domestic animals **44**(2): 320-325.

Sun, X., Y. Su, Y. He, J. Zhang, W. Liu, H. Zhang, Z. Hou, J. Liu and J. Li (2015). "New strategy for in vitro activation of primordial follicles with mTOR and PI3K stimulators." Cell Cycle **14**(5): 721-731.

Sunico, C. R., D. González-Forero, G. Domínguez, J. M. García-Verdugo and B. Moreno-López (2010). "Nitric oxide induces pathological synapse loss by a protein kinase G-, Rho kinase-dependent mechanism preceded by myosin light chain phosphorylation." Journal of Neuroscience **30**(3): 973-984.

Surks, H. K. (2007). cGMP-dependent protein kinase I and smooth muscle relaxation: a tale of two isoforms, Am Heart Assoc.

Šutovský, P., J. E. Fléchon, B. Fléchon, J. Motlik, N. Peynot, P. Chesné and Y. Heyman (1993). "Dynamic changes of gap junctions and cytoskeleton during in vitro culture of cattle oocyte cumulus complexes." Biology of reproduction **49**(6): 1277-1287.

Šutovský, P., J. E. Fléchon and A. Pavlok (1995). "F-actin is involved in control of bovine cumulus expansion." Molecular reproduction and development **41**(4): 521-529.

Sutton, M., R. Gilchrist and J. Thompson (2003). "Effects of in-vivo and in-vitro environments on the metabolism of the cumulus–oocyte complex and its influence on oocyte developmental capacity." Human reproduction update **9**(1): 35-48.

Svoboda, P., V. Franke and R. M. Schultz (2015). Sculpting the transcriptome during the oocyte-to-embryo transition in mouse. Current topics in developmental biology, Elsevier. **113:** 305-349.

Szybek, K. (1972). "In-vitro maturation of oocytes from sexually immature mice." Journal of Endocrinology **54**(3): 527-528.

Tajima, K., A. Dantes, Z. Yao, K. Sorokina, F. Kotsuji, R. Seger and A. Amsterdam (2003). "Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1." The Journal of Clinical Endocrinology & Metabolism **88**(5): 2288-2299.

Teixeira Filho, F. L., E. C. Baracat, T. H. Lee, C. S. Suh, M. Matsui, R. J. Chang, S. Shimasaki and G. F. Erickson (2002). "Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome." The Journal of Clinical Endocrinology & Metabolism **87**(3): 1337-1344.

Telfer, E. E. and M. McLaughlin (2011). In vitro development of ovarian follicles. Seminars in reproductive medicine, © Thieme Medical Publishers.

Telfer, E. E. and M. B. Zelinski (2013). "Ovarian follicle culture: advances and challenges for human and nonhuman primates." Fertility and sterility **99**(6): 1523-1533.

Tevosian, S. G., K. H. Albrecht, J. D. Crispino, Y. Fujiwara, E. M. Eicher and S. H. Orkin (2002). "Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2." Development **129**(19): 4627-4634.

Themmen, A. P. and I. T. Huhtaniemi (2000). "Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function." Endocrine reviews **21**(5): 551-583.

Thomas, F. H., R. S. Ismail, J.-Y. Jiang and B. C. Vanderhyden (2008). "Kit ligand 2 promotes murine oocyte growth in vitro." Biology of reproduction **78**(1): 167-175.

Thomas, F. H. and B. C. Vanderhyden (2006). "Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth." Reproductive Biology and Endocrinology **4**(1): 19.

Tingen, C. M., S. K. Bristol-Gould, S. E. Kiesewetter, J. T. Wellington, L. Shea and T. K. Woodruff (2009). "Prepubertal primordial follicle loss in mice is not due to classical apoptotic pathways." Biology of Reproduction **81**(1): 16-25.

Tomizawa, S.-I., J. Nowacka-Woszuk and G. Kelsey (2013). "DNA methylation establishment during oocyte growth: mechanisms and significance." International Journal of Developmental Biology **56**(10-11-12): 867-875.

Törnell, J., M. Brännström and T. Hillensjö (1984). "Different effects of cyclic nucleotide derivatives upon the rat oocyte-cumulus complex in vitro." Acta physiologica scandinavica **122**(4): 507-513.

Tran, T. N., K. Brettingham-Moore, C. P. Duong, C. Mitchell, N. J. Clemons and W. A. Phillips (2013). "Molecular changes in the phosphatidylinositide 3-kinase (PI3K) pathway are common in gastric cancer." Journal of surgical oncology **108**(2): 113-120.

Trigatti, B., H. Rayburn, M. Viñals, A. Braun, H. Miettinen, M. Penman, M. Hertz, M. Schrenzel, L. Amigo and A. Rigotti (1999). "Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology." Proceedings of the National Academy of Sciences **96**(16): 9322-9327.

Tsafriri, A., S. H. Pomerantz and C. P. Channing (1976). "Inhibition of oocyte maturation by porcine follicular fluid: partial characterization of the inhibitor." Biology of reproduction **14**(5): 511-516.

Tsutsumi, O., K. Satoh, Y. Taketani and T. Kato (1992). "Determination of enzyme activities of energy metabolism in the maturing rat oocyte." Molecular reproduction and development **33**(3): 333-337.

Tsutsumi, O., T. Yano, K. Satoh, M. Mizuno and T. Kato (1990). "Studies of hexokinase activity in human and mouse oocyte." American Journal of Obstetrics & Gynecology **162**(5): 1301-1304.

Tulsiani, D. (2012). Introduction to mammalian reproduction, Springer Science & Business Media.

Ueda, K., M. Murata-Hori, M. Tatsuka and H. Hosoya (2002). "Rho-kinase contributes to diphosphorylation of myosin II regulatory light chain in nonmuscle cells." Oncogene **21**(38): 5852.

Vaccari, S., K. Horner, L. M. Mehlmann and M. Conti (2008). "Generation of mouse oocytes defective in cAMP synthesis and degradation: endogenous cyclic AMP is essential for meiotic arrest." Developmental biology **316**(1): 124-134.

Vaccari, S., J. L. Weeks, M. Hsieh, F. S. Menniti and M. Conti (2009). "Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes." Biology of reproduction **81**(3): 595-604.

Vainio, S., M. Heikkilä, A. Kispert, N. Chin and A. P. McMahon (1999). "Female development in mammals is regulated by Wnt-4 signalling." Nature **397**(6718): 405.

Van Blerkom, J., P. W. Davis and J. Lee (1995). "Fertilization and early embryolgoy: ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer." Human Reproduction **10**(2): 415-424.

Van Tol, H., M. Van Eijk, C. Mummery, R. Van Den Hurk and M. Bevers (1996). "Influence of FSH and hCG on the resumption of meiosis of bovine oocytes surrounded by cumulus cells connected to membrana granulosa." Molecular Reproduction and Development: Incorporating Gamete Research **45**(2): 218-224.

Vanderhyden, B. (2002). "Molecular basis of ovarian development and function." Front Biosci **7**(9): d2-006.

Vanderhyden, B., J. Cohen and P. Morley (1993). "Mouse oocytes regulate granulosa cell steroidogenesis." Endocrinology **133**(1): 423-426.

Vanderhyden, B. C. and E. A. Macdonald (1998). "Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development." Biology of reproduction **59**(6): 1296-1301.

Vanderhyden, B. C. and A. M. Tonary (1995). "Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by A factor (s) secreted by the oocyte." Biology of Reproduction **53**(6): 1243-1250.

Varani, S., J. A. Elvin, C. Yan, J. DeMayo, F. J. DeMayo, H. F. Horton, M. C. Byrne and M. M. Matzuk (2002). "Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility." Molecular endocrinology **16**(6): 1154-1167.

Varelas, X. (2014). "The Hippo pathway effectors TAZ and YAP in development, homeostasis and disease." Development **141**(8): 1614-1626.

Vitt, U., M. Hayashi, C. Klein and A. Hsueh (2000). "Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles." Biology of reproduction **62**(2): 370-377.

Vivarelli, E., M. Conti, M. De Felici and G. Siracusa (1983). "Meiotic resumption and intracellular cAMP levels in mouse oocytes treated with compounds which act on cAMP metabolism." Cell differentiation **12**(5): 271-276.

Vlahos, C. J., W. F. Matter, K. Y. Hui and R. F. Brown (1994). "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)." Journal of Biological Chemistry **269**(7): 5241-5248.

von Gise, A., Z. Lin, K. Schlegelmilch, L. B. Honor, G. M. Pan, J. N. Buck, Q. Ma, T. Ishiwata, B. Zhou and F. D. Camargo (2012). "YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy." Proceedings of the National Academy of Sciences **109**(7): 2394-2399.

Wang, P., B. Mao, W. Luo, B. Wei, W. Jiang, D. Liu, L. Song, G. Ji, Z. Yang and Y.-Q. Lai (2014). "The alteration of Hippo/YAP signaling in the development of hypertrophic cardiomyopathy." Basic research in cardiology **109**(5): 435.

Wang, S., G. Ning, X. Chen, J. Yang, H. Ouyang, H. Zhang, P. Tai, X. Mu, B. Zhou and M. Zhang (2008). "PDE5 modulates oocyte spontaneous maturation via cGMP-cAMP but not cGMP-PKG signaling." Front Biosci **13**(7087.7095).

Wang, W., J. Huang and J. Chen (2011). "Angiomotin-like proteins associate with and negatively regulate YAP1." Journal of Biological Chemistry **286**(6): 4364-4370.

Wang, Y., J. Li, C. Ying Wang, A. H. Yan Kwok and F. C. Leung (2007). "Epidermal growth factor (EGF) receptor ligands in the chicken ovary: I. Evidence for heparin-binding EGF-like growth factor (HB-EGF) as a potential oocyte-derived signal to control granulosa cell proliferation and HB-EGF and kit ligand expression." Endocrinology **148**(7): 3426-3440.

Wang, Y., Z. Teng, G. Li, X. Mu, Z. Wang, L. Feng, W. Niu, K. Huang, X. Xiang and C. Wang (2015). "Cyclic AMP in oocytes controls meiotic prophase I and primordial folliculogenesis in the perinatal mouse ovary." Development **142**(2): 343-351.

Wassarman, P. M. (1988). "Zona pellucida glycoproteins." Annual review of biochemistry **57**(1): 415-442.

Wassarman, P. M. and G. E. Letourneau (1976). "RNA synthesis in fully-grown mouse oocytes." Nature **261**(5555): 73.

Wassarman, P. M. and E. S. Litscher (2013). Biogenesis of the mouse egg's extracellular coat, the zona pellucida. Current topics in developmental biology, Elsevier. **102:** 243-266.

Wassarman, P. M. and E. S. Litscher (2013). "Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility." International Journal of Developmental Biology **56**(10-11-12): 833-839.

Wassarman, P. M. and S. Mortillo (1991). Structure of the mouse egg extracellular coat, the zona pellucida. International review of cytology, Elsevier. **130:** 85-110.

Wert, S. E. and W. J. Larsen (1989). "Meiotic resumption and gap junction modulation in the cultured rat cumulus-oocyte complex." Gamete research **22**(2): 143-162.

Westergaard, L. and C. Y. Anhdersen (1989). "Epidermal growth factor (EGF) in human preovulatory follicles." Human Reproduction **4**(3): 257-260.

White, T., D. Paul, D. Goodenough and R. Bruzzone (1995). "Functional analysis of selective interactions among rodent connexins." Molecular Biology of the Cell **6**(4): 459-470.

Whitelaw, P., C. Smyth, C. Howles and S. Hillier (1992). "Cell-specific expression of aromatase and LH receptor mRNAs in rat ovary." Journal of molecular endocrinology **9**(3): 309-312.

Wickramasinghe, D., K. M. Ebert and D. F. Albertini (1991). "Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes." Developmental biology **143**(1): 162-172.

Wigglesworth, K., K.-B. Lee, M. J. O'Brien, J. Peng, M. M. Matzuk and J. J. Eppig (2013). "Bidirectional communication between oocytes and ovarian follicular somatic cells is required for meiotic arrest of mammalian oocytes." Proceedings of the National Academy of Sciences: 201314829.

Winterhager, E. and G. M. Kidder (2015). "Gap junction connexins in female reproductive organs: implications for women's reproductive health." Human reproduction update **21**(3): 340-352.

Woodruff, T. K. (2015). "Oncofertility: a grand collaboration between reproductive medicine and oncology." Reproduction: REP-15-0163.

Xin, M., Y. Kim, L. B. Sutherland, M. Murakami, X. Qi, J. McAnally, E. R. Porrello, A. I. Mahmoud, W. Tan and J. M. Shelton (2013). "Hippo pathway effector Yap promotes cardiac regeneration." Proceedings of the National Academy of Sciences **110**(34): 13839-13844.

Xu, J., M. Lawson, R. Yeoman, K. Pau, S. Barrett, M. Zelinski and R. Stouffer (2011). "Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: effects of gonadotrophins, oxygen and fetuin." Human Reproduction **26**(5): 1061-1072.

Yan, C., P. Wang, J. DeMayo, F. J. DeMayo, J. A. Elvin, C. Carino, S. V. Prasad, S. S. Skinner, B. S. Dunbar and J. L. Dube (2001). "Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function." Molecular endocrinology **15**(6): 854-866.

Yang, Q., P. Allard, M. Huang, W. Zhang and H. J. Clarke (2010). "Proteasomal activity is required to initiate and to sustain translational activation of messenger RNA encoding the stem-loop-binding protein during meiotic maturation in mice." Biology of reproduction **82**(1): 123-131.

Yao, H. H.-C., W. Whoriskey and B. Capel (2002). "Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis." Genes & development **16**(11): 1433-1440.

Yi, Y.-J., E. Nagyova, G. Manandhar, R. Procházka, M. Sutovsky, C.-S. Park and P. Sutovsky (2008). "Proteolytic activity of the 26S proteasome is required for the meiotic resumption, germinal vesicle breakdown, and cumulus expansion of porcine cumulus-oocyte complexes matured in vitro." Biology of reproduction **78**(1): 115-126.

Ying, Y., X.-M. Liu, A. Marble, K. A. Lawson and G.-Q. Zhao (2000). "Requirement of Bmp8b for the generation of primordial germ cells in the mouse." Molecular endocrinology **14**(7): 1053-1063.

Ying, Y. and G.-Q. Zhao (2001). "Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse." Developmental biology **232**(2): 484-492.

Yoshida, H., N. Takakura, H. Kataoka, T. Kunisada, H. Okamura and S.-I. Nishikawa (1997). "Stepwise requirement ofc-kit tyrosine kinase in mouse ovarian follicle development." Developmental biology **184**(1): 122-137.

Yoshino, O., H. E. McMahon, S. Sharma and S. Shimasaki (2006). "A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse." Proceedings of the National Academy of Sciences **103**(28): 10678-10683.

Yu, F.-X., Y. Zhang, H. W. Park, J. L. Jewell, Q. Chen, Y. Deng, D. Pan, S. S. Taylor, Z.-C. Lai and K.-L. Guan (2013). "Protein kinase A activates the Hippo pathway to modulate cell proliferation and differentiation." Genes & development **27**(11): 1223-1232.

Yu, F.-X., B. Zhao, N. Panupinthu, J. L. Jewell, I. Lian, L. H. Wang, J. Zhao, H. Yuan, K. Tumaneng and H. Li (2012). "Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling." Cell **150**(4): 780-791.

Zaidel-Bar, R., G. Zhenhuan and C. Luxenburg (2015). "The contractome–a systems view of actomyosin contractility in non-muscle cells." J Cell Sci: jcs. 170068.

Zamah, A., M. Hsieh, J. Chen, J. Vigne, M. Rosen, M. Cedars and M. Conti (2010). "Human oocyte maturation is dependent on LH-stimulated accumulation of the epidermal growth factor-like growth factor, amphiregulin." Human reproduction **25**(10): 2569-2578.

Zamboni, L. (1970). "Ultrastructure of mammalian oocytes and ova." Biology of reproduction(Suppl. 2): 44-63.

Zanconato, F., M. Forcato, G. Battilana, L. Azzolin, E. Quaranta, B. Bodega, A. Rosato, S. Bicciato, M. Cordenonsi and S. Piccolo (2015). "Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth." Nature cell biology **17**(9): 1218.

Zeleznik, A., A. Midgley Jr and L. Reichert Jr (1974). "Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo." Endocrinology **95**(3): 818-825.

Zeng, H.-t., Z. Ren, L. Guzman, X. Wang, M. L. Sutton-McDowall, L. J. Ritter, M. De Vos, J. Smitz, J. G. Thompson and R. B. Gilchrist (2013). "Heparin and cAMP modulators interact during pre-in vitro maturation to affect mouse and human oocyte meiosis and developmental competence." Human Reproduction **28**(6): 1536-1545.

Zhang, F.-P., M. Poutanen, J. Wilbertz and I. Huhtaniemi (2001). "Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice." Molecular endocrinology **15**(1): 172-183.

Zhang, H., S. Risal, N. Gorre, K. Busayavalasa, X. Li, Y. Shen, B. Bosbach, M. Brännström and K. Liu (2014). "Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice." Current Biology **24**(21): 2501-2508.

Zhang, M., Y.-Q. Su, K. Sugiura, K. Wigglesworth, G. Xia and J. J. Eppig (2011). "Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro." Endocrinology **152**(11): 4377-4385.

Zhang, M., Y.-Q. Su, K. Sugiura, G. Xia and J. J. Eppig (2010). "Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes." Science **330**(6002): 366-369.

Zhang, M., Y. Tao, G. Xia, H. Xie, H. Hong, F. Wang and L. Lei (2005). "Atrial natriuretic peptide negatively regulates follicle-stimulating hormone-induced porcine oocyte maturation and

cumulus expansion via cGMP-dependent protein kinase pathway." Theriogenology **64**(4): 902-916.

Zhang, M., Y. Tao, B. Zhou, H. Xie, F. Wang, L. Lei, L. Huo, Q. Sun and G. Xia (2005). "Atrial natriuretic peptide inhibits the actions of FSH and forskolin in meiotic maturation of pig oocytes via different signalling pathways." Journal of molecular endocrinology **34**(2): 459-472.

Zhang, W.-C., Y.-J. Peng, G.-S. Zhang, W.-Q. He, Y.-N. Qiao, Y.-Y. Dong, Y.-Q. Gao, C. Chen, C.-H. Zhang and W. Li (2010). "Myosin light chain kinase is necessary for tonic airway smooth muscle contraction." Journal of Biological Chemistry **285**(8): 5522-5531.

Zhang, X., X. Q. Wu, S. Lu, Y. L. Guo and X. Ma (2006). "Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles." Cell research **16**(10): 841.

Zhang, Y., Z. Zhang, X. Y. Xu, X. S. Li, M. Yu, A. M. Yu, Z. H. Zong and B. Z. Yu (2008). "Protein kinase A modulates Cdc25B activity during meiotic resumption of mouse oocytes." Developmental dynamics: an official publication of the American Association of Anatomists **237**(12): 3777-3786.

Zhao, B., L. Li, Q. Lu, L. H. Wang, C.-Y. Liu, Q. Lei and K.-L. Guan (2011). "Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein." Genes & development **25**(1): 51-63.

Zhao, B., L. Li, K. Tumaneng, C.-Y. Wang and K.-L. Guan (2010). "A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCFβ-TRCP." Genes & development **24**(1): 72-85.

Zhao, B., X. Wei, W. Li, R. S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu and L. Li (2007). "Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control." Genes & development **21**(21): 2747-2761.

Zhao, B., X. Ye, J. Yu, L. Li, W. Li, S. Li, J. Yu, J. D. Lin, C.-Y. Wang and A. M. Chinnaiyan (2008). "TEAD mediates YAP-dependent gene induction and growth control." Genes & development **22**(14): 000-000.

Zhou, D., C. Conrad, F. Xia, J.-S. Park, B. Payer, Y. Yin, G. Y. Lauwers, W. Thasler, J. T. Lee and J. Avruch (2009). "Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene." Cancer cell **16**(5): 425-438.

Zhou, D., Y. Zhang, H. Wu, E. Barry, Y. Yin, E. Lawrence, D. Dawson, J. E. Willis, S. D. Markowitz and F. D. Camargo (2011). "Mst1 and Mst2 protein kinases restrain intestinal stem cell proliferation and colonic tumorigenesis by inhibition of Yes-associated protein (Yap) overabundance." Proceedings of the National Academy of Sciences: 201110428.

Zuccotti, M., P. Giorgi Rossi, A. Martinez, S. Garagna, A. Forabosco and C. Redi (1998). "Meiotic and developmental competence of mouse antral oocytes." Biology of reproduction **58**(3): 700-704.

Zuelke, K. A. and B. Brackett (1992). "Effects of luteinizing hormone on glucose metabolism in cumulus-enclosed bovine oocytes matured in vitro." Endocrinology **131**(6): 2690-2696.

Zwick, E., P. O. Hackel, N. Prenzel and A. Ullrich (1999). "The EGF receptor as central transducer of heterologous signalling systems." Trends in pharmacological sciences **20**(10): 408-412.