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Élucidation du rôle de la voie Hippo dans l'ovaire chez la souris

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Résumé

La voie de signalisation Hippo est une voie conservée entre espèces avec des rôles bien établis dans le développement embryonnaire, l'homéostasie tissulaire et le cancer. La voie ne possède ni ligand ni récepteur spécifique, mais semble être régulée par une variété de signaux extracellulaires et intracellulaires qui diffèrent selon le type cellulaire. L'activation de la voie Hippo débute avec la phosphorylation de MST1/2 qui phosphoryle et lie la protéine adaptatrice SAV1. Ensemble, ils phosphorylent et activent LATS1/2 et la protéine adaptatrice MOB1. Ce complexe phosphoryle et inactive les effecteurs principaux de la voie, c'est-à-dire les co-activateurs transcriptionnels YAP et TAZ. Inactivation de la voie permet à YAP et TAZ de se déplacer vers le noyau et de se lier à des facteurs de transcription, notamment ceux de la famille TEAD, afin de moduler la transcription de gènes cibles impliqués dans la prolifération cellulaire et l'inhibition de l'apoptose. De plus en plus de publications suggèrent l'implication de la voie Hippo dans l'ovaire postnatal, cependant, les facteurs qui régulent la voie et les rôles spécifiques de ses effecteurs demeurent inconnus. L'objectif global des deux études présentées dans cette thèse était d'élucider la régulation et les rôles de LATS1, LATS2, YAP et TAZ spécifiquement dans les cellules de la granulosa. Les résultats de la première étude ont démontré que l'hormone lutéinisante (LH) induit la phosphorylation de LATS1 et YAP et que cette dernière s'effectue par l'action de la protéine kinase A (PKA). De plus, nous avons identifié qu'en absence de *Yap/Taz*, la LH est incapable d'induire la transcription de ses gènes cibles et que ceci serait dû, au moins en partie, à la perte de l'expression du gène codant pour le récepteur à la LH (*Lhcgr*). Dans notre deuxième étude, la génération et les analyses de souris *Lats1*$^{flox/flox}$*;* *Lats2*$^{flox/flox}$*;CYP19*-cre ont révélé que LATS1/2 sont essentiels pour le maintien du destin des cellules de la granulosa. En effet, en absence de *Lats1/2*, celles-ci perdent leur identité et leur fonction, subissent une transition épithéliale-mésenchymale (EMT) et se transdifférencient en cellules de Sertoli, en ostéoblastes ainsi qu'en cellules dérivées de la crête neurale. Nous soupçonnons que ces processus cellulaires surviennent à cause d'une activité transcriptionnelle aberrante induite par une accumulation de YAP/TAZ. Ces deux études dévoilent de nouveaux rôles pour les effecteurs de la voie Hippo dans la cascade de signalisation de la LH et dans le maintien de la gonade femelle, en plus d'établir une solide base de connaissances sur laquelle les études subséquentes visant l'élucidation des mécanismes en cause pourront s'appuyer.

Mots clés : la voie Hippo, *Lats1, Lats2, Yap, Taz*, l'ovaire, les cellules de la granulosa, les souris transgéniques

Abstract

The Hippo signaling pathway is an evolutionarily conserved pathway with well-defined roles in embryonic development, tissue homeostasis, and cancer. The Hippo pathway has no specific ligands or receptors but is regulated by a variety of extracellular and intracellular cues that vary depending on the cell type. Activation of the Hippo pathway begins with phosphorylation of MST1/2 that phosphorylate and bind to an adaptor protein SAV1. Together, they phosphorylate and activate LATS1/2 and its adaptor protein MOB1. This complex then phosphorylates and inactivates the key downstream effectors of the pathway, the transcriptional co-activators YAP and TAZ. Disruption of Hippo signaling allows YAP and TAZ to translocate to the nucleus to bind notably to members of the TEAD family of transcription factors to mediate the transcription of target genes that promote cell proliferation and inhibit apoptosis. An increasing amount of evidence in the literature suggests a role for Hippo signaling in the postnatal ovary, however, regulators of (and specific roles for) Hippo effectors remain unknown. The global objective of this thesis was therefore to characterize Hippo signaling in the murine ovary by investigating the regulation of and roles of LATS1, LATS2, YAP, and TAZ specifically in granulosa cells. Results from our first study identified that luteinizing hormone (LH) activates Hippo signaling by inducing the phosphorylation of LATS1 and YAP and that this occurs via protein kinase A (PKA). In addition, we found that LH is unable to induce the transcription of its target genes in the absence of *Yap/Taz*, and that this might be due in part to the loss of the expression of the gene encoding the LH receptor (*Lhcgr*). In our second study, generation and analyses of *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$*;CYP19*-cre mice revealed that LATS1/2 are critical mediators of granulosa cell fate maintenance and in their absence, granulosa cells lose their identity and function, undergo epithelial-to-mesenchymal transition (EMT), and transdifferentiate into Sertoli-like cells, osteoblasts, and neural crest cell derivatives. We suspect that these cell processes occur as a result of aberrant transcriptional activity induced by an overaccumulation of YAP/TAZ. This thesis presents novel and exciting findings that confirm important roles for Hippo pathway effectors in the LH signaling cascade and in the maintenance of the female gonad, as well as pave the way for future studies that will elucidate the mechanisms underlying these processes.

Key words: Hippo pathway, *Lats1, Lats2, Yap, Taz*, ovary, granulosa cells, transgenic mice

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List of abbreviations

Adamts1	A disintegrin and metalloproteinase with thrombospondin-like motifs-1
Amh	Anti-Müllerian hormone
Apc	Adenomatous polyposis coli
Areg	Amphiregulin
AS	Activation segment motif
Axin	Axis inhibition protein 1/2
Bcl-2	B cell lymphoma 2
Birc	Baculoviral inhibitor of apoptosis repeat containing
Bmp	Bone morphogenetic protein
Btc	Betacellulin
β-TrCP	β-transducin repeat-containing protein E3 ubiquitin ligase
cAMP	Cyclic adenosine monophosphate
CCN	Family of regulatory proteins including Cyr61, Ctgf, Nov
Ccnd1	Cyclin D1
Ccnd2	Cyclin D2
Cdk	Cyclin dependent kinase
Cdkn	Cyclin dependent kinase inhibitor
Cebpb	CAAT enhancer binding protein beta
cGMP	Cyclic guanosine monophosphate
CK1	Casein kinase 1
CL	Corpus luteum
COC	Cumulus cell oocyte complex
Creb	cAMP responsive element binding protein
Ctgf	Connective tissue growth factor, or Ccn2
Cyp11a1	Cytochrome P450 family 11 subfamily A member 1
Cyp17a1	Cytochrome P450 family 17 subfamily A member 1
Cyp19a1	Cytochrome P450 family 19 subfamily A member 1 or aromatase
Cyr61	Cysteine-rich 61, or Ccn1
DHEA	Dehydroepiandrosterone
Dpp	Days post-partum
Dvl	Dishevelled
E	Embryonic day
E2	Estradiol or 17-βestradiol
ECM	Extracellular matrix
Egfr	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
Ep2	Prostaglandin E receptor 2
Ereg	Epiregulin
Erk	Extracellular signal-regulated kinase
Esr1/2	Estrogen receptor 1 and 2
Fgf9	Fibroblast growth factor 9
Figla	Factor in the germline α

Foxl2	Forkhead box L2
Foxo1/3	Forkhead box protein 01
FSH	Follicle-stimulating hormone
Fshr	FSH receptor
Fshb	FSH beta subunit
Fzd	Frizzled
Gdf	Growth differentiation factor
Gja1	Gap junction protein 1, or connexin 43
Gja4	Gap junction protein 4, or connexin 37
GnRH	Gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GSK3β	Glycogen synthase kinase 3 β
Has2	Hyaluronan synthase 2
Hif1a	Hypoxia inducible factor 1 alpha subunit
HM	Hydrophobic motif
Hpg	Hypothalamic-pituitary-gonadal
Hpo	Hippo
HSD	Hydroxysteroid dehydrogenase
hTERT	Human telomerase reverse transcriptase
IaI	Inter-alpha trypsin inhibitor
ICM	Inner cell mass
Igf1	Insulin-like growth factor 1
IHC	Immunohistochemistry
Il6	Interleukin 6
Inha	Inhibin alpha subunit
Kit	Kit receptor
Kitl	Kit ligand
Lats1/2	Large tumor suppressors 1 and 2
LH	Luteinizing hormone
Lhcgr	LH/choriogonadotropin receptor
Lhb	LH beta subunit
LPA	Lysophosphatidic acid
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
MAPK	Mitogen-activated protein kinase
Mats	Mob-as-tumor-suppressor
mESCs	Mouse embryonic stem cells
Mob1	MOB kinase activator 1A and 1B
Mst1/2	Mammalian STE20-like protein kinase 1 and 2
mTORC1	Mammalian target of rapamycin complex 1
Nf2	Merlin, or neurofibromin 2
Nobox	Newborn ovary homeobox
Nov	Nephroblastoma overexpressed, or Ccn3
Nr5a2	Nuclear receptor member 5a2, or Lhr1

Nrip1	Nuclear receptor interaction protein 1
OSE	Ovarian surface epithelium
P4	Progesterone
PCR	Polymerase chain reaction
PDE3A	Phosphodiesterase
PGC	Primordial germ cell
PGE1/2	Prostaglandins E1 and E2
Pgr	Progesterone receptor
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PP1A/2A	Protein phosphatase 1A or 2A
PPARγ	Peroxisome proliferator-activated receptor gamma
Prlr	Prolactin receptor
Pten	Phosphatase and tensin homolog
Ptgs2	Prostaglandin-endoperoxide synthase 2
Ptp	Protein tyrosine phosphatase
Ptx3	Pentraxin 3
Rspo1	R-spondin-1
RT-qPCR	Quantitative reverse transcription PCR
Runx2	Runt related transcription factor 2
S1P	Sphingosine-1 phosphate
Sav	Salvador
Sav1	Salvador homologue 1
Scrib	Scribble
Sd	Scalloped
Ser	Serine
Sohlh1/2	Spermatogenesis and oogenesis basic helix-loop-helix
Sox9	SRY-Box 9
Src	Sarcoma
Sry	Sex-determining region on Y chromosome
StAR	Steroidogenic acute regulatory protein
Stra8	Retinoic acid gene 8
Taz	Transcriptional co-activator with PDZ-binding motif, or Wwtr1
Tbx5	T-box 5
Tcf/Lef	T-cell factor/lymphoid enhancer factor
TE	Trophectoderm
Tead1-4	TEA domain family members 1-4
TGF-β	Transforming growth factor beta
Thr	Threonine
Tnfaip6	TNF-α-induced protein 6
Tsc1/2	Tuberous sclerosis complex 1 or 2
Vcan	Versican
Vegf	Vascular endothelial growth factor

WB	Western blotting
Wnt4	Wingless-type MMTV integration site family, member 4
Wts	Warts
Yap	Yes-associated protein
Yki	Yorkie
Zeb1	Zinc finger E-box-binding homeobox 1

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Chapter 1. Literature review

1 Introduction

The study of ovarian follicle development and its regulation by gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) has been an active area of research over the course of several decades. It is well-known that FSH stimulates granulosa cell proliferation and estradiol production, while LH regulates ovulation and formation of the corpus luteum (CL)(Richards, 1980). FSH and LH bind to their respective receptors, FSHR and LH/choriogonadotropin receptor (LHCGR). This activates a variety of signaling cascades that lead to the transcription of FSH and LH target genes, respectively, which are ultimately responsible for mediating their effects (Richards & Pangas, 2010b). There still, however, remain important gaps in our knowledge regarding, for instance, the factors that link FSHR and LHCGR signaling pathways to the transcription of their respective target genes. This has driven the investigation of novel signaling pathways (known for their roles in other contexts) and their potential involvement in folliculogenesis.

One such pathway is the Hippo pathway, that is widely recognized as a regulator of organ size and tissue growth in embryonic development; its roles in the adult are still being uncovered (Zhao et al, 2010a). The Hippo pathway does not possess any specific ligands or receptors but instead, is regulated by a wide variety of cues (Piccolo et al, 2014). Recent studies have hinted at roles for Hippo effectors in the ovary. The generation of knockout mouse models for certain Hippo pathway effectors have revealed that in these animals, female fertility is severely affected (Hossain et al, 2007; St John et al, 1999). Other studies suggest that Hippo effectors might be involved in ovarian follicle development and ovarian cancer (Fu et al, 2014; Kawamura et al, 2013; St John et al, 1999). Very few studies, however, have investigated the specific roles of Hippo signaling effectors over the course of ovarian follicle development. No studies to date have investigated how Hippo signaling is regulated in granulosa cells.

This thesis describes two studies that addressed these questions: 1) How is Hippo signaling regulated in ovarian granulosa cells? and 2) What roles do Hippo effectors play in ovarian follicle development? using conditional knockout mouse models and primary granulosa cell cultures.

1.1 Anatomy of the mouse female reproductive tract

The female reproductive tract consists of two ovaries, two oviducts, two uterine horns, one uterine body, the cervix, the vagina, a ventral and two dorsal labia, and the vulva. The ovaries are located at the caudal pole of the kidneys (Bertolin & Murphy, 2014). Each ovary is enclosed within an ovarian bursa, that is suspended from the dorsal body wall by the mesovarium. Blood vessels and nerves pass through a small opening in the ovarian bursa that enter the ovary at the ovarian hilus (Rendi et al, 2012).

Each oviduct provides a passageway for the cumulus oocyte complexes (COCs) to travel from the ovary at the time of ovulation to the ipsilateral uterine horn (Evans & DeLahunta, 2004). The oviducts are narrow, coiled tubes, approximately 1.8cm long composed of three anatomic portions: the infundibulum, the ampulla, and the isthmus (Rendi et al, 2012). The infundibulum is the funnel-shaped, fimbriated end of the oviduct that catches the COCs at ovulation. The ampulla is the middle segment where fertilization occurs. The isthmus is the posterior end of the oviduct that opens into the uterine horn (Evans & DeLahunta, 2004). The oviducts are suspended from the dorsal body wall by the mesotubarium (that is continuous with the mesovarium, ovarian bursa, and mesometrium)(Rendi et al, 2012).

The uterus is a tubular organ composed of two horns that join distally to form one body (FIGURE 1.1)(Pasquini et al, 2007). The uterus is suspended by the mesometrium from the dorsal body wall. The uterine body opens up into the cervix, which is continuous with the vagina. The vestibule is common to both urinary and genital systems that connects the vagina to the vulva, which is the external genital orifice (Pasquini et al, 2007).

The ovaries are vascularized by ovarian arteries and veins. The ovarian arteries arise from the aorta distally from the renal arteries (Evans & DeLahunta, 2004). The ovarian veins leave the ovary and enter the caudal vena cava. The uterus is vascularized by the uterine branch of the ovarian artery and the uterine artery (Bertolin & Murphy, 2014; Evans & DeLahunta, 2004).

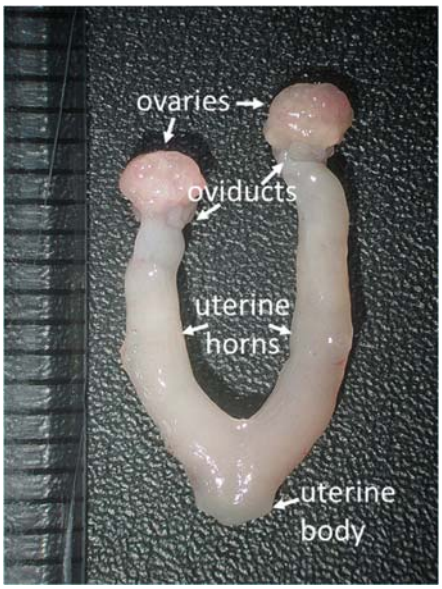


FIGURE 1.1 Anatomy of the mouse female reproductive tract

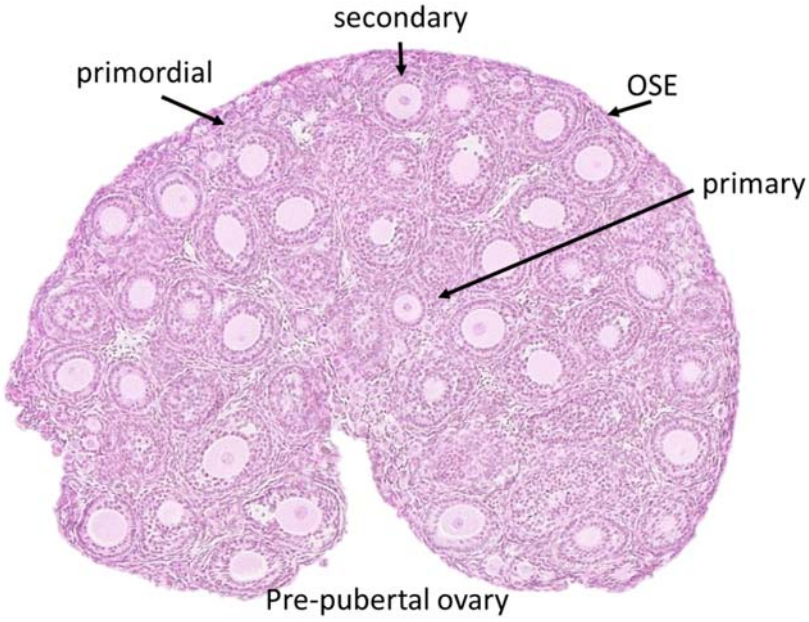
1.2 Histology of the mouse ovary

The ovarian surface epithelium (OSE) is a simple cuboidal to columnar epithelium that covers the surface of the ovary (Rendi et al, 2012). A dense connective tissue located right below the OSE is the tunica albuginea. Once primordial follicles begin to form directly beneath the OSE, the ovary becomes organized into two morphologically distinct compartments, the outer cortex and the inner medulla (Rendi et al, 2012; Wilhelm et al, 2007). The cortex contains the ovarian follicles at all stages of development, corpora lutea (CLs), and connective tissue. As follicles develop, they migrate towards the medulla (Sforza et al, 2003). The medulla contains blood vessels, nerves, lymphatics, smooth muscle fibers, and connective tissue fibers (Pasquini et al, 2007).

An ovarian follicle is made up of a central germ cell (oocyte) surrounded by varying layers of somatic cells (granulosa and theca) depending on the stage of follicle development. A primordial follicle contains a small oocyte surrounded by a layer of squamous pre-granulosa cells (Rendi et al, 2012). Once the primordial follicle is activated, the pre-granulosa cells differentiate into cuboidal granulosa cells to form primary follicles. The oocyte secretes a matrix of glycosylated zona proteins that directly surrounds itself, called the zona pellucida (Rankin et al, 2000; Richards et al, 2015a). A secondary follicle contains two or more layers of granulosa

cells that are surrounded by a layer of theca cells, the two separated by a basal lamina. The follicle (and oocyte) grow considerably in size as granulosa and theca cells proliferate. Dispersed areas of interstitial fluid develop between granulosa cells and form the antrum, marking the antral stage of follicle development. The theca cell layer differentiates into the steroidogenic theca interna and the theca externa (Pangas & Rajkovic, 2015; Richards et al, 2015a). Adjacent to the theca externa lies a second basal lamina (Richards et al, 2015a).

A large proportion of small antral follicles are not selected to continue maturation and undergo atresia (Richards, 1980). The preovulatory follicle is characterized by a large antrum and granulosa cells that have differentiated into two distinct populations: the cumulus cells surround the oocyte forming the COC while the mural granulosa cells line the basement membrane of the follicle. At ovulation, the follicle wall breaks down and releases the COC. After ovulation, the remaining follicular cells hypertrophy, differentiate into luteal cells, and become vascularized, forming the corpus luteum (CL). An ovary from a pre-pubertal mouse (whose first ovulations occur around 30 days post-partum (30 dpp)) contains primordial, primary, and secondary follicles (Bertolin & Murphy, 2014). An ovary from an adult mouse contains follicles of all stages, with CLs in different stages of regression (FIGURE 1.2).



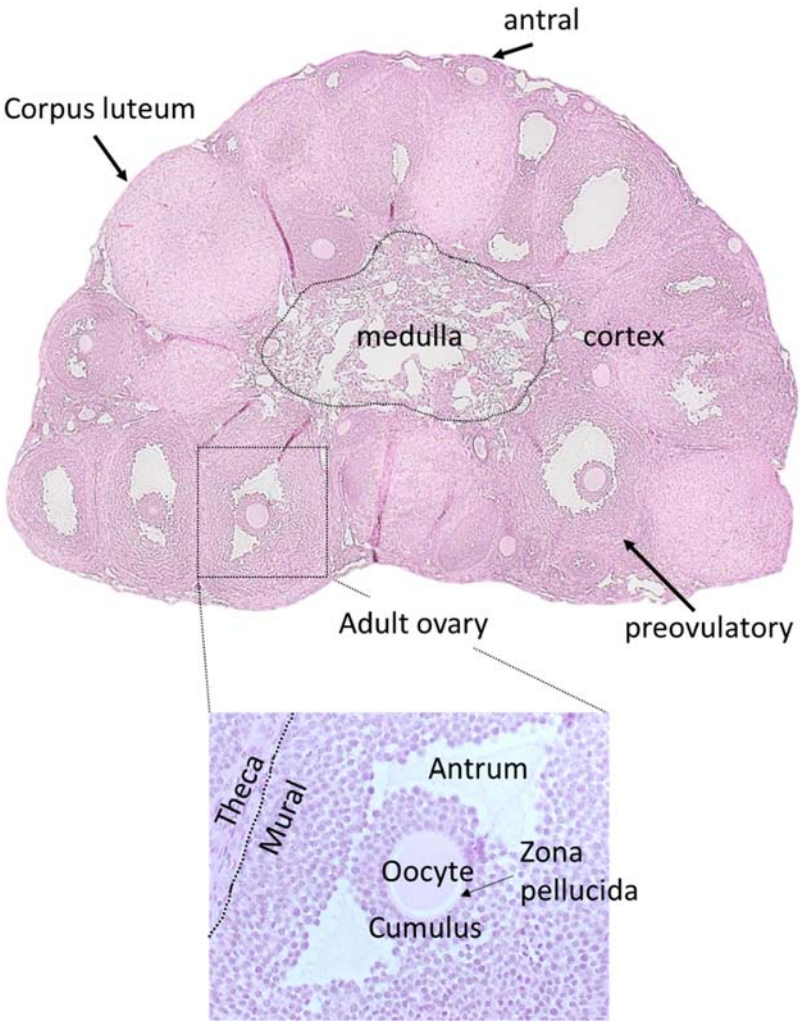


FIGURE 1.2 Histology of ovaries from pre-pubertal and adult mice

1.3 Embryology of the mouse ovary

1.3.1 Female sex determination

As in other mammals, sex determination in the mouse begins in bipotential precursor cells within the fetal gonad (Wilhelm et al, 2007). Expression of the Y-linked gene sex-determining region on Y chromosome (*Sry*) around embryonic day 10.5 (e10.5) leads to activation of its target gene *Sox9,* which initiates the differentiation of bipotential precursor cells into Sertoli cells and thus, the differentiation of the bipotential gonad into a testis (Gubbay et al,

1990; Koopman et al, 1991; Vidal et al, 2001). Overexpression of *Sox9* or *Sry* in XX mice is sufficient to drive male sexual development (Koopman et al, 1991; Vidal et al, 2001).

In the absence of *Sry* or *Sox9*, the bipotential precursor cells differentiate into granulosa cells (Wilhelm et al, 2007). While no single ovarian-determining factor has been identified in the female gonad, evidence suggests that ovarian development is orchestrated by multiple genes (Wilhelm et al, 2007). Primary female sex-determining factors were identified using knockout mouse models in which loss-of-function of these genes led to partial or complete sex reversal. These genes are primarily components of the Wnt/β-catenin signaling pathway, including *Wnt4*, *Rspo1*, β-catenin, in addition to *Foxl2* (Richards & Pangas, 2010b). At e11.0, *Wnt4* is expressed in the indifferent gonad, is downregulated around e11.5 in the male gonad and upregulated in the female gonad (Vainio et al, 1999; Wilhelm et al, 2007). *Wnt4* knockout XX mice develop testicular-like structures (Vainio et al, 1999). At e11.5, *Foxl2* is activated exclusively in the female gonad with the highest expression occurring in pre-granulosa cells (Ottolenghi et al, 2005; Ottolenghi et al, 2007). *Foxl2* knockout XX mice lack ovarian follicles while still containing oocytes, and develop testicular cord-like structures (Ottolenghi et al, 2005). Ablation of *Rspo1* (which stabilizes β-catenin) from XX mice leads to partial gonadal sex reversal and oocyte loss (Chassot et al, 2008; Tomizuka et al, 2008). Conditional expression of stable β-catenin in XY mice induces partial sex reversal, with testes containing ovarian-like structures devoid of germ cells (Maatouk et al, 2008). Interestingly, double *Wnt4/Foxl2* knockout XX mice undergo complete sex reversal from an ovary to a functional testis containing tubules and spermatogonia, suggesting that separate mechanisms regulate somatic cell vs germ cell specification in the ovary (Ottolenghi et al, 2007). While the expression of female-specific markers has been identified (starting around e11.5), the precise moment at which follicle differentiation occurs is unclear.

During this critical period, a battle between the expression of male vs female-specific genes determines the fate of the bipotential gonad. SOX9 antagonizes β-catenin to prevent ovarian development in males (Chassot et al, 2008). β-catenin and FOXL2 antagonize SOX9 to prevent testis development in females (Matzuk & Burns, 2012). WNT4 and FGF9 (a male-specific gene that enhances *Sox9* expression) antagonize one another (FIGURE 1.3)(Kim et al, 2006). Whether there is a hierarchy in terms of dominance of one factor over another (for

example if one male-specific gene and one female-specific gene are simultaneously expressed, will the gonad develop as a male or female?) has yet to be determined.

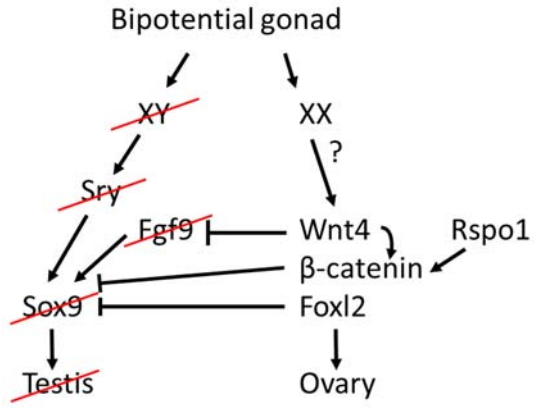


FIGURE 1.3 Female sex determination. *Inspired by Schlessinger et al 2010.*

1.3.2 Female gonadal development

During embryonic development, the bipotential gonads are derived from the intermediate mesoderm and develop next to the urogenital ridge (Richards & Pangas, 2010a; Richards & Pangas, 2010b; Wilhelm et al, 2007). The urogenital ridge is made up of three sections that include the pronephros (that will give rise to the adrenals), the mesonephros (the future gonads and genital ducts), and the metanephros (the future kidney)(Wilhelm et al, 2007). More specifically, the gonads develop on the surface of the mesonephros around e9.5-10.5 as paired thickenings of the epithelium in conjunction with cells from the mesonephros and the coelomic epithelium, which give rise to the somatic precursor cells (Bertolin & Murphy, 2014; Tanaka & Nishinakamura, 2014; Wilhelm et al, 2007).

Primordial germ cells (PGCs) are specified from epiblast cells (that arise from the inner cell mass) around e6.0-8.0, they proliferate and migrate from the region of the hindgut to colonize the genital ridge around e9.5-11.5 (Richards & Pangas, 2010b; Wilhelm et al, 2007). Colonization of the bipotential gonad by PGCs is followed by the differentiation of somatic precursor cells into granulosa cells (Richards & Pangas, 2010b; Tanaka & Nishinakamura, 2014; Wilhelm et al, 2007). Between e10.5-13.5, PGCs undergo mitosis without complete cytokinesis, which forms syncytia of germ cells, called germ cell cysts, that are connected by cytoplasmic bridges (Pepling & Spradling, 2001). In females, meiosis is induced in PGCs by

retinoic acid induction of retinoic acid gene 8 (*Stra8*)(Koubova et al, 2006). PGCs enter meiosis around e13.5, arrest at the diplotene stage of meiotic prophase I at approximately e17.5, and remain arrested until the LH surge induces the resumption of meiosis in pubertal mice (FIGURE 1.4)(Bertolin & Murphy, 2014; Pepling & Spradling, 2001; Richards & Pangas, 2010b).

Members of the transforming growth factor beta (TGF-β) superfamily appear to be the major regulators of PGC proliferation and survival. Bone morphogenetic protein (BMP) -4 and -7 promote PGC proliferation, in contrast to activin and TGF-β that inhibit proliferation, while follistatin (an activin inhibitor) is required for PGC survival (Pesce et al, 2002; Richards et al, 1999; Richards & Pangas, 2010b; Ross et al, 2007; Yao et al, 2004).

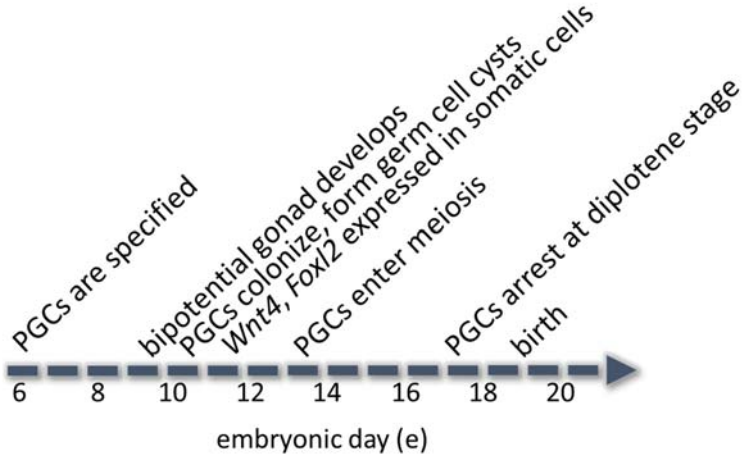


FIGURE 1.4 Timeline of female gonadal development

1.4 Physiology of ovarian follicle development

1.4.1 The regulation of ovarian follicle development by the hypothalamic-pituitary-gonadal (HPG) axis

Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and acts on the anterior pituitary to stimulate the secretion of FSH and LH from gonadotrope cells. FSH and LH are dimeric glycoproteins composed of a common α subunit and a unique β subunit (Pangas & Rajkovic, 2015). Once released in the bloodstream, FSH and LH reach the ovary to stimulate the production of steroid hormones depending on the phase of the estrous cycle. The secretion

of FSH and LH is controlled (in part) by ovarian-derived hormones (steroidal and non-steroidal) that exert positive and negative feedback mechanisms on the hypothalamus and pituitary (Messinis, 2006).

In mice, the estrous cycle lasts 4-5 days and is divided into phases: proestrus (lasting approximately 32.4h) and estrus (20.7h), metestrus (21.8h), and diestrus (21.8h)(Bertolin & Murphy, 2014; Van Ebbenhorst Tengbergen, 1955). During proestrus, FSH and LH stimulate antral follicles to produce increasing levels of estradiol (E2), which exerts a positive feedback on the hypothalamus and pituitary to induce the preovulatory LH surge (Richards, 1980; Richards, 1994). During estrus, ovulation occurs 12-16h after the LH surge (Richards, 2005). Lower estradiol levels during the other phases of the cycle, exert a negative feedback at the level of the hypothalamus and the pituitary to suppress gonadotropin secretion (Herbison, 2015). During metestrus, the CL produces increasing amounts of progesterone (P4), which inhibits LH secretion. During diestrus (in the absence of mating), the CL regresses and the levels of P4 decrease, which releases LH inhibition, allowing GnRH secretion and proestrus to continue (Bertolin & Murphy, 2014).

Granulosa cells produce activins, inhibins (A and B isoforms), and follistatins that regulate pituitary function. As their names suggest, activin stimulates FSH secretion while inhibin inhibits FSH by antagonizing activin (McArdle & Roberson, 2015). Follistatin binds and inhibits activin activity; it is expressed starting in antral stage follicles (Pangas & Rajkovic, 2015). Inhibin B appears to be produced by small follicles and its levels are inversely correlated with FSH levels during the estrous cycle, while inhibin A levels correlate with estradiol production (FIGURE 1.5)(Pangas & Rajkovic, 2015).

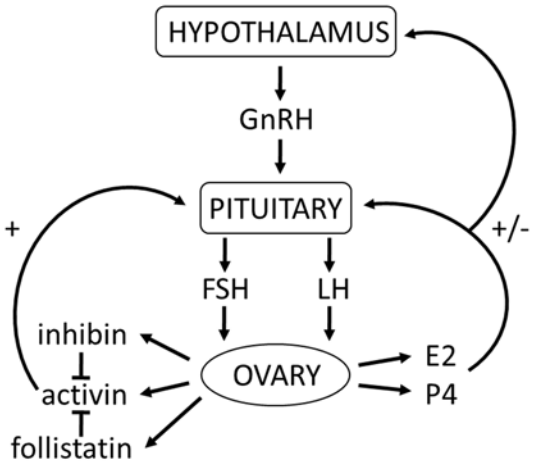


FIGURE 1.5 Hypothalamic-pituitary-gonadal axis

1.4.2 Gonadotropin signaling in the ovary

FSH and LH bind their respective receptors, the FSHR and LHCGR, which are $G_{\alpha s}$ protein-coupled receptors located on plasma membranes of their target cells (Pangas & Rajkovic, 2015). FSHR is expressed exclusively on granulosa cells (starting in preantral stage follicles), while LHCGR is located on theca cells (starting in secondary stage follicles), mural granulosa cells of large preovulatory follicles, and luteal cells (Hunzicker-Dunn & Mayo, 2015; Richards, 1980; Richards & Midgley, 1976). Gonadotropin binding to their receptors initiates an intracellular signaling cascade leading to the transcription of FSH and LH-specific target genes. Why FSHR expression is limited to granulosa cells in the female remains unknown.

1.4.2.1 FSH signaling

FSH regulates the expression of around 500 target genes that mediate granulosa cell proliferation, survival, estradiol synthesis, and differentiation (Hunzicker-Dunn & Mayo, 2015; Richards & Pangas, 2010b). FSH exerts its functions primarily through the cAMP/PKA pathway. FSH binds to FSHR to activate adenylyl cyclase, which converts ATP to cAMP (Simoni et al, 1997). Cyclic AMP accumulates, binds to, and activates PKA (Taylor, 1989). PKA phosphorylates most notably CREB(Ser133), which has binding sites on the promoters of some FSH target genes, including inhibin alpha (*Inha*) and *Cyp19a1* (Carlone & Richards, 1997; Pei et al, 1991). PKA also rapidly phosphorylates histone H3, which is suspected to promote the

transcription of FSH target genes (by facilitating the access of transcription factors to loosened chromatin)(Hunzicker-Dunn & Mayo, 2015)

FSH signals via cAMP/PKA to activate ERK. More specifically in preantral granulosa cells, PKA stimulates the phosphorylation/inactivation of protein tyrosine phosphatase (PTP) to dissociate it from ERK, which allows ERK to be activated by RAS/RAF/MEK (Cottom et al, 2003). ERK phosphorylates or promotes the phosphorylation of transcription factors and co-activators involved in FSH target gene expression, including *Ccnd2* (Hunzicker-Dunn & Mayo, 2015; Kayampilly & Menon, 2004). The mechanism responsible for regulating RAS/RAF/MEK (upstream of PKA) in granulosa cells is unclear, but appears to involve calcium entry into the cell (Hunzicker-Dunn & Mayo, 2015)

FSH signals via cAMP/PKA to activate PI3K in immature granulosa cells, which activates AKT (FIGURE 1.6)(Gonzalez-Robayna et al, 2000). Important AKT targets include HIF1α, FOXO1 and FOXO3, and GSK3β. HIF1α is a transcription factor required by FSH to induce the transcription of *Lhcgr*, *Vegf*, and *Inha*. FOXO1 and FOXO3 are transcription factors that are negatively regulated by AKT; phosphorylation by AKT targets them for degradation. In their active state (in the absence of PI3K/AKT signaling), they promote cell cycle arrest, and repress genes involved in steroidogenesis and *Lhcgr* expression. Finally, the protein kinase GSK3β is also inactivated by AKT; in its active state, GSK3β regulates glucose production, cell survival, and cell motility (Alam et al, 2004; Cunningham et al, 2003; Diehl et al, 1998; Hunzicker-Dunn & Mayo, 2015). The regulation of PI3K/AKT signaling (upstream of PKA) in granulosa cells is still under investigation, but seems to involve insulin-like growth factor 1 (IGF1) activation of its receptor (Hunzicker-Dunn & Mayo, 2015).

FSH is also able to activate signaling cascades independently from cAMP/PKA in granulosa cells, including PI3K, RAS, and GSK3β (Gonzalez-Robayna et al, 2000; Richards & Pangas, 2010b; Wayne et al, 2007). Their importance relative to cAMP/PKA signaling for the induction of FSH target genes is unclear.

Many questions still remain unanswered regarding FSH signaling in granulosa cells. For instance, is PKA is responsible for mediating the majority of FSH target genes and if so, by interacting with which signaling pathways, and how? While many transcription factors and regulators responsible for regulating the transcription of several FSH target genes have been identified, the majority still remain unknown (Hunzicker-Dunn & Mayo, 2015).

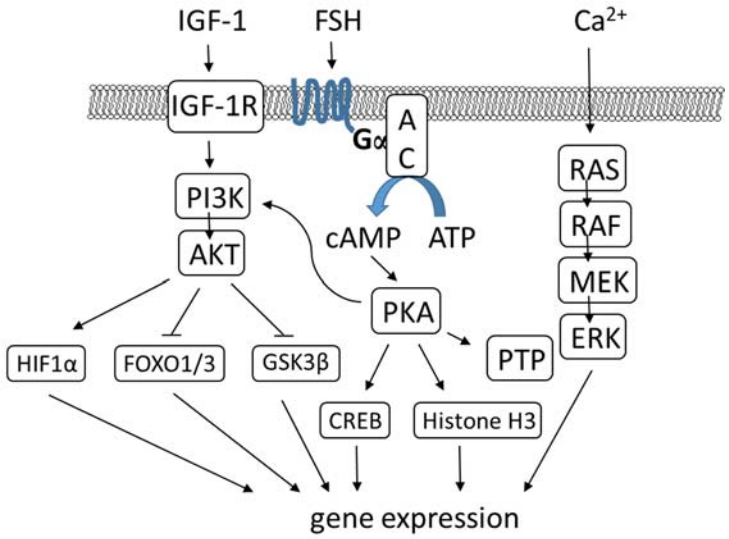


FIGURE 1.6 FSH signaling in granulosa cells. *Inspired by Hunzicker-Dunn 2015.*

1.4.2.2 LH signaling

LH regulates the expression of more than 500 target genes that mediate oocyte maturation, cumulus expansion, ovulation, and luteinization in preovulatory follicles (Richards & Pangas, 2010b). LH signals via cAMP/PKA to induce the expression of epidermal growth factor receptor (EGFR) ligands amphiregulin (*Areg*), betacellulin (*Btc)*, and epiregulin (*Ereg*) in mural granulosa cells (Conti et al, 2006). These ligands are first synthesized as transmembrane precursors, are cleaved by a metalloproteinase, and then shed as mature proteins (Hsieh & Conti, 2005). They bind EGFRs on mural granulosa and cumulus cells, which activates RAS and ERK1/2 signaling (Fan et al, 2008b). ERK1/2 turns off the FSH genetic program (including *Inha, Cyp19a1, Lhcgr*)(Fan et al, 2009a). ERK1/2 also activates transcription factors CAAT enhancer binding protein beta (*Cebpb*), nuclear receptor member 5a2 (*Nr5a2*), and the progesterone receptor (*Pgr*), which are required for the transcription of specific target genes involved in oocyte maturation, cumulus expansion, ovulation, and luteinization (FIGURE 1.7)(Duggavathi et al, 2008; Fan et al, 2009b). One critical CEBPβ target is *Ptgs2*, which is responsible for prostaglandin E2 (PGE2) synthesis. In a positive feedback loop, *Areg* induces the expression of *Ptgs2*, which promotes PGE2 synthesis, allowing it to bind to its receptor on cumulus cells to promote increased *Areg* expression (Shimada et al, 2006).

Briefly, cumulus expansion and oocyte maturation involves the induction of *Has2, Ptx3,* and *Tnfaip6* in cumulus granulosa cells (Ochsner et al, 2003a). Ovulation requires induction of *Ptgs2* and *Pgr* in cumulus and mural granulosa cells (Lim et al, 1997; Lydon et al, 1995). Luteinization (that converts mural granulosa cells and theca cells into luteal cells) results in the expression of *Cyp11a1,* Steroidogenic acute regulatory protein (*Star*), and the downregulation of FSH target genes that promote proliferation (Espey & Richards, 2002; Goldring et al, 1987; Hunzicker-Dunn & Mayo, 2015). Disruption of EGFR or ERK1/2 signaling is sufficient to block oocyte maturation, cumulus expansion, ovulation, and luteinization (Fan et al, 2009b; Hsieh et al, 2011), illustrating the fact that these two signaling pathways are upstream regulators essential for mediating all of the events triggered by the LH surge.

Although LH primarily signals via cAMP, LH also exerts its effects in a PKA-independent manner to activate RAS, p38 MAPK (MAPK14), and PI3K/AKT signaling. LH signals via a SRC tyrosine kinase to activate RAS, which also leads to the activation of MEK1 and ERK1/2 (Wayne et al, 2007). LH signaling induces a rapid phosphorylation of p38 MAPK (MAPK14) in preovulatory granulosa cells that is involved in COC expansion (Sela-Abramovich et al, 2005). LH has been found to synergize with IGF1 to activate PI3K/AKT signaling, however, the mechanism by which this occurs remains unknown (Vanhaesebroeck et al, 2010). As with FSH signaling, the relative importance of PKA-independent signaling in comparison to PKA-dependent signaling in the transcription of LH target genes remains to be determined.

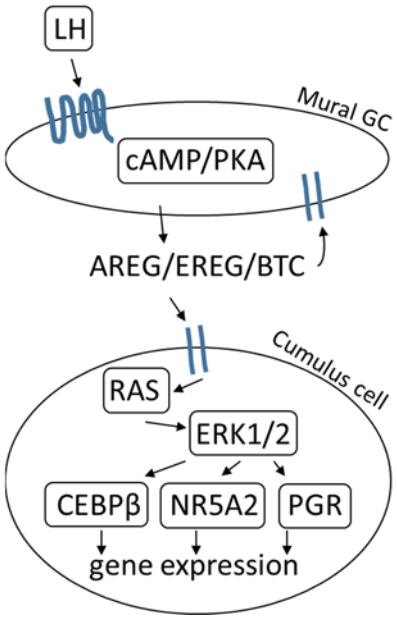


FIGURE 1.7 LH signaling in the preovulatory follicle. *Inspired by Conti et al 2006.*

1.4.3 Follicle development

1.4.3.1 Crosstalk between germ cells and gonadal somatic cells during follicle development

Communication between the oocyte and granulosa cells (cumulus cells and mural granulosa cells) throughout all stages of follicle development ensures that oogenesis and folliculogenesis are coordinated, leading to the release of a fertilizable oocyte. This is especially critical during the early stages of follicle development when intra-ovarian factors drive follicle growth (Pangas & Rajkovic, 2015). Communication is primarily achieved via gap junctions and paracrine signaling. Gap junctions are composed of connexin proteins that form membrane channels between cells which allow passage of ions and small molecules. Gap junction protein 1 (GJA1, or connexin 43) forms gap junctions between granulosa cells, while GJA4 (connexin 37) forms gap junctions between the oocyte and granulosa cells (Ackert et al, 2001). Paracrine signaling is when one cell type produces a signal (ex. growth factor) that binds receptors on a neighboring cell type to elicit a response (Russell et al, 2016). One classic example of this involves the expression of KIT ligand (KITL) by granulosa cells that act on the tyrosine kinase

receptor KIT localized on the plasma membrane of the oocyte. Binding of KITL to KIT activates PI3K signaling within the oocyte to stimulate oocyte growth (Thomas & Vanderhyden, 2006).

1.4.3.2 Primordial follicle formation

Primordial follicle formation begins with the breakdown of the cytoplasmic bridges that make up the germ cell cysts between e17.5 and 4dpp, and is accompanied by significant germ cell loss (with approximately only one third of oocytes surviving past this stage)(Pangas & Rajkovic, 2015; Pepling & Spradling, 2001; Richards & Pangas, 2010a). Squamous pre-granulosa cells migrate and surround the oocytes to form primordial follicles, that become enclosed within a basement membrane (Pepling & Spradling, 2001; Rajah et al, 1992; Tingen et al, 2009; Wilhelm et al, 2007). This pool of dormant primordial follicles provides the source of germ cells available for recruitment during the reproductive lifespan of the female, known as the ovarian reserve (Pangas & Rajkovic, 2015; Richards, 1980; Richards & Pangas, 2010a). The mechanisms responsible for germ cell cyst breakdown and primordial follicle formation are incompletely understood, but appear to be regulated, at least in part, by members of the TGF-β family, oocyte-specific transcription factors, and steroid hormone signaling.

The proper formation of a primordial follicle requires complete germ cell cyst breakdown so that pre-granulosa cells can enclose a single oocyte. Numerous factors regulate this process, as evidenced by the mouse models that exhibit multi-oocyte follicles (which is the result of incomplete germ cell cyst breakdown), including granulosa cell-specific conditional knockouts of activin, *Bmp15* knockouts in oocytes, and overexpression of *Inha* in granulosa cells (Jorgez et al, 2004; McMullen et al, 2001; Pangas et al, 2007; Yan et al, 2001).

Primordial follicle formation requires a healthy oocyte, which depends on the expression of oocyte-specific transcription factors. Ablation of factor in the germline alpha (*Figla*) results in significant oocyte loss and a decrease in the formation of primordial follicles (Soyal et al, 2000). In addition, ablation of *Sohlh1, Sohlh2*, or *Nobox* also causes increased oocyte loss and disrupts the formation of primordial and primary follicles (FIGURE 1.8)(Choi et al, 2008; Pangas et al, 2006a; Rajkovic et al, 2004).

Appropriate levels of hormone signaling is also crucial. For instance, treatment of newborn mouse ovaries with estrogens, estrogen-mimetics, or progesterone inhibits the

formation of primordial follicles (Chen et al, 2007; Tingen et al, 2009). One hypothesis that has been advanced is that elevated levels of estrogens and progesterone during pregnancy maintain germ cell cysts, but at birth, the drop in circulating hormones triggers germ cell cyst breakdown (Chen et al, 2007). Any pathologies therefore that cause aberrant elevated levels of steroid hormones can prevent primordial follicle formation.

1.4.3.3 Primary follicle development

Primordial follicles have three possible fates: to remain dormant, to be activated to join the growing follicle pool, or to directly undergo atresia (McGee & Hsueh, 2000; Reddy et al, 2010). Cohorts of primordial follicles (approximately 3-6) become activated daily to develop to the primary follicle stage (Fortune, 2003; Richards & Pangas, 2010a). Primary follicles are detectable by 3 dpp and consist of a growing oocyte surrounded by proliferating cuboidal granulosa cells (Bertolin & Murphy, 2014; Pangas & Rajkovic, 2015; Richards, 1980).

Activation of primordial follicles appears to be regulated by mTORC1 and PI3K/AKT signaling in granulosa cells (AKT acts upstream of mTORC1 by repressing its inhibitor, TSC1/2)(Reddy et al, 2010). Inhibition of mTORC1 signaling in squamous pre-granulosa cells prevented their differentiation into cuboidal granulosa cells and maintained oocytes in a quiescent state, whereas overactivation of mTORC1 signaling accelerated granulosa cell differentiation and prematurely activated primordial follicles (Zhang et al, 2014a).

Continuous inhibitory signals are required in order to maintain primordial follicles in a dormant state and therefore preserve the ovarian reserve. Oocyte-specific ablation of *Foxo3a* (negatively regulated by AKT), *Pten* (a PI3K inhibitor), or *Tsc1/2* (an mTORC1 inhibitor) caused widespread premature activation of primordial follicles and consequently depletion of the primordial follicle reserve (Adhikari et al, 2009; Castrillon et al, 2003; Reddy et al, 2008). How PTEN/PI3K/AKT signaling is regulated in this context remains unknown.

Inhibitory signals also originate from granulosa cells. Total knockout of *Foxl2* impaired squamous pre-granulosa cell differentiation into cuboidal granulosa cells, with oocytes undergoing premature growth, leading to follicle depletion and oocyte death (Schmidt et al, 2004; Uda et al, 2004). Ablation of anti-Müllerian hormone (*Amh*) resulted in overactivation of primordial follicles and premature primordial follicle depletion (FIGURE 1.8)(Durlinger et al,

1999). Despite what we do know, the regulation of the dormant primordial follicle pool remains one of the least understood mechanisms in ovarian physiology.

1.4.3.4 Secondary follicle development

By 7 dpp, secondary follicles can be detected within the ovary, which consist of a growing oocyte surrounded by more than a single layer of proliferating granulosa cells and a second somatic cell layer consisting of theca cells. The regulation of theca cell differentiation is poorly understood, but appears to involve the differentiation of interstitial cells mediated by granulosa cell-derived signals (Gardiner & Swain, 2015). Follicle development is still dependent at this stage on intra-ovarian factors; the oocyte continues to supply cumulus cells with growth factors, while the latter contribute to maintaining meiotic arrest in the oocyte (Monniaux, 2016).

Loss-of-function of either gap junction proteins or growth factor signaling impede follicle growth beyond the primary stage. Ablation of *Gja1* from granulosa cells caused follicle arrest at the primary follicle stage (Ackert et al, 2001), in contrast to ablation of *Gja4* from oocytes caused arrest at the preantral follicle stage (Simon et al, 1997). Loss-of-function of KIT caused follicle arrest at the primary follicle stage (John et al, 2009). Ablation of growth differentiation factor 9 (*Gdf9*) resulted in follicle arrest at the primary follicle stage, in which large oocytes were surrounded by a single layer of cuboidal granulosa cells (FIGURE 1.8)(Dong et al, 1996). These conditional knockout models illustrate, without any doubt, how communication between the oocyte and granulosa cells is essential in order for normal follicle development to proceed.

1.4.3.5 Antral follicle development

Antral follicles become apparent around 13 dpp, wherein the oocyte has completed its growth and becomes suspended in the antrum surrounded by cumulus granulosa cells (Bertolin & Murphy, 2014; Conti et al, 2006; Pangas & Rajkovic, 2015; Richards, 1980). At the antral stage, follicles are now dependent on gonadotropins, FSH and LH, for their continued development (Pangas & Rajkovic, 2015; Richards & Pangas, 2010a). A small pool of growing follicles is capable of responding to FSH and LH to reach the preovulatory stage around 21 dpp

(Pepling & Spradling, 2001). LH stimulates theca cells to produce androgens and FSH stimulates granulosa cells to aromatize androgens into estradiol, which then exerts a positive feedback on the HPG axis to trigger the LH surge (Richards, 1980; Richards, 1994). Estradiol acts in synergy with FSH to stimulate granulosa cell proliferation (by induction of *Ccnd2* and stimulation of IGF1 synthesis) and granulosa cell differentiation (by induction of *Lhcgr,* increased CYP19A1 activity, and increased inhibin production)(Pangas & Rajkovic, 2015). In the absence of FSH stimulation, widespread apoptosis of granulosa cells in early antral follicles occurs, resulting in follicular atresia (Chun et al, 1996; Richards, 1980). *Fshb*, *Fshr*, *Ccnd2*, *Lhb*, and *Igf1* knockout mice are all unable to form antral follicles and arrest at the preantral stage (Dierich et al, 1998; Kumar et al, 1997; Ma et al, 2004; Sicinski et al, 1996; Zhou et al, 1997). *Lhcgr* knockout mice do not develop past the antral stage (Lei et al, 2001). Constitutive activation of *Foxo1* (a downstream effector of FSH and IGF1) in granulosa cells results in suppression of genes involved in granulosa cell proliferation (*Ccnd2*), steroidogenesis (*Cyp19a1*, cholesterol biosynthesis), and gonadotropin signaling (*Fshr, Lhcgr*) (Liu et al, 2009b; Park et al, 2005; Richards & Pangas, 2010b).

TGF-β/SMAD signaling stimulated by growth factors produced by granulosa cells, is particularly critical during the preantral and antral stages of follicle development. Activin enhances FSH activity and signals through SMAD2/3/4 to promote granulosa cell proliferation (by regulating *Ccnd2*) and steroidogenesis (by regulating *Cyp19a1*)(Hunzicker-Dunn & Maizels, 2006; Park et al, 2005). Ablation of activin subunits lead to premature luteinization of preantral follicles and accumulation of CLs (Pangas et al, 2007). Conditional knockouts of *Smad2/3* or *Smad4* exhibit increased preantral follicle atresia, premature luteinization, defective cumulus cells, and decreased ovulation (Li et al, 2008; Pangas et al, 2006b). On the other hand, conditional knockout of follistatin (an activin inhibitor) leads to follicle depletion by eight months of age (Jorgez et al, 2004). Ablation of *Inha* (an activin antagonist) results in granulosa cell proliferation in the absence of oocyte growth, and consequently the absence of late-stage follicles (FIGURE 1.8)(Myers et al, 2009). Clearly, gonadotropin-dependent follicle development (past the antral stage) involves a complex network in which hormones enhance or repress the effects of FSH to induce key target genes that drive granulosa cell proliferation, differentiation, survival, and steroidogenesis. While several of the key mediators required for growth beyond the antral stage have been identified, many still have yet to be discovered.

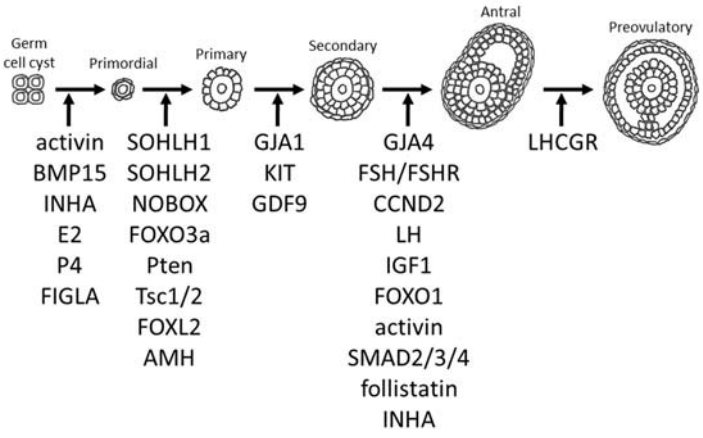


FIGURE 1.8 Regulation of folliculogenesis.
Inspired by Richards and Pangas 2010, and Pangas 2015.

1.4.3.6 Events initiated by the LH surge

The LH surge acts on large preovulatory follicles to trigger key events that lead to the release of a fertilisable oocyte, which include cumulus expansion, oocyte maturation, ovulation, and luteinization (Richards et al, 2015b). Only preovulatory follicles are able to respond to the LH surge and ovulate because only their mural granulosa cells selectively express high levels of LHCGR that bind LH/hCG (Jeppesen et al, 2012). While LH signaling via its receptor to activate EGFR signaling is well-established (Espey & Richards, 2002; Park et al, 2004; Shimada et al, 2006), many of the downstream targets have yet to be identified.

1.4.3.6.1 Oocyte maturation

Throughout follicle development, oocyte meiotic arrest is maintained by elevated cAMP and cGMP levels within the oocyte (Conti et al, 2012). cAMP/PKA maintains oocyte arrest by inhibiting the CDK1-Cyclin B complex (Chesnel & Eppig, 1995). cAMP levels are regulated by phosphodiesterase 3A (PDE3A) that breaks down cAMP and adenylyl cyclase 3 that converts ATP to cAMP (Vaccari et al, 2008). cGMP originates from cumulus cells that reach the oocyte via gap junctions (made up of GJA4) to inhibit PDE3A (Simon et al, 1997).

The LH surge causes a disruption of GJA4 leading to a drop in cGMP in the oocyte, which derepresses PDE3A. PDE3A, now active, can hydrolyze cAMP. The drop in cAMP

derepresses CDK1-Cyclin B, allowing meiosis to resume (FIGURE 1.9)(Conti et al, 2012; Oh et al, 2010). This results in the breakdown of the oocyte nuclear membrane (commonly referred to as germinal vesicle breakdown)(Hunzicker-Dunn & Mayo, 2015). The oocyte completes meiosis I, releases the first polar body, and progresses to metaphase II where it arrests again until fertilization (Pangas & Rajkovic, 2015; Richards, 1980).

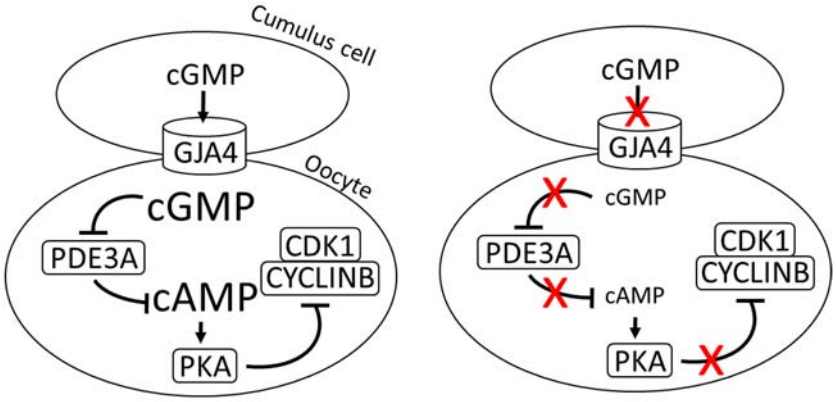


FIGURE 1.9 Maintenance of oocyte meiotic arrest (on left) vs resumption of meiosis (on right). *Inspired by Hunzicker-Dunn and Mayo 2015.*

1.4.3.6.2 Cumulus expansion

Cumulus cells produce a complex hyaluronan-rich extracellular matrix that is stabilized by numerous hyaluronan-binding proteins, in which the cumulus cells dissociate from one another and migrate outwards away from the oocyte in a process referred to as cumulus expansion (Richards, 2005; Richards et al, 2015a). The main drivers of cumulus expansion include granulosa cell expression of hyaluronan synthase 2 (*Has2*), EGFR ligands, prostaglandin synthase 2 (*Ptgs2*), and oocyte-derived *Gdf9* and *Bmp15* (Richards et al, 2015b).

In mural granulosa cells, LH induces the expression of EGFR ligands *Areg*, *Ereg*, and *Btc*, which promote expression of *Ptgs2*, leading to the production of prostaglandins PGE1/2 that act on cumulus cells (Park et al, 2004; Shimada et al, 2006). A *Ptgs2* autoregulatory loop promotes increased expression of EGFR ligands to further enhance its own expression (Shimada et al, 2006). EGFR ligands will then act in an autocrine and paracrine manner. In the latter, they regulate the expression of *Has2*, which synthesizes hyaluronan polymers that are stabilized by versican (VCAN), A disintegrin and metalloproteinase with thrombospondin-like motifs-1

(ADAMTS1), TNF-α-induced protein 6 (TNFAIP6), inter-alpha trypsin inhibitor (IAI), and pentraxin 3 (PTX3)(Richards, 2005). *Areg*, *Ereg*, *Btc* signaling through EGFR are sufficient to drive cumulus expansion (Park et al, 2004). Disruption of *Ptgs2*, *Has2*, *Adamts1*, *Tnfaip6*, or *Ptx3* impairs cumulus expansion and oocytes remain trapped within luteinized follicles (Dinchuk et al, 1995; Mittaz et al, 2004; Ochsner et al, 2003a; Sugiura et al, 2009; Varani et al, 2002). The phenotype of these mice illustrates how COC expansion/ovulation *vs* luteinization are regulated by separate mechanisms and that a proper sequence of events is essential in order for ovulation to occur.

Oocyte-derived *Gdf9* and *Bmp15* promote cumulus cell expression of genes that will provide the oocyte with the metabolic substrates it requires, such as cholesterol and glucose (Su et al, 2008; Sugiura et al, 2007). Ablation of *Bmp15* results in defective cumulus cell expansion and reduced ovulation (Su et al, 2004).

In addition, several innate immune response components also mediate cumulus expansion, including Toll-like receptors 2 and 4, and interleukin-6 (IL6)(Richards et al, 2008); IL6 alone is sufficient to stimulate COC expansion (FIGURE 1.10)(Liu et al, 2009a).

1.4.3.6.3 Ovulation

Ovulation entails the rupture of the preovulatory follicle wall and the release of the COC from the ovary into the oviduct (Richards et al, 2008). The breakdown of the follicle wall is carried out by proteases and collagenases that loosen and dissolve the numerous layers separating the COC from the peritoneal cavity (Richards et al, 2015a; Robker et al, 2000). Smooth muscle contractions in the theca layer and intra-follicular positive pressure assist the release of the COC out of the ruptured follicle (Richards et al, 2015a; Rose et al, 1999). Genes that mediate ovulation must be expressed prior to genes that mediate luteinization, otherwise the oocyte remains trapped within a luteinized follicle (Robker et al, 2000).

Two transcription factors are key for the processes of ovulation and luteinization, NR5A2 and CEBPB. Conditional knockout of either *Nr5a2* or *Cebpb* in granulosa cells exhibit impaired ovulation and luteinization (Duggavathi et al, 2008; Sterneck et al, 1997). LH also induces expression of the transcriptional co-regulator *Nrip1* that regulates expression of *Areg* (Nautiyal et al, 2010). *Nrip1* knockout mice also fail to ovulate (Tullet et al, 2005).

The LH surge rapidly induces the expression of two key genes, *Pgr* and *Ptgs2* that mediate ovulation-related events (Park & Mayo, 1991; Wong & Richards, 1991). In cumulus cells PTGS2 binds to one of its receptors, EP2, and induces the expression of *Tnfaip6* (Ochsner et al, 2003b). PGR induces the expression of proteases such as *Adamts1* and cathepsin L (FIGURE 1.10)(Robker et al, 2000). Beyond these two proteases, the other key proteases that mediate the "breakdown" of the follicle wall remain to be identified. Mice deficient in *Pgr*, *Ptgs2*, or *Ep2* are anovulatory (Dinchuk et al, 1995; Hizaki et al, 1999; Lydon et al, 1995).

1.4.3.6.4 Luteinization

Following the release of the COC, the remaining mural granulosa and theca cells in the follicle stop proliferating (exit the cell cycle), become hypertrophic, and terminally differentiate into luteal cells that synthesize P4 (Richards et al, 1998). This process requires the expression of transcription factors *Cebpb*, *Nr5a2*, and *Runx2*, steroidogenic enzymes *Cyp11a1* and *Star, Prlr*, cell cycle inhibitor genes *Cdkn1a/b*, and downregulation of FSH genes involved in follicle maturation (*Ccnd2, Fshr, Esr2, Cyp19a1, Inha, Foxo1*)(Hunzicker-Dunn & Mayo, 2015).

Major remodelling occurs with the breakdown of the basal lamina between the theca and granulosa cells and the infolding of the follicle wall. Several proteases are involved in extracellular matrix (ECM) remodeling: serine proteases, matrix metalloproteinases, their tissue inhibitors, and ADAMTS-1 (Curry & Osteen, 2003). This process can be described as an inflammatory response given that many innate immunity-related molecules (cytokines, chemokines) are recruited, the previously avascular granulosa layer becomes infiltrated with new blood vessels, and large amounts of prostaglandins (mostly PGE2) are produced (FIGURE 1.10)(Richards et al, 2008; Richards et al, 2015a; Robker et al, 2000) .

The differentiated luteal cells will then form the CL. The CL has a limited lifespan which depends on whether pregnancy occurs or not. If pregnancy occurs, the CL is functional and secretes P4 throughout the duration of gestation to maintain pregnancy (Bertolin & Murphy, 2014; Stouffer & Hennebold, 2015). In rodents, "pseudopregnancy" can occur in which imitation of mating by cervical stimulation at estrus induces the development of a functional CL that produces progesterone for 12-14 days. In the absence of either pregnancy or pseudopregnancy, no functional CL is formed in rodents, and the insufficient amount of

progesterone produced is unable to induce uterine decidualization (Stouffer & Hennebold, 2015).

Luteotropic factors are essential to develop and maintain the functional CL, which include prolactin (from the anterior pituitary), prolactin-like hormones (from the uterine decidua and placenta), and estradiol (produced locally) (Binart et al, 2000). Luteolysis is the process by which prostaglandin F2α (the "uterine luteolytic factor") promotes the loss of CL function and (eventually) structure either at the end of an ovarian cycle (in the absence of pregnancy) or at the end of gestation, allowing for the start of the subsequent ovarian cycle (Horton & Poyser, 1976). Luteal regression is marked by a drop in progesterone production (Niswender et al, 1994). In the mouse, three or more generations of non-functional CLs may be present during the same cycle (Stouffer & Hennebold, 2015).

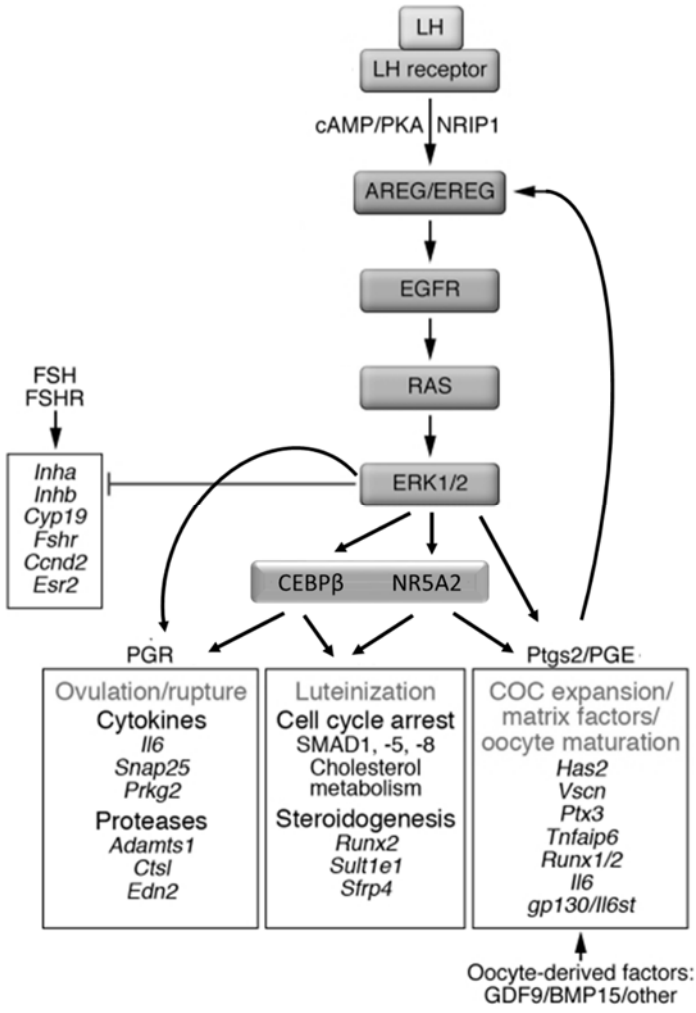


FIGURE 1.10 LH signaling that drives oocyte maturation, cumulus expansion, ovulation, and luteinization. *Revised from Richards and Pangas 2010.*

1.4.4 Steroidogenesis

1.4.4.1 Estradiol

Ovarian estrogen synthesis requires theca and granulosa cells that express cell-specific enzymes to convert cholesterol into estrogens (Richards, 1980). The main ovarian sex steroid hormones are 18-carbon estrogens, 19-carbon androgens, and 21-carbon progestins (McKenna, 2015). Steroidogenic enzymes/proteins can be classified into three classes: cytochromes P450

(CYP11A1, CYP17A1, CYP19A1), oxidoreductases (17βHSD, 3βHSD), and transport proteins (STAR)(Auchus, 2015).

Cholesterol is the precursor of all steroids (Auchus, 2015). Within granulosa and theca cells, STAR transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where CYP11A1 is located (Auchus, 2015; Clark et al, 1994). The first (rate-limiting) step is the side-chain cleavage of 27-carbon cholesterol to 21-carbon pregnenolone by CYP11A1 (Miller & Auchus, 2011). Pregnenolone is converted to progesterone by 3βHSD2 (Miller & Auchus, 2011). In theca cells only, CYP17A1 converts pregnenolone to 17-hydroxypregnenolone and then to DHEA (Gupta et al, 2003). Progesterone is converted to androstenedione by CYP17A1 (Gupta et al, 2003). Oxidoreductase reactions carried out by 17βHSD1 and 3βHSD2 convert DHEA to androstenedione and testosterone (Miller & Auchus, 2011). These two 19-carbon androgens are then aromatized to 18-carbon estrone or 17β-estradiol (commonly referred to as estradiol) by CYP19A1 in granulosa cells only (Simpson et al, 1994). An additional step converts estrone to estradiol by 17βHSD1 (FIGURE 1.11)(Miller & Auchus, 2011).

Steroidogenesis is stimulated by gonadotropins. In theca cells, LH binds to its receptor to induce conversion of cholesterol to androgens (Hillier et al, 1991). In granulosa cells, FSH binds to its receptor to induce progesterone production and CYP19A1 expression (Escamilla-Hernandez et al, 2008).

A distinction between granulosa vs theca cell expression of steroidogenic enzymes dictates which hormones are produced within each cell type. Granulosa cells do not express CYP17A1 and therefore are unable to convert progesterone to androgens. Thecal cells do not express CYP19A1, which explains why they are unable to aromatize androgens to estrogens (Auchus, 2015).

Estradiol binds to estrogen receptors 1 and 2 (ESR1/2) that are nuclear transcription factors expressed in the hypothalamus, anterior pituitary, and ovary (Couse et al, 1999). Within the ovary, ESR1 is predominantly expressed in theca cells, while ESR2 is expressed in granulosa cells (Couse & Korach, 2001).

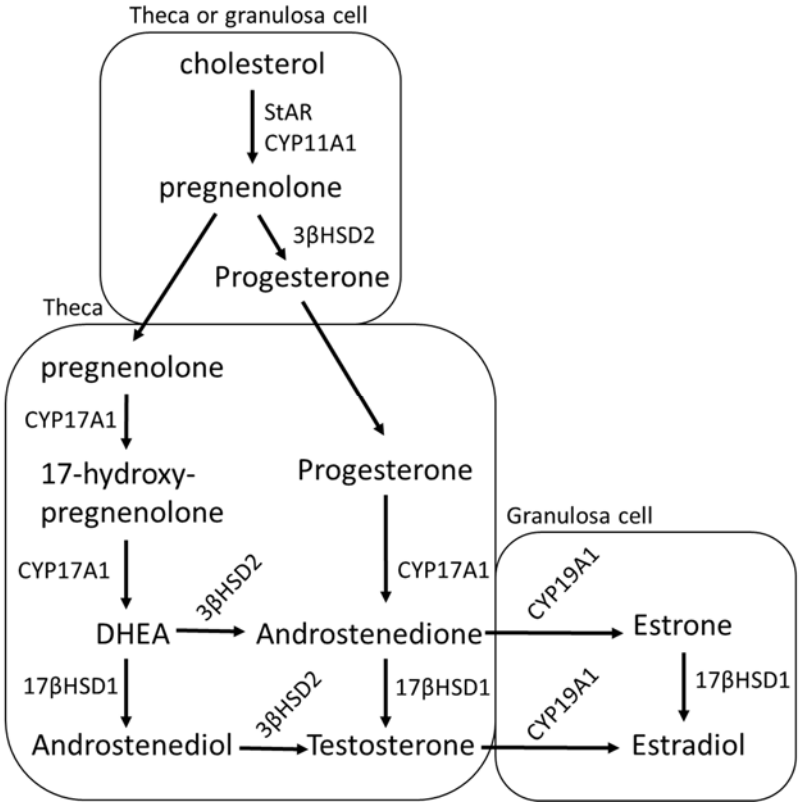


FIGURE 1.11 Estradiol biosynthesis. *Adapted from Auchus 2015.*

1.4.4.2 Progesterone

After ovulation, granulosa and theca cells within the ovulated follicle luteinize to form a functional CL that secretes larger amounts of P4 (Richards, 1980). The same steps required for progesterone synthesis in granulosa and theca cells apply to progesterone synthesis in luteal cells: cholesterol is transported by STAR to CYP11A1, which cleaves it to pregnenolone, which is then converted to progesterone by 3βHSD2 (FIGURE 1.12)(Auchus, 2015). This occurs under the control of LH (Goldring et al, 1987).

In the ovary, PGRs are expressed in theca cells of large preovulatory follicles, OSE, stroma, and transiently in mural granulosa cells of preovulatory follicles (induced by the LH surge) (Gava et al, 2004). *Pgr* expression is low during folliculogenesis except during a short 4-6h window after the LH surge (Park & Mayo, 1991). Its primary role (as described in section

1.4.3.6.3 Ovulation) is to mediate follicle rupture by inducing expression of key proteases that break down the follicle wall and the ECM (Lydon et al, 1995).

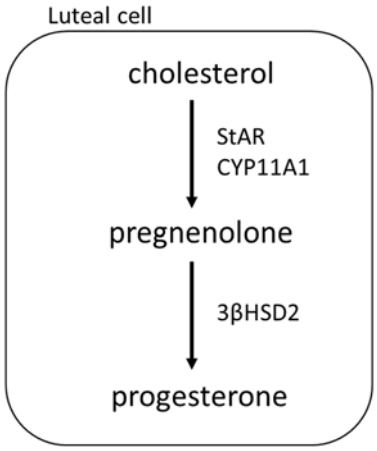


FIGURE 1.12 Progesterone biosynthesis. *Adapted from Auchus 2015.*

As outlined above, many critical steps during ovarian follicle development still remain incompletely understood. These gaps in our knowledge are what prompt us to investigate novel signaling pathways that are well-known in other contexts but whose study in the ovary is only just beginning. The Hippo signaling pathway is one such pathway, as outlined below.

2 Hippo signaling pathway

2.1 Introduction

How are cells instructed to stop growing when an organ has reached its proper size? How do cells know whether to proliferate or to differentiate? How do cells detect that a part of an organ is missing? How do damaged tissues regenerate? These are all long-standing questions that have puzzled researchers for many years until recently; with the discovery of the Hippo pathway, these enigmas are beginning to be resolved.

2.2 Discovery of the Hippo signaling pathway

Components of the Hippo pathway were first discovered in 1995 in genetic screens for tumor suppressor genes in the fruit fly *Drosophila melanogaster*. The screens identified two kinases Hippo (Hpo) and Warts (Wts), and the scaffold proteins Salvador (Sav) and Mob-as-tumor-suppressor (Mats)(Justice et al, 1995; Lai et al, 2005; Tapon et al, 2002; Wu et al, 2003). Loss-of-function mutations in any of these genes led to massive tissue overgrowth as a result of excessive cell proliferation and resistance to apoptosis. For example, the eye discs of *hpo*-null flies developed into oversized eyes and heads that resembled a hippopotamus hide, which eventually inspired the name of the pathway (Udan et al, 2003). Hpo-Sav formed a kinase complex that phosphorylated and activated Wts-Mats kinase complex, which was named the "Salvador-Warts-Hippo" (SWH) pathway (Wei et al, 2007). Yorkie (Yki) was later identified as a Wts-binding protein whose activity is negatively regulated by Hpo and Wts (Huang et al, 2005). Yki is unable to bind DNA directly, which led to the discovery of Scalloped (Sd), a member of the TEA domain (TEAD) family of transcription factors, as the mediator required for Yki to exert its functions (Wu et al, 2008).

In parallel with the discovery of the SWH pathway in *Drosophila*, several of the mammalian homologs were also being identified, however, it would take years before they became linked to what is now known as the Hippo pathway. In 1994, Yes-associated protein (YAP) was identified as a Yes tyrosine kinase-binding protein (Sudol, 1994). In 1998, mammalian STE20-like protein kinase 1 (MST1) was found to promote apoptosis (Graves et al, 1998). In 1999, large tumor suppressor 1 (LATS1) was found to regulate the cell cycle (Tao et

al, 1999). In 2000, transcriptional co-activator with PDZ-binding motif (TAZ) was identified as a transcriptional co-activator that binds to 14-3-3 proteins (Kanai et al, 2000). In 2001, four members of the TEAD family of transcription factors were found to interact with YAP (Vassilev et al, 2001).

Beginning around 2005, the mammalian Hippo pathway components were found to be evolutionarily conserved. Studies identified two homologs for Hpo (MST1 and 2), one homolog for Sav (SAV1), two homologs for Wts (LATS1 and 2), two homologs for Mats (MOB1A and B), two homologs for Yki (YAP and TAZ), and four homologs for Sd (TEAD1-4)(TABLE 2.1)(Callus et al, 2006; Chan et al, 2005; Dong et al, 2007; Lei et al, 2008; Praskova et al, 2008). Several human proteins were able to rescue their corresponding *Drosophila* mutants *in vivo*, confirming the functional conservation of these genes between species (Lai et al, 2005; Tao et al, 1999; Wu et al, 2003).

DROSOPHILA	**MAMMALS**
HIPPO (HPO)	MST1 (or STK4) MST2 (or STK3)
WARTS (WTS)	LATS1 LATS2
SALVADOR (SAV)	SAV1 (or WW45)
MOB-AS-TUMOR-SUPPRESSOR (MATS)	MOB1A MOB1B (or collectively MOB1)
YORKIE (YKI)	YAP TAZ (or WWTR1)
SCALLOPED (SD)	TEAD1 TEAD2 TEAD3 TEAD4

TABLE 2.1 Evolutionarily conserved Hippo pathway components

2.3 Canonical Hippo signaling pathway

The Hippo pathway is composed of a protein kinase cascade that ultimately regulates the protein stability and subcellular localization of the transcriptional co-activators, YAP and TAZ. The core kinase cassette is composed of two pairs of serine/threonine (Ser/Thr) kinases MST1/2 and LATS1/2, as well as adaptor proteins SAV1 and MOB1 (Pan, 2010; Piccolo et al, 2014).

When Hippo is activated by a variety of intracellular and extracellular cues, activated MST1/2 undergo autophosphorylation at Thr183 and Thr180, respectively, bind and phosphorylate SAV1 (at an unidentified site)(Callus et al, 2006; Praskova et al, 2004). Together they phosphorylate and activate LATS1(Thr1079)/ LATS2(Thr1041), which undergo autophosphorylation on Ser909/Ser872, respectively, enabled by MOB1 binding (Chan et al, 2005; Hergovich et al, 2006a). MST1/2 phosphorylate MOB1 on Thr12/Thr35 to enhance its interaction with LATS1/2 (Praskova et al, 2008). Together they phosphorylate and inactivate YAP and TAZ. LATS1/2 can phosphorylate YAP on five serine residues (Ser61, 109, 127, 164, and 381) and TAZ on four (Ser66, 89, 117, and 311) within HXRXXS consensus motifs (Lei et al, 2008; Zhao et al, 2007). Phosphorylation of YAP on Ser127 or TAZ on Ser89 generates a binding site for 14-3-3 protein leading to their sequestration in the cytoplasm (Basu et al, 2003; Dong et al, 2007; Kanai et al, 2000). Phosphorylation of YAP on Ser397 or TAZ on Ser311 primes YAP/TAZ for subsequent phosphorylation by casein kinase 1 (CK1), which activates a degradation motif (called a phosphodegron) leading to the recruitment of β-transducin repeat-containing protein (β-TrCP) E3 ubiquitin ligase, ubiquitination, and proteasome-mediated degradation (Liu et al, 2010; Zhao et al, 2010b).

When Hippo is inactivated, unphosphorylated YAP and TAZ translocate to the nucleus and bind to transcription factors, such as TEAD1-4, to regulate the transcription of target genes involved notably in cell proliferation and inhibition of apoptosis (FIGURE 2.1)(Vassilev et al, 2001; Zhao et al, 2008).

While over 40 proteins have been implicated as components of Hippo signaling, they all intersect at the level of the core Hippo kinases. One such protein is Merlin (encoded by the tumor suppressor gene *Nf2*), that is an upstream activator of Hippo signaling (Yin et al, 2013). Its importance is highlighted by the fact that ablation of *Nf2* results in phenotypes resembling the ablation of any of the four members of the core Hippo kinase cassette (Zhang et al, 2010).

It is important to note that not all of the core Hippo pathway components are involved in all contexts. For example, conditional ablation of *Mst1/2* in the mouse liver caused YAP overexpression leading to hepatocellular carcinoma, independently from LATS1/2 (Zhou et al, 2009). There is no hard and fast rule that applies to all cell types when it comes to regulation of and outputs of the Hippo signaling pathway, as detailed below.

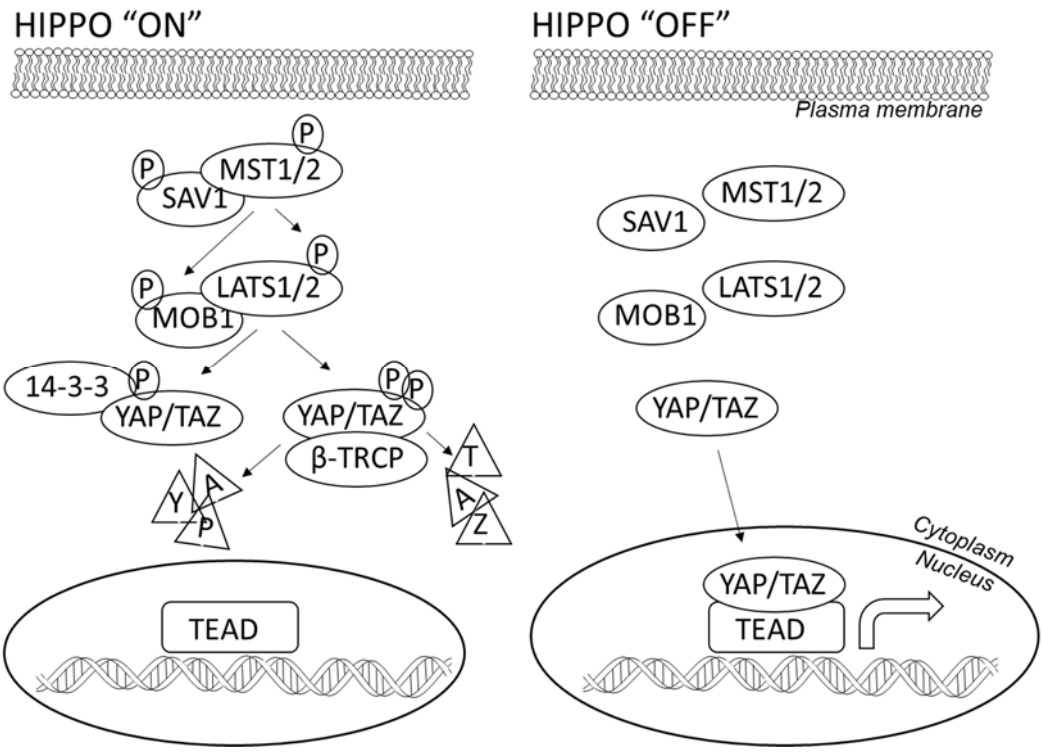


FIGURE 2.1 Canonical Hippo signaling pathway

2.4 Biological functions of Hippo in the mouse

2.4.1 Germline knockouts

Hippo pathway components have been identified as essential mediators during embryogenesis, given that they regulate a myriad of critical cell processes including cell proliferation, differentiation, apoptosis, and pluripotency (Piccolo et al, 2014). Indeed, total knockouts of *Yap, Yap/Taz, Tead1/2, Tead4, Lats2, Mst1/2, Sav1, Mob1,* and *Nf2* are all embryonic lethal in mice (Lee et al, 2008; McClatchey et al, 1997; McPherson et al, 2004; Morin-Kensicki et al, 2006; Nishio et al, 2012; Nishioka et al, 2009; Sawada et al, 2008; Song et al, 2010; Yagi et al, 2007). More specifically, *Yap/Taz* knockout mice do not survive past the 16-32 cell stage while *Yap* knockout mice die at e8.5 from several development defects (Morin-Kensicki et al, 2006; Nishioka et al, 2009). Few *Taz* knockout mice survive to adulthood (≈25%) but then die from pulmonary and polycystic kidney disease (Hossain 2007, Makita 2008).

2.4.2 Early embryonic development

A distinction between YAP and TAZ expression is already apparent at the blastocyst stage, the moment when the first cell fate decision is made. The outer cells that develop into the trophectoderm (TE; that gives rise to the placenta) contained nuclear YAP and TEAD4 that expressed TE-specific genes, while the inner cells that give rise to the inner cell mass (ICM; the future embryo) contained cytoplasmic YAP and TAZ (Nishioka et al, 2009). Interestingly, overexpression of *Yap* and *Tead4* was sufficient to induce the expression of TE-specific genes in the ICM (Nishioka et al, 2009), which highlights their significant role in determining cell fate.

2.4.3 Stem cells

In mouse embryonic stem cells (mESCs), YAP and TAZ promote pluripotency and inhibit differentiation (Tamm et al, 2011). YAP exerts these effects in part via its interaction with TEADs. Activated YAP interacted with TEAD2 on the promoters of pluripotency genes *Sox2, Oct4,* and *Nanog* to induce their expression, which promoted cell proliferation and prevented differentiation of mESCs (Lian et al, 2010). Restricting YAP and TEAD2 expression was sufficient to promote differentiation of mESCs (Tamm et al, 2011). The effects of YAP/TAZ on stemness derive also from interactions with TGF-β/SMAD signaling (see section *2.6.2 Crosstalk with the TGF-β/SMAD pathway*).

2.4.4 Organ development, homeostasis, and regeneration

Hippo pathway components play critical roles in organogenesis, homeostasis, and regeneration in a number of tissue types. As a general rule, knockdown of Hippo tumor suppressor genes (*Nf2, Mst1/2, Sav1, Lats1/2,* or *Mob1*) or overexpression of *Yap/Taz* promote expansion (proliferation) of progenitor cells and inhibit differentiation, resulting in tissue (over)growth notably in the liver, heart, and intestines.

2.4.4.1 Liver

The mouse liver is an archetypal organ to illustrate how Hippo effectors regulate cell proliferation and differentiation to control organ size and tissue growth. In the embryonic mouse liver, conditional knockout of *Mst1/2, Sav1,* or *Nf2,* or *Yap* activation promoted expansion of progenitor cells resulting in liver overgrowth (Camargo et al, 2007; Lu et al, 2010; Zhang et al, 2010; Zhou et al, 2011). In the adult liver, activation of *Yap* in hepatocytes induced severe liver hypertrophy (increase in size) due to hepatocyte hyperplasia (increase in cell number), while *Yap* ablation promoted apoptosis (Camargo et al, 2007; Zhang et al, 2010). Liver injury rapidly activated YAP to induce progenitor cell proliferation and inhibit hepatocyte differentiation, while ablation of *Yap* following tissue injury inhibited hepatocyte proliferation (Bai et al, 2012; Su et al, 2015).

2.4.4.2 Heart

In the embryonic mouse heart, conditional knockout of *Mst1/2, Lats2,* or *Sav1* or *Yap* overexpression led to increased cardiomyocyte proliferation and cardiomegaly (Heallen et al, 2011; von Gise et al, 2012; Xin et al, 2011). Conditional ablation of *Yap* in cardiomyocytes decreased cell proliferation, causing embryonic death at e10.5 due to myocardial hypoplasia (von Gise et al, 2012; Xin et al, 2011). Postnatal ablation of *Yap* promoted cardiomyocyte apoptosis and decreased cardiomyocyte proliferation, causing dilated cardiomyopathy and death (Del Re et al, 2013).

Traditionally, the heart has been considered to be a non-regenerative organ, however, recent studies have shown that damaged heart tissue has (limited) regenerative capacity that is mediated by Hippo. Ablation of *Sav1* or *Lats1*/2, or overexpression of *Yap* stimulated the proliferation of adult cardiomyocytes in response to tissue damage (Heallen et al, 2013; Xin et al, 2013), while *Yap* ablation impaired this response (Del Re et al, 2013).

2.4.4.3 Intestines

Roles for Hippo effectors in the intestines differ slightly from that in other organs. In mouse intestines, conditional knockout of *Mst1/2 or Sav1,* inhibition of *Lats1/2*, or activation of *Yap* promoted expansion of intestinal stem cells (without inducing an increase in organ size).

Differentiation occurred when *Yap* was inactivated (Camargo et al, 2007; Imajo et al, 2015; Lee et al, 2008; Zhou et al, 2011).

YAP appears to play critical roles in intestinal regeneration. YAP is expressed in the nuclei of intestinal progenitor cells within intestinal crypts and following tissue injury, YAP protein levels increased within the regenerating crypts, while *Yap* deletion from the intestinal epithelium prevented regeneration (Cai et al, 2010; Camargo et al, 2007).

2.4.5 Cancer

As described in the previous section, either conditional deletion of Hippo tumor suppressor genes (*Nf2, Mst1/2, Sav1, Lats1/2, Mob1*) or *Yap/Taz* activation lead to uncontrolled cell proliferation and massive tissue overgrowth. Prolonged dysregulation of these same components is sufficient to drive tumor development (Dong et al, 2007; Lee et al, 2010; Zhang et al, 2010; Zhou et al, 2009). In humans, several studies have linked either reduced tumor suppressor activity or elevated (nuclear) YAP and TAZ levels to the development of numerous types of cancer, including ovarian, colorectal, gastric carcinoma, hepatocellular carcinoma, esophageal, non-small-cell lung, breast, glioblastoma, melanoma, mesothelioma, osteosarcoma, and pancreatic ductal adenocarcinoma (Harvey et al, 2013; Piccolo et al, 2014). However, dysregulation of YAP/TAZ cannot be attributed to mutations in the core Hippo tumor suppressor genes. The only well-established mutation is in *Nf2*, which occurs in one out of 25 000 people and leads to tumor development in the nervous system, skin, and ophthalmological lesions (Asthagiri et al, 2009). No other mutations to date have been identified for core Hippo proteins with such high frequency. While the mechanisms driving the dysregulation of YAP/TAZ in cancer still remain elusive, their oncogenic functions are well described.

Hanahan and Weinberg established the “hallmarks of cancer”, which describe the biological properties acquired by cancer cells, including uncontrolled proliferation, evasion of apoptosis, indefinite replicative potential, angiogenesis, and metastasis (Hanahan & Weinberg, 2011). The oncogenic functions of YAP and TAZ as mediated in part by their target genes, can be categorized into several of these “hallmarks” and explain how they drive cell transformation.

2.4.5.1 Uncontrolled proliferation

The best-established role for YAP/TAZ is to promote cell proliferation. Overexpression of YAP or TAZ promoted excessive cell proliferation in conditional knockout mouse models of Hippo tumor suppressor genes and in a variety of cell lines (Dong et al, 2007; Lee et al, 2010; Lei et al, 2008; Overholtzer et al, 2006). This cell process was attributed to YAP/TAZ interaction with TEADs. TEADs were required for YAP-dependent cell growth and target gene expression, which include members of the CCN family of matricellular proteins that promote cell proliferation (Zhao et al, 2008). Another set of YAP/TAZ target genes that promote cell proliferation include those that regulate progression through the cell cycle, including *c-Myc*, *Foxm1,* and *Ccnd1* (Dong et al, 2007; Mizuno et al, 2012).

2.4.5.2 Evasion of apoptosis

YAP and TAZ help cells to evade apoptosis normally induced by a variety of cues. YAP inhibited apoptosis induced by anoikis, which is defined as programmed cell death caused by cell detachment from the cell substrate, in a mechanism that may involve EMT (which represses anoikis) and activation of ERK (a well-established pathway that promotes cell survival) (Zhao et al, 2012). Overexpression of YAP prevented apoptosis normally induced by FAS (a mediator of the extrinsic apoptotic pathway) in the mouse liver, (potentially) by inducing the expression of negative regulators of apoptosis (Dong et al, 2007). Overexpression of YAP also promoted the development of cancer cell resistance to chemotherapeutic drugs in nontransformed mammary epithelial cells (by an unidentified mechanism)(Overholtzer et al, 2006), while TAZ knockdown sensitized breast cancer cells to chemotherapeutic drugs (in a mechanism involving *Cyr61* and *Ctgf*)(Lai et al, 2011).

Several YAP/TAZ target genes encode proteins that have anti-apoptotic functions, which explains how cells that overexpress *Yap/Taz* are able to evade apoptosis. These include the anti-apoptotic genes B cell lymphoma 2 (*Bcl-2*) family members, baculoviral inhibitor of apoptosis repeat containing (*Birc*) family members, and CCN family members, *Ctgf* and *Cyr61*, which inhibited apoptosis and promoted resistance to chemotherapy in hepatocytes (Dong et al, 2007; Huo et al, 2013; Lai et al, 2011).

2.4.5.3 Indefinite replicative potential

YAP and TAZ promote pluripotency and inhibit differentiation in embryonic stem cells via their interaction with TEAD and/or SMAD transcription factors (see section *2.4.3 Stem cells*). Similar mechanisms (that potentially involve upregulated telomerase expression) may endow cancer cells with the ability to proliferate indefinitely. Activation of TAZ endowed cancer stem cell abilities to non-transformed cells and was required for self-renewal in breast cancer cells (Bartucci et al, 2014; Cordenonsi et al, 2011). One study made a critical link between YAP and telomerase activity. It showed that YAP bound a transcription factor (called ZEB1) to promote the transcription of human telomerase reverse transcriptase (hTERT*;* which promotes telomere length) in breast cancer cell lines (Yu et al, 2018), suggesting that hTERT is a YAP target gene in this context.

2.4.5.4 Angiogenesis

Recent studies have described YAP/TAZ expression and function in endothelial cells, which are directly responsible for the formation of new blood vessels. Ablation of *Yap/Taz* in mouse endothelial cells illustrated their critical role in endothelial cell proliferation and metabolism (Kim et al, 2017). They appear to exert these effects, in part, via vascular endothelial growth factor (VEGF)-VEGFR2 signaling, given that YAP/TAZ were identified as downstream mediators of this pathway in angiogenesis (Wang et al, 2017b).

2.4.5.5 Metastasis

In order for metastasis to occur, epithelial cancer cells first undergo epithelial-to-mesenchymal transition (EMT), which allows them to detach from neighboring cells via the loss of E-cadherin (Yi et al, 2015). YAP/TAZ have been implicated in EMT through their regulation by (and interaction with) several key proteins. Loss of the cell polarity protein Scribble (SCRIB) led to overexpression of TAZ, which promoted EMT and the expansion of cancer stem cells in mammary epithelial cells (Cordenonsi et al, 2011). Overexpression of Twist (another EMT driver) activated TAZ, leading to migration and invasion of breast cancer cells (Wang et al, 2016). The mechanism mediating EMT might be through YAP/TAZ interactions with the transcription factors Snail/Slug (encoded by *Snai1* and *Snai2*; which promote EMT by

repressing the cell adhesion molecule E-cadherin). Snail/Slug formed complexes with YAP/TAZ to activate YAP/TAZ/TEAD and YAP/TAZ/RUNX2 target genes, which promoted skeletal stem cell renewal (Tang et al, 2016).

All of the above mentioned studies illustrate the many ways in which overexpression of YAP and TAZ can help cells acquire cancer cell traits. Given that the majority of these studies are descriptive only, the specific mechanisms underlying these processes have yet to be elucidated.

2.5 Regulation of the Hippo pathway

The Hippo pathway has no specific ligands or cell surface receptors but is regulated by a wide variety of intracellular and extracellular cues that vary depending on the cell context. These signals can originate from cell-cell contact, cell-cell adhesion, cell polarity, mechanical signals, G protein-coupled receptors (GPCRs), or the actin cytoskeleton (TABLE 2.2).

2.5.1 Cell-cell contact

When grown in culture, normal cells stop proliferating once they become confluent. This is referred to as cell contact inhibition and is a characteristic that cancer cells are able to overcome (Eagle & Levine, 1967). One of the first regulators of Hippo signaling to be discovered was indeed cell-cell contact. When cells are grown in culture at low cell density, YAP and TAZ are active and nuclear. At high cell density, LATS is activated and phosphorylates/inactivates YAP (Zhao et al, 2007). Overexpression of YAP endowed cells with the ability to overcome cell contact inhibition and continue to proliferate, while inhibition of YAP restored this function in human cancer cell lines (Zhao et al, 2007).

2.5.2 Cell-cell adhesion

Adherens junctions (AJs) are protein complexes that mediate cell-cell adhesion between epithelial cells (Hartsock & Nelson, 2008). AJ proteins E-cadherin and α-catenin as well as associated proteins Kibra, Merlin, and Ajuba have all been linked to Hippo and may be involved in the activation of Hippo signaling initiated by cell contact. E-cadherin associated with α-

catenin at AJs, the latter of which retained YAP bound to 14-3-3 protein in the cytoplasm (Schlegelmilch et al, 2011). Knockdown of E-cadherin or α-catenin promoted nuclear localization of YAP (Yang et al, 2015). In confluent mammalian epithelial cells, Merlin was localized near AJs to recruit protein scaffolds (such as Kibra) to form a complex with LATS and YAP, which inactivated YAP (Yin et al, 2013; Yu et al, 2010). Loss of one of these scaffold proteins (such as knockdown of *Kibra*) was sufficient to reduce YAP phosphorylation (Xiao et al, 2011). Ajuba proteins were also recruited to AJs in confluent cultures and interacted with LATS and SAV but instead to inhibit YAP phosphorylation (Das Thakur et al, 2010).

2.5.3 Cell polarity

A defining characteristic of epithelial cells is their polarity, which entails that they possess distinct apical, lateral, and basal plasma membrane domains (Yang et al, 2015). A number of cell polarity proteins including SCRIB, Crumbs complex (CRB), and angiomotins (AMOT) regulate Hippo signaling in response to increases in cell density. SCRIB localized to the lateral plasma membrane and recruited MST1/2, LATS1/2, and TAZ to activate Hippo signaling (Cordenonsi et al, 2011). CRB localized to the apical plasma membrane recruited AMOTs, which activated LATS and bound YAP to sequester it in the cytoplasm (Paramasivam et al, 2011; Varelas et al, 2010b; Yi et al, 2011; Zhao et al, 2011). Loss of CRB, AMOT, or SCRIB allowed YAP/TAZ to translocate to the nucleus (Cordenonsi et al, 2011; Varelas et al, 2010b; Zhao et al, 2011).

2.5.4 Mechanical inputs

Mechanical signals can originate from changes in cell shape, in cell attachment, or in the rigidity of the ECM, and represents a second major regulator of Hippo signaling (after cell contact). A cell that stretches over a large area undergoes high mechanical stress, which promoted YAP/TAZ to localize to the nucleus, while a cell confined to a small area (exposed to low mechanical stress) contained cytoplasmic YAP/TAZ (Dupont et al, 2011). Cell attachment activated YAP/TAZ while anoikis inactivated YAP/TAZ (Zhao et al, 2012). Cells grown on a stiff matrix (rigid ECM) promoted YAP/TAZ activation and nuclear localization while a soft ECM inactivated YAP/TAZ and excluded them from the nucleus (Dupont et al, 2011).

2.5.5 G protein-coupled receptors

Extracellular ligands bind and activate GPCRs, which are receptors with seven transmembrane domains, which then activate intracellular G proteins. Different ligands activate different combinations of G protein subunits to either activate or repress Hippo signaling (Rao et al, 2016). For example, lysophosphatidic acid (LPA, a component found in serum) and sphingosine-1 phosphate (S1P) signaled through $G\alpha_{12/13}$ to inactivate Hippo signaling, while glucagon and epinephrine signaled through $G\alpha_s$ to activate Hippo signaling (Yu et al, 2012).

2.5.6 Actin cytoskeleton

The majority of the above-mentioned signals modulate Hippo signaling through activation of Rho GTPases (signaling G proteins that regulate actin cytoskeletal organization) that induce the polymerization of globular actin (G-actin) to filamentous actin (F-actin), which activates YAP/TAZ (Dupont et al, 2011; Johnson & Halder, 2014). For instance, GPCRs transmit their signals through Rho-GTPases and the actin cytoskeleton to regulate Hippo signaling. Indeed, a complex signaling cascade was identified that was initiated by S1P binding to its GPCR, which activated Rho-GTPase, induced F-actin polymerization, and ultimately led to nuclear YAP accumulation (Miller et al, 2012).

While many regulators have been identified that activate or repress Hippo signaling, these signals have yet to be attributed to specific cellular contexts (i.e. in the ovary, it is unknown what the predominant regulators of Hippo signaling might be).

CATEGORY	SIGNAL	MEDIATORS	EFFECT	REFERENCES
CELL CONTACT	Low cell density		Activates YAP/TAZ	Zhao 2007
	High cell density		Inactivates YAP/TAZ	Zhao 2007
ADHERENS JUNCTION PROTEINS	High cell density	E-cadherin and α-catenin	Inactivates YAP	Schlegelmich 2011, Yang 2015
	High cell density	Merlin and Kibra	Inactivates YAP	Yu 2010, Yin 2013
	High cell density	Ajuba	Activates YAP	Das Thakur 2010
POLARITY PROTEINS	High cell density	Scribble	Inactivates TAZ	Cordenonsi 2011
	High cell density	Crumbs and Amot	Inactivates YAP	Varelas 2010, Paramasivam 2011, Yi 2011, Zhao 2011
MECHANICAL INPUTS	High stress		Activates YAP/TAZ	Dupont 2011
	Low stress		Inactivates YAP/TAZ	Dupont 2011
	Cell attachment		Activates YAP/TAZ	Zhao 2012
	Anoikis		Inactivates YAP/TAZ	Zhao 2012
	Rigid ECM		Activates YAP/TAZ	Dupont 2011
	Soft ECM		Inactivates YAP/TAZ	Dupont 2011
GPCR	LPA, S1P		Activates YAP/TAZ	Yu 2012, Miller 2012
	Glucagon, epinephrine		Inactivates YAP/TAZ	Yu 2012
ACTIN CYTOSKELETON	Actin polymerization	Rho GTPase	Activates YAP/TAZ	Dupont 2011, Miller 2012

TABLE 2.2 Regulators of Hippo signaling

2.6 Crosstalk with other pathways

2.6.1 Crosstalk between the Hippo and Wnt/β-catenin pathways

Wnt/β-catenin is an extensively characterized pathway that regulates cell fate, differentiation, and proliferation in embryonic development, tissue homeostasis, and cancer (Clevers & Nusse, 2012). In canonical Wnt signaling (i.e. dependent on β-catenin), Wnt ligands bind to cell-surface frizzled (FZD) receptors and low-density lipoprotein receptor-related

protein 5/6 (LRP5/6) co-receptors to activate an intracellular signaling cascade that ultimately regulates the stability of the transcriptional co-activator β-catenin (Clevers & Nusse, 2012). β-catenin stability is controlled by a cytoplasmic destruction complex composed of the scaffold proteins Axin1/2 and APC, and the kinases GSK3β and CK1. In the absence of Wnt signaling, cytoplasmic β-catenin is recruited to the destruction complex, phosphorylated by GSK3β and CK1, ubiquitinated by β-TrCP, and degraded by the proteasomal pathway. When Wnt signaling is on, Wnt ligands (ex. WNT3A) bind to FZD/LRP5/6, recruit the scaffold protein dishevelled (Dvl) and Axin, which disassembles the destruction complex allowing β-catenin to be stabilized and translocate to the nucleus to bind T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to drive the transcription of Wnt target genes (Clevers, 2006).

Several Hippo and Wnt effectors have been shown to interact at different levels within the cell to regulate the stability, subcellular localization, and transcriptional (co-)activity of β-catenin, YAP, and TAZ. These can be grouped according to where the interactions occur within the cell: in the cytoplasm, as part of the cytoplasmic destruction complex, or in the nucleus. It is important to note that although reports may appear contradictory, it would seem that the manner in which the Wnt and Hippo pathways interact vary depending on the cellular context.

One of the earliest reports linking Wnt and Hippo signaling described how phospho-TAZ bound DVL in the cytoplasm and inhibited its phosphorylation by CK1, which blocked Wnt signaling (Varelas et al, 2010a). On the other hand, disruption of Hippo signaling decreased the interaction between TAZ-DVL, promoted DVL phosphorylation, increased nuclear levels of TAZ and β-catenin, and ultimately promoted the transcription of Wnt target genes (Varelas et al, 2010a). An additional level of interaction in the cytoplasm was characterized, in which WNT3A promoted protein phosphatase 1A (PP1A)-mediated TAZ dephosphorylation and hence activation, thereby promoting osteogenesis in a mouse fibroblast cell line (Byun et al, 2014).

Individual links between YAP, TAZ, β-catenin, and β-TrCP were described before YAP and TAZ were identified as key components of the Wnt cytoplasmic destruction complex. GSK3β phosphorylation of β-catenin brought together TAZ and β-TrCP, leading to TAZ degradation (Azzolin et al, 2012). Phospho-YAP(S127) bound β-catenin in the cytoplasm and blocked its nuclear translocation, and thus inhibited Wnt signaling (Imajo et al, 2012). It was the ground-breaking study by Azzolin *et al.* that elucidated the mechanism responsible for

recruiting β-TrCP to the complex was not β-catenin but rather YAP and TAZ (Azzolin et al, 2014). They demonstrated that in the absence of Wnt signaling, YAP and TAZ are bound to Axin1 in the destruction complex and recruit β-TrCP, which ubiquitinates β-catenin, leading to its degradation (Azzolin et al, 2014). When Wnt signaling is turned on, YAP, TAZ, and β-catenin are released from the complex and translocate to the nucleus to regulate the transcription of target genes (Azzolin et al, 2014; Azzolin et al, 2012). Along the same lines, loss of YAP/TAZ permits activation of β-catenin. This was shown in *Taz* knockout mice, in which β-catenin accumulated in polycystic kidney cells (Varelas et al, 2010a), as well as in mouse ES cells, in which the loss of *Yap/Taz* allowed β-catenin to maintain self-renewal (Azzolin et al, 2014).

Wnt and Hippo transcriptional co-activators cooperate to regulate the transcription of target genes. β-catenin, YAP and the TBX5 transcription factor formed a complex to activate anti-apoptotic genes *Birc5* and *Bcl2l2* in β-catenin-driven cancer cell lines (Rosenbluh et al, 2012). YAP-TEAD and β-catenin/TCF/LEF cooperated to induce the transcription of target genes *Sox2* and *Snai2* in cardiomyocytes (FIGURE 2.2)(Heallen et al, 2011). An additional level of control was identified in which β-catenin/TCF bound an enhancer element on the *Yap* gene in colon cancer cells, suggesting that *Yap* is a direct Wnt/β-catenin target gene (Bottomly et al, 2010; Konsavage et al, 2012).

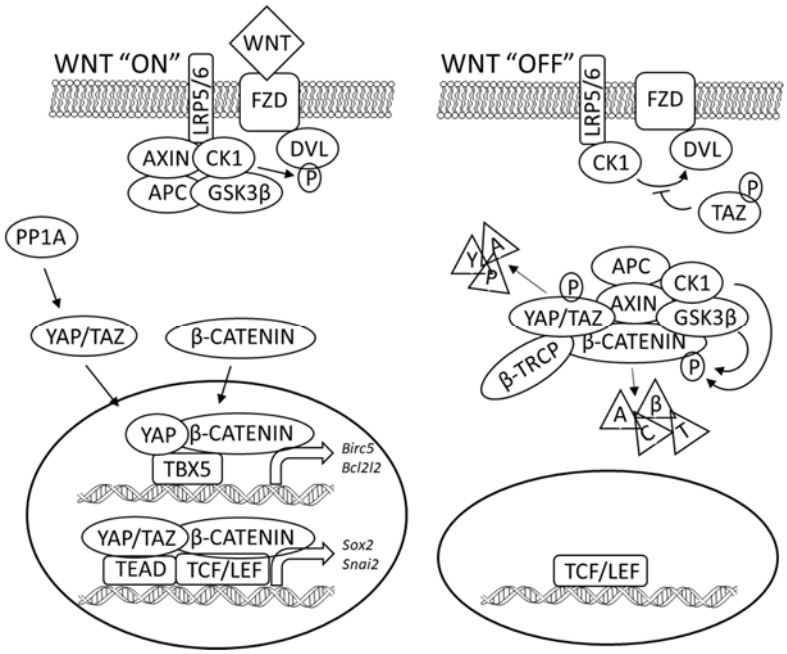


FIGURE 2.2 Crosstalk between the Hippo and Wnt/β-catenin pathways

2.6.2 Crosstalk between the Hippo and TGF-β/SMAD pathways

The TGF-β superfamily includes TGFβs, BMPs, GDFs, and activin/inhibin ligands that regulate critical functions such as pluripotency and differentiation, in development and in cancer (Varelas et al, 2008). The TGF-β ligand binds to its cell surface serine/threonine kinase receptors, which activates an intracellular signaling cascade composed of SMAD proteins. SMADs are generally categorized into three groups: the receptor-associated SMADs (R-SMADs) that are activated by receptor complexes and include SMAD1, 2, 3, 5, 8; the common mediator SMAD4, which forms a transcription factor complex with R-SMADs to regulate ligand-specific gene expression; and the inhibitory SMADs, SMAD6 and -7, which antagonize TGF-β signaling by either binding SMAD4 or blocking activation of R-SMADs (Li et al, 2008; Pangas, 2012).

YAP/TAZ and SMADs have been found to interact at different levels within the cell to regulate their subcellular localization and transcriptional activity in the context of stem cell maintenance and tumorigenesis.

Cytoplasmic YAP and TAZ inhibit TGFβ/SMAD signaling in a variety of cell contexts. High cell density detected by CRB promoted phosphorylated YAP/TAZ to accumulate in the

cytoplasm and bind SMAD2/3, which inhibited TGF-β signaling in murine mammary epithelial cells. Disruption of YAP/TAZ phosphorylation (or disruption of CRB), induced YAP/TAZ and SMAD2/3 translocation into the nucleus to promote EMT (Varelas et al, 2010b). YAP bound SMAD7 in the cytoplasm to enhance inhibition of SMAD3/4 signaling in a fibroblast cell line (Ferrigno et al, 2002).

YAP/TAZ and SMADs form a variety of nuclear complexes in response to different TGF-β ligands to regulate the transcription of genes involved in stem cell maintenance and cancer. TGF-β stimulation promoted the interaction between TAZ and SMAD2/3/4 complex in the nucleus to drive the expression of pluripotency genes *Oct4* and *Nanog* in human embryonic stem cells; in the absence of TAZ, SMAD2/3/4 were unable to remain in the nucleus and as a result, this allowed differentiation to occur (Varelas et al, 2008; Varelas et al, 2010b). Similarly, BMP promoted the interaction between YAP and SMAD1 to regulate BMP target gene expression required for maintaining pluripotency in mESCs (FIGURE 2.3)(Alarcon et al, 2009). YAP/TAZ/TEAD and SMAD2/3 interacted in the nucleus to drive transcription of target genes responsible for promoting tumorigenesis in breast cancer cells (Hiemer et al, 2014).

Based on the numerous interactions between Hippo and Wnt or TGF-β effectors previously identified in a variety of cell types, in the (future) study of how the Hippo pathway is regulated in a given cellular context, it will be imperative to take these potential interactions into account.

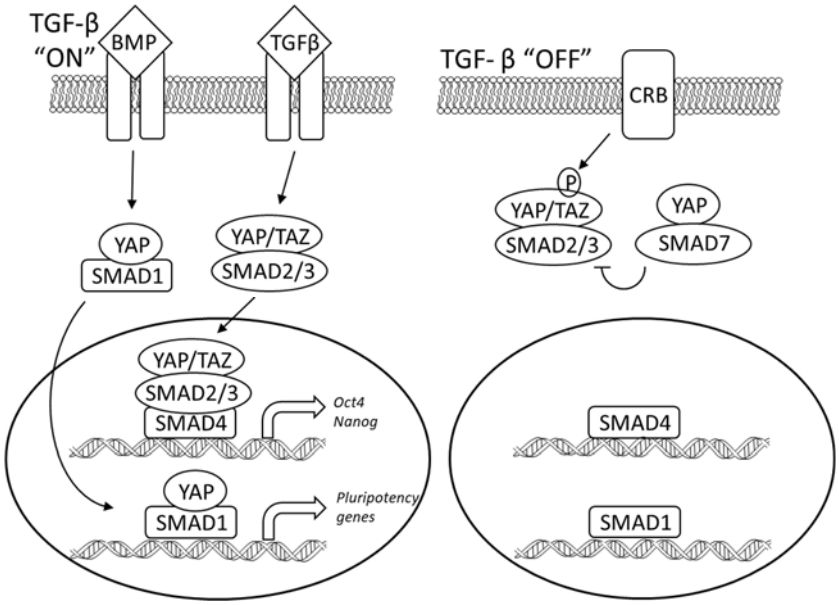


FIGURE 2.3 Crosstalk between the Hippo and TGF-β/SMAD pathways

2.7 Large tumor suppressors

LATS1/2 represent the master regulators of the Hippo pathway as they are directly responsible for regulating the stability and subcellular localization of YAP and TAZ and are expressed in all tissue types (Hergovich, 2013; Visser & Yang, 2010). LATS1/2 have also been shown to exert functions unrelated to canonical Hippo signaling, as well as to be regulated by mechanisms not involving MST1/2.

2.7.1 Regulation of LATS

LATS1/2 are members of the serine/threonine AGC class of protein kinases. They are regulated on two conserved phosphorylation sites: the activation segment motif (AS; Ser909/Ser872) and the hydrophobic motif (HM; Thr1079/Thr1041)(Hergovich et al, 2006b). MST1/2 phosphorylate the HM while the AS is an autophosphorylation site. Phosphorylation of LATS at both AS and HM motifs is required for LATS activity (Chan et al, 2005). LATS kinase activity can be reversed by protein phosphatase 2A (PP2A), that dephosphorylates the AS and HM motifs (Chan et al, 2005). Beyond phosphorylation, LATS has been found to be regulated by additional mechanisms.

A few reports have identified how *Lats1/2* are regulated at the transcriptional level. Interestingly, a negative feedback loop was identified in which activated YAP-TEAD directly promoted *Lats2* transcription (Park et al, 2016). The transcription factor p53 promoted transcription of *Lats2* in response to an antineoplastic drug (Aylon et al, 2006). Several microRNAs negatively regulated *Lats2* expression (Fang et al, 2012; Lin et al, 2013). In several types of cancer, promoter hypermethylation was an important mechanism of *Lats1/2* downregulation (Jiang et al, 2006; Takahashi et al, 2005).

LATS1 protein stability is regulated by different proteins: NUAK1 (involved in cell senescence) phosphorylated LATS1 to regulate its stability (Humbert et al, 2010); Itch, NEDD4, and WWP1 E3 ligases ubiquitinated LATS1 leading to its degradation (Ho et al, 2011; Salah et al, 2013; Yeung et al, 2013).

Direct binding of LATS to cell adhesion and cell polarity proteins can either activate or repress its kinase activity. Binding to SCRIB, AMOTs, and KIBRA activated LATS kinases, while binding to Ajuba proteins appeared to repress LATS activity (Cordenonsi et al, 2011; Das Thakur et al, 2010; Paramasivam et al, 2011; Xiao et al, 2011).

Subcellular localization is a mechanism that regulates LATS1 activity, as shown by MOB1-mediated activation of LATS1, which directed LATS1 to the membrane and enhanced its activity (Hergovich et al, 2006a).

2.7.2 Functions of LATS

LATS1 and LATS2 are homologous proteins and exhibit many overlapping functions (Hergovich, 2013; Visser & Yang, 2010). However, small structural differences between LATS1 and LATS2 do exist, and these may account for certain differences in function. For example, LATS1 has two PPXY motifs (that bind WW domain proteins such as YAP and TAZ) while LATS2 has only one (Hao et al, 2008). Beyond YAP/TAZ, LATS directly phosphorylates other proteins and these interactions illustrate additional roles for LATS beyond canonical Hippo signaling in the regulation of cell cycle checkpoints and the maintenance of genetic stability.

The role for LATS1/2 as inhibitors of cell proliferation is well established. LATS appear to mediate this in part by regulating cell cycle components, such as cyclin dependent kinases

(CDKs). LATS1 blocked the G_2/M transition by binding the G_2/M regulator, CDK1, which inhibited cell proliferation (Tao et al, 1999). LATS2 blocked the G_1/S transition by inhibiting the Cyclin E/CDK2 complex (Li et al, 2003).

Malfunction of cell cycle checkpoints not only affects cell proliferation but also genetic stability. Both LATS1 and LATS2 regulated levels of p53 to control the G1 tetraploidy checkpoint (Aylon et al, 2006; Iida et al, 2004). Loss of either *Lats1* or *Lats2* resulted in aneuploidy that was associated with centrosomal defects (that drive the formation of spindle poles during mitosis) and aberrant cytokinesis (Iida et al, 2004; McPherson et al, 2004).

2.8 YAP and TAZ

YAP and TAZ are two transcriptional co-activators that are the key downstream effectors of the Hippo pathway, and as such are directly responsible for the majority of Hippo pathway functions. YAP and TAZ are ubiquitously expressed (Zhao et al, 2010a). Their molecular structure allows them to interact with a wide variety of proteins and thus exert a vast array of functions.

2.8.1 Molecular structure of YAP and TAZ

YAP and TAZ are homologous and possess similar domains which allow them to interact with a variety of proteins. YAP domains include: a proline-rich N-terminal, TEAD-binding, 14-3-3 binding, two WW, SH3-binding motif, coiled-coil, transcription activation, and a C-terminal PDZ-binding motif (Zhao et al, 2010a). In comparison to YAP, TAZ lacks the proline-rich domain, the second WW domain, and the SH3-binding motif (Zhao et al, 2010a). The WW domains of YAP/TAZ recognize PPXY motifs on several binding proteins, including LATS1/2, AMOTs, RUNX1/2, p73, and TBX5 (Piccolo et al, 2014). The TEAD family of transcription factors do not possess the PPXY motif and interact instead with the TEAD-binding domain of YAP/TAZ (Li et al, 2010). The coiled-coil domain facilitates interactions with SMAD2/3 (Varelas et al, 2008). The differences in their respective domains explain why YAP and TAZ cannot bind to all of the same proteins. For example, YAP interacts with Yes tyrosine kinase through the SH3-binding motif; TAZ lacks this binding motif and has not been shown to bind to Yes (FIGURE 2.4)(Kodaka & Hata, 2015; Sudol, 1994).

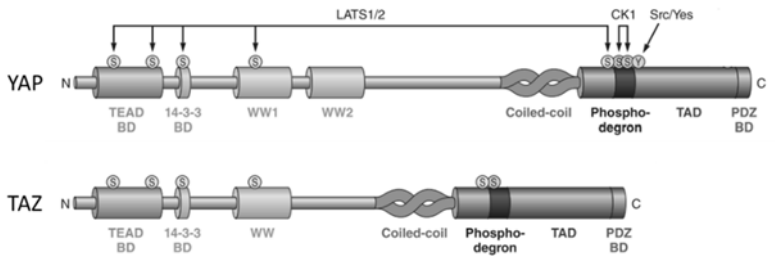


FIGURE 2.4 Molecular structure of YAP and TAZ. *Adapted from Piccolo et al 2014.*

2.8.2 Regulation of YAP/TAZ

YAP and TAZ activity is dependent on their phosphorylation status and subcellular localization. Unphosphorylated YAP/TAZ translocate to the nucleus to co-activate the transcription of target genes, while phosphorylated YAP/TAZ localize to the cytoplasm and are (transcriptionally) inactive. Protein phosphatases, PP1A and PP2A act as positive regulators of YAP/TAZ as they reverse phosphorylation by LATS. PP1A dephosphorylates YAP at Ser127 and TAZ at Ser89 and Ser311; PP2A dephosphorylates YAP at Ser127 (Cai & Xu, 2013; Liu et al, 2011). Regulation of *Yap/Taz* at the transcriptional level does not appear to be a major mode of regulation (Kodaka & Hata, 2015).

2.8.3 YAP and TAZ as transcriptional co-activators

YAP and TAZ are transcriptional co-activators that do not possess DNA-binding domains (making them unable to bind DNA directly) and must bind to transcription factors to regulate the transcription of target genes (Mauviel et al, 2012; Zhao et al, 2010a). YAP/TAZ bind primarily to TEAD1-4, which are required for the majority of YAP/TAZ functions, including cell contact inhibition, EMT, cell transformation, cell survival, and trophectoderm development (Nishioka et al, 2009; Ota & Sasaki, 2008; Zhang et al, 2009a; Zhao et al, 2008).

YAP and TAZ are able to bind alternative transcription factors depending on the cellular context, including SMADs, RUNX2, PPARγ, p73, and TBX5. Interactions with SMADs and TBX5 have already been described in sections *2.6.1* and *2.6.2*. TAZ bound RUNX2 to promote osteogenesis in mesenchymal stem cells and bound PPARγ concomitantly to repress transcription of adipogenic genes (Cui et al, 2003; Hong et al, 2005). Interestingly, YAP can either inhibit or promote RUNX2-mediated transcription depending on the cell type (Vitolo et

al, 2007; Zaidi et al, 2004). DNA damage induced phosphorylation of YAP, which enhanced its interaction with the tumor suppressor p73 to drive transcription of pro-apoptotic genes (Strano et al, 2001).

2.8.4 YAP/TAZ target genes

YAP/TAZ/TEAD target genes were initially discovered by microarray analyses performed on a variety of cell types. These analyses identified the oncogene *c-Myc* and the anti-apoptotic genes *Birc2* and *Birc5* in mouse livers; *Birc5* and *Itgb2* in breast epithelial cells; and *Ankrd1* and *Ctgf* in cancer-associated fibroblasts (Calvo et al, 2013; Dong et al, 2007; Hao et al, 2008). It became quite clear based on those analyses that the majority of target genes are induced in a cell-type specific manner. The best established Hippo target genes to date include: *Ctgf, Cyr61, Ankrd1, Axl, Sox9, Areg, Birc2/5, Itgb2, and c-Myc* (Kodaka & Hata, 2015; Mauviel et al, 2012; Ota & Sasaki, 2008; Pan, 2010; Park & Guan, 2013; Piccolo et al, 2014; Pobbati & Hong, 2013; Zhao et al, 2010a; Zhao et al, 2008). Following those analyses, functional roles for YAP/TAZ/TEAD target genes were identified.

Ctgf is by far the most frequently referenced YAP/TAZ/TEAD target gene and is used in assays to confirm YAP/TAZ activity (Zhang et al, 2009a; Zhao et al, 2008). Due to the ability of CCN proteins to bind a variety of cell surface receptors, they are able to regulate a wide array of functions including cell proliferation, survival, adhesion, senescence, and migration (Leask & Abraham, 2006). For example, CTGF was required for YAP-induced cell proliferation and anchorage-independent growth in human breast epithelial cells (Zhao et al, 2008). CYR61 and CTGF promoted taxol resistance in MCF10A breast cancer cells (Lai et al, 2011). Moreover, CTGF inhibited apoptosis in liver cells (Urtasun et al, 2011).

AXL is a receptor tyrosine kinase that promoted YAP-mediated cell proliferation and invasion in hepatocellular carcinoma cells (Xu et al, 2011). SOX9 is a transcription factor whose expression, when induced by *Yap* activation, endowed non-transformed esophageal cells with cancer stem cell characteristics (Song et al, 2014). AREG is an EGFR ligand that promoted cell proliferation and migration in MCF10A breast epithelial cells (Yang et al, 2012; Zhang et al, 2009b; Zhao et al, 2010a).

Today, with the advancement of RNA-Seq and ChIP-Seq techniques, direct YAP/TAZ target genes will be more readily identifiable in the various cellular contexts.

2.9 Hippo signaling in the ovary

There is an increasing amount of evidence in the literature that implicates the Hippo signaling pathway in ovarian physiology and ovarian cancer. Numerous total and conditional knockout mouse models have been generated for the purpose of identifying physiological roles of the Hippo pathway effectors.

Studies from *Lats1* knockout females revealed that *Lats1* is essential for normal fertility, follicle development to the antral stage, and acts as a tumor suppressor in the ovary (St John et al, 1999). Investigation of the roles of LATS1 at earlier stages of follicle development found that it is critical for germ cell maintenance, proper primordial follicle formation, and activation (Sun et al, 2015). TAZ was also found to be necessary for normal fertility, as *Taz* knockout female mice produced smaller litter sizes, however the cause of the decreased fertility was not investigated (Hossain et al, 2007). Conditional knockout of the direct Hippo target gene, *Ctgf,* revealed that it is required for normal follicle development, ovulation, and luteolysis (Nagashima et al, 2011).

Hippo pathway components are expressed in the ovary over the course of follicle development. MST1/2, SAV1, and LATS1/2 are expressed in the cytoplasm of granulosa cells, theca cells, and oocytes of primordial to antral follicles, with some expression in CLs (Kawamura et al, 2013; Sun et al, 2015). TAZ expression was nuclear and cytoplasmic in oocytes and granulosa cells of primordial to antral follicles, and in CLs (Kawamura et al, 2013; Sun et al, 2015). YAP expression was mostly cytoplasmic in oocytes and granulosa cells from primordial to antral follicles (Sun et al, 2015).

Hippo components regulate critical mediators of granulosa cell function. LATS1 directly phosphorylated FOXL2, a gene essential for granulosa cell fate maintenance, which enhanced the repression of the stereoidogenic gene *Star* (Pisarska et al, 2010). *Areg*, another direct Hippo target gene (identified in breast epithelial cells), is a critical gene responsible for cumulus expansion and oocyte maturation (Zhang et al, 2009b).

The ground-breaking study that created a clear link between Hippo signaling and ovarian follicle development in the mouse came from the Hsueh laboratory. They fragmented and allo-transplanted immature murine ovaries and showed that fragmentation stimulated actin polymerization, which decreased phospho-YAP levels, promoted translocation of YAP into the nucleus of granulosa cells of primary and secondary follicles, and increased expression of target genes. This led to the stimulation of ovarian follicle development and oocyte maturation (Kawamura et al, 2013). Follicle development (induced by fragmentation) was blocked when mice were pretreated with verteporfin (a small molecule inhibitor of the YAP-TEAD interaction), further reinforcing a role for Hippo in folliculogenesis. In a subsequent study, the same group used actin polymerization-promoting drugs on grafted ovaries to promote YAP translocation into the nucleus, which also promoted follicle growth (Cheng et al, 2015).

The Hippo pathway, composed of several tumor suppressor genes, also clearly plays a role in ovarian cancer. *Lats1* knockout mice develop ovarian stromal cell tumors by 3 months of age (St John et al, 1999). YAP was overexpressed in granulosa cell tumors with elevated nuclear expression, which promoted cell proliferation, migration, and the expression of *Cyp19a1* (Fu et al, 2014). YAP was also overexpressed in epithelial ovarian cancers, and promoted cell proliferation, migration, anchorage-independent growth, and evasion of apoptosis (Hall et al, 2010). TAZ promoted cell migration in epithelial ovarian cancer cells (Jeong et al, 2013). YAP/TEAD promoted ovarian cancer initiating cells to self-renew and regulate transcription of genes involved in stemness and chemoresistance (Xia et al, 2014).

In conclusion, there is evidence to support a role for Hippo signaling in ovarian follicle development, however, more questions than answers exist at this point in time. For instance, do gonadotropins regulate Hippo signaling in the ovary, and if so, how and when? Is canonical Hippo signaling (involving the core Hippo components) active in ovarian granulosa cells and if so, what purpose does it serve? What specific roles do Hippo effectors play in granulosa cells? It was therefore the goal of this thesis to elucidate the roles and regulation of the Hippo pathway in the ovary.

Chapter 2. Hypotheses and objectives

The Hippo signaling pathway has been investigated primarily in the context of development, tissue homeostasis, and cancer in several tissue types. Accumulating evidence in the literature suggests that Hippo signaling might have a role to play in the postnatal ovary. Notably, Hippo effectors appear to be required for normal female fertility, ovarian follicle development, and ovarian cancer. However, many fundamental questions have yet to be answered: 1) What regulates Hippo signaling in ovarian granulosa cells? 2) Is canonical Hippo signaling active in granulosa cells? 3) What roles do individual Hippo pathway effectors play in the ovary?

Based on evidence from the literature, our general hypothesis was that the Hippo pathway is required for normal ovarian follicle development in the mouse.

To study this hypothesis, we had two primary objectives: 1) To identify regulators of Hippo signaling in granulosa cells, and 2) To identify physiological roles for LATS1, LATS2, YAP, and TAZ in granulosa cells. Results pertaining to objectives 1 and 2 are described in Chapter 3; results pertaining to objective 2 are described in Chapter 4.

Chapter 3. Article 1

***Yap* and *Taz* are required for the granulosa cell response to luteinizing hormone**

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This manuscript is in the final phase of preparation to be submitted to *Endocrinology*.

Author contributions: MT contributed to the experimental design, performed experiments, analyzed the results, and wrote the article; representing a relative contribution of 90%. PG contributed to the experimental design and performed experiments. CR performed experiments. DB contributed to the experimental design, analyzed the results, and wrote the article.

3.1 ABSTRACT

Very little is known regarding the roles of the Hippo signaling pathway in the ovary. Studies suggest that Hippo effectors are implicated in female fertility, in regulating ovarian follicle growth, and in ovarian cancer, however, the specific roles of the key Hippo downstream effectors, YAP and TAZ, in the ovary remain unknown. We therefore hypothesized that YAP and TAZ activity in granulosa cells is critical for normal ovarian follicle development. To study this, we treated C57BL/6J mice with eCG with or without hCG on a time course and discovered that hCG activates Hippo signaling by inducing a transient increase in phospho-LATS1(Thr1079), and phospho-YAP(Ser127 and Ser397), and YAP expression levels in granulosa cells *in vivo*. Pre-treatment of granulosa cells with inhibitors against well-established signaling pathway effectors acting downstream of the LH receptor suggest that LH signals via PKA to activate Hippo signaling. To study whether *Yap/Taz* are necessary for LH signaling, we knocked down *Yap* and *Taz* individually in primary granulosa cell cultures and discovered that loss of either gene blunted the induction of LH target genes *Btc, Star, Pgr*, and *Tnfaip6*. This appeared to be, at least in part, as a result of the loss of *Lhcgr* expression. Overall, these results identified a novel regulatory role for LH on Hippo signaling in granulosa cells and demonstrated that *Yap* and *Taz* are required for LH to exert some of its effects.

3.2 INTRODUCTION

Hippo is a highly conserved intracellular signaling pathway best known for the roles it plays in multiple cell- and tissue types during embryonic development, regulating processes such as organ size determination, cell fate specification, proliferation, apoptosis, and cell migration (Pan, 2010; Piccolo et al, 2014; Zhao et al, 2010a). The Hippo pathway consists of a core kinase cascade that is not regulated by a specific ligand or receptor. Rather, it integrates a multitude of signals derived from the intracellular and extracellular environment, such as the establishment of cell-cell contacts, cell polarity, mechanical forces such as shear stress, and cellular stresses including nutrient deprivation and hypoxia (Jho, 2018). These signals converge to activate the kinases MST1 and MST2 (the mammalian homologs of *Drosophila* Hippo, after which the

pathway is named). MST1/2 then function in a redundant manner to phosphorylate (and thereby activate) the kinases LATS1 and LATS2. The latter kinases, which are also functionally redundant, act in turn to phosphorylate the transcriptional co-regulators YAP and TAZ (also known as WWTR1), leading to either their sequestration in the cytoplasm or their proteasomal degradation. Absence or loss of upstream signal (such as by disruption of cell-cell contacts) disrupts the Hippo kinase cascade, allowing YAP and TAZ proteins to escape phosphorylation/degradation and accumulate within the cell. Following translocation to the nucleus, YAP/TAZ can bind to several transcription factors, notably those of the TEAD family, resulting in the modulation of the transcriptional activity of a variety of target genes in a cell type- and context-specific manner (Ota & Sasaki, 2008; Zhang et al, 2009a; Zhao et al, 2008). Although able to perform the same functions, the biological roles of YAP and TAZ are not entirely redundant, as evidenced by the divergent phenotypes of *Yap* and *Taz* knockout mice. Whereas *Yap*-null mice die during early embryogenesis (e8.5) from a range of profound developmental defects, ≈25% of *Taz* knockout mice are born live and survive until adulthood, although they develop polycystic kidney disease and pulmonary emphysema phenotypes that hinder their postnatal development and health (Hossain et al, 2007; Makita et al, 2008; Morin-Kensicki et al, 2006). The discrepancies between the phenotypes could be due to differences between YAP and TAZ with regards to their patterns of expression and/or regulation.

Several lines of evidence now indicate that the Hippo pathway may play an important role in ovarian follicle development. An early study reported that female *Lats1* knockout mice have reduced fertility, possibly due to a defect in follicle development (St John et al, 1999). It should be noted however that *Lats1*-null mice suffer from multiple phenotypic abnormalities that result in decreased viability, runting, and endocrine disruptions including reduced pituitary LH synthesis, rendering it unclear whether the ovarian defects observed in the *Lats1* knockout model are entirely ovary-autonomous. More recently, LATS1 has been identified as a kinase of the transcription factor FOXL2 (Pisarska et al, 2010). Phosphorylation of FOXL2 by LATS1 enhances its ability to suppress the transcriptional activity of the steroidogenesis regulator *Star* in granulosa cells (Pisarska et al, 2010). *Lats1* may therefore function as a regulator of ovarian steroidogenesis and follicle development via mechanisms unrelated to the canonical Hippo pathway. Both *Yap* and *Taz* are expressed in the mouse ovary (Kawamura et al, 2013). *Taz* expression localizes mainly to the cytoplasm of granulosa cells (GCs) at all stages of follicle

development and at lower levels in the corpus luteum (Kawamura et al, 2013). Female *Taz*-null mice have been reported to be infertile (Hossain et al, 2007), but whether this infertility is of ovarian origin has not been studied. *Yap* expression is strongest in the cytoplasm of GCs from primordial to antral follicles (Sun et al, 2015). One recent study has shown that *Yap* is overexpressed in human granulosa cell tumors relative to GCs present in normal follicles (Fu et al, 2014). This study further showed that knockdown of *Yap* expression in a granulosa tumor cell line significantly reduced cell proliferation, migration, *Cyp19a1* expression and estrogen synthesis. The relevance of these findings to the biology of normal GCs remains to be determined.

The first direct evidence of the involvement of the Hippo pathway in follicle development came from two studies that examined the mechanisms underlying follicle growth that occurs following ovarian fragmentation and allo-transplantation (Cheng et al, 2015; Kawamura et al, 2013). This follicle growth was associated with the disruption of Hippo signaling, a decrease in YAP phosphorylation and an increase in the expression of YAP-TEAD transcriptional targets, including the growth factor *Ctgf* (also known as *Ccn2*). Treatment with verteporfin (a small molecule that inhibits the interaction between YAP and TEAD (Liu-Chittenden et al, 2012)) blocked fragmentation-induced increases in *Ctgf* expression and follicle growth (Kawamura et al, 2013). Conversely, treatment of the grafted ovaries with drugs that enhance actin polymerization increased nuclear YAP expression, along with *Ctgf* mRNA levels and follicle growth (Cheng et al, 2015). Neutralization of CCN2 suppressed 75% of fragmentation-induced allograft growth (Kawamura et al, 2013), suggesting that *Ctgf* is a major YAP-TEAD target gene responsible for follicle growth. Importantly, a previous study of *Ctgf* function in GCs using conditional knockout models showed that *Ctgf* is required for normal follicle development and female fertility in mice (Nagashima et al, 2011).

On the basis of the aforementioned studies, we hypothesized that YAP/TAZ could be required for follicle development in the physiological context. In this report, we show that a surge of Hippo signaling and YAP expression occurs in GCs following hCG/LH treatment, and that this surge appears to be PKA-dependent. Knockdown of *Yap/Taz* in primary cultures blunted the ability of GCs to respond to LH. This effect was attributed to a partial loss of *Lhcgr* expression in *Yap* or *Taz*-depleted cells. Together, our data show a novel role for *Yap* and *Taz*

in determining LH responsiveness, and suggest that Hippo signaling may play a broad role in regulating gonadotropin-driven follicle development.

3.3 MATERIALS AND METHODS

ANIMAL MODELS AND TISSUE COLLECTION

C57BL/6J wild-type mice (referred to as WT) were purchased from The Jackson Laboratory and mice bearing floxed alleles for *Yap/Taz* (referred to as $Yap^{flox/flox};Taz^{flox/flox}$) were graciously provided by Eric Olson at UT Southwestern. Genotyping analyses were performed on DNA extracted from tail biopsies with the following oligonucleotides: *Taz* forward 5'-GGCTTGTGACAAAGAACCTGGGGCTATCTGAG-3', *Taz* reverse 5'-CCCACAGTTAAA TGCTTCTCCCAAGACTGGG-3' (generates a floxed band of 655bp and a WT band of 496bp), *Yap* forward 5'-ACATGTAGGTCTGCATGCCAGAGGAGG-3', *Yap* reverse 5'AGGCTGAG ACAGGAGGATCTCTGTGAG-3' (generates a floxed band of 600bp and a WT band of 457bp). The PCR conditions were: 2 min at 94ºC for one cycle, 30 sec at 94ºC, 30 sec at 60ºC, and 40 sec at 72ºC for 35 cycles, and 4 min at 72ºC for one cycle.

Immature (22- to 25-day old) female mice were stimulated with equine chorionic gonadotropin (eCG; 5 IU i.p.; Folligon; Intervet), followed or not 48h later by an ovulatory dose of human chorionic gonadotropin (hCG; 5 IU i.p.; Chorulon; Intervet). Intact ovaries obtained 48h after eCG, and 4, 8, 12, 16, 24, 48h after hCG were fixed in 10% formalin for immunohistochemical analyses. Ovaries collected 48h after eCG, and 4, 8, 12h after hCG were punctured to release GCs and flash frozen for immunoblotting and real-time PCR (RT-qPCR) analyses. All animal procedures were approved by the institutional animal care and use committee and conformed to the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

CELL CULTURE

Immature WT female mice were stimulated with eCG for 48h and then their ovaries were collected. Ovaries were placed in HBSS and punctured using 26 gauge needles to release the GCs (Zeleznik et al, 1974). GCs were seeded onto 96-well plates at a density of 0.5 ovaries

per well in MEM media (ThermoFisher Scientific) supplemented with sodium pyruvate (0.25mM, ThermoFisher Scientific), L-glutamine (3mM, Wisent Inc.), Pen-Strep (Wisent), and 1% fetal bovine serum (FBS; Wisent) for 3 hours at 37ºC. Cells were serum starved for 2 hours in MEM media at 37ºC before treatment with hLH (National Hormone & Peptide Program/LA Biomedical Research Institute) at 50ng/ml for 5, 15, 30, 60 min, 2, 4, 6 hours.

Alternatively, cells were pre-treated with inhibitors against PKA (H-89, 50μM for 30 min, Tocris #2910; PKA inhibitor 14-22 amide, 50μM for 30 min, EMD Millipore # 476485), MEK1/2 (UO126, 10μM for 60 min, Selleckchem #S1102), or AKT1/2/3 (MK-2206, 10μM for 60 min, Selleckchem #S1078) prior to treatment with hLH at 50ng/ml for 30 min or 2 hours.

Yap$^{flox/flox}$, *Taz*$^{flox/flox}$, and *Yap*$^{flox/flox}$;*Taz*$^{flox/flox}$ granulosa cells primed with eCG for 48h were seeded onto 96-well plates (0.5 ovaries/well) in MEM medium supplemented with 2% FBS for 4 hours before infection with either Ad5-CMV-eGFP (control) or Ad5-CMV-Cre-eGFP (cre-expressing adenovirus; Vector Development Lab, Baylor College of Medicine) for 18 or 24h in 2% FBS using a MOI 50 (this generated a 10-fold knockdown of *Yap/Taz* and an infection efficiency of 80%). After infection, cells were serum starved for 2h before treatment with hLH at 50ng/ml for 30 min to 2 hours.

For all of the experiments described above, cells were then flash frozen for subsequent immunoblotting and real-time RT-PCR analyses.

REAL-TIME RT-PCR ANALYSES

Total RNA from granulosa cells was extracted using the RNeasy mini kit (Qiagen) and RNA was reverse transcribed using the SuperScript VILO cDNA synthesis kit (ThermoFisher Scientific), according to the manufacturer's instructions. Real-time qPCR was done with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Real-Time System/ C1000 Touch Thermal Cycler (Bio-Rad). PCR reactions consisted of 2.3 ul of H_20, 6 pmol of each forward and reverse gene-specific primer, and 7.5 ul of SYBR Green Supermix. The thermal cycling program consisted of 3 min at 95ºC once, 45 sec at 95ºC, 30 sec at 60ºC, and 15 sec at 72ºC for 39 cycles. Relative mRNA levels were determined using the mathematical model established by Pfaffl (Pfaffl 2001), which quantifies gene expression of a target gene relative to a reference gene (we used *Rpl19*) and Bio-Rad CFX Manager software. Primer sequences for specific genes can be found in Table 1.

IMMUNOHISTOCHEMISTRY

Immunohistochemical (IHC) analyses were performed on formalin-fixed, paraffin-embedded, 3 μm ovarian sections. Sections were probed with primary antibodies against Phospho-LATS1(Thr1079) and Phospho-YAP(S127); antibody sources can be found in Table 2. Vectastain Elite ABC HRP Kit (Vector Laboratories, # PK-6101) was used as directed by the manufacturer, followed by staining using the DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, #SK-4100), and counterstaining with hematoxylin before mounting.

IMMUNOBLOTTING

Granulosa cells isolated from ovaries treated with eCG for 48 hours followed by hCG on a time course or frozen granulosa cells (as described above) were lysed in SDS loading buffer, resolved on 10% SDS-polyacrylamide gels, and transferred onto Immobilon-P PVDF membrane (Millipore, IPVH00010). Membranes were blocked with 5% non-fat dry milk, and sequentially probed with the antibodies mentioned above in addition to LATS1, YAP, Phospho-YAP(S397), Phospho-AKT(S473), AKT, Phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204), p44/42 MAPK(Erk1/2), Phospho-CREB(S133), CREB (see Table 2 for sources), diluted in 5% bovine serum albumin (Bioshop Canada Inc. #ALB001) overnight at 4^{o}C, and β-actin diluted in 5% milk for 1 hour at room temperature. Membranes were then probed with anti-rabbit IgG HRP Conjugate diluted in 5% milk for 1 hour at room temperature. Immunosignal was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, WBKLS0500), the images were captured with ChemiDoc MP Imaging System (Bio-Rad) and analyzed with Image Lab 5.0 software (Bio-Rad).

STATISTICAL ANALYSES

Data are presented as means ± SEM. Effects of gonadotropins on Hippo pathway effectors and target genes, and effects of loss of *Yap* and *Taz* on gonadotropin responsiveness were analyzed by One-Way ANOVA followed by Tukey's multiple comparisons test to identify differences between groups. $P \leq 0.05$ was considered statistically significant. Effect of loss of *Yap, Taz,* and *Yap/Taz* on *Lhcgr* expression was analyzed by Student's t-test. Analyses were done using GraphPad Prism version 6.01 (GraphPad Software Inc.,) software.

3.4 RESULTS

LH activates the Hippo signaling pathway in granulosa cells *in vivo* and *in vitro*

To study the expression of Hippo pathway effectors throughout follicle development, ovaries were obtained from immature mice (22-25d) that had been treated on a time course with eCG for up to 48h to promote follicle growth, followed (or not) by hCG treatment for up to 12h to induce ovulation/luteinization. YAP, TAZ, LATS1 and LATS2 protein levels and phosphorylation were analyzed in whole ovaries by immunohistochemistry and in isolated granulosa cells by immunoblotting. eCG treatment did not result in clear changes in the expression levels of any Hippo pathway effectors (not shown). However, hCG treatment resulted in a transient increase in LATS1(Thr1079) and YAP (Ser127 and Ser397) phosphoproteins at 4h post-hCG (Fig. 1A). Similar results were obtained by immunohistochemistry, which showed an increase in phospho-LATS1(Thr1079) and phospho-YAP(S127) levels between 0h and 4h post-hCG in the granulosa cells of antral follicles (Fig. 1B). Total TAZ levels were not affected by hCG, whereas total YAP levels increased modestly in tandem with the increase in YAP phosphoproteins (Fig. 1A). LATS2 protein levels were beneath the detection threshold.

RT-qPCR analyses of Hippo pathway effectors in granulosa cells showed that hCG induced modest increases in *Lats1* and *Lats2* mRNA levels 12h post-treatment, whereas *Yap* and *Taz* transcript levels were not affected. Interestingly, *Tead1* and *Tead4* mRNA levels increased transiently in tandem with the increase in YAP/phospho-YAP/phospho-LATS1 at 4h post-hCG, as did the mRNA levels of the Hippo pathway target genes *Ctgf* and *Areg* (Fig. 1C).

We also investigated the regulation of Hippo signaling by LH *in vitro.* Granulosa cells were isolated from eCG-primed immature mice, placed in culture, and treated with LH on a time course. As observed *in vivo*, transient increases in phospho-YAP(Ser127) and phospho-LATS1(Thr1079) were observed in response to LH, with peak levels being observed 30 minutes after treatment (Figs. 2, 3, S2). Taken together, these results suggest that LH/hCG signaling induces a transient surge in Hippo pathway activity.

LH appears to signal via PKA to phosphorylate YAP

To determine how LH activates Hippo signaling, we evaluated three signaling pathways that are activated downstream of the LH receptor, cAMP/PKA, PI3K-AKT, and MAPK/ERK (Richards & Pangas, 2010b). eCG-primed wild-type granulosa cells were placed in culture and pretreated (or not) with the PKA inhibitors H-89 and PKI, the AKT1/2/3 inhibitor MK-2206, or the MEK1/2 inhibitor UO126, followed (or not) by treatment with LH. Treatment with inhibitors had no effect on basal levels of YAP phosphorylation (Fig. 3). LH treatment increased YAP phosphorylation at Ser127 and Ser397, which was partially inhibited by H89 (Fig 3, S2) and PKI (Fig. S2). MK-2206 or UO126 alone (Fig. 3) or in combination (Fig. S1), had no effect on LH-induced YAP phosphorylation. Likewise, concomitant pretreatment with H-89 and either MK-2206 or UO126 did not have a greater effect than H-89 alone (Fig. 3, S1). All inhibitors were effective, as they were able to inhibit LH-dependent phosphorylation of CREB (H-89, PKI), ERK (UO126) and AKT (MK-2206) (Fig 3, S1). Together, these results suggest that LH acts via the PKA pathway to activate Hippo signaling in granulosa cells.

Loss of *Yap* and *Taz* blunts LH responsiveness

To determine whether LH requires *Yap* and *Taz* to exert its effects, granulosa cells were isolated from mice bearing floxed alleles for *Yap, Taz*, or both *Yap* and *Taz*, placed in culture and infected with adenoviruses to drive expression of eGFP (Ad-eGFP, control) or cre recombinase (Ad-cre, to inactivate the floxed alleles), followed (or not) by treatment with LH. In this model, knockdowns of *Yap* and *Taz* mRNA levels of ≈10-fold were achieved by 18 hours following adenovirus treatment (Fig. 4A, B, C). Although loss of *Yap*, *Taz* or both did not affect the basal level of expression of any LH target genes, LH-induced target gene mRNA expression was significantly impaired. Loss of *Yap* blunted the response of *Btc*, *Star*, *Pgr* and *Tnfaip6* to LH (Fig. 4A), whereas loss of *Taz* blunted the induction of *Areg*, *Btc, Star, Pgr, Ptgs2*, and *Tnfaip6* (Fig. 4B). Similar (but not greater) effects were obtained when *Yap* and *Taz* were knocked down concomitantly (Fig. 4C), suggesting that *Yap* and *Taz* function in a non-redundant manner in granulosa cells, as both must be present to permit normal LH induction of its target genes. Our results therefore demonstrate that both *Yap* and *Taz* are required for LH signaling in granulosa cells.

Yap and *Taz* are required for *Lhcgr* expression

To investigate a potential mechanism whereby loss of *Yap* and *Taz* results in blunted induction of LH target genes, we examined *Lhcgr,* whose expression is induced in mural granulosa cells in preovulatory follicles and is essential for LH signaling. Knockdown of *Yap*, *Taz,* or both *in vitro* resulted in a significant drop in *Lhcgr* mRNA levels (Fig. 5), indicating that *Yap* and *Taz* are required for the expression of *Lhcgr* in granulosa cells.

3.5 DISCUSSION

The results from this study show for the first time that LH is a regulator of Hippo signaling in ovarian granulosa cells and that it appears to signal via PKA to activate Hippo. Additionally, we show that *Yap* and *Taz* are necessary for the expression of *Lhcgr* and consequently the induction of LH target genes. These results provide an additional level of understanding and complexity to the components of the signaling cascade that are initiated after the LH surge, and provide a solid foundation for future studies of Hippo signaling in the ovary.

Our results demonstrate that LH activates Hippo signaling by inducing the phosphorylation of LATS1 at Thr1079 and YAP at Ser127 and Ser397. Phosphorylation of YAP at Ser127 and Ser397 are the two most critical sites for YAP inactivation by LATS (Zhao et al, 2009). Phosphorylation at Ser127 is associated with YAP binding to 14-3-3, which retains YAP in the cytoplasm, while phosphorylation of YAP at Ser397 primes YAP for subsequent phosphorylation by CK1, leading to ubiquitin-mediated degradation (Piccolo et al, 2014; Zhao et al, 2010b). Given that there is no discernable change in total YAP protein levels following activation of Hippo, it is possible that YAP subcellular localization (as dictated by phospho-YAP(S127)) is the predominant mechanism regulating its activity, such that YAP transcriptional co-activity is rapidly turned on and off by shuttling YAP in and out of the nucleus, respectively.

Our results suggest that LH signals via PKA to phosphorylate YAP. Although results from the Lim laboratory showed that PKA directly phosphorylates LATS to promote phosphorylation of YAP(Ser397) in mouse embryonic fibroblasts (Kim et al, 2013), we were unable to generate evidence of this in the present study. Indeed, our data indicates that LH induces the phosphorylation of LATS1 at Thr1079, which is the specific phosphorylation site

by MST1/2 (Chan et al, 2005; Hergovich et al, 2006a), indicating that PKA may act upstream of LATS to affect the activity of MST1/2 or a kinase with similar specificity. Further experiments are underway that will validate LH-induced Hippo activity via PKA.

Our results demonstrate that following the depletion of *Yap/Taz*, LH is unable to adequately induce the expression of its target genes in granulosa cells. This appears to be at least in part as a result of the loss of *Lhcgr* expression; the mechanism by which this occurs remains unknown. It is possible that *Lhcgr* is a direct target gene of YAP/TAZ. Indeed, several YAP/TAZ binding partners, including transcription factors RUNX1, TBX5, PAX3, MYOD, and GLI3 are all expressed in the ovary (Uhlen et al, 2015) and can bind the *Lhcgr* promoter (Cartharius et al, 2005). Although granulosa cell dependence on YAP/TAZ for *Lhcgr* expression remains the simplest explanation as to why loss of *Yap/Taz* results in a blunted LH response, there is also evidence in the literature to suggest that EGFR/ERK and AKT signaling are modulated by YAP/TAZ. Notably, TAZ regulates the EGFR/AKT/ERK pathway in glioblastoma (Yang et al, 2016), while inhibition of YAP led to inhibition of the EGFR/PI3K/AKT pathway in breast cancer cells (Andrade et al, 2017). Further studies will therefore be required to determine if YAP/TAZ affect LH signaling mainly by controlling *Lhcgr* expression, or if they also act downstream of LHCGR to modulate the activity of downstream pathways. Our observation that Hippo signaling is induced by LH certainly supports the latter hypothesis.

Alternatively, the loss of *Lhcgr* expression might be due to YAP/TAZ crosstalk with the Wnt/β-catenin signaling pathway. YAP, TAZ, and β-catenin have been shown to interact in the nucleus to regulate the transcriptional co-activation of Hippo and Wnt target genes. For example, Azzolin *et al.* identified that TAZ is necessary for the transcription of several Wnt target genes (Azzolin et al, 2012), while Heallen *et al.* demonstrated that both YAP/TEAD and β-catenin/TCF bind to the promoters of *Sox2* and *Snai2* to regulate their transcription (Heallen et al, 2011). Interestingly, a direct link was made between β-catenin and *Lhcgr* expression. FSH stimulated the PKA-dependent phosphorylation of β-catenin at Ser552 and Ser675 to induce the expression of *Lhcgr*. In addition, phospho-β-catenin(Ser 552 and Ser675) and its transcription factor binding partner, TCF3, were bound to the *Lhcgr* promoter in response to FSH, identifying *Lhcgr* as a direct β-catenin target gene (Law et al, 2013). Whether loss of *Yap/Taz* impacted

canonical Wnt signaling in our model and thereby affected *Lhcgr* expression will be grounds for further investigation.

One important question that arises is which role(s) do(es) Hippo play in ovarian follicle development during the late stages of follicle development? Initially, we sought to evaluate this by generating granulosa cell-specific knockout mice for *Lats1/2* and *Yap/Taz* (using Tg(*CYP19A1*-cre)1Jri strain that drives recombination in antral stage follicles (Fan et al, 2008b)). Unexpectedly, mice either exhibited partial sex reversal (manuscript in preparation) or recombination efficiency was poor (unpublished data), precluding informative *in vivo* functional studies. Our *in vitro* results showed that LH induces a transient peak of Hippo activity and that in the absence of *Yap/Taz, Lhcgr* mRNA expression drops. Taken together, this might represent a mechanism whereby LH uses the Hippo pathway to downregulate its own receptor in order to modulate the LH response. The mechanism by which *Lhcgr* gets downregulated following the LH surge is incompletely understood, but appears to be as a result of increased mRNA degradation rather than decreased synthesis (Menon & Menon, 2014; Menon et al, 2010). How the stability of *Lhcgr* mRNA might be linked to *Yap/Taz* represents another avenue of investigation to address in the future.

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3.7 REFERENCES

1. Andrade, D., M. Mehta, J. Griffith, J. Panneerselvam, A. Srivastava, T. D. Kim, R. Janknecht, T. Herman, R. Ramesh and A. Munshi (2017). "YAP1 inhibition radiosensitizes triple negative breast cancer cells by targeting the DNA damage response and cell survival pathways." Oncotarget **8**(58): 98495-98508.

2. Azzolin, L., F. Zanconato, S. Bresolin, M. Forcato, G. Basso, S. Bicciato, M. Cordenonsi and S. Piccolo (2012). "Role of TAZ as mediator of Wnt signaling." Cell **151**(7): 1443-1456.
3. Cartharius, K., K. Frech, K. Grote, B. Klocke, M. Haltmeier, A. Klingenhoff, M. Frisch, M. Bayerlein and T. Werner (2005). "MatInspector and beyond: promoter analysis based on transcription factor binding sites." Bioinformatics **21**(13): 2933-2942.
4. Chan, E. H., M. Nousiainen, R. B. Chalamalasetty, A. Schafer, E. A. Nigg and H. H. Sillje (2005). "The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1." Oncogene **24**(12): 2076-2086.
5. 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." Faseb j **29**(6): 2423-2430.
6. Fu, D., X. Lv, G. Hua, C. He, J. Dong, S. M. Lele, D. W. Li, Q. Zhai, J. S. Davis and C. Wang (2014). "YAP regulates cell proliferation, migration, and steroidogenesis in adult granulosa cell tumors." Endocr Relat Cancer **21**(2): 297-310.
7. 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.
8. Hergovich, A., D. Schmitz and B. A. Hemmings (2006). "The human tumour suppressor LATS1 is activated by human MOB1 at the membrane." Biochem Biophys Res Commun **345**(1): 50-58.
9. Hossain, Z., S. M. Ali, H. L. Ko, J. Xu, C. P. Ng, K. Guo, Z. Qi, S. Ponniah, W. Hong and W. Hunziker (2007). "Glomerulocystic kidney disease in mice with a targeted inactivation of Wwtr1." Proc Natl Acad Sci U S A **104**(5): 1631-1636.
10. Jho, E. H. (2018). "Hippo signaling: Special issue of BMB Reports in 2018." BMB Rep **51**(3): 105.
11. Kawamura, K., Y. Cheng, N. Suzuki, M. Deguchi, Y. Sato, S. Takae, C. H. Ho, N. Kawamura, M. Tamura, S. Hashimoto, Y. Sugishita, Y. Morimoto, Y. Hosoi, N. Yoshioka, B. Ishizuka and A. J. Hsueh (2013). "Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment." Proc Natl Acad Sci U S A **110**(43): 17474-17479.
12. 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." Embo j **32**(11): 1543-1555.
13. Law, N. C., J. Weck, B. Kyriss, J. H. Nilson and M. Hunzicker-Dunn (2013). "Lhcgr expression in granulosa cells: roles for PKA-phosphorylated beta-catenin, TCF3, and FOXO1." Mol Endocrinol **27**(8): 1295-1310.
14. 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 Dev **26**(12): 1300-1305.
15. Makita, R., Y. Uchijima, K. Nishiyama, T. Amano, Q. Chen, T. Takeuchi, A. Mitani, T. Nagase, Y. Yatomi, H. Aburatani, O. Nakagawa, E. V. Small, P. Cobo-Stark, P. Igarashi, M. Murakami, J. Tominaga, T. Sato, T. Asano, Y. Kurihara and H. Kurihara (2008). "Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ." Am J Physiol Renal Physiol **294**(3): F542-553.

16. Menon, K. M. and B. Menon (2014). "Regulation of luteinizing hormone receptor expression by an RNA binding protein: role of ERK signaling." Indian J Med Res **140 Suppl**: S112-119.
17. Menon, K. M., B. Menon, L. Wang, T. Gulappa and M. Harada (2010). "Molecular regulation of gonadotropin receptor expression: relationship to sterol metabolism." Mol Cell Endocrinol **329**(1-2): 26-32.
18. Morin-Kensicki, E. M., B. N. Boone, M. Howell, J. R. Stonebraker, J. Teed, J. G. Alb, T. R. Magnuson, W. O'Neal and S. L. Milgram (2006). "Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65." Mol Cell Biol **26**(1): 77-87.
19. Nagashima, T., J. Kim, Q. Li, J. P. Lydon, F. J. DeMayo, K. M. Lyons and M. M. Matzuk (2011). "Connective tissue growth factor is required for normal follicle development and ovulation." Mol Endocrinol **25**(10): 1740-1759.
20. 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.
21. Pan, D. (2010). "The hippo signaling pathway in development and cancer." Dev Cell **19**(4): 491-505.
22. Piccolo, S., S. Dupont and M. Cordenonsi (2014). "The Biology of YAP/TAZ: Hippo Signaling and Beyond." Physiol Rev **94**(4): 1287-1312.
23. Pisarska, M. D., F. T. Kuo, I. K. Bentsi-Barnes, S. Khan and G. M. Barlow (2010). "LATS1 phosphorylates forkhead L2 and regulates its transcriptional activity." Am J Physiol Endocrinol Metab **299**(1): E101-109.
24. Richards, J. S. and S. A. Pangas (2010). "The ovary: basic biology and clinical implications." J Clin Invest **120**(4): 963-972.
25. St John, M. A., W. Tao, X. Fei, R. Fukumoto, M. L. Carcangiu, D. G. Brownstein, A. F. Parlow, J. McGrath and T. Xu (1999). "Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction." Nat Genet **21**(2): 182-186.
26. Uhlen, M., L. Fagerberg, B. M. Hallstrom, C. Lindskog, P. Oksvold, A. Mardinoglu, A. Sivertsson, C. Kampf, E. Sjostedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani, C. A. Szigyarto, J. Odeberg, D. Djureinovic, J. O. Takanen, S. Hober, T. Alm, P. H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J. M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen and F. Ponten (2015). "Proteomics. Tissue-based map of the human proteome." Science **347**(6220): 1260419.
27. Yang, R., Y. Wu, J. Zou, J. Zhou, M. Wang, X. Hao and H. Cui (2016). "The Hippo transducer TAZ promotes cell proliferation and tumor formation of glioblastoma cells through EGFR pathway." Oncotarget **7**(24): 36255-36265.
28. Zeleznik AJ, Midgley AR, Jr., Reichert LE, Jr. (1974) Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. *Endocrinology* **95:** 818-825
29. Zhang, H., C. Y. Liu, Z. Y. Zha, B. Zhao, J. Yao, S. Zhao, Y. Xiong, Q. Y. Lei and K. L. Guan (2009). "TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition." J Biol Chem **284**(20): 13355-13362.

30. Zhao, B., J. Kim, X. Ye, Z. C. Lai and K. L. Guan (2009). "Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein." Cancer Res **69**(3): 1089-1098.
31. Zhao, B., L. Li, Q. Lei and K. L. Guan (2010). "The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version." Genes Dev **24**(9): 862-874.
32. 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 SCF(beta-TRCP)." Genes Dev **24**(1): 72-85.
33. Zhao, B., X. Ye, J. Yu, L. Li, W. Li, S. Li, J. Yu, J. D. Lin, C. Y. Wang, A. M. Chinnaiyan, Z. C. Lai and K. L. Guan (2008). "TEAD mediates YAP-dependent gene induction and growth control." Genes Dev **22**(14): 1962-1971.

3.8 FIGURES

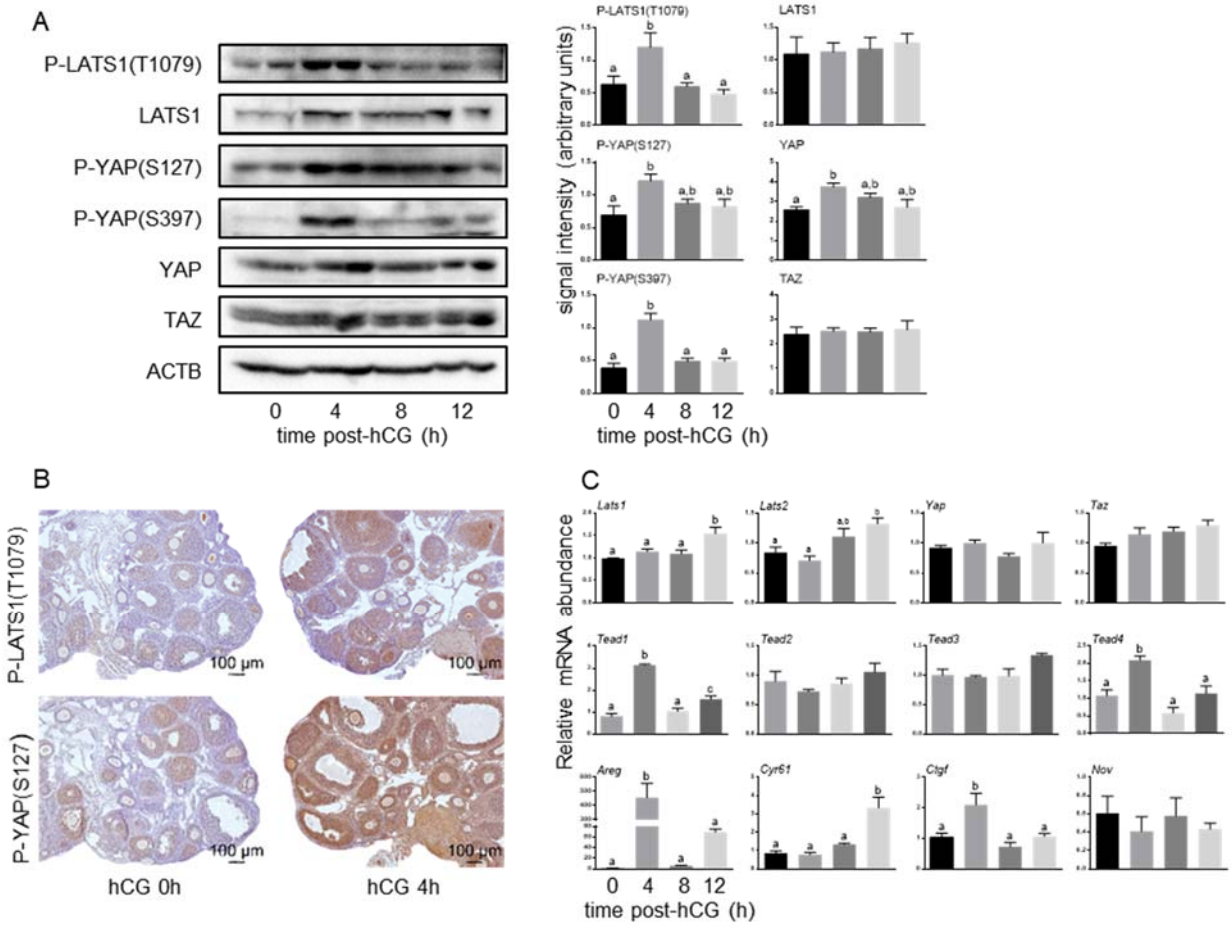

Figure 1. LH activates the Hippo pathway in granulosa cells *in vivo*

A) Immature (22-25d) female mice were injected with 5IU eCG i.p. 44-48h prior to administration of 5IU hCG i.p. Ovaries were collected on a time course and granulosa cells were isolated by needle puncture. Representative immunoblots show 2 replicates per time point while quantification was done on 4 replicates per time point. β-Actin (ACTB) was used as the loading control.

B) Representative images of phospho-LATS1(Thr1079) and phospho-YAP(S127) immunohistochemistry on ovarian sections from immature mice primed with 5IU eCG for 44-48h prior to administration or not of 5IU hCG for 4h.

C) RT-qPCR analysis of Hippo pathway effectors and reported Hippo target genes was performed on isolated granulosa cells derived from eCG-primed immature mice treated with hCG on a time course (n=3 mice/time point). All data were normalized to the housekeeping

gene *Rpl19.* Data are represented as means ± SEM. Different letters above histograms indicate significant differences between groups. $P \leq 0.05$.

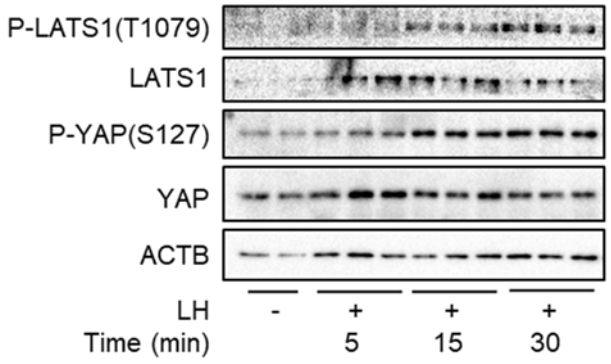

Figure 2. LH activates the Hippo pathway in granulosa cells *in vitro*

Primary cultured granulosa cells were treated with or without 50ng/ml LH on a time course and the expression of the indicated proteins was evaluated by Western blotting. Representative immunoblots show 3 replicates per time point (2 replicates for control). β-Actin (ACTB) was used as the loading control.

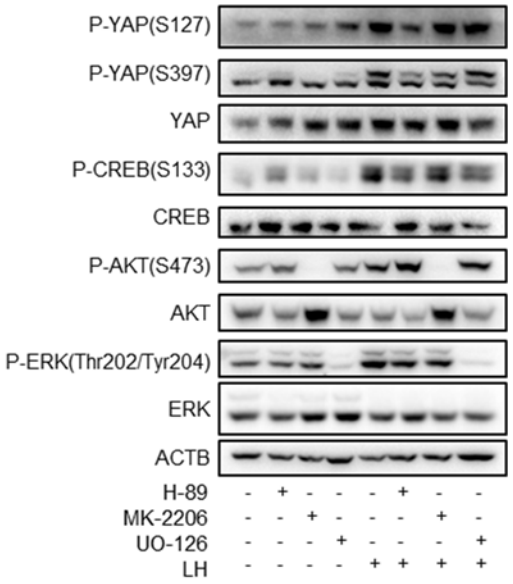

Figure 3. LH acts via PKA to phosphorylate YAP

Primary cultured granulosa cells were treated with or without 50μM H-89, 10μM MK-2206, or 10μM UO126 for 30-60 min followed by treatment with or without 50ng/ml LH for 30 min. Representative immunoblots show 1 replicate per treatment. CREB, AKT, and ERK are well-established substrates of PKA, AKT (by auto-phosphorylation), and MEK signaling in granulosa cells and confirmed the inhibition by H-89, MK-2206, and UO126, respectively. β-Actin (ACTB) was used as the loading control.

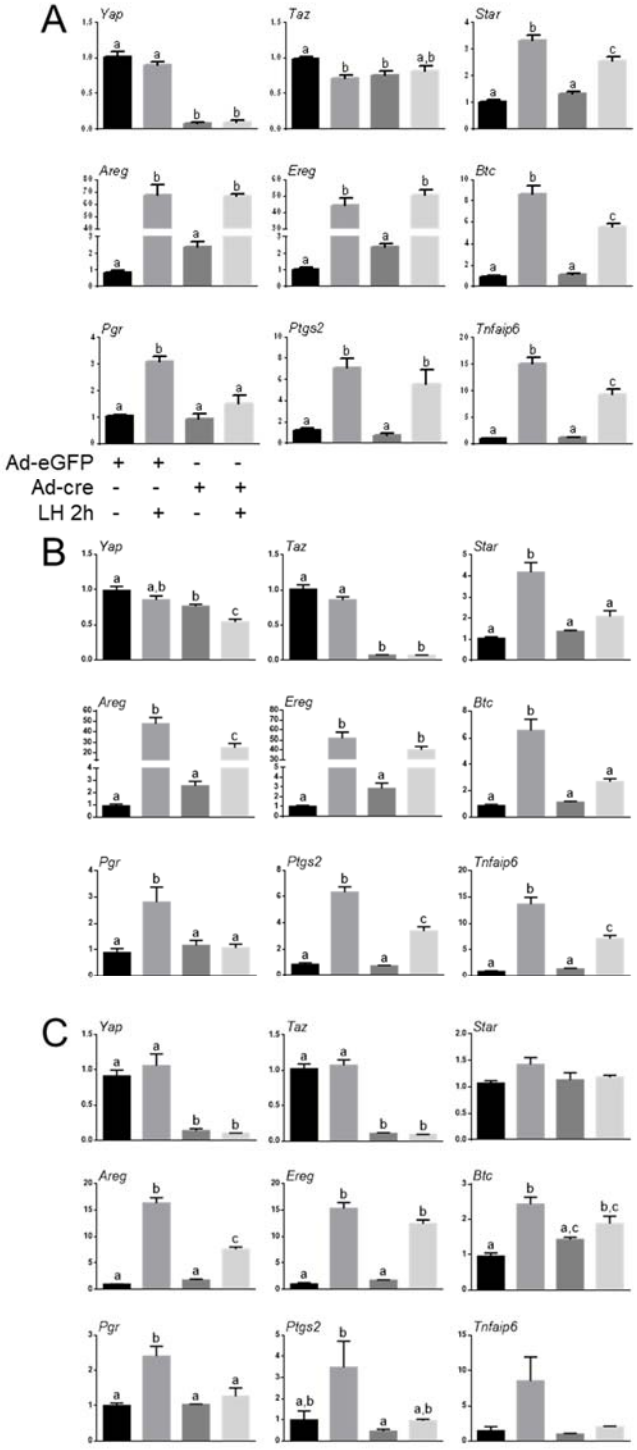

Figure 4. Loss of *Yap* and *Taz* blunts LH responsiveness

A) RT-qPCR analysis was performed on primary cultured *Yap* floxed granulosa cells infected with adenoviruses expressing either eGFP (as control; Ad-eGFP) or cre (Ad-cre; to knockdown

Yap) for 18 hours followed by treatment with or without 50ng/ml LH for 2 hours (n=4 replicates/treatment).

B) RT-qPCR analysis was performed on primary cultured *Taz* floxed granulosa cells and

C) *Yap*/*Taz* floxed granulosa cells infected with Ad-eGFP or Ad-cre followed by treatment with or without 50ng/ml LH for 2 hours (n=4 replicates/treatment). All data were normalized to the housekeeping gene *Rpl19.* Data are represented as means ± SEM. Different letters above histograms indicate significant differences between groups. $P \leq 0.05$.

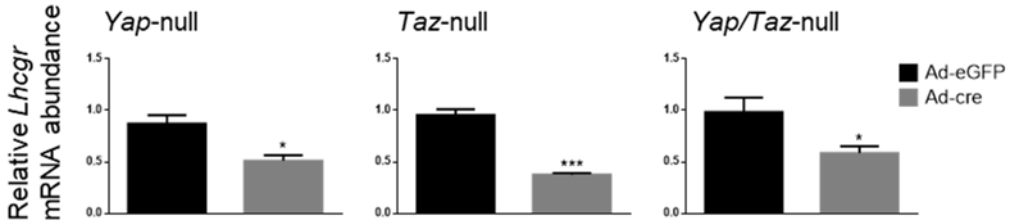

Figure 5. *Yap* and *Taz* are required for the expression of *Lhcgr*

RT-qPCR analysis was performed on primary cultured *Yap, Taz,* and *Yap/Taz* floxed granulosa cells infected with adenoviruses expressing either eGFP (Ad-eGFP; as control) or cre (Ad-cre) for 18 hours (n=4 replicates/time point). All data were normalized to the housekeeping gene *Rpl19.* Data are represented as means ± SEM. *P≤0.05 and ***P≤0.001, statistically significant differences between groups.

3.9 SUPPLEMENTAL FIGURES

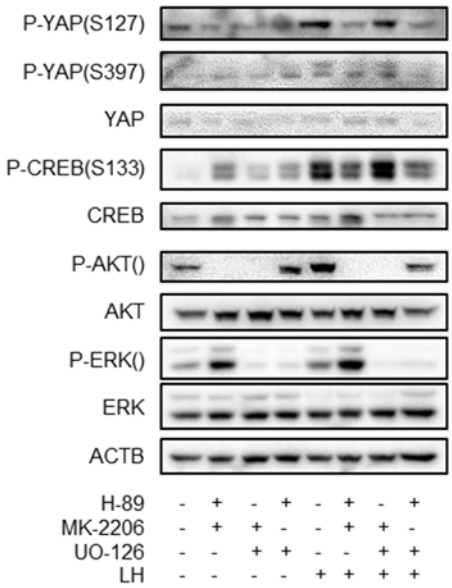

Figure S1. No additional effects on LH-induced YAP phosphorylation are observed when inhibitors are used in combination. Primary cultured granulosa cells were treated with or without 50μM H-89, 10μM MK-2206, or 10μM UO126 in combination for 30-60 min followed by treatment with or without 50ng/ml LH for 30 min. Representative immunoblots show 1 replicate per treatment.

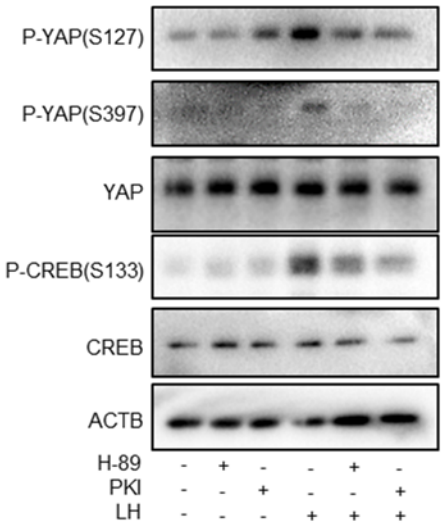

Figure S2. Two PKA inhibitors similarly reduce LH-induced YAP phosphorylation (Ser127 and Ser397). Primary cultured granulosa cells were treated with or without 50μM H-89 or 50μM PKA inhibitor 14-22 amide (PKI) for 30 min followed by treatment with or without 50ng/ml LH for 30 min. Representative immunoblots show 1 replicate per treatment.

3.10 TABLES

Primer	Sequence (5' to 3')
Areg F	CTCGCAGCTATTGGCATCGGCA
Areg R	TGGCATGCACAGTCCCGTTT
Btc F	GCATCCATGGGAGATGCCGCTT
Btc R	ACCACTATCAAGCAGACCACCAGG
Ctgf F	GAGGAAAACATTAAGAAGGGCAAAA
Ctgf R	CCGCAGAACTTAGCCCTGTA
Cyr61 F	TTGACCAGACTGGCGCTCT
Cyr61 R	AGTTTTGCTGCAGTCCTCGT
Ereg F	ACGTTGCGTTGACAGTGATTCTCAT
Ereg R	GGTCCCCTGAGGTCACTCTCTCAT
Lats1 F	AGCAGCACGTAGAGAACGTC
Lats1 R	TCTCATTTGATCCTGGGCATCT
Lats2 F	TGCACTGGATTCAGGTGGACTCA
Lats2 R	GAGAATGTGCCAGGCACCTCT
Lhcgr F	GCTGGAGTCCATTCAGACGCTCA
Lhcgr R	AGCATCTGGTTCTGGAGTACATTG
Nov F	AGAGTTGTTCTGAGATGAGACCC
Nov R	CCCTCTGGAACCATGCAAATG
Pgr F	TCCAGGTGACCCATGAGGAA
Pgr R	TTGCCTTGATCAATTCGCGG
Ptgs2 F	CCTGAAGCCGTACACATCATTTGA
Ptgs2 R	AGGCACTTGCATTGATGGTGGCT
Rpl19 F	CTGAAGGTCAAAGGGAATGTG
Rpl19 R	GGACAGAGTCTTGATGATCTC
StAR F	GATTAAGGCACCAAGCTGTGCTG
StAR R	CTGCTGGCTTTCCTTCTTCCAGC
Tead1 F	CCTGGCTATCTATCCGCCGT
Tead1 R	CCCGTTCTGAGTTTGATGTATCT
Tead2 F	ACCATCCTCCAGGTTGTGAC
Tead2 R	CCTCGTTCACTGGTGGAGAC
Tead3 F	GCATTAAGGCTATGAACCTGGAC
Tead3 R	TTTGGGCAGACGACATGGAT
Tead4 F	GGTGTATGGAGCCCCGAAAT
Tead4 R	CGATCAGCTCATTCCGACCATA
Taz F	ACTGGCCAGAGATACTTCCTTAATC
Taz R	AGGCTGATTCATCACCTTCCTG
Tnfaip6 F	TGAAGGTGGTCGTCTCGCAACC
Tnfaip6 R	TCCACAGTTGGGCCCAGGTTTCA

Yap F	GACGCTGATGAATTCTGCCTCA
Yap R	CATGGCAAAACGAGGGTCC

Table 1. Primer list

Antigen	**Cat#**	**Company**
Anti-rabbit IgG HRP Conjugate	W401B	Promega
Akt(pan)	4691	Cell signaling
β-actin HRP	47778	Santa Cruz
CREB	9197	Cell signaling
p44/42 MAPK (Erk1/2)	4695	Cell signaling
LATS1	3477	Cell signaling
P-Akt (Ser473)	4058	Cell signaling
P-CREB (Ser133)	9198	Cell signaling
P-p44/42 MAPK (Erk1/2)(Thr202/Tyr204)	4370	Cell signaling
P-LATS1 (Thr1079)	8654	Cell signaling
P-YAP (Ser127)	13008	Cell signaling
P-YAP (Ser397)	13619	Cell signaling
TAZ	4883	Cell signaling
YAP	14074	Cell signaling

Table 2. Antibody list

Chapter 4. Article 2

***Lats1* and *Lats2* are required for granulosa cell fate maintenance**

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This manuscript is in the final phase of preparation to be submitted to *Development*.

Author contributions: MT contributed to the experimental design, performed experiments, analyzed the results, and wrote the article; representing a relative contribution of 90%. CR performed experiments. MP analyzed the results. RJ generated the experimental animals. DB contributed to the experimental design, analyzed the results, and wrote the article.

4.1 ABSTRACT

An increasing amount of evidence in the literature suggests that the Hippo signaling pathway is required for normal ovarian follicle development, however the precise roles of the key Hippo effectors in ovarian cells remain unknown. The present study focuses on the roles of the kinases Large Tumor Suppressors 1 and 2 (LATS1/2) that are directly responsible for inactivating the transcriptional co-activators YAP and TAZ. To study this, we generated granulosa cell-specific knockout mice, *Lats1*$^{flox/flox}$*;CYP19*-cre, *Lats2*$^{flox/flox}$*;CYP19*-cre, and *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$*; CYP19*-cre, and quite unexpectedly, discovered that combined depletion of *Lats1/2* induced a loss of granulosa cell FOXL2 expression, promoted epithelial-to-mesenchymal transition (EMT), and transdifferentiation of granulosa cells into Sertoli-like cells and osteoblasts. Knockdown of *Lats1/2* was performed *in vitro* using *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$ granulosa cells infected with adenoviruses to drive the expression of either eGFP (Ad-eGFP; as control) or cre (Ad-cre, to knockdown *Lats1* and *Lats2*). *Lats1/2*-depleted granulosa cells no longer expressed granulosa cell-specific genes, but expressed Sertoli, osteoblast, neural crest, and stem cell-specific genes, reinforcing the fact that reprogramming of the granulosa cell fate had occurred. In addition, *Lats1/2*-depleted granulosa cells were unable to respond adequately to LH to induce the expression of LH target genes *Areg*, *Ereg*, *Btc*, *Ptgs2,* and *Star*, illustrating that granulosa cell function was also lost. Together, these results demonstrate for the first time a novel and exciting role for *Lats1/2* as critical mediators in the maintenance of the granulosa cell genetic program as well as suggest a potential role for *Lats1/2* in sex determination.

4.2 INTRODUCTION

Hippo is an evolutionarily conserved signaling pathway with a well-established role in organ size determination (Zhao et al, 2010a). It also acts to regulate processes such as cell fate determination, differentiation, proliferation, and apoptosis in a variety of cell types during embryogenesis (Pan, 2010; Piccolo et al, 2014). Hippo has no known specific ligands or receptors, but rather is activated by intracellular and extracellular cues, including the

establishment of cell-cell contacts and cytoskeletal changes (Dupont et al, 2011; Zhao et al, 2007). The pathway consists of a core kinase cascade beginning with the mammalian STE20-like protein kinases 1 and -2 (MST1/2). MST1 and -2 function in a redundant manner to phosphorylate the scaffold protein salvador (SAV1) and MOB kinase activator 1A and -1B (MOB1A/B)(Callus et al, 2006; Praskova et al, 2008). Whereas the interaction between phospho-SAV and MST1/2 serves to enhance kinase activity, phospho-MOB1A/B binds an autoinhibitory motif within the kinases large tumor suppressor 1 and -2 (LATS1/2), which permits their subsequent phosphorylation (and activation) by MST1/2 (Chan et al, 2005; Pearce et al, 2010). LATS1 and -2 both function to phosphorylate the transcriptional co-regulators Yes-associated protein (YAP) and transcriptional co-activator with PDZ binding motif (TAZ, also known as WWTR1). Upon phosphorylation, YAP and TAZ are sequestered in the cytoplasm and/or degraded by the cellular proteasomal machinery (Basu et al, 2003; Dong et al, 2007; Liu et al, 2010; Zhao et al, 2010b; Zhao et al, 2007). Disruption of the Hippo kinase cascade allows YAP and TAZ proteins to escape phosphorylation, accumulate within the cell and translocate to the nucleus, where they can bind to several transcription factors, notably those of the TEAD and RUNX families (Vassilev et al, 2001; Vitolo et al, 2007; Zhao et al, 2008). This results in the modulation of the transcriptional activity of a variety of target genes in a cell type- and context-specific manner (Pan, 2010; Piccolo et al, 2014).

The Hippo pathway is required to direct cell fate specification starting at very early stages of preimplantation embryonic development. Notably, a series of studies have shown that Hippo signaling must be differentially regulated in the inner and outer cells of the morula to permit their adoption of the inner cell mass or trophectoderm cell fates (Sasaki, 2017). The outer cells contain nuclear YAP that co-activates TEAD4 and the expression of trophectoderm-specific genes while the inner cells contain cytoplasmic YAP (Nishioka et al, 2009). Inactivation of *Tead4* leads to all cells adopting the inner cell mass fate (Nishioka et al, 2008). Hippo subsequently acts in different progenitor cell types to direct fate specification in a variety of embryonic and adult tissues (Fu et al, 2017). For instance, in the liver, the differentiation of hepatoblasts/progenitor cells into either hepatocytes or cholangiocytes is determined by YAP/TAZ expression and activity (Lee et al, 2016; Nguyen et al, 2015). Likewise, YAP/TAZ activity is necessary and sufficient for the pluripotent progenitor cells of the optic vesicle to adopt the retinal pigment epithelial cell fate (Miesfeld et al, 2015). In the kidney, Hippo

signaling also appears to direct progenitor cells to give rise either to nephron epithelial cells or myofibroblasts (McNeill & Reginensi, 2017). The ability of the Hippo pathway to direct cell fate decisions is further illustrated by its ability to induce transdifferentiation in cells already committed to a particular fate. For instance, YAP overexpression in adult hepatocytes causes them to transdifferentiate into biliary epithelial cells (Yimlamai et al, 2014). Likewise, Hippo signaling can alter cancer cell fate decisions, such as the transdifferentiation of lung adenocarcinoma to squamous cell carcinoma (Wang et al, 2017a).

With most research to date having focused on Hippo's involvement in embryogenesis and cancer, Hippo signaling in post-developmental, physiological contexts has only recently become intensively studied. In the ovary, early evidence of a role for Hippo signaling in follicle development came with the phenotypic analysis of *Lats1* knockout mice. The latter were found to be subfertile, and their ovaries contained reduced numbers of antral follicles, no corpora lutea, and developed stromal tumors later in life (St John et al, 1999). More recently, Kawamura *et al* studied follicle growth that is induced by ovarian injury, such as that which occurs when ovarian wedge resection, drilling, or grafting procedures are used in the context of infertility treatments. Using a mouse ovary fragmentation and allo-transplantation model, they showed that follicle growth is associated with the disruption of Hippo pathway signaling, as evidenced by a decrease in YAP phosphorylation and an increase in the mRNA levels of YAP-TEAD transcriptional targets (Kawamura et al, 2013). The latter study also showed that fragmentation-induced follicle growth could be blocked with verteporfin, a small molecule inhibitor of the interaction between YAP and TEAD (Liu-Chittenden et al, 2012). In a follow-up study, the same group showed that drugs that promote actin polymerization could enhance follicle growth, and do so by increasing nuclear accumulation of YAP and mRNA levels of its transcriptional targets (Cheng et al, 2015). Together these studies suggest that Hippo signaling is a negative regulator of follicle growth, at least in the context of ovarian injury.

Based on the aforementioned studies, we sought to determine if Hippo signaling plays a role in the context of physiological, gonadotropin-driven ovarian follicle development. Using a conditional gene targeting approach to inactivate *Lats1* and *Lats2* in ovarian granulosa cells, we unexpectedly found that loss of Hippo signaling causes rapid loss of granulosa cell fate. The targeted cells underwent epithelial-to-mesenchymal transition (EMT) with transdifferentiation into multiple cell types, notably leading to the formation of seminiferous tubules and bone.

Aberrant YAP/TAZ-mediated transcriptional activity of genes not normally expressed in the ovary that drive male sex determination and osteogenesis was suspected to be the mechanism underlying the transdifferentiation into multiple cell lineages. Together, our findings indicate a previously unsuspected role for Hippo signaling in maintaining granulosa cell fate, and further suggest that the Hippo pathway can impact the process of sex determination.

4.3 MATERIALS AND METHODS

ANIMAL MODEL

Mice bearing floxed alleles for *Lats1*, *Lats2*, and *Lats1/2* (*Lats1*$^{tm1.1Jfm}$ and *Lats2*$^{tm1.1Jfm}$, hereafter *Lats1*$^{flox/flox}$, *Lats2*$^{flox/flox}$, and *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$) were graciously provided by Randy Johnson (University of Texas). These mice were mated to the Tg(*CYP19A1*-cre)1Jri (hereafter *CYP19*-cre) strain (courtesy of Jan Gossen, Organon)(Fan et al, 2008b). Genotyping analyses were performed on DNA extracted from tail biopsies with the following oligonucleotides *Lats1*: forward 5'-TTGTTGCTGGTGTTGTTTCC-3', *Lats1* reverse 5'-ATGAATGAACCTGAGGCTGC-3' (generates a floxed band of 400 bp and a WT band of 250 bp), *Lats2* forward 5'-ATCCTAGCACTCAGGAGGCA-3', *Lats2* reverse 5'-ACACATTCCCCTCCACTGAC-3' (generates a floxed band of 400 bp and a WT band of 250 bp). The PCR conditions were: 3 min at 94ºC for one cycle, 15 sec at 94ºC, 30 sec at 55ºC, and 45 sec at 72ºC for 35 cycles, and 10 min at 72ºC for one cycle. A separate set of primers and conditions were used to detect the knockout bands: *Lats1* forward 5'-AGGATGTAGTGAAGGCGTGTAAC-3', *Lats1* reverse 5'-AGACCTCGTCGCACAGAATG-3' (generates a knockout band of 231 bp), *Lats2* forward 5'-CTATCGCTAGGCTGTTCCCAC-3', *Lats2* reverse 5'-CTGAGCAACGACTCCAGGAAC-3' (generates a knockout band of 258 bp). The PCR conditions were: 3 min at 94ºC for one cycle, 15 sec at 94ºC, 30 sec at 56ºC, and 45 sec at 72ºC for 35 cycles, and 10 min at 72ºC for one cycle. All animal procedures were approved by the institutional animal care and use committee and conformed to the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

MATING TRIAL

Six week-old *Lats1*$^{flox/flox}$*;CYP19*-cre, *Lats2*$^{flox/flox}$*;CYP19*-cre, and *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$*; CYP19*-cre female mice and control littermates *Lats1*$^{flox/flox}$*, Lats2*$^{flox/flox}$*,* and *Lats1*$^{flox/flox}$*/Lats2*$^{flox/flox}$ were placed in cages with 6 week-old C57BL/6J males for 6 months. Cages were monitored daily to record intervals between litters, litter sizes at birth, and at weaning. Males were removed after 6 months and the experiment concluded 22 days later (to wait for the final litter).

TISSUE COLLECTION

Ovaries were collected from 6 day, 10 day, 3 week, 1 month, 2 month, 4 month, and 5 month-old *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre female mice and control littermates and either flash-frozen followed by homogenization for WB or RT-qpCR analyses, fixed in 10% formalin, or embedded in OCT and frozen for IHC analyses. BrdU powder (Sigma-Aldrich #B5002) was reconstituted in sterile saline and administered at 100mg/kg i.p. for 3 hours prior to tissue collection.

CELL CULTURE

Ovaries from *Lats1*$^{flox/flox}$*, Lats2*$^{flox/flox}$*,* and *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ female mice primed with equine chorionic gonadotropin (eCG; 5 IU i.p.; Folligon; Intervet) for 44-48h were collected, placed in HBSS, and punctured using 26 gauge needles to release the GCs (Zeleznik et al, 1974). GCs were seeded onto 96-well plates (0.5 ovaries/well) in MEM medium (ThermoFisher Scientific) supplemented with sodium pyruvate (0.25mM, ThermoFisher Scientific), L-glutamine (3mM, Wisent Inc.), Pen-Strep (Wisent Inc.), and 2% fetal bovine serum (FBS; Wisent Inc.) for 4 hours before infection with either Ad5-CMV-eGFP (control; Ad-eGFP) or Ad5-CMV-Cre-eGFP (cre-expressing adenovirus; Ad-cre; Vector Development Lab, Baylor College of Medicine) for 18, 24, and 30 hours in 2% FBS with a MOI 50 (this generated a ≈65- and ≈15-fold knockdown of *Lats1* and *Lats2,* respectively). Cells were flash frozen for subsequent analyses by WB, RT-qPCR, and microarray analyses. Alternatively, after 18 hours of infection, cells were serum starved for 2 hours before treatment with hLH (National Hormone & Peptide Program/LA Biomedical Research Institute) at 50ng/ml for 2 hours and flash frozen for subsequent RT-qPCR analyses.

REAL-TIME RT-PCR AND MICROARRAY ANALYSES

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed using the SuperScript VILO cDNA synthesis kit (ThermoFisher Scientific) according to the manufacturer's instructions. Real-time qPCR was done with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Real-Time System/ C1000 Touch Thermal Cycler (Bio-Rad). PCR reactions consisted of 2.3 ul of H_20, 6 pmol of each forward and reverse gene-specific primer, and 7.5 ul of SYBR Green Supermix. The thermal cycling program consisted of 3 min at 95°C once, 45 sec at 95°C, 30 sec at 60°C, and 15 sec at 72°C for 39 cycles. Relative mRNA levels were determined using Bio-Rad CFX Manager software, with the mathematical model according to Pfaffl (Pfaffl, 2001), with *Rpl19* as the housekeeping gene. Primer sequences for specific genes can be found in Table S1. The specificity of all primer pairs was confirmed by sequencing.

Total RNA derived from $Lats1^{flox/flox};Lats2^{flox/flox}$ granulosa cells infected for 12 or 30 hours with either Ad-eGFP or Ad-cre was performed in duplicate for microarray analyses. Mouse Clariom S (Affymetrix) was used and all steps were done by the McGill University and Génome Québec Innovation Centre. Data were pre-processed using the Affymetrix Gene Expression Console software, and differential analysis of gene expression was done by the R package limma followed by a *t-test*. The Canadian Centre for Computational Genomics (C3G) assisted with data analysis. Functional annotation was performed using the web tool DAVID Bioinformatics Resources 6.8 (Huang da et al, 2009). A P value cut-off of 0.05 and a 2-fold change cut-off were used to identify differentially expressed genes.

IMMUNOHISTOCHEMISTRY

Immunohistochemical (IHC) analyses were performed on formalin-fixed, paraffin-embedded, 3 μm ovarian sections. Sections were probed with primary antibodies against LATS1, YAP, Phospho-YAP(S127), TAZ, SOX9, vimentin, CTNNB1, Cleaved Caspase-3, and Connexin 43(GJA1) (Cell Signaling Technology, #3477, 14074, 13008, 4883, 82630, 5741, 8480, 9661, 3512), LATS2 (Biorbyt #6306), FOXL2 (courtesy of Dagmar Wilhelm), and BrdU (Dako #M0744). Vectastain Elite ABC HRP Kit (Vector Laboratories, # PK-6101) was used as directed by the manufacturer, followed by staining using the DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, #SK-4100), and counterstaining with hematoxylin before mounting.

For mouse primary antibodies, the Mouse on Mouse (M.O.M.) Detection Kit (Vector Laboratories Inc., #PK-2200) was used according to the manufacturer's instructions. IHC for S100 (Agilent (Dako) #Z0311) was performed using the LabVision autostainer (ThermoFisher) and counterstained the slides with congo red (BioGenex). IHC for Alpl was performed on 4 μm ovarian sections embedded in OCT (Sakura Finetek USA). Slides were fixed in 0,2% gluteraldehyde in cold PBS for 10 mins, washed in AP buffer twice for 5 mins (100 mM Tris·HCl, pH 9.5, 100 mM NaCl, 10 mM MgCl2), then incubated in BCIP/NBT solution (100 mM Tris·HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40, 337 μg/ml NBT, 175 μg/ml BCIP; Sigma-Aldrich #72091) for 15 mins. Slides were counterstained with Nuclear Fast Red (5% sulfate d'aluminium, 0,1% Nuclear Fast Red; EMD Millipore #7602-8) for 5 min followed by standard dehydration steps.

IMMUNOBLOTTING

Frozen granulosa cells (as described above) or homogenized whole ovaries were lysed in SDS loading buffer, resolved on 10% SDS-polyacrylamide gels, and transferred onto Immobilon-P PVDF membrane (Millipore, IPVH00010). Membranes were blocked with 5% non-fat dry milk, and sequentially probed with the antibodies mentioned above, in addition to Phospho-YAP(S397) (Cell Signaling #13619) and Phospho-TAZ(S89) (Santa Cruz, *discontinued*), diluted in 5% bovine serum albumin (Bioshop Canada Inc. #ALB001) overnight at 4ºC, and β-actin (C4) HRP (Santa Cruz #47778) diluted in 5% milk for 1 hour at room temperature. Membranes were then probed with anti-rabbit IgG HRP Conjugate (Promega #W401B) diluted in 5% milk for 1 hour at room temperature. Immunosignal was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore, WBKLS0500), the images were captured with ChemiDoc MP Imaging System (Bio-Rad), and analyzed with Image Lab 5.0 software (Bio-Rad).

STEROID HORMONE MEASUREMENTS

Serum was collected from 3 week, 2 month, and 4-month-old adult mice. Serum estradiol (E2) and progesterone (P4) levels were measured by enzyme-linked immunosorbent assay (Calbiotech and IBL, respectively). Serum FSH and LH levels were determined by

multiplex testing (EMD Millipore) and radioimmunoassay (in-house protocol). All assays were performed by the Ligand Assay and Analysis Core at the University of Virginia.

STATISTICAL ANALYSES

Data is presented as mean ± SEM. Effects of *Lats1* and *Lats2* knockdown on LH responsiveness were analyzed by One-Way ANOVA followed by Tukey post-test to identify differences between groups. Effects of *Lats1* and *Lats2* ablation on ovarian size, serum hormone levels, and gene expression were analyzed by Student's t-test. $P \leq 0.05$ was considered statistically significant. Analyses were done using GraphPad Prism version 6.01 (GraphPad Software Inc.) software.

4.4 RESULTS

Ovarian tissue overgrowth and infertility in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice

To study the roles of *Lats1* and *-2* in the ovary, granulosa cell-specific conditional knockout mice were generated by mating strains bearing *Lats1* and *Lats2* floxed alleles to the *CYP19*-cre strain (Fan et al, 2008a; Park et al, 2016). Both *Lats1*$^{flox/flox}$;*CYP19*-cre and *Lats2*$^{flox/flox}$;*CYP19*-cre females were fertile, and had no obvious ovarian defects (not shown). Hypothesizing that the lack of phenotypic abnormalities in these mice was due to functional redundancy of *Lats1* and *Lats2* in granulosa cells, we generated *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice to inactivate both genes concomitantly. To assess fertility, five *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre females were placed in 6-month breeding trials with wild-type males. One of these mice produced two small litters, whereas the others were sterile (Table 1). Examination of the ovaries of *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice at different ages showed a dysregulation of postnatal development, with growth occurring at an accelerated pace and ovarian weights attaining ≈10-fold that of controls by 2 months of age, but not increasing significantly thereafter (Fig. 1A). The ovaries of adult *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice had a distinctive lobular appearance and hardened consistency (Fig. 1B). Analyses of serum hormone levels in 4 month-old mice showed that progesterone levels were ≈6-fold lower in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre animals relative to controls (2.51±0.73ng/ml *vs*

15.10±4.09ng/ml, mean±SEM, n = 8/genotype, P <0.01), and that estradiol levels were beneath the radioimmunoassay detection threshold. Conversely, vastly increased circulating FSH and LH levels were found in adult *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre females (Fig. S1), together suggesting that ovarian failure had occurred.

Depletion of *Lats1* and *Lats2* causes loss of granulosa cell identity, epithelial-to-mesenchymal transition, and transdifferentiation into Sertoli-like cells

Histopathologic analyses of ovaries from 6 day-old *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice showed no differences relative to controls (not shown). However, at 10 days of age (corresponding to the earliest reported expression of the *CYP19*-cre transgene (Fan et al, 2008b)), striking abnormalities were observed. Many follicles were found to contain a new population of cells featuring large nuclei, prominent nucleoli, and abundant cytoplasm (Fig. 2A). These changes occurred mostly in follicles located in the ovarian medulla, where the first wave of follicle development normally occurs (Sforza et al, 2003). Immunohistochemistry analyses revealed that the new cell type did not express the granulosa cell marker FOXL2 (Fig. 2B). Rather, it expressed the mesenchymal cell marker vimentin (Fig. 2C). Interestingly, ≈10-20% of the cells were also positive for the Sertoli cell marker SOX9 (Fig. 2D). BrdU incorporation assays demonstrated that these cells were not proliferative (Fig. 2E), and CASP3 immunohistochemistry (Fig. 2F) analyses showed that weren't undergoing apoptosis, unlike the adjacent granulosa cells. Together, these results suggest that the granulosa cells in the ovaries of *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice undergo EMT and transdifferentiate into a heterogeneous cell population. Increased ovarian size in these mice was apparently due to the larger size of the transdifferentiated cells relative to granulosa cells and to their evasion of apoptosis, but was not due to increased cell proliferation.

To determine how loss of *Lats1* and *Lats2* affected Hippo signaling in the ovaries of *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice, immunohistochemistry and immunoblotting analyses of Hippo pathway effectors and RT-qPCR analyses of Hippo target gene expression were conducted. These analyses confirmed that the transdifferentiated cells do not express *Lats1* or *Lats2* (Fig. 3A, B). Depletion of *Lats1/2* resulted in decreased phosphorylation of YAP at Ser127, and consequent increases in YAP (and TAZ) protein levels relative to the adjacent

normal-looking granulosa cells (Fig. 3C-E). Although recent reports have suggested that the stability of YAP/TAZ and that of the WNT pathway effector CTNNB1 (β-catenin) are interdependent (Azzolin et al, 2014; Azzolin et al, 2012), CTNNB1 levels were found to be decreased in the transdifferentiated cells (Fig. 3F). Similar decreases in LATS1, LATS2, phospho-YAP (both Ser 127 and Ser 397) and phospho-TAZ (Ser89) as well as increases in total YAP and TAZ levels were observed by immunoblot analyses of whole ovaries from 4 week-old *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice, relative to age-matched controls (Fig. 3G). The mRNA levels of the YAP/TAZ target genes *Cyr61, Ctgf,* and *Nov* were also increased in the ovaries of *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice (Fig. 3H). These results link the transdifferentiation and EMT processes observed in the *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre model to a disruption in the Hippo kinase cascade, with resultant accumulation of YAP/TAZ proteins and transactivation of their target genes.

Multi-lineage transdifferentiation in the ovaries of *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice

After 10 days of age, transdifferentation progressed throughout the granulosa cell population in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre mice, leading to ovaries almost entirely devoid of normal follicles. By one month of age, the ovaries were mainly composed of spindle-shaped cells organized into follicle-like structures delineated by basal lamina and with necrotic centers (Fig. 4A). The necrotic material appeared to consist of decaying granulosa cells and oocytes. GJA1 immunohistochemistry revealed that a broad loss of gap junctions occurred through the transdifferentiation process (Fig. 4B). As granulosa cells and oocytes rely on extensive gap junction networks for nutrient transport through the (avascular) granulosa cell layer, the remaining (non-transdifferentiated) granulosa cells and oocytes may therefore have degenerated due to nutrient deprivation.

By 3 months of age, a new cell population was occasionally observed within the follicle-like structures, featuring abundant eosinophilic granules in the cytoplasm (Fig. 4C), possibly of a neurosecretory or immune lineage. Furthermore, ≈10% of cells were found to be positive for S100 (Fig. 4D), a marker of cell types derived from the neural crest. Strikingly, by 4 months of age, many follicle-like structures composed of spindle-shaped cells were still present, but the majority of the ovary was occupied by a partially mineralized osteoid matrix (Fig. 4E).

Osteoblasts and osteocytes were clearly visible (Fig. 4E), and which stained positive for alkaline phosphatase (Fig. 4F). Cord-like structures were often found, consisting of prominent basal lamina lined with large ovoid cells with cytoplasmic veils extending into the lumen (Fig. 4G). The latter cells stained positive for SOX9 (Fig. 4H), and the structures were therefore defined as seminiferous tubules, although germ cells were absent. At 5 months of age, the ovaries were composed nearly entirely of bone, with presence of osteoblasts, osteoclasts, and bone marrow replete with hematopoietic cells (Fig. 4I).

Reprogramming of the granulosa cell line occurs in *Lats1/2*-depleted cells

To further investigate the molecular mechanisms underlying the phenotypic changes observed in the *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre model, we developed a primary granulosa cell culture system in which *Lats1* and *Lats2* could be inactivated acutely. Granulosa cells isolated from eCG-primed immature *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ mice were placed in culture and infected with adenoviruses to drive expression of either eGFP (Ad-eGFP, control) or cre (Ad-cre, to recombine the floxed alleles). Ad-cre treatment for 30 hours resulted in ≈65- and ≈15-fold reductions in *Lats1* and *Lats2* mRNA levels, respectively, and was accompanied by a loss of LATS1 protein and YAP phosphorylation, along with increased in total YAP levels (Fig. 5A, B). This was also accompanied by a dramatic loss in the expression of genes associated with granulosa cell differentiation and function, including *Wnt4*, *Fshr*, *Lhcgr,* and *Cyp19a1* (Fig. 5A). Loss of granulosa cell function was further evidenced by the blunted response of *Lats1/2*-depleted granulosa cells to LH, characterized by a significantly impaired induction of LH target genes such as *Areg*, *Ereg*, *Btc*, *Ptgs2,* and *Star* (Fig. 5C). Whereas the LH response was also blunted to some extent in granulosa cells in which *Lats1* or *Lats2* alone were depleted (Fig S2A, S2B), the effect was not nearly as pronounced as when they were depleted concomitantly, suggesting that *Lats1* and *Lats2* function in a partially redundant manner in this context. In addition to the loss of granulosa cell gene expression and function, *Lats1/2*-depleted granulosa cells also had dramatically increased mRNA levels of genes associated with Sertoli cells (Fig. 6). This was not observed in granulosa cells deficient in *Lats1* or *Lats2* alone (Fig. S2C). *Lats1/2*-depleted granulosa cells expressed increased levels of genes associated with osteoblasts and neural cell lineages, as well as markers of stem/progenitor cell types (Fig. 6). Together, these data indicate that the culture system faithfully replicated the granulosa cell

transdifferentiation phenotype observed *in vivo* in the *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre model. They further suggest that loss of granulosa cell differentiation/fate occurs rapidly following the loss of *Lats1/2*, and concomitantly with multi-lineage transdifferentiation.

To gain further insight into the cellular and molecular processes that were altered following *Lats1/2*-depletion in granulosa cells, microarray analyses were conducted 12 and 30 hours following Ad-cre/Ad-eGFP treatment. An increase of two-fold or greater was found in the expression of 58 transcripts, and a decrease for 101 transcripts, at 12h post-treatment (threshold: $P < 0.01$). These numbers increased to 174 and 328, respectively, by 30h. Functional analysis of the array data using DAVID (Huang da et al, 2009) notably identified large groups of genes involved in regulating apoptosis and the cell cycle (selected biological process GO terms from the 30 hour data set are presented in Table 2), providing a potential basis for the abrogation of proliferation and apoptosis observed in the *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre model. Also consistent with the *in vivo* transdifferentiation phenotype, transcripts related to osteoblastic differentiation, male and female gonad development, ossification, and spermatogenesis were identified by the GO term analysis (Table 2). Unexpectedly however, groups of genes related to adrenal gland, kidney and liver development and keratinocyte differentiation were also identified, suggesting that loss of *Lats1/2* affected the expression of the genetic programs of a number of cell lineages (Table 2).

4.5 DISCUSSION

Very little is known regarding the specific roles for Hippo signaling effectors in the ovary however the work by the Hsueh group suggests that the Hippo pathway might mediate granulosa cell proliferation during ovarian follicle development (Kawamura et al, 2013). Whereas the present study did not achieve its original goal of elucidating the roles of *Lats1/2* in the process of follicle development, it unexpectedly revealed that *Lats1/2* play a fundamental role in maintaining granulosa cell identity and function. In *Lats1/2*-depleted cells, granulosa cells transdifferentiate into Sertoli-like cells, osteoblasts, and cells derived from the neural crest. This paper identifies a novel link between Hippo signaling and maintenance of the granulosa cell genetic program in the adult ovary, however, the precise mechanisms by which this occurs

still remain to be uncovered. In addition, the elucidation of the roles of *Lats1/2* in follicle development may ultimately require an approach that avoids complete loss of LATS activity (such as the use of hypomorphic alleles), to permit an analysis of follicle development without alteration of granulosa cell fate.

In mammalian fetal development, a fine balance between the expression of female *vs* male-specific genes drives the differentiation of bipotential precursor cells into either granulosa or Sertoli cells, respectively. During ovarian differentiation, female-specific CTNNB1 reinforced by WNT4 and RSPO1 suppresses male-specific *Sox9* expression (Maatouk et al, 2008). In our model, ovarian specification occurs normally in the presence of *Lats1/2*, however, once *Lats1/2* are knocked down, the ovarian phenotype is partially reversed, a process which is regulated by a slightly different set of genes.

The maintenance of granulosa cell identity is an active process in the adult gonad and requires continuous expression of ovarian-specific genes that repress testicular gene expression. In a normal XX mouse, granulosa-cell specific FOXL2 binds to the *Sox9* promoter to repress its transcription in order to maintain granulosa cell fate (Uhlenhaut et al, 2009). We show that disruption of Hippo signaling by knockdown of *Lats1/2* results in transdifferentiation from granulosa to Sertoli-like cells, which marks the first time that such a link has been established between the Hippo pathway and the maintenance of gonadal sex. In our model, loss of a single gene, *Foxl2*, may account for the resulting phenotype observed in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$; *CYP19*-cre ovaries. Loss of *Foxl2* alone has been shown to induce sex reversal in XX mice. In a *Foxl2*-inducible knockout mouse model, loss of *Foxl2* induced the expression of *Sox9* in the ovary leading to transdifferentiation of granulosa cells into Sertoli cells and the formation of seminiferous tubules (Uhlenhaut et al, 2009). The question then becomes, what is the connection between loss of *Lats1/2* and loss of *Foxl2* expression? One report has suggested a link between *Yap/Taz* and the maintenance of the male cell fate. In *Yap/Taz*-null Sertoli cells, male sex-differentiation genes (*Dhh, Dmrt1,* and *Sox9*) were downregulated and the female sex-differentiation gene, *Wnt4*, was upregulated, however, no sex reversal was observed (Levasseur et al, 2017). We hypothesize that the loss of FOXL2 expression in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$; *CYP19*-cre ovaries may be due to repression by SOX9, which has been shown to be a direct YAP-TEAD target gene in some cellular contexts, and is significantly upregulated in our model (Song et al, 2014). In the absence of *Lats1/2*, the overaccumulation of YAP might be driving

the transcription of this atypical target in granulosa cells. An alternative hypothesis is that loss of FOXL2 expression is mediated by a loss of CTNNB1. This idea stems from a recent article that showed that CTNNB1 binds to its transcription factor TCF/LEF, which directly binds to the *Foxl2* promoter to induce its transcription (Li et al, 2017). This is supported by findings in our model in which we show that in *Lats1/2*-depleted granulosa cells, CTNNB1 expression is lost. It would be possible to test either hypothesis by performing a ChIP-qPCR on *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells infected with Ad-eGFP or Ad-cre, to determine whether more YAP is bound to TEAD on the *Sox9* promoter, or if less CTNNB1 is bound to TCF/LEF on the *Foxl2* promoter in *Lats1/2*-depleted granulosa cells relative to controls.

EMT occurs over the course of development, in wound healing, and in cancer. EMT involves the loss of epithelial cell characteristics such as cell polarity and cell adhesion and the acquisition of mesenchymal cell characteristics such as spindle cell shape, cell migration, and stemness (Chen et al, 2017). In our model, a subset of *Lats1/2*-depleted granulosa cells transition into vimentin-positive, spindle-shaped, mesenchymal stem-like cells. Both *Yap* and *Taz* have been implicated in EMT (Lei et al, 2008; Zhang et al, 2014b). In fact, YAP forms a complex with members of the TGF-β family, SMAD2/3/4, and is required for upregulation of transcription factors *Snai1, Snai2*, and *Twist*, that drive EMT (Zhang et al, 2014b). Indeed, *Snai1* is upregulated *in vivo* and *in vitro* in *Lats1/2*-depleted granulosa cells. Again, we suspect that the overaccumulation of YAP/TAZ in *Lats1/2*-depleted cells is responsible for promoting EMT in a mechanism that might involve SMAD2/3/4. This hypothesis could be tested by performing another ChIP-qPCR on *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells infected with Ad-eGFP or Ad-cre, to determine whether more YAP/TAZ are bound to SMAD2/3/4 on the *Snai1* promoter in *Lats1/2*-null granulosa cells relative to controls.

In our model, once a subset of *Lats1/2*-depleted cells have undergone EMT, we suspect that they have acquired multipotent capabilities. Indeed, *Lats1/2*- depleted cells express stem-cell markers *in vitro* that are not normally expressed in granulosa cells. From this point forward, cells have the potential to differentiate into different cell types, such as osteoblasts. Granulosa cell transdifferentiation into osteoblasts is not without precedent in the literature. In a mouse model expressing dominant stable mutant CTNNB1 in granulosa cells, mice developed granulosa cell tumors that contained areas of ossification (Boerboom et al, 2005). Again, we looked at YAP/TAZ binding partners to potentially explain our phenotype. RUNX2 is well-

established binding partner for YAP/TAZ, and is a critical mediator of osteogenesis (Teplyuk et al, 2008). In fact, TAZ-mediated induction of *Bglap* (osteocalcin) via RUNX2 has been shown to drive osteoblast differentiation in mesenchymal stem cells (Cui et al, 2003). We suspect that elevated levels of TAZ in our model are inducing aberrant transcription of osteoblast-promoting genes via the RUNX2 transcription factor. Additional mediators of osteogenesis are *Snai1* and *Snai2*, which are genes encoding the proteins SNAIL and SLUG. YAP and TAZ have been reported to form a complex with SNAIL and SLUG and together bind to RUNX2. In fact, deletion of both *Snai1* and -*2* was shown to inhibit the expression of RUNX2 target genes that are essential for osteogenesis (Tang et al, 2016). A third potential driver of osteogenesis is the YAP/TAZ/TEAD target gene, *Ctgf*, which is essential for bone formation as evidenced by *Ctgf*-KO mice that develop skeletal deformities and have reduced ossification (Heath et al, 2008), and which was upregulated *in vivo* and *in vitro*. Our study therefore represents a potential molecular insight into granulosa-to-osteoblast transdifferentiation.

Another cell population within *Lats1/2*- depleted ovaries express a marker of cell types derived from the neural crest, which give rise to cells of the nervous system (Hindley et al, 2016). Relatively few studies have studied Hippo signaling in the context of nervous system development. Of note, YAP and its ortholog Yorkie were required for astrocytic differentiation (Huang et al, 2016), and to dictate the terminal differentiation of neuroblasts, respectively (Poon et al, 2016). It is possible that the differentiation of mesenchymal stem-like cells into neural crest cell types is mediated by *Ctgf,* that is expressed during embryonic brain development and appears to play a role in differentiation of neural crest cells (Malik et al, 2015). To test whether *Ctgf* is driving the differentiation of the *Lats1/2*- depleted mesenchymal stem cell-like cells into either osteoblasts or neural cell types, we could treat $Lats1^{flox/flox}$;$Lats2^{flox/flox}$ granulosa cells infected with Ad-eGFP or Ad-cre with CTGF antibody and recombinant protein and then evaluate whether this alone is sufficient to neutralize or enhance, respectively, the expression of osteoblast and neural crest cell-specific genes. A ChIP-qPCR could also be performed on $Lats1^{flox/flox}$;$Lats2^{flox/flox}$; *CYP19*-cre ovaries (or controls) to determine whether more TAZ is bound to RUNX2 on the *Bglap* (or other osteogenic genes) promoter in *Lats1/2*- depleted ovaries relative to controls.

In conclusion, this is the first report demonstrating that disruption of the Hippo pathway can have such a profound effect on maintaining the gonadal sex that it results in phenotypic sex

reversal. Not only do granulosa cells transdifferentiate into Sertoli-like cells, but they are also reprogrammed to differentiate into multiple cell lineages, illustrating a critical role for LATS as barriers to reprogramming. These findings invite future investigation of the potential roles of Hippo in the physiological process of sex determination during embryogenesis and in the context of induced pluripotent stem cells.

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4.7 REFERENCES

1. Azzolin, L., T. Panciera, S. Soligo, E. Enzo, S. Bicciato, S. Dupont, S. Bresolin, C. Frasson, G. Basso, V. Guzzardo, A. Fassina, M. Cordenonsi and S. Piccolo (2014). "YAP/TAZ incorporation in the beta-catenin destruction complex orchestrates the Wnt response." Cell **158**(1): 157-170.
2. Azzolin, L., F. Zanconato, S. Bresolin, M. Forcato, G. Basso, S. Bicciato, M. Cordenonsi and S. Piccolo (2012). "Role of TAZ as mediator of Wnt signaling." Cell **151**(7): 1443-1456.
3. 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." Mol Cell **11**(1): 11-23.
4. Boerboom, D., M. Paquet, M. Hsieh, J. Liu, S. P. Jamin, R. R. Behringer, J. Sirois, M. M. Taketo and J. S. Richards (2005). "Misregulated Wnt/beta-catenin signaling leads to ovarian granulosa cell tumor development." Cancer Res **65**(20): 9206-9215.

5. 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." FEBS J **273**(18): 4264-4276.

6. Chan, E. H., M. Nousiainen, R. B. Chalamalasetty, A. Schafer, E. A. Nigg and H. H. Sillje (2005). "The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1." Oncogene **24**(12): 2076-2086.

7. Chen, T., Y. You, H. Jiang and Z. Z. Wang (2017). "Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis." J Cell Physiol **232**(12): 3261-3272.

8. 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." Faseb j **29**(6): 2423-2430.

9. Cui, C. B., L. F. Cooper, X. Yang, G. Karsenty and I. Aukhil (2003). "Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ." Mol Cell Biol **23**(3): 1004-1013.

10. 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.

11. Dupont, S., L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore and S. Piccolo (2011). "Role of YAP/TAZ in mechanotransduction." Nature **474**(7350): 179-183.

12. Fan, H. Y., Z. Liu, N. Cahill and J. S. Richards (2008). "Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells." Mol Endocrinol **22**(9): 2128-2140.

13. Fan, H. Y., M. Shimada, Z. Liu, N. Cahill, N. Noma, Y. Wu, J. Gossen and J. S. Richards (2008). "Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation." Development **135**(12): 2127-2137.

14. Fu, V., S. W. Plouffe and K. L. Guan (2017). "The Hippo pathway in organ development, homeostasis, and regeneration." Curr Opin Cell Biol **49**: 99-107.

15. Heath, E., D. Tahri, E. Andermarcher, P. Schofield, S. Fleming and C. A. Boulter (2008). "Abnormal skeletal and cardiac development, cardiomyopathy, muscle atrophy and cataracts in mice with a targeted disruption of the Nov (Ccn3) gene." BMC Dev Biol **8**: 18.

16. Hindley, C. J., A. L. Condurat, V. Menon, R. Thomas, L. M. Azmitia, J. A. Davis and J. Pruszak (2016). "The Hippo pathway member YAP enhances human neural crest cell fate and migration." Sci Rep **6**: 23208.

17. Huang da, W., B. T. Sherman and R. A. Lempicki (2009). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." Nat Protoc **4**(1): 44-57.

18. Huang, Z., J. Hu, J. Pan, Y. Wang, G. Hu, J. Zhou, L. Mei and W. C. Xiong (2016). "YAP stabilizes SMAD1 and promotes BMP2-induced neocortical astrocytic differentiation." Development **143**(13): 2398-2409.

19. Kawamura, K., Y. Cheng, N. Suzuki, M. Deguchi, Y. Sato, S. Takae, C. H. Ho, N. Kawamura, M. Tamura, S. Hashimoto, Y. Sugishita, Y. Morimoto, Y. Hosoi, N. Yoshioka, B. Ishizuka and A. J. Hsueh (2013). "Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment." Proc Natl Acad Sci U S A **110**(43): 17474-17479.

20. Lee, D. H., J. O. Park, T. S. Kim, S. K. Kim, T. H. Kim, M. C. Kim, G. S. Park, J. H. Kim, S. Kuninaka, E. N. Olson, H. Saya, S. Y. Kim, H. Lee and D. S. Lim (2016). "LATS-YAP/TAZ controls lineage specification by regulating TGFbeta signaling and Hnf4alpha expression during liver development." Nat Commun **7**: 11961.

21. 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." Mol Cell Biol **28**(7): 2426-2436.

22. Levasseur, A., M. Paquet, D. Boerboom and A. Boyer (2017). "Yes-associated protein and WW-containing transcription regulator 1 regulate the expression of sex-determining genes in Sertoli cells, but their inactivation does not cause sex reversal." Biol Reprod **97**(1): 162-175.

23. Li, Y., L. Zhang, Y. Hu, M. Chen, F. Han, Y. Qin, M. Chen, X. Cui, S. Duo, F. Tang and F. Gao (2017). "beta-Catenin directs the transformation of testis Sertoli cells to ovarian granulosa-like cells by inducing Foxl2 expression." J Biol Chem **292**(43): 17577-17586.

24. 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 Dev **26**(12): 1300-1305.

25. Liu, C. Y., Z. Y. Zha, X. Zhou, H. Zhang, W. Huang, D. Zhao, T. Li, S. W. Chan, C. J. Lim, W. Hong, S. Zhao, Y. Xiong, Q. Y. Lei and K. L. Guan (2010). "The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase." J Biol Chem **285**(48): 37159-37169.

26. Maatouk, D. M., L. DiNapoli, A. Alvers, K. L. Parker, M. M. Taketo and B. Capel (2008). "Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal." Hum Mol Genet **17**(19): 2949-2955.

27. Malik, A. R., E. Liszewska and J. Jaworski (2015). "Matricellular proteins of the Cyr61/CTGF/NOV (CCN) family and the nervous system." Front Cell Neurosci **9**: 237.

28. McNeill, H. and A. Reginensi (2017). "Lats1/2 Regulate Yap/Taz to Control Nephron Progenitor Epithelialization and Inhibit Myofibroblast Formation." J Am Soc Nephrol **28**(3): 852-861.

29. Miesfeld, J. B., G. Gestri, B. S. Clark, M. A. Flinn, R. J. Poole, J. R. Bader, J. C. Besharse, S. W. Wilson and B. A. Link (2015). "Yap and Taz regulate retinal pigment epithelial cell fate." Development **142**(17): 3021-3032.

30. Nguyen, Q., R. A. Anders, G. Alpini and H. Bai (2015). "Yes-associated protein in the liver: Regulation of hepatic development, repair, cell fate determination and tumorigenesis." Dig Liver Dis **47**(10): 826-835.

31. Nishioka, N., K. Inoue, K. Adachi, H. Kiyonari, M. Ota, A. Ralston, N. Yabuta, S. Hirahara, R. O. Stephenson, N. Ogonuki, R. Makita, H. Kurihara, E. M. Morin-Kensicki, H. Nojima, J. Rossant, K. Nakao, H. Niwa and H. Sasaki (2009). "The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass." Dev Cell **16**(3): 398-410.

32. Nishioka, N., S. Yamamoto, H. Kiyonari, H. Sato, A. Sawada, M. Ota, K. Nakao and H. Sasaki (2008). "Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos." Mech Dev **125**(3-4): 270-283.

33. Pan, D. (2010). "The hippo signaling pathway in development and cancer." Dev Cell **19**(4): 491-505.

34. Park, G. S., H. Oh, M. Kim, T. Kim, R. L. Johnson, K. D. Irvine and D. S. Lim (2016). "An evolutionarily conserved negative feedback mechanism in the Hippo pathway reflects functional difference between LATS1 and LATS2." Oncotarget.

35. Pearce, L. R., D. Komander and D. R. Alessi (2010). "The nuts and bolts of AGC protein kinases." Nat Rev Mol Cell Biol **11**(1): 9-22.

36. Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

37. Piccolo, S., S. Dupont and M. Cordenonsi (2014). "The Biology of YAP/TAZ: Hippo Signaling and Beyond." Physiol Rev **94**(4): 1287-1312.

38. Poon, C. L., K. A. Mitchell, S. Kondo, L. Y. Cheng and K. F. Harvey (2016). "The Hippo Pathway Regulates Neuroblasts and Brain Size in Drosophila melanogaster." Curr Biol **26**(8): 1034-1042.

39. Praskova, M., F. Xia and J. Avruch (2008). "MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation." Curr Biol **18**(5): 311-321.

40. Sasaki, H. (2017). "Roles and regulations of Hippo signaling during preimplantation mouse development." Dev Growth Differ **59**(1): 12-20.

41. Sforza, C., L. Vizzotto, V. F. Ferrario and A. Forabosco (2003). "Position of follicles in normal human ovary during definitive histogenesis." Early Hum Dev **74**(1): 27-35.

42. Song, S., J. A. Ajani, S. Honjo, D. M. Maru, Q. Chen, A. W. Scott, T. R. Heallen, L. Xiao, W. L. Hofstetter, B. Weston, J. H. Lee, R. Wadhwa, K. Sudo, J. R. Stroehlein, J. F. Martin, M. C. Hung and R. L. Johnson (2014). "Hippo coactivator YAP1 upregulates

SOX9 and endows esophageal cancer cells with stem-like properties." Cancer Res **74**(15): 4170-4182.

43. St John, M. A., W. Tao, X. Fei, R. Fukumoto, M. L. Carcangiu, D. G. Brownstein, A. F. Parlow, J. McGrath and T. Xu (1999). "Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction." Nat Genet **21**(2): 182-186.

44. Tang, Y., T. Feinberg, E. T. Keller, X. Y. Li and S. J. Weiss (2016). "Snail/Slug binding interactions with YAP/TAZ control skeletal stem cell self-renewal and differentiation." Nat Cell Biol **18**(9): 917-929.

45. Teplyuk, N. M., M. Galindo, V. I. Teplyuk, J. Pratap, D. W. Young, D. Lapointe, A. Javed, J. L. Stein, J. B. Lian, G. S. Stein and A. J. van Wijnen (2008). "Runx2 regulates G protein-coupled signaling pathways to control growth of osteoblast progenitors." J Biol Chem **283**(41): 27585-27597.

46. Uhlenhaut, N. H., S. Jakob, K. Anlag, T. Eisenberger, R. Sekido, J. Kress, A. C. Treier, C. Klugmann, C. Klasen, N. I. Holter, D. Riethmacher, G. Schutz, A. J. Cooney, R. Lovell-Badge and M. Treier (2009). "Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation." Cell **139**(6): 1130-1142.

47. Vassilev, A., K. J. Kaneko, H. Shu, Y. Zhao and M. L. DePamphilis (2001). "TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm." Genes Dev **15**(10): 1229-1241.

48. Vitolo, M. I., I. E. Anglin, W. M. Mahoney, Jr., K. J. Renoud, R. B. Gartenhaus, K. E. Bachman and A. Passaniti (2007). "The RUNX2 transcription factor cooperates with the YES-associated protein, YAP65, to promote cell transformation." Cancer Biol Ther **6**(6): 856-863.

49. Wang, R., Y. Li, E. Hu, F. Kong, J. Wang, J. Liu, Q. Shao, Y. Hao, D. He and X. Xiao (2017). "S100A7 promotes lung adenocarcinoma to squamous carcinoma transdifferentiation, and its expression is differentially regulated by the Hippo-YAP pathway in lung cancer cells." Oncotarget **8**(15): 24804-24814.

50. Yimlamai, D., C. Christodoulou, G. G. Galli, K. Yanger, B. Pepe-Mooney, B. Gurung, K. Shrestha, P. Cahan, B. Z. Stanger and F. D. Camargo (2014). "Hippo pathway activity influences liver cell fate." Cell **157**(6): 1324-1338.

51. Zeleznik AJ, Midgley AR, Jr., Reichert LE, Jr. (1974) Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. *Endocrinology* **95:** 818-825

52. Zhang, H., A. von Gise, Q. Liu, T. Hu, X. Tian, L. He, W. Pu, X. Huang, L. He, C. L. Cai, F. D. Camargo, W. T. Pu and B. Zhou (2014). "Yap1 is required for endothelial to mesenchymal transition of the atrioventricular cushion." J Biol Chem **289**(27): 18681-18692.

53. Zhao, B., L. Li, Q. Lei and K. L. Guan (2010). "The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version." Genes Dev **24**(9): 862-874.

54. 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 SCF(beta-TRCP)." Genes Dev **24**(1): 72-85.

55. Zhao, B., X. Wei, W. Li, R. S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, P. Zheng, K. Ye, A. Chinnaiyan, G. Halder, Z. C. Lai and K. L. Guan (2007). "Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control." Genes Dev **21**(21): 2747-2761.

56. Zhao, B., X. Ye, J. Yu, L. Li, W. Li, S. Li, J. Yu, J. D. Lin, C. Y. Wang, A. M. Chinnaiyan, Z. C. Lai and K. L. Guan (2008). "TEAD mediates YAP-dependent gene induction and growth control." Genes Dev **22**(14): 1962-1971.

4.8 FIGURES

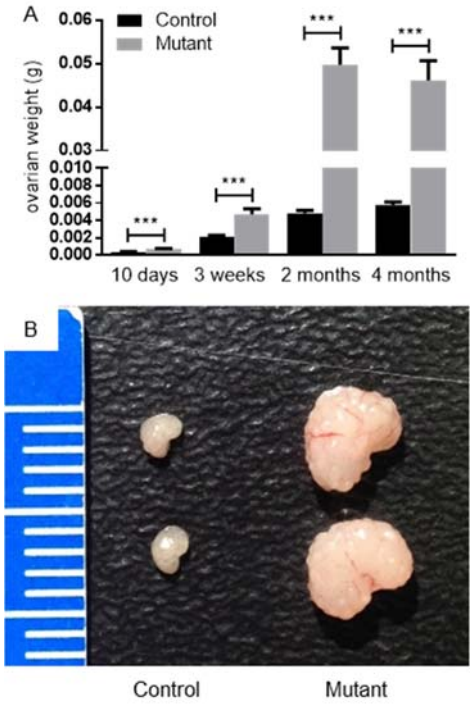

Figure 1. *Lats1*$^{flox/flox}$*;Lats2f*$^{lox/flox}$*;CYP19*-cre ovaries. A) Ovarian weights of *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$ (control) and *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$*;CYP19*-cre (mutant) (n=16 ovaries/time point) with representative images of 2 month-old ovaries in (B). Data are means ±SEM. ***P≤0.001, statistically significant differences between groups. Scale bar units represent millimeters.

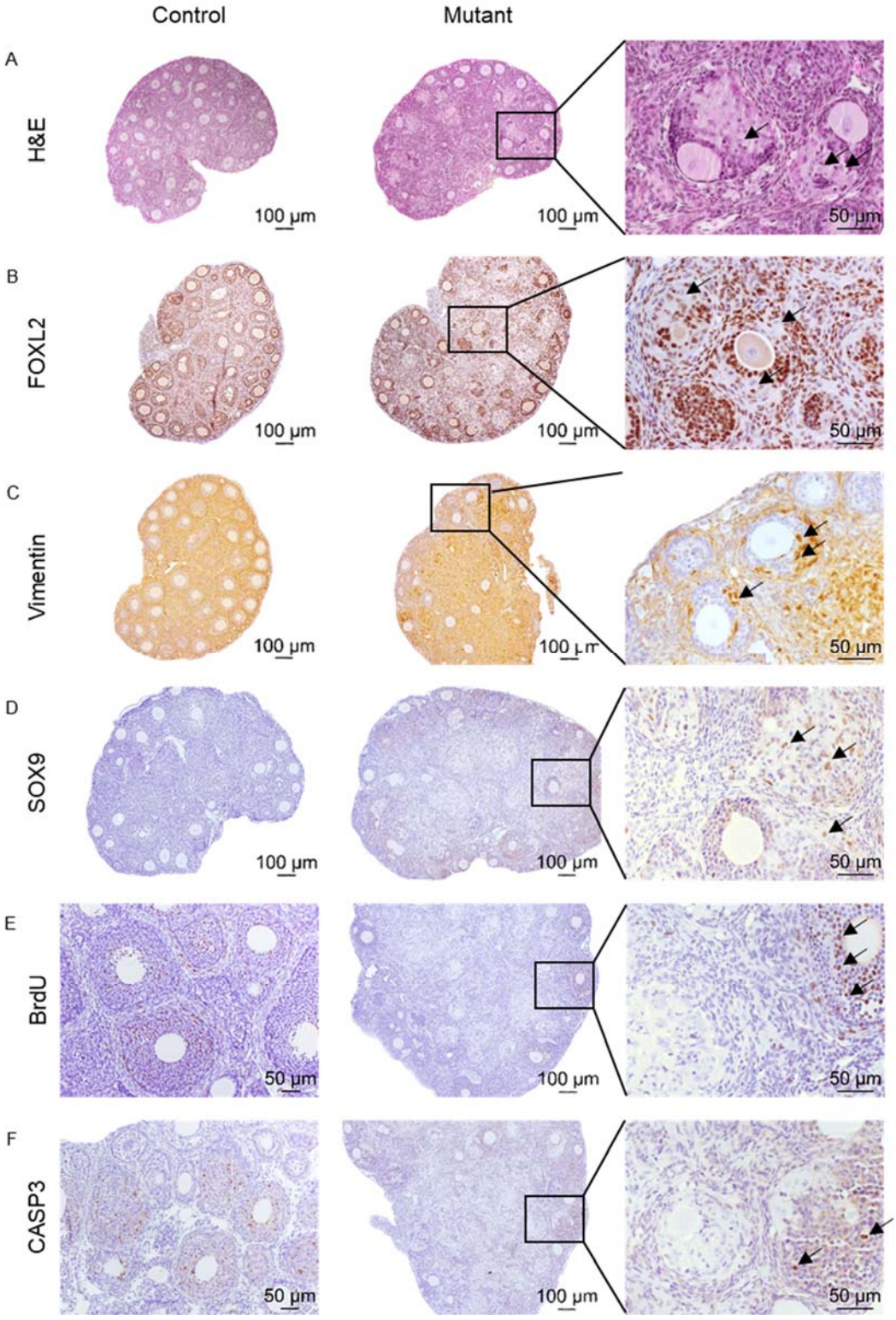
Control
Mutant
A
H&E
100 μm
100 μm
50 μm
B
FOXL2
100 μm
100 μm
50 μm
C
Vimentin
100 μm
100 μm
50 μm
D
SOX9
100 μm
100 μm
50 μm
E
BrdU
50 μm
100 μm
50 μm
F
CASP3
50 μm
100 μm
50 μm

Figure 2. Depletion of *Lats1* and *Lats2* causes loss of granulosa cell identity, epithelial-to-mesenchymal transition, transdifferentiation into Sertoli-like cells, a lack of proliferation, and evasion of apoptosis

A) Representative images of *Lats1*$^{flox/flox}$;*Lats2f*$^{lox/flox}$ (control) and *Lats1*$^{flox/flox}$;*Lats2f*$^{lox/flox}$; *CYP19*-cre (mutant) ovaries at 10 dpp. In zoom: follicles exhibit a new cell type (arrows). B) FOXL2 IHC; in zoom: the new cells are FOXL2-negative (arrows). C) Vimentin IHC, in zoom: the new cells are vimentin-positive (arrows). D) SOX9 IHC, in zoom: occasional new cells are SOX9-positive (arrows). E) BrdU incorporation assay on ovaries at 21 dpp. In zoom: normal-looking granulosa cells stain positive for BrdU (arrows); the new cells do not. F) Cleaved Caspase-3 (CASP3) IHC at 21 dpp. In zoom: normal-looking granulosa cells stain positive for CASP3 (arrows); the new cells do not.

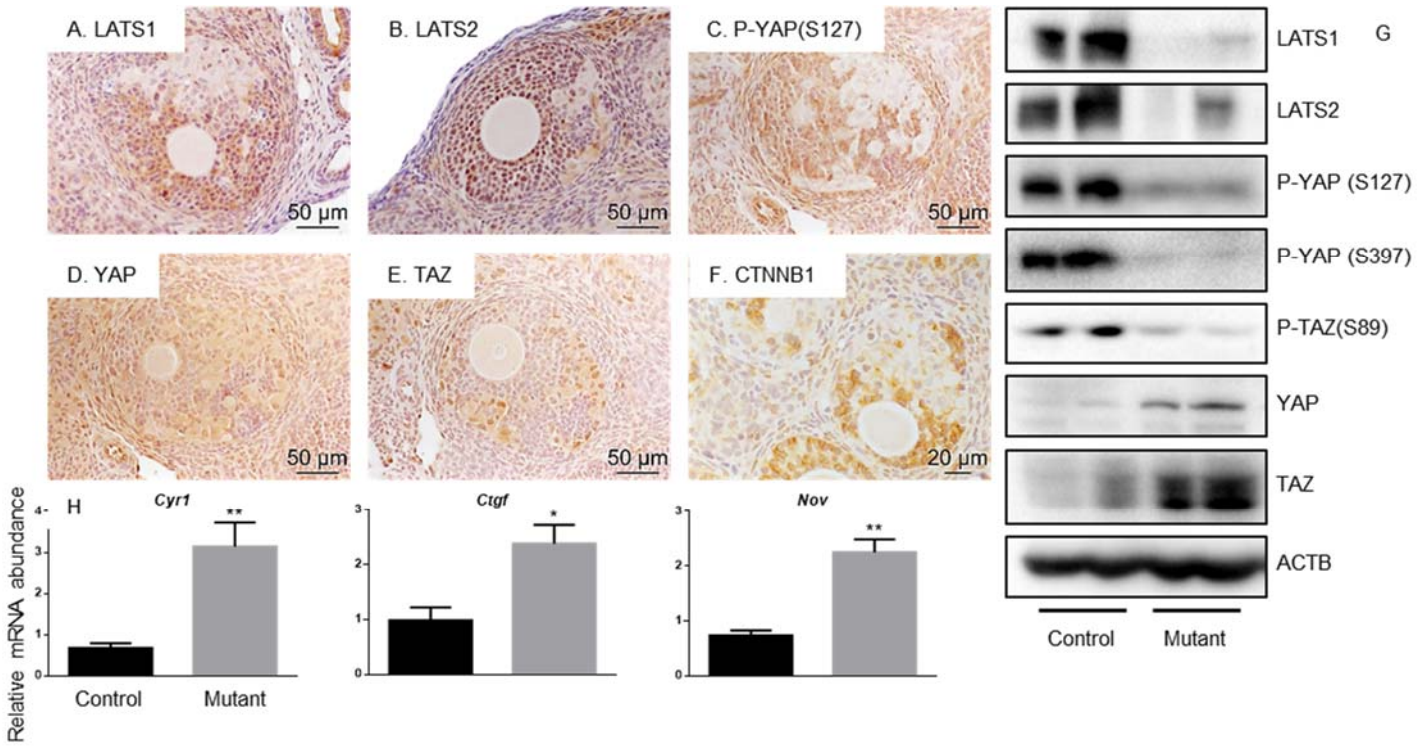

Figure 3. Hippo signaling is disrupted in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre ovaries. Representative images of *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre (mutant) ovaries. A) LATS1 immunohistochemistry of ovarian sections from 21 day-old mutant mice, B) LATS2, C) Phospho-YAP(Ser127), D) YAP, E) TAZ, and F) CTNNB1. G) Representative immunoblots of whole 4-week-old control and mutant ovaries show n=2 ovaries/genotype. H) RT-qPCR was performed on whole 2 month-old ovaries from control vs mutant mice to determine mRNA levels of YAP/TAZ/TEAD target genes (n=4 ovaries/genotype). Data were normalized to the housekeeping gene *Rpl19*. Data are means ±SEM. *P≤0.05 and **P≤0.01, statistically significant differences between groups.

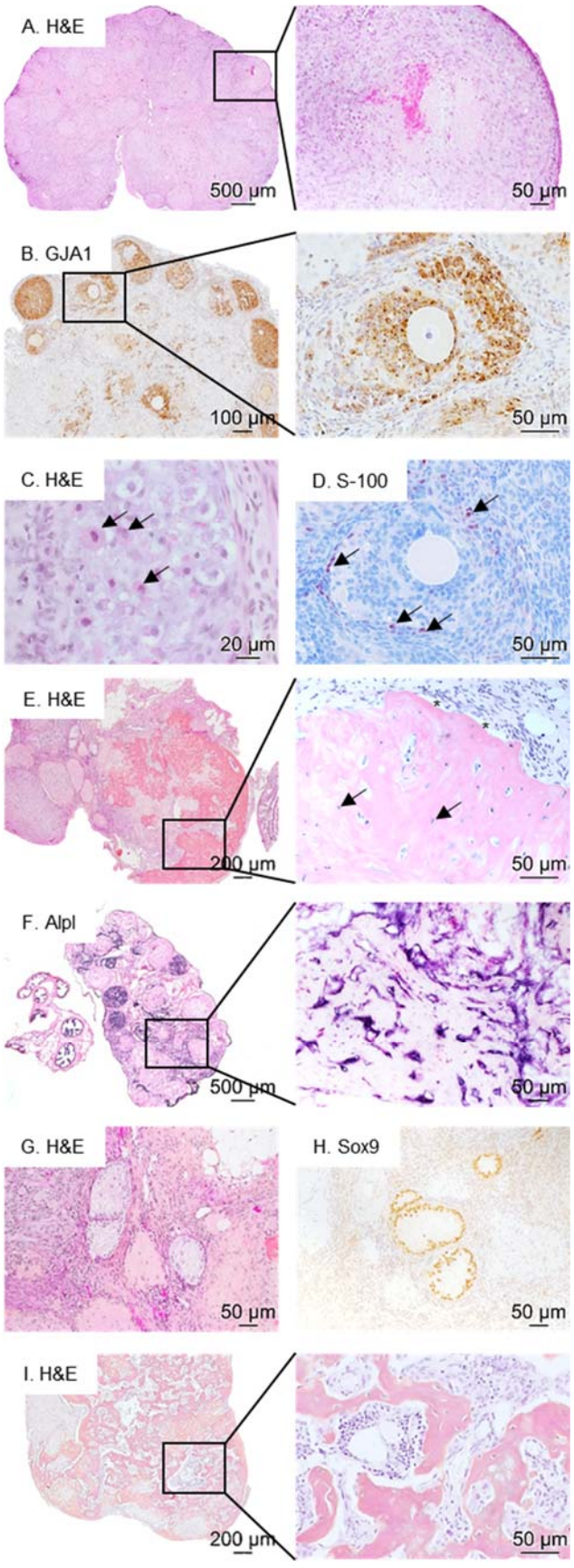
A. H&E
500 μm
50 μm
B. GJA1
100 μm
50 μm
C. H&E
20 μm
D. S-100
50 μm
E. H&E
200 μm
50 μm
F. Alpl
500 μm
50 μm
G. H&E
50 μm
H. Sox9
50 μm
I. H&E
200 μm
50 μm

Figure 4. *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$*;CYP19*-cre granulosa cells transdifferentiate into multiple cell lineages.

A) Representative image of *Lats1*$^{flox/flox}$*;Lats2*$^{flox/flox}$*;CYP19*-cre (mutant) ovary at 30 dpp. In zoom: follicle-like structure containing a necrotic center. B) GJA1 (connexin 43) IHC on ovarian sections from 21 day-old mutant mice. In zoom: new cell populations lack the gap junction protein GJA1. C) Representative image of a follicle-like structure containing abundant eosinophilic cytoplasmic granules (arrows) from 3-month old mutant mice. D) S-100 IHC on ovarian sections from 30 day-old mutant mice. Occasional cells (of the new cell populations) stain positive for S-100 (arrows). E) Representative image of mutant ovaries at 4 months. In zoom: osteoblasts (*) and osteocytes (arrows) are clearly visible. F) Alkaline phosphatase (Alpl) IHC. In zoom: osteoblasts and osteocytes stain positive for Alpl. G) Representative image of cord-like structures. H) The cells lining the cord-like structures stain positive for SOX9. I) Representative image of mutant ovaries at 5 months of age that are almost entirely composed of bone and bone marrow (in zoom).

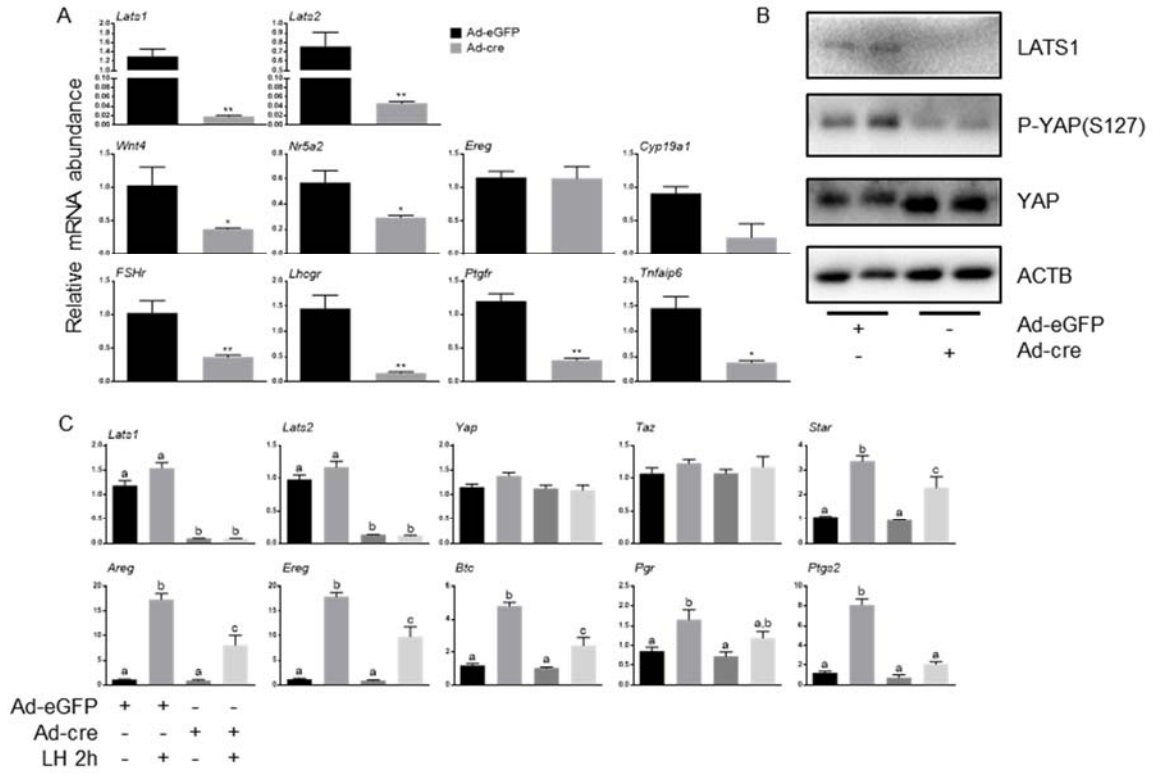

Figure 5. Knockdown of *Lats1* and *Lats2 in vitro* causes a loss of granulosa cell identity and function. A) *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells were infected with either Ad-eGFP or Ad-cre for 30 hours and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19* (n=3 replicates/treatment). Data are means ±SEM. *P≤0.05 and **P≤0.01, statistically significant differences between groups. B) *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells were infected with either Ad-eGFP or Ad-cre for 24 hours and the expression of the indicated proteins was evaluated by immunoblot analysis (n=2 replicates/treatment are shown). C) *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells were infected with either Ad-eGFP or Ad-cre for 18 hours, followed by treatment with 50ng/ml LH for 2 hours, and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19* (n=4 replicates/treatment). Data are means ±SEM. Different letters show statistically significant differences between groups (P≤0.05).

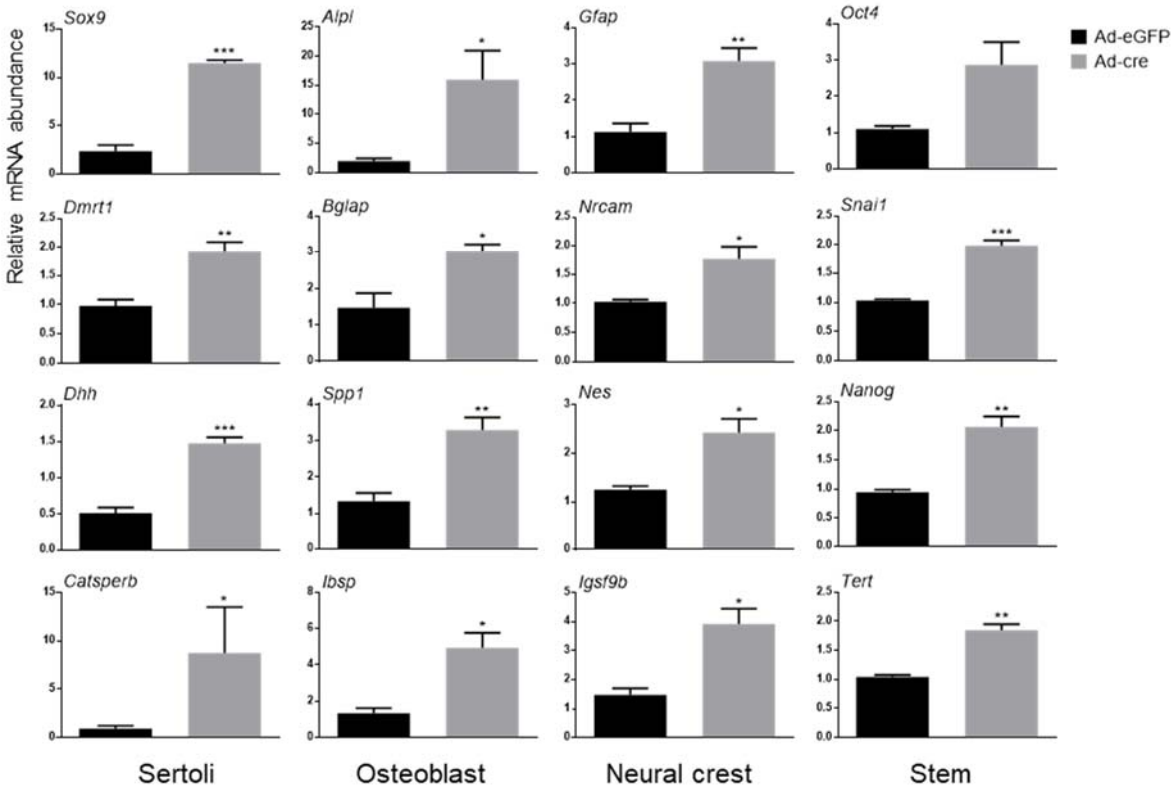

Figure 6. Knockdown of *Lats1* and *Lats2* in granulosa cells *in vitro* induces transdifferentiation into multiple cell lineages. A) *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells were infected with either Ad-eGFP or Ad-cre for 30 hours and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19* (n=3 replicates/treatment). Data are means ±SEM. *P≤0.05, **P≤0.01, **P≤0.001, statistically significant differences between groups.

4.9 SUPPLEMENTAL FIGURES

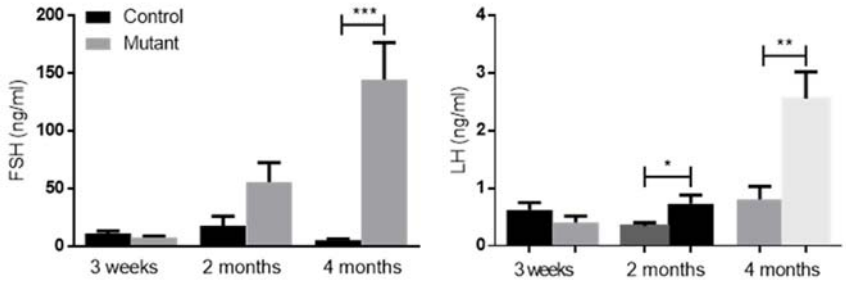

Figure S1. FSH and LH serum levels from *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ (control) vs *Lats1*$^{flox/flox}$; *Lats2*$^{flox/flox}$;*CYP19*-cre (mutant) female mice. N=6 mice/time point. Data are means ±SEM. *P≤0.05, **P≤0.01, **P≤0.001, statistically significant differences between groups.

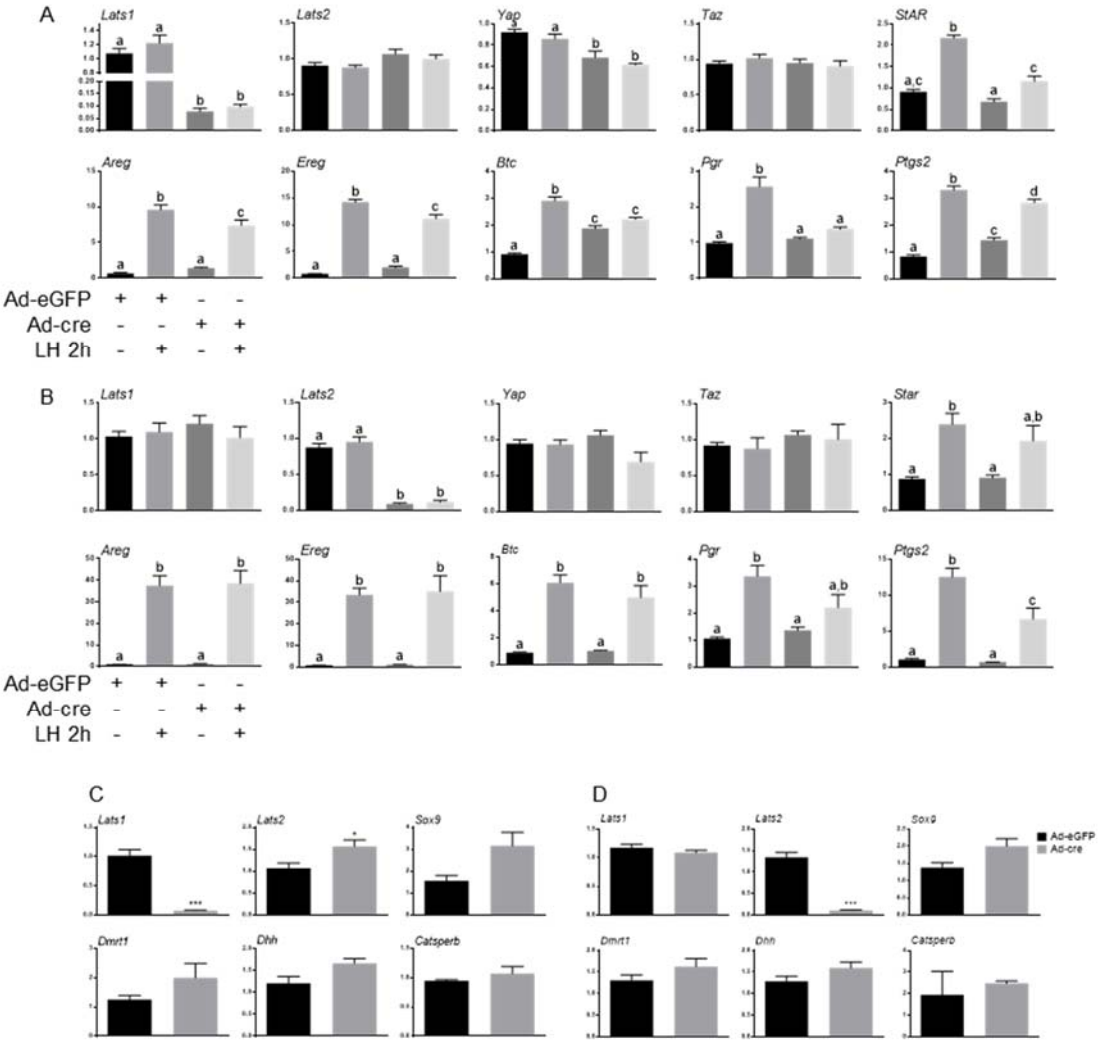

Figure S2. Knockdown of *Lats1* or *Lats2* alone has less effect on the transcription of LH target genes or on markers of Sertoli cells (relative to knockdown of *Lats1/2*). A) *Lats1*$^{flox/flox}$ granulosa cells were infected with either Ad-eGFP or Ad-cre for 18 hours followed by treatment with 50ng/ml LH for 2 hours and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19* (n=4 replicates/treatment). Different letters show statistically significant differences between groups. B) idem to A except using *Lats2*$^{flox/flox}$ granulosa cells. C) *Lats1*$^{flox/flox}$ granulosa cells were infected with either Ad-eGFP or Ad-cre for 18 hours and mRNA levels of the indicated genes were determined by RT-qPCR and normalized to the housekeeping gene *Rpl19* (n=4 replicates/treatment). D) idem to C except

using *Lats2*$^{flox/flox}$ granulosa cells. Data are means ±SEM. *P≤0.05, ***P≤0.001, statistically significant differences between groups. n=4 replicates/treatment.

4.10 TABLES

	Control	Mutant
Mating pairs (n)	7	5
Total litters (n)	52	2
Total pups (n)	471	12

Table 1. Mating trials

Biological process (Functional Annotation)	Count	P Value	Fold Enrichment
Cell-cell adhesion	19	1.37E-05	3.39
G1/S transition of mitotic cell cycle	10	7.89E-05	5.47
Negative regulation of apoptotic process	34	1.78E-04	2.02
Osteoblast differentiation	12	6.32E-04	3.51
Negative regulation of cell proliferation	24	1.24E-03	2.1
Cell adhesion	28	1.25E-03	1.96
Adrenal gland development	6	1.44E-03	7.01
Regulation of cell cycle	11	1.99E-03	3.27
Positive regulation of apoptotic process	21	2.50E-03	2.11
Angiogenesis	15	1.24E-02	2.11
Male gonad development	9	1.69E-02	2.75
Kidney development	10	1.85E-02	2.51
Blood vessel morphogenesis	5	1.89E-02	4.84
G2/M transition of mitotic cell cycle	5	1.89E-02	4.84
Ossification	8	2.45E-02	2.79
Hippo signaling	4	2.58E-02	6.16
Positive regulation of cell proliferation	25	3.25E-02	1.56
Spermatogenesis	20	3.68E-02	1.64
Keratinocyte differentiation	7	3.75E-02	2.82
Liver development	8	4.77E-02	2.42
Female gonad development	4	4.83E-02	4.84

Table 2. Selected biological process GO terms from the microarray data set.

Microarray analyses were performed on *Lats1*$^{flox/flox}$;*Lats2* $^{flox/flox}$ granulosa cells infected with Ad-eGFP *vs* Ad-cre for 30 hours. GO terms reflect both up- and down-regulated genes.

4.11 SUPPLEMENTAL TABLE

Primer	Sequence (5' to 3')
Alpl F	GGTCACAGCAGTTGGTAGCTT
Alpl R	TAATTGACGTTCCGATCCTGAGTG
Areg F	CTCGCAGCTATTGGCATCGGCA
Areg R	TGGCATGCACAGTCCCGTTT
Bglap F	GGACCATCTTTCTGCTCACTC
Bglap R	CTTGGACATGAAGGCTTTGTCA
Btc F	GCATCCATGGGAGATGCCGCTT
Btc R	ACCACTATCAAGCAGACCACCAGG
Catsperb F	GCAGGCACATACCACCTGAA
Catsperb R	GGAGGTCTGTAGTTTATCGGCA
Ctgf F	GAGGAAAACATTAAGAAGGGCAAAA
Ctgf R	CCGCAGAACTTAGCCCTGTA
Cyp19a1 F	CTGAACATCGGAAGAATGCACAG
Cyp19a1 R	GAGTAGATAGGCCACACTTCTTC
Cyr61 F	TTGACCAGACTGGCGCTCT
Cyr61 R	AGTTTTGCTGCAGTCCTCGT
Dhh F	CGCCTGATGACAGAGCGTT
Dhh R	AGTGGAGTGAATCCTGTGCG
Dmrt1 F	TGGCAGATGAAGACCTCAGAGAG
Dmrt1 R	CGAGAACACACTGGCTTTGGC
Ereg F	ACGTTGCGTTGACAGTGATTCTCAT
Ereg R	GGTCCCCTGAGGTCACTCTCTCAT
FSHR F	ATTCTGGAAGGCCTCAGGGTTGAT
FSHR R	TGGATGTCATCACTGGCTGTGTCA
Gfap F	GAGAACAACCTGGCTGCGTA
Gfap R	CGGAGTTCTCGAACTTCCTCC
Ibsp F	GGACTGCCGAAAGGAAGGTT
Ibsp R	TTTTCATCGAGAAAGCACAGGC
Igsf9b F	CACCTCACTGTCATCGGCAC
Igsf9b R	AATGTCTGCTCGTAGCCTCC
Lats1 F	AGCAGCACGTAGAGAACGTC
Lats1 R	TCTCATTTGATCCTGGGCATCT
Lats2 F	TGCACTGGATTCAGGTGGACTCA
Lats2 R	GAGAATGTGCCAGGCACCTCT
Lhcgr F	GCTGGAGTCCATTCAGACGCTCA
Lhcgr R	AGCATCTGGTTCTGGAGTACATTG
Nanog F	ACCTGAGCTATAAGCAGGTTAAGA
Nanog R	TGAATCAGACCATTGCTAGTCTTC
Nes F	GCTACATACAGGACTCTGCTGG
Nes R	GGTGCTGGTCCTCTGGTATC
Nov F	AGAGTTGTTCTGAGATGAGACCC

Nov R	CCCTCTGGAACCATGCAAATG
Nr5a2 F	ATGGGAAGGAAGGGACAATC
Nr5a2 R	TGCAGGTTCTCCAGGTTCTT
Nrcam F	CCGTGCAGAAACGGAGACT
Nrcam R	AAAATTCGTTTTCGTGCCGC
Oct4 F	CCATGTTTCTGAAGTGCCCG
Oct4 R	ACCATACTCGAACCACATCCTTC
Pgr F	TCCAGGTGACCCATGAGGAA
Pgr R	TTGCCTTGATCAATTCGCGG
Ptgfr F	GAGGAAAGAGAGGTGGAACCC
Ptgfr R	TCGGAGTGCAGACATCTCG
Ptgs2 F	CCTGAAGCCGTACACATCATTTGA
Ptgs2 R	AGGCACTTGCATTGATGGTGGCT
Rpl19 F	CTGAAGGTCAAAGGGAATGTG
Rpl19 R	GGACAGAGTCTTGATGATCTC
Snai1 F	CCACACTGGTGAGAAGCCATTC
Snai1 R	GACATGCGGGAGAAGGTTCG
Sox9 F	AGGAAGTCGGTGAAGAACGG
Sox9 R	GGACCCTGAGATTGCCCAGA
Spp1 F	TGCTTTTGCCTGTTTGGCAT
Spp1 R	TGCAGGCTGTAAAGCTTCTTCT
StAR F	GATTAAGGCACCAAGCTGTGCTG
StAR R	CTGCTGGCTTTCCTTCTTCCAGC
Taz F	ACTGGCCAGAGATACTTCCTTAATC
Taz R	AGGCTGATTCATCACCTTCCTG
Tert F	CCTTTGACCAGCGTGTTAGG
Tert R	TGTCATTCCTGGATTCTTGACCTT
Tnfaip6 F	TGAAGGTGGTCGTCTCGCAACC
Tnfaip6 R	TCCACAGTTGGGCCCAGGTTTCA
Wnt4 F	CGAGCAATTGGCTGTACCTGG
Wnt4 R	GGCCTTTGAGTTTCTCGCAC
Yap F	GACGCTGATGAATTCTGCCTCA
Yap R	CATGGCAAAACGAGGGTCC

Table S1. Primer list

Chapter 5. General discussion

5.1 Why study Hippo signaling in the ovary?

My PhD thesis investigated how Hippo signaling in the mouse ovary is regulated and the roles of the Hippo pathway effectors LATS1, LATS2, YAP, and TAZ in ovarian follicle development. The major findings from our studies identified that LH activates the Hippo pathway and appears to do so by signaling via PKA, that *Yap* and *Taz* are required to mediate the granulosa cell response to LH, and that *Lats1/2* are required to maintain postnatal granulosa cell fate and function. How this project came about was as a logical next step for the Boerboom laboratory but was also inspired by evidence in the literature that supported a role for Hippo signaling in the ovary.

For over a decade, the focus of the Boerboom laboratory has been to study the roles of Wnt/β-catenin signaling pathway effectors in ovarian follicle development and cancer. An increasing amount of reports in the literature has identified clear links between Wnt and Hippo signaling in the context of embryonic development and cancer in numerous tissue types, which led us to believe that this might also be the case in ovarian physiology. While investigating the interactions between Wnt and Hippo signaling in the ovary was not an objective of this thesis, it was nonetheless important to take this relationship into consideration when trying to interpret our results (for example, how the loss of *Lhcgr* expression might be linked to the loss of phospho-β-catenin activity in the absence of *Yap/Taz*). To study a similar developmental signaling pathway in the context of ovarian follicle development was a relatively easy transition to make, given that we already had the expertise and material required to carry out the majority of experiments, including many of the mouse strains.

Another important reason that led to our decision to study the Hippo pathway was that an increasing number of reports in the literature hinted at a role for Hippo signaling in the ovary. Ablation of Hippo effectors were found to reduce fertility and even be implicated in ovarian cancer, while disruption of Hippo signaling was shown to promote follicle growth. However, specific roles for these effectors had not been investigated in the context of ovarian follicle development.

Given that Hippo has no specific ligands or receptors and that its regulation varies greatly depending on the cell type, we wondered how Hippo might be regulated specifically in granulosa cells. We hypothesized that if Hippo signaling is indeed critical for follicle development, then gonadotropins that govern late stages of follicle development, were likely

candidates as regulators of Hippo signaling. All of the effectors that mediate the transcription of FSH and LH target genes downstream of FSHR and LHCGR have yet to be uncovered and we hypothesized that the Hippo pathway might be a part of that missing link.

For all of these reasons, we designed two studies that would attempt to address all of these issues, as described in this thesis.

5.2 Discussion topics for Article 1

5.2.1 Challenges

While undertaking this study, we faced several challenges in terms of managing experimental variables, a lack of specific agonists/antagonists, a lack of quality antibodies, the absence of proven target genes in granulosa cells, and generating useful mouse models.

It is well known that Hippo signaling is regulated by a variety of cues, which makes studying Hippo signaling *in vitro* very challenging. Variables that potentially came into play when working with primary granulosa cell cultures (that have been shown to affect Hippo signaling) include: cell density, use of serum in the culture medium, rigidity of the cell culture plate, cell attachment, and mechanical disruption (i.e. isolation of granulosa cells by needle puncture). It was possible to control for some of these variables, for example, by testing for the optimal cell density (a density at which Hippo signaling is not yet activated), and by "serum starving" the cells prior to treatment, however, it was not possible to control for the other variables in order to attenuate their potential impact on Hippo signaling.

Based on the same premise, that Hippo signaling has no specific ligands but is regulated by a variety of cues, this entails that there are no Hippo-specific agonists that exist that we could have used as positive controls. In addition, at the time of the study, very few YAP/TAZ-specific inhibitors were commercially available. The best described is the small molecule verteporfin that inhibits the interaction between YAP and TEAD, however, its mechanism of action is not clear (Liu-Chittenden et al, 2012). Another small molecule antagonist, C19, strongly inhibits Hippo, but also inhibits Wnt and TGF-β pathways, making it a non-specific inhibitor (Basu et al, 2014). More novel small molecule antagonists that target the YAP/TAZ/TEAD interaction

are appearing in the literature, however, whether they are truly specific inhibitors remains to be seen.

Given that Hippo signaling is a relatively recently discovered signaling pathway, quality antibodies are still lacking. At the time of the study, there was no commercially available phospho-LATS2 antibody that worked for IHC or WB, and the only two phospho-TAZ antibodies commercially available were both discontinued halfway through the study.

Microarray analyses have been performed on numerous cell types and have identified direct Hippo target genes that appear to vary depending on the cell type. Given that no such study has been performed to date on granulosa cells, mRNA-Seq was attempted in order to identify granulosa cell-specific Hippo target genes. Unfortunately, after several unsuccessful attempts, we opted instead to perform microarray analyses on *Yap*$^{flox/flox}$*;Taz*$^{flox/flox}$ primary cultured granulosa cells infected with either Ad-eGFP or Ad-cre for 18h; these results are still pending. Therefore, we were faced with the challenge of evaluating Hippo signaling in the absence of direct downstream targets that could have served to validate the presence or absence of Hippo activity. We tested out several reported Hippo target genes (identified in other cell types) and identified *Ctgf* and *Cyr61* as candidate target genes by RT-qPCR; these have yet to be evaluated by microarray.

While not the primary objective of the study, we did attempt to study the physiological roles of YAP and TAZ around the antral stage of follicle development by generating *Yap*$^{flox/flox}$*;CYP19*-cre, *Taz*$^{flox/flox}$*;CYP19*-cre, and *Yap*$^{flox/flox}$*;Taz*$^{flox/flox}$;*CYP19*-cre mice but unfortunately, the recombination efficiency was poor in granulosa cells and consequently there was no resulting reproductive phenotype for any of the models. This prohibited our ability to study *Yap* and *Taz in vivo* and obligated us to pursue our study using primary granulosa cell cultures.

5.2.2 In retrospect

The characterization of FSH as a regulator of Hippo signaling in granulosa cells was set aside in order to focus entirely on LH regulation of Hippo signaling, given that the preliminary FSH results were not as conclusive as the LH results. If time had permitted it, it would have

been interesting to continue to pursue that avenue of investigation because it would have allowed us to paint a more complete picture of Hippo signaling over the course of follicle development.

5.2.3 Perspectives

The results obtained in Article 1 inspired several ideas for follow-up experiments. Having identified that LH regulates Hippo signaling, it would be very interesting to uncover what physiological role(s) *Yap/Taz* play at that stage of follicle development. One method would be to use the *PR*-cre strain of mice (that is under control of the promoter of the progesterone receptor locus (Soyal et al, 2005)) to generate $Yap^{flox/flox}$;$Taz^{flox/flox}$;*PR*-cre which would inactivate *Yap/Taz* primarily in preovulatory follicles, and then perform a full fertility profile on these mice (mating trials, ovulation rates, COC expansion analysis, and serum hormone measurements).

It would be worthwhile to investigate precisely what happens to YAP/TAZ when they are phosphorylated by LATS1 4h post hCG. While we did collect ovaries treated with hCG on a time course and performed IHC to probe for Hippo effectors, we were unable to distinguish whether YAP/TAZ significantly changed subcellular localization or not. One method would be to perform double immunofluorescence for YAP/TAZ combined to a nuclear marker, which would make it possible to quantify the relative amount of nuclear vs cytoplasmic YAP/TAZ over time (following treatment with hCG on a time course).

It would be important to complete the characterization of Hippo signaling downstream of LH by evaluating other core members of the Hippo pathway including MST1/2, SAV1, and MOB1 (phospho- and total protein) by WB and IHC on mice treated with hCG on a time course. We suspect that Hippo signaling is activated downstream of PKA, but is this along the same cascade that leads to activation of EGFR/ERK or an independent one? It would be possible to evaluate this by knocking down *Yap*/*Taz* and determining whether this affects the phosphorylation of EGFR/ERK signaling effectors, or not, by Western blotting.

5.2.4 Comparison to the literature

Two of our main findings are indirectly supported by evidence in the literature, including the identification of LH as a regulator of Hippo signaling that appears to signal via PKA and the

redundancy of Hippo pathway effectors. In one study, inhibition of $G_{\alpha s}$-PKA signaling led to inhibition of Hippo signaling and activation of YAP in basal cell carcinoma (Iglesias-Bartolome et al, 2015). In another study, GPCR signaling activated PKA, which directly phosphorylated LATS to promote phosphorylation of YAP(Ser397) in mouse embryonic fibroblasts (Kim et al, 2013). Given that the LH receptor is a classic example of a $G_{\alpha s}$PCR, and that GPCR signaling is a well-established regulator of Hippo signaling, our identification of LH as a Hippo regulator fits within that logic.

Based on our results, *Yap* and *Taz* appear to exert non-redundant functions in granulosa cells. Ablation of either *Yap* or *Taz in vitro* resulted in a blunted response to LH, with a more marked inhibition in the absence of *Taz*. This is in contrast to *Lats1/2*, in which *in vivo* knockdown of *Lats1* or *Lats2* alone had no phenotype, suggesting that *Lats1* and *Lats2* have redundant functions in this context. The *in vitro* results in which *Lats1/2* were knocked down are more confounding, but seem to suggest non-redundant functions for *Lats1/2* in regulating the gonadotropin response, but redundant functions in regulating the expression of Sertoli markers. Both of these findings can be corroborated by evidence in the literature that has shown redundant and non-redundant roles for both of these homologous pairs of proteins depending on the cellular context (Hossain et al, 2007; McPherson et al, 2004; Morin-Kensicki et al, 2006; St John et al, 1999).

5.3 Discussion topics Article 2

5.3.1 Further discussion topics

There are two topics that were not addressed in the article but require some clarification nonetheless, pertaining to the differentiation process and the oocyte phenotype. A subject of debate is whether loss of *Lats1/2* is inducing de-differentiation *vs* transdifferentiation of granulosa cells into other cell types. De-differentiation has been described in the context of induced pluripotent stem cells, in which terminally differentiated cells acquire pluripotent characteristics. Transdifferentiation implies that a direct differentiation occurs from one mature cell type to another, often between two cell types that share a common cell precursor (Zhou & Melton, 2008). It is very difficult in practice to distinguish between the two processes. The

majority of reports that describe partial sex reversal phenotypes assume that a process of transdifferentiation is occurring from granulosa to Sertoli or vice versa, mostly based on temporal expression of female vs male-specific genes, without further validation (Uhlenhaut et al, 2009). In our case, a heterogeneous cell population arises from granulosa cells, making it difficult to account for the development of each cell type. The fact that there is a general increase in stem cell markers within *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre ovaries suggests a process of de-differentiation. In contrast, the fact that both SOX9 and FOXL2 are expressed at 10 dpp, albeit in different cells, suggests that transdifferentiation is also occurring. These findings support the likelihood that both processes are occurring simultaneously within the heterogeneous granulosa cell-derived population.

While it was not the focus of our study and was not a cell type targeted by the cre-driver, it cannot be overlooked that knockdown of *Lats1/2* also affected the oocytes. Morphologic changes consistent with apoptotic oocytes were evident in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre ovaries, including shrinkage (as evidenced by the retraction of the oocyte cell membrane from the zona pellucida) and membrane blebbing (Wu et al, 2000). By 21 dpp, we observe large "orphan" oocytes not surrounded by any normal-looking granulosa cells and by 30 dpp, the ovary is entirely devoid of oocytes. Premature loss of granulosa cells surrounding the oocyte is a well-known inducer of oocyte apoptosis, given that this deprives the oocyte of growth factors, nutrients, and survival factors necessary for maintaining its health (Tiwari et al, 2015). Therefore, the rapid loss of oocytes can be explained as an indirect effect secondary to the loss of normal granulosa cells.

5.3.2 Challenges

We did face certain setbacks and challenges over the course of this study. In order to study physiological roles for *Lats1/2* in granulosa cells at an earlier stage of follicle development, we generated *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*AMHR2*-cre mice (Jorgez et al, 2004). Unfortunately, while fascinating phenotypes occurred within derivatives of the Müllerian duct and in the OSE, recombination was poor in granulosa cells and therefore revealed nothing about the potential roles for *Lats1/2* in this cell context.

Knockdown of *Lats1/2* in granulosa cells using the *CYP19*-cre strain generated mice whose ovaries developed a rapid and complex cellular phenotype that evolved over time to ultimately obliterate any normal ovarian architecture. This created certain difficulties for the characterization of the phenotype. For instance, we were unable to isolate and study granulosa cells from the mutant ovaries, given that granulosa cells rapidly differentiated into different cell types starting at 10 dpp. It was difficult to differentiate between direct vs indirect effects induced by ablation of *Lats1/2*. For example, the new cell population exhibited a loss of GJA1; does this imply that *Lats1/2* are required for the formation/maintenance of gap junctions or is this an indirect effect? In addition, although we expected the ablation of *Lats1/2* to cause reproductive defects or even ovarian cancer, the unanticipated phenotype ultimately caused us to stray from the intended purpose of the study and to evaluate processes such as sex reversal, transdifferentiation, EMT, stem cells, and osteogenesis.

5.3.3 In retrospect

We were unable to optimize some techniques, notably co-immunoprecipitation, despite trying many different protocols, kits, and antibodies. This technique was employed in *Lats1/2*-depleted granulosa cells to evaluate whether more YAP was bound to TEAD relative to *Lats1/2*-intact cells; which we suspected was driving the aberrant transcription of *Sox9,* as previously described in esophageal cancer cells (Song et al, 2014). Similarly, we wanted to evaluate in these same cells, whether more TAZ was bound to RUNX2 (relative to controls), which we suspected was driving the aberrant transcription of *Bglap*, as described in an osteosarcoma cell line (Cui et al, 2003). We suspect that the primary reason for our failure is that we tried to pulldown endogenous proteins from primary cell cultures; all reports of co-immunoprecipitation in the literature describe using this technique with immortalized cell lines. Working with immortalized cell lines makes it possible to introduce large amounts of a given protein of interest to then determine which proteins are bound to that protein. Briefly, this can be done by transfecting cells with a vector expressing YAP, for example, that is fused to an antigen tag, immunoprecipitating YAP by using an antibody against the tag, and then by WB, probing against suspected YAP binding partners. While the downside of this technique is that it does

not represent physiological interactions, it would have been useful to first confirm that our technique worked, before using it in a more physiological context, such as primary cell cultures.

5.3.4 Perspectives

Future experiments that would add a mechanistic component to our study include investigating the driver responsible for initiating the ovarian phenotype in our model as well as identifying the sex-determining gene responsible for the transdifferentiation of granulosa-to-Sertoli-like cells. We suspect that excessive YAP/TAZ are effectively responsible for driving the ovarian phenotype in *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$;*CYP19*-cre ovaries; the alternative being that knockdown of *Lats1/2* is affecting another substrate. To do this, granulosa cells could be placed in culture and transfected with expression vectors that overexpress *Yap/Taz* to determine whether this is sufficient to drive the knockdown of granulosa cell-specific genes and the expression of Sertoli, osteoblast, stem, and neural crest cell-specific genes.

It would be important to investigate further the mechanism driving the transdifferentiation of granulosa cells into Sertoli-like cells. Ablation of several female-specific genes have been reported to induce granulosa cell transdifferentiation including *Foxl2, Wnt4, Esr1/2,* and *Rspo1* (Chassot et al, 2008; Couse et al, 1999; Uhlenhaut et al, 2009; Vainio et al, 1999). We suspect that YAP/TAZ are either directly activating male-specific genes (*Sox9, Dmrt1)* or alternatively, repressing female-specific genes. One method to evaluate this would be to perform ChIP-Seq on *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells infected with Ad-eGFP or Ad-cre for 30 hours, to pulldown YAP or TAZ and to sequence all the binding sites to identify the gene promoters that are bound by YAP/TAZ. This would with any luck correspond to the direct YAP/TAZ target gene(s) driving the transdifferentiation process.

While unrelated to our original objectives, it would be fascinating to study the potential multipotency of *Lats1/2*-depleted granulosa cells. To do this, *Lats1*$^{flox/flox}$;*Lats2*$^{flox/flox}$ granulosa cells would be placed in culture, infected with Ad-eGFP or Ad-cre and then cultured with, for example, osteogenic differentiation medium to observe whether these cells are able to develop into osteoblasts. This would confirm an earlier report that identified LATS as barriers to pluripotency in human cells (Qin et al, 2012) as well as potentially provide a new source of cells to those that work with induced pluripotent stem cells (iPSCs).

5.3.5 Comparison to the literature

Our report is not the first to describe *in vivo* cell lineage reprogramming, however, our results are novel because they identified that knockdown of *Lats1/2*, which are not known to be sex-determining genes, is driving this process. This makes it more complicated to investigate the mechanism whereby partial sex reversal is occurring in the absence of *Lats1/2*. If we compare our model to other models in which loss-of-function of female sex-determining genes (*Wnt4, Rspo1, Foxl2*, etc.) drove the transdifferentiation of granulosa-to-Sertoli cells, we find that ours is most similar to the inducible model of *Foxl2* ablation in adult mice, in terms of ovarian phenotype and gene expression (Uhlenhaut et al, 2009). Investigation into the precise link between LATS1/2 and FOXL2, whether loss of LATS1/2 is directly responsible for the loss of FOXL2 expression or whether it is by YAP/TAZ-mediated upregulation of *Sox9* (that would repress *Foxl2* expression), remains to be determined.

The plasticity of granulosa cells has already been reported in the literature. Human and porcine granulosa cells that were placed in culture de-differentiated after several days and then using specific growth differentiation media were induced to differentiate into osteoblasts, chondrocytes, and neurons (Kossowska-Tomaszczuk et al, 2009; Mattioli et al, 2012; Oki et al, 2012). The mechanism by which this occurred, however, was not elucidated. Our findings relating to knockdown of *Lats1/2* may therefore provide insight into a mechanism whereby de-differentiation occurs in other cell types.

5.4 A global model of Hippo signaling in the ovary

5.4.1 Comparison of results from both studies

Results from both studies support the idea that Hippo signaling is indeed active in murine granulosa cells and more specifically, that LATS1/2 phosphorylate YAP/TAZ. Both studies also support a post-translational mode of regulation for LATS1/2 over YAP/TAZ (by phosphorylation). Indeed, in response to LH treatment or ablation of *Lats1/2*, YAP/TAZ phosphorylation is induced, while *Yap/Taz* relative mRNA levels remain stable. Given that Hippo signaling has been found to vary between cell types, for example, in mouse embryonic

fibroblasts, in which MST1/2 directly regulate YAP/TAZ independently from LATS1/2, this establishes a solid foundation for future research of Hippo signaling in this cell type.

One confounding result that arose when comparing the two studies is that ablation of both *Lats1/2* and *Yap/Taz* followed by LH treatment resulted in blunted LH induction of target genes. One would expect that if LATS1/2 directly inactivate YAP/TAZ, that the opposite effects would be observed, however, this can be explained by two independent theories. In the absence of *Lats1/2*, we suspect that a global reprogramming of the granulosa cell genetic program has occurred such that they can no longer function as granulosa cells (i.e. to respond to LH by inducing the transcription of LH target genes). In the case of *Yap/Taz* ablation, we suspect that the loss of *Lhcgr* expression is responsible for the inability of granulosa cells to induce the expression of LH target genes.

Results from both studies suggest that *Ctgf* is a YAP/TAZ/TEAD target gene in granulosa cells. This is not surprising, given that *Ctgf* has been identified as a direct Hippo target gene in a large number of cell types and is frequently used to confirm YAP/TAZ activity. Microarray results performed on $Yap^{flox/flox};Taz^{flox/flox}$ granulosa cells infected with either Ad-eGFP or Ad-cre will be able to support this finding.

5.4.2 Importance of findings for the scientific community

Results from the present studies have the potential not only to further the understanding of signaling pathways involved in ovarian follicle development, but also to affect the fields of sex determination and stem cell biology. Our novel finding that *Yap* and *Taz* are required for LH to induce the expression of its target genes in granulosa cells in a mechanism that involves (at least in part) regulation of *Lhcgr* expression provides one more piece of the puzzle required to resolve the extraordinarily complex signaling cascade that is initiated by the LH surge. We showed that Hippo signaling is indeed active in murine granulosa cells and this information alone provides groundwork for future studies investigating physiological roles for Hippo effectors in ovarian physiology, and can also be useful to study how disruption of the Hippo pathway might be involved in ovarian pathology, such as ovarian cancer.

The unexpected identification of LATS1/2 as essential mediators of granulosa cell fate maintenance introduces a whole new player into the battle of the (maintenance of the) sexes,

that is not a primary sex-determining gene but most likely regulates one (such as *Foxl2* or *Sox9*), and may provide insight into the mechanisms responsible for disorders of sex development. Finally, the discovery that knockdown of *Lats1/2* induces EMT and the expression of stem cell markers in granulosa cells further exemplifies the fact that terminally differentiated cells do retain some degree of plasticity and can potentially serve as models for the study of induced pluripotent stem cells.

Together, results from the present thesis provide a solid foundation and justify the continued study of Hippo signaling in granulosa cells.

Chapter 6. Final conclusions

Results from this thesis undeniably serve to advance the understanding of Hippo signaling in ovarian follicle development, of which little information exists to date. In our first study, we identified that LH is an upstream regulator of Hippo signaling and that it appears to act via PKA. We showed that canonical Hippo signaling is present in granulosa cells, which entails the activation of LATS1/2 that phosphorylate and inactivate YAP/TAZ. We showed that *Yap* and *Taz* are essential for LH to induce the expression of its target genes, and that this might be as a result of the loss of *Lhcgr* expression. These results have allowed us to create a hypothetical signaling cascade downstream of LHCGR that will need to be confirmed by additional studies (Figure 5.1).

In our second study, we identified a novel role for LATS1/2 as essential mediators of granulosa cell fate maintenance. In *Lats1/2*-depleted granulosa cells, we showed that granulosa cells lose their identity and function, undergo EMT, and transdifferentiate into Sertoli-like cells, osteoblasts, and neural crest cell derivatives. We believe that an overaccumulation of YAP/TAZ is inducing the aberrant transcription of target genes responsible for driving these cellular processes. We have likely only reached the tip of the iceberg with regards to uncovering the roles of the Hippo pathway in the ovary and I sincerely hope that the results from the present studies will encourage the continuation of this investigation.

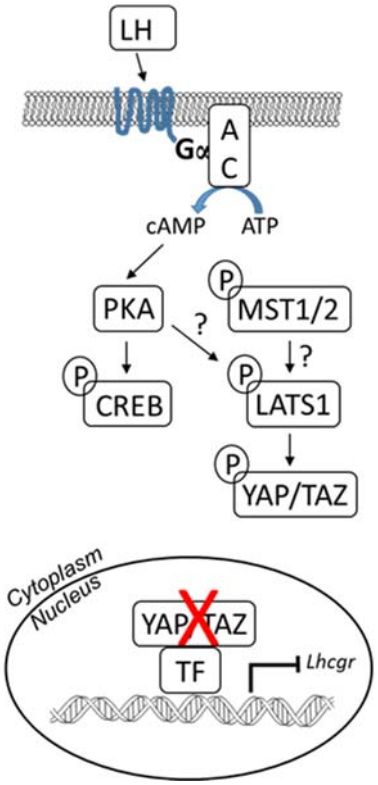

Figure 6.1 Hypothetical model of Hippo signaling in mouse granulosa cells

References

1. Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ, Kidder GM (2001) Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Developmental biology* **233:** 258-270

2. Adhikari D, Flohr G, Gorre N, Shen Y, Yang H, Lundin E, Lan Z, Gambello MJ, Liu K (2009) Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. *Molecular human reproduction* **15:** 765-770

3. Alam H, Maizels ET, Park Y, Ghaey S, Feiger ZJ, Chandel NS, Hunzicker-Dunn M (2004) Follicle-stimulating hormone activation of hypoxia-inducible factor-1 by the phosphatidylinositol 3-kinase/AKT/Ras homolog enriched in brain (Rheb)/mammalian target of rapamycin (mTOR) pathway is necessary for induction of select protein markers of follicular differentiation. *The Journal of biological chemistry* **279:** 19431-19440

4. Alarcon C, Zaromytidou AI, Xi Q, Gao S, Yu J, Fujisawa S, Barlas A, Miller AN, Manova-Todorova K, Macias MJ, Sapkota G, Pan D, Massague J (2009) Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell* **139:** 757-769

5. Andrade D, Mehta M, Griffith J, Panneerselvam J, Srivastava A, Kim TD, Janknecht R, Herman T, Ramesh R, Munshi A (2017) YAP1 inhibition radiosensitizes triple negative breast cancer cells by targeting the DNA damage response and cell survival pathways. *Oncotarget* **8:** 98495-98508

6. Asthagiri AR, Parry DM, Butman JA, Kim HJ, Tsilou ET, Zhuang Z, Lonser RR (2009) Neurofibromatosis type 2. *Lancet* **373:** 1974-1986

7. Auchus RJ (2015) Human Steroid Biosynthesis. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik A.J. (ed), Vol. 1, 4th edn, 8, pp 295-312. London, UK: Academic Press

8. Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M (2006) A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes & development* **20:** 2687-2700

9. Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V, Fassina A, Cordenonsi M, Piccolo S (2014) YAP/TAZ incorporation in the beta-catenin destruction complex orchestrates the Wnt response. *Cell* **158:** 157-170

10. Azzolin L, Zanconato F, Bresolin S, Forcato M, Basso G, Bicciato S, Cordenonsi M, Piccolo S (2012) Role of TAZ as mediator of Wnt signaling. *Cell* **151:** 1443-1456

11. Bai H, Zhang N, Xu Y, Chen Q, Khan M, Potter JJ, Nayar SK, Cornish T, Alpini G, Bronk S, Pan D, Anders RA (2012) Yes-associated protein regulates the hepatic response after bile duct ligation. *Hepatology* **56:** 1097-1107

12. Bartucci M, Dattilo R, Moriconi C, Pagliuca A, Mottolese M, Federici G, Benedetto AD, Todaro M, Stassi G, Sperati F, Amabile MI, Pilozzi E, Patrizii M, Biffoni M, Maugeri-Sacca M, Piccolo S, De Maria R (2014) TAZ is required for metastatic activity and chemoresistance of breast cancer stem cells. *Oncogene*

13. Basu D, Lettan R, Damodaran K, Strellec S, Reyes-Mugica M, Rebbaa A (2014) Identification, mechanism of action, and antitumor activity of a small molecule inhibitor of hippo, TGF-beta, and Wnt signaling pathways. *Molecular cancer therapeutics* **13:** 1457-1467

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

15. Bertolin K, Murphy BD (2014) Reproductive Tract Changes During the Mouse Estrous Cycle. In *The Guide to Investigation of Mouse Pregnancy, First Edition*, pp 85-94. 2014 Elsevier Inc.

16. Binart N, Helloco C, Ormandy CJ, Barra J, Clement-Lacroix P, Baran N, Kelly PA (2000) Rescue of preimplantatory egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration. *Endocrinology* **141:** 2691-2697

17. Boerboom D, Paquet M, Hsieh M, Liu J, Jamin SP, Behringer RR, Sirois J, Taketo MM, Richards JS (2005) Misregulated Wnt/beta-catenin signaling leads to ovarian granulosa cell tumor development. *Cancer research* **65:** 9206-9215

18. Bottomly D, Kyler SL, McWeeney SK, Yochum GS (2010) Identification of {beta}-catenin binding regions in colon cancer cells using ChIP-Seq. *Nucleic acids research* **38:** 5735-5745

19. Byun MR, Hwang JH, Kim AR, Kim KM, Hwang ES, Yaffe MB, Hong JH (2014) Canonical Wnt signalling activates TAZ through PP1A during osteogenic differentiation. *Cell death and differentiation* **21:** 854-863

20. Cai H, Xu Y (2013) The role of LPA and YAP signaling in long-term migration of human ovarian cancer cells. *Cell Commun Signal* **11:** 31

21. Cai J, Zhang N, Zheng Y, de Wilde RF, Maitra A, Pan D (2010) The Hippo signaling pathway restricts the oncogenic potential of an intestinal regeneration program. *Genes & development* **24:** 2383-2388

22. Callus BA, Verhagen AM, Vaux DL (2006) Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. *FEBS J* **273:** 4264-4276

23. Calvo F, Ege N, Grande-Garcia A, Hooper S, Jenkins RP, Chaudhry SI, Harrington K, Williamson P, Moeendarbary E, Charras G, Sahai E (2013) Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nature cell biology* **15:** 637-646

24. Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R, Brummelkamp TR (2007) YAP1 increases organ size and expands undifferentiated progenitor cells. *Current biology : CB* **17:** 2054-2060

25. Carlone DL, Richards JS (1997) Functional interactions, phosphorylation, and levels of 3',5'-cyclic adenosine monophosphate-regulatory element binding protein and steroidogenic factor-1 mediate hormone-regulated and constitutive expression of aromatase in gonadal cells. *Molecular endocrinology (Baltimore, Md)* **11:** 292-304

26. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21:** 2933-2942

27. Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA (2003) Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science (New York, NY)* **301:** 215-218

28. Chan EH, Nousiainen M, Chalamalasetty RB, Schafer A, Nigg EA, Sillje HH (2005) The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* **24:** 2076-2086

29. Chassot AA, Ranc F, Gregoire EP, Roepers-Gajadien HL, Taketo MM, Camerino G, de Rooij DG, Schedl A, Chaboissier MC (2008) Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Human molecular genetics* **17:** 1264-1277

30. Chen T, You Y, Jiang H, Wang ZZ (2017) Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis. *Journal of cellular physiology* **232:** 3261-3272

31. Chen Y, Jefferson WN, Newbold RR, Padilla-Banks E, Pepling ME (2007) Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology* **148:** 3580-3590

32. Cheng Y, Feng Y, Jansson L, Sato Y, Deguchi M, Kawamura K, Hsueh AJ (2015) Actin polymerization-enhancing drugs promote ovarian follicle growth mediated by

the Hippo signaling effector YAP. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **29:** 2423-2430

33. Chesnel F, Eppig JJ (1995) Synthesis and accumulation of p34cdc2 and cyclin B in mouse oocytes during acquisition of competence to resume meiosis. *Molecular reproduction and development* **40:** 503-508

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

35. Chun SY, Eisenhauer KM, Minami S, Billig H, Perlas E, Hsueh AJ (1996) Hormonal regulation of apoptosis in early antral follicles: follicle-stimulating hormone as a major survival factor. *Endocrinology* **137:** 1447-1456

36. Clark BJ, Wells J, King SR, Stocco DM (1994) The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *The Journal of biological chemistry* **269:** 28314-28322

37. Clevers H (2006) Wnt/beta-catenin signaling in development and disease. *Cell* **127:** 469-480

38. Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. *Cell* **149:** 1192-1205

39. Conti M, Hsieh M, Park JY, Su YQ (2006) Role of the epidermal growth factor network in ovarian follicles. *Molecular endocrinology (Baltimore, Md)* **20:** 715-723

40. Conti M, Hsieh M, Zamah AM, Oh JS (2012) Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Molecular and cellular endocrinology* **356:** 65-73

41. Cordenonsi M, Zanconato F, Azzolin L, Forcato M, Rosato A, Frasson C, Inui M, Montagner M, Parenti AR, Poletti A, Daidone MG, Dupont S, Basso G, Bicciato S, Piccolo S (2011) The Hippo transducer TAZ confers cancer stem cell-related traits on breast cancer cells. *Cell* **147:** 759-772

42. Cottom J, Salvador LM, Maizels ET, Reierstad S, Park Y, Carr DW, Davare MA, Hell JW, Palmer SS, Dent P, Kawakatsu H, Ogata M, Hunzicker-Dunn M (2003) Follicle-stimulating hormone activates extracellular signal-regulated kinase but not extracellular signal-regulated kinase kinase through a 100-kDa phosphotyrosine phosphatase. *The Journal of biological chemistry* **278:** 7167-7179

43. Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ, Korach KS (1999) Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science (New York, NY)* **286:** 2328-2331

44. Couse JF, Korach KS (2001) Contrasting phenotypes in reproductive tissues of female estrogen receptor null mice. *Ann N Y Acad Sci* **948:** 1-8

45. Cui CB, Cooper LF, Yang X, Karsenty G, Aukhil I (2003) Transcriptional coactivation of bone-specific transcription factor Cbfa1 by TAZ. *Molecular and cellular biology* **23:** 1004-1013

46. Cunningham MA, Zhu Q, Unterman TG, Hammond JM (2003) Follicle-stimulating hormone promotes nuclear exclusion of the forkhead transcription factor FoxO1a via phosphatidylinositol 3-kinase in porcine granulosa cells. *Endocrinology* **144:** 5585-5594

47. Curry TE, Jr., Osteen KG (2003) The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocrine reviews* **24:** 428-465

48. Das Thakur M, Feng Y, Jagannathan R, Seppa MJ, Skeath JB, Longmore GD (2010) Ajuba LIM proteins are negative regulators of the Hippo signaling pathway. *Current biology : CB* **20:** 657-662

49. Del Re DP, Yang Y, Nakano N, Cho J, Zhai P, Yamamoto T, Zhang N, Yabuta N, Nojima H, Pan D, Sadoshima J (2013) Yes-associated protein isoform 1 (Yap1) promotes cardiomyocyte survival and growth to protect against myocardial ischemic injury. *The Journal of biological chemistry* **288:** 3977-3988

50. Diehl JA, Cheng M, Roussel MF, Sherr CJ (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes & development* **12:** 3499-3511

51. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P (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 of the United States of America* **95:** 13612-13617

52. Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, et al. (1995) Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* **378:** 406-409

53. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383:** 531-535

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

55. Duggavathi R, Volle DH, Mataki C, Antal MC, Messaddeq N, Auwerx J, Murphy BD, Schoonjans K (2008) Liver receptor homolog 1 is essential for ovulation. *Genes & development* **22:** 1871-1876

56. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S, Elvassore N, Piccolo S (2011) Role of YAP/TAZ in mechanotransduction. *Nature* **474:** 179-183

57. Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP (1999) Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology* **140:** 5789-5796

58. Eagle H, Levine EM (1967) Growth regulatory effects of cellular interaction. *Nature* **213:** 1102-1106

59. Escamilla-Hernandez R, Little-Ihrig L, Orwig KE, Yue J, Chandran U, Zeleznik AJ (2008) Constitutively active protein kinase A qualitatively mimics the effects of follicle-stimulating hormone on granulosa cell differentiation. *Molecular endocrinology (Baltimore, Md)* **22:** 1842-1852

60. Espey LL, Richards JS (2002) Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. *Biology of reproduction* **67:** 1662-1670

61. Evans HE, DeLahunta A (2004) *Guide to the Dissection of the Dog*, 6th edn. Missouri: Elsevier.

62. Fan HY, Liu Z, Cahill N, Richards JS (2008a) Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells. *Molecular endocrinology (Baltimore, Md)* **22:** 2128-2140

63. Fan HY, Liu Z, Paquet M, Wang J, Lydon JP, DeMayo FJ, Richards JS (2009a) Cell type-specific targeted mutations of Kras and Pten document proliferation arrest in granulosa cells versus oncogenic insult to ovarian surface epithelial cells. *Cancer research* **69:** 6463-6472

64. Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, Richards JS (2009b) MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science (New York, NY)* **324:** 938-941

65. Fan HY, Shimada M, Liu Z, Cahill N, Noma N, Wu Y, Gossen J, Richards JS (2008b) Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. *Development (Cambridge, England)* **135:** 2127-2137

66. Fang L, Du WW, Yang W, Rutnam ZJ, Peng C, Li H, O'Malley YQ, Askeland RW, Sugg S, Liu M, Mehta T, Deng Z, Yang BB (2012) MiR-93 enhances angiogenesis and metastasis by targeting LATS2. *Cell cycle (Georgetown, Tex)* **11:** 4352-4365

67. Ferrigno O, Lallemand F, Verrecchia F, L'Hoste S, Camonis J, Atfi A, Mauviel A (2002) Yes-associated protein (YAP65) interacts with Smad7 and potentiates its inhibitory activity against TGF-beta/Smad signaling. *Oncogene* **21:** 4879-4884

68. Fortune JE (2003) The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal reproduction science* **78:** 135-163

69. Fu D, Lv X, Hua G, He C, Dong J, Lele SM, Li DW, Zhai Q, Davis JS, Wang C (2014) YAP regulates cell proliferation, migration, and steroidogenesis in adult granulosa cell tumors. *Endocrine-related cancer* **21:** 297-310

70. Fu V, Plouffe SW, Guan KL (2017) The Hippo pathway in organ development, homeostasis, and regeneration. *Current opinion in cell biology* **49:** 99-107

71. Gardiner JR, Swain A (2015) Sex Determination and Differentiation. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik AJ (eds), Vol. 1, 4th edn, 7, pp 267-292. London, UK: Academic Press

72. Gava N, Clarke CL, Byth K, Arnett-Mansfield RL, deFazio A (2004) Expression of progesterone receptors A and B in the mouse ovary during the estrous cycle. *Endocrinology* **145:** 3487-3494

73. Goldring NB, Durica JM, Lifka J, Hedin L, Ratoosh SL, Miller WL, Orly J, Richards JS (1987) Cholesterol side-chain cleavage P450 messenger ribonucleic acid: evidence for hormonal regulation in rat ovarian follicles and constitutive expression in corpora lutea. *Endocrinology* **120:** 1942-1950

74. Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS (2000) Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-lnduced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. *Molecular endocrinology (Baltimore, Md)* **14:** 1283-1300

75. Graves JD, Gotoh Y, Draves KE, Ambrose D, Han DK, Wright M, Chernoff J, Clark EA, Krebs EG (1998) Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *The EMBO journal* **17:** 2224-2234

76. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R (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:** 245-250

77. Gupta MK, Guryev OL, Auchus RJ (2003) 5alpha-reduced C21 steroids are substrates for human cytochrome P450c17. *Arch Biochem Biophys* **418:** 151-160

78. Hall CA, Wang R, Miao J, Oliva E, Shen X, Wheeler T, Hilsenbeck SG, Orsulic S, Goode S (2010) Hippo pathway effector Yap is an ovarian cancer oncogene. *Cancer research* **70:** 8517-8525

79. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* **144:** 646-674

80. Hao Y, Chun A, Cheung K, Rashidi B, Yang X (2008) Tumor suppressor LATS1 is a negative regulator of oncogene YAP. *The Journal of biological chemistry* **283:** 5496-5509

81. Hartsock A, Nelson WJ (2008) Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochimica et biophysica acta* **1778:** 660-669

82. Harvey KF, Zhang X, Thomas DM (2013) The Hippo pathway and human cancer. *Nature reviews Cancer* **13:** 246-257

83. Heallen T, Morikawa Y, Leach J, Tao G, Willerson JT, Johnson RL, Martin JF (2013) Hippo signaling impedes adult heart regeneration. *Development (Cambridge, England)* **140:** 4683-4690

84. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, Martin JF (2011) Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science (New York, NY)* **332:** 458-461

85. Heath E, Tahri D, Andermarcher E, Schofield P, Fleming S, Boulter CA (2008) Abnormal skeletal and cardiac development, cardiomyopathy, muscle atrophy and cataracts in mice with a targeted disruption of the Nov (Ccn3) gene. *BMC developmental biology* **8:** 18

86. Herbison AE (2015) Physiology of the Adult Gonadotropin-Releasing Hormone Neuronal Network. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik A.J. (ed), Vol. 1, 4th edn, 11, pp 399-467. London, UK: Academic Press

87. Hergovich A (2013) Regulation and functions of mammalian LATS/NDR kinases: looking beyond canonical Hippo signalling. *Cell & bioscience* **3:** 32

88. Hergovich A, Schmitz D, Hemmings BA (2006a) The human tumour suppressor LATS1 is activated by human MOB1 at the membrane. *Biochemical and biophysical research communications* **345:** 50-58

89. Hergovich A, Stegert MR, Schmitz D, Hemmings BA (2006b) NDR kinases regulate essential cell processes from yeast to humans. *Nature reviews Molecular cell biology* **7:** 253-264

90. Hiemer SE, Szymaniak AD, Varelas X (2014) The transcriptional regulators TAZ and YAP direct transforming growth factor beta-induced tumorigenic phenotypes in breast cancer cells. *The Journal of biological chemistry* **289:** 13461-13474

91. Hillier SG, Yong EL, Illingworth PJ, Baird DT, Schwall RH, Mason AJ (1991) Effect of recombinant activin on androgen synthesis in cultured human thecal cells. *The Journal of clinical endocrinology and metabolism* **72:** 1206-1211

92. Hindley CJ, Condurat AL, Menon V, Thomas R, Azmitia LM, Davis JA, Pruszak J (2016) The Hippo pathway member YAP enhances human neural crest cell fate and migration. *Scientific reports* **6:** 23208

93. Hizaki H, Segi E, Sugimoto Y, Hirose M, Saji T, Ushikubi F, Matsuoka T, Noda Y, Tanaka T, Yoshida N, Narumiya S, Ichikawa A (1999) Abortive expansion of the cumulus and impaired fertility in mice lacking the prostaglandin E receptor subtype EP(2). *Proceedings of the National Academy of Sciences of the United States of America* **96:** 10501-10506

94. Ho KC, Zhou Z, She YM, Chun A, Cyr TD, Yang X (2011) Itch E3 ubiquitin ligase regulates large tumor suppressor 1 stability [corrected]. *Proceedings of the National Academy of Sciences of the United States of America* **108:** 4870-4875

95. Hong JH, Hwang ES, McManus MT, Amsterdam A, Tian Y, Kalmukova R, Mueller E, Benjamin T, Spiegelman BM, Sharp PA, Hopkins N, Yaffe MB (2005) TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. *Science (New York, NY)* **309:** 1074-1078

96. Horton EW, Poyser NL (1976) Uterine luteolytic hormone: a physiological role for prostaglandin F2alpha. *Physiological reviews* **56:** 595-651

97. Hossain Z, Ali SM, Ko HL, Xu J, Ng CP, Guo K, Qi Z, Ponniah S, Hong W, Hunziker W (2007) Glomerulocystic kidney disease in mice with a targeted inactivation of Wwtr1. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 1631-1636

98. Hsieh M, Conti M (2005) G-protein-coupled receptor signaling and the EGF network in endocrine systems. *Trends in endocrinology and metabolism: TEM* **16:** 320-326

99. Hsieh M, Thao K, Conti M (2011) Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation. *PloS one* **6:** e21574

100. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4:** 44-57

101. Huang J, Wu S, Barrera J, Matthews K, Pan D (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122:** 421-434

102. Huang Z, Hu J, Pan J, Wang Y, Hu G, Zhou J, Mei L, Xiong WC (2016) YAP stabilizes SMAD1 and promotes BMP2-induced neocortical astrocytic differentiation. *Development (Cambridge, England)* **143:** 2398-2409

103. Humbert N, Navaratnam N, Augert A, Da Costa M, Martien S, Wang J, Martinez D, Abbadie C, Carling D, de Launoit Y, Gil J, Bernard D (2010) Regulation of ploidy and senescence by the AMPK-related kinase NUAK1. *The EMBO journal* **29:** 376-386

104. Hunzicker-Dunn M, Maizels ET (2006) FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. *Cellular signalling* **18:** 1351-1359

105. Hunzicker-Dunn M, Mayo K (2015) Gonadotropin Signaling in the Ovary. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik A.J. (ed), Vol. 1, 4th edn, 20, pp 895-945. London, UK: Academic Press

106. Huo X, Zhang Q, Liu AM, Tang C, Gong Y, Bian J, Luk JM, Xu Z, Chen J (2013) Overexpression of Yes-associated protein confers doxorubicin resistance in hepatocellullar carcinoma. *Oncol Rep* **29:** 840-846

107. Iglesias-Bartolome R, Torres D, Marone R, Feng X, Martin D, Simaan M, Chen M, Weinstein LS, Taylor SS, Molinolo AA, Gutkind JS (2015) Inactivation of a Galpha(s)-PKA tumour suppressor pathway in skin stem cells initiates basal-cell carcinogenesis. *Nature cell biology* **17:** 793-803

108. Iida S, Hirota T, Morisaki T, Marumoto T, Hara T, Kuninaka S, Honda S, Kosai K, Kawasuji M, Pallas DC, Saya H (2004) Tumor suppressor WARTS ensures genomic integrity by regulating both mitotic progression and G1 tetraploidy checkpoint function. *Oncogene* **23:** 5266-5274

109. Imajo M, Ebisuya M, Nishida E (2015) Dual role of YAP and TAZ in renewal of the intestinal epithelium. *Nature cell biology* **17:** 7-19

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

111. Jeong GO, Shin SH, Seo EJ, Kwon YW, Heo SC, Kim KH, Yoon MS, Suh DS, Kim JH (2013) TAZ mediates lysophosphatidic acid-induced migration and proliferation of epithelial ovarian cancer cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* **32:** 253-263

112. Jeppesen JV, Kristensen SG, Nielsen ME, Humaidan P, Dal Canto M, Fadini R, Schmidt KT, Ernst E, Yding Andersen C (2012) LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *The Journal of clinical endocrinology and metabolism* **97:** E1524-1531

113. Jho EH (2018) Hippo signaling: Special issue of BMB Reports in 2018. *BMB reports* **51:** 105

114. Jiang Z, Li X, Hu J, Zhou W, Jiang Y, Li G, Lu D (2006) Promoter hypermethylation-mediated down-regulation of LATS1 and LATS2 in human astrocytoma. *Neurosci Res* **56:** 450-458

115. John GB, Shidler MJ, Besmer P, Castrillon DH (2009) Kit signaling via PI3K promotes ovarian follicle maturation but is dispensable for primordial follicle activation. *Developmental biology* **331:** 292-299

116. Johnson R, Halder G (2014) The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. *Nature reviews Drug discovery* **13:** 63-79

117. Jorgez CJ, Klysik M, Jamin SP, Behringer RR, Matzuk MM (2004) Granulosa cell-specific inactivation of follistatin causes female fertility defects. *Molecular endocrinology (Baltimore, Md)* **18:** 953-967

118. Justice RW, Zilian O, Woods DF, Noll M, Bryant PJ (1995) The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes & development* **9:** 534-546

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

120. Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, Ho CH, Kawamura N, Tamura M, Hashimoto S, Sugishita Y, Morimoto Y, Hosoi Y, Yoshioka

N, Ishizuka B, Hsueh AJ (2013) Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. *Proceedings of the National Academy of Sciences of the United States of America* **110:** 17474-17479

121. Kayampilly PP, Menon KM (2004) Inhibition of extracellular signal-regulated protein kinase-2 phosphorylation by dihydrotestosterone reduces follicle-stimulating hormone-mediated cyclin D2 messenger ribonucleic acid expression in rat granulosa cells. *Endocrinology* **145:** 1786-1793

122. Kim J, Kim YH, Kim J, Park DY, Bae H, Lee DH, Kim KH, Hong SP, Jang SP, Kubota Y, Kwon YG, Lim DS, Koh GY (2017) YAP/TAZ regulates sprouting angiogenesis and vascular barrier maturation. *The Journal of clinical investigation* **127:** 3441-3461

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

124. Kim Y, Kobayashi A, Sekido R, DiNapoli L, Brennan J, Chaboissier MC, Poulat F, Behringer RR, Lovell-Badge R, Capel B (2006) Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. *PLoS biology* **4:** e187

125. Kodaka M, Hata Y (2015) The mammalian Hippo pathway: regulation and function of YAP1 and TAZ. *Cellular and molecular life sciences : CMLS* **72:** 285-306

126. Konsavage WM, Jr., Kyler SL, Rennoll SA, Jin G, Yochum GS (2012) Wnt/beta-catenin signaling regulates Yes-associated protein (YAP) gene expression in colorectal carcinoma cells. *The Journal of biological chemistry* **287:** 11730-11739

127. Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* **351:** 117-121

128. Kossowska-Tomaszczuk K, De Geyter C, De Geyter M, Martin I, Holzgreve W, Scherberich A, Zhang H (2009) The multipotency of luteinizing granulosa cells collected from mature ovarian follicles. *Stem cells (Dayton, Ohio)* **27:** 210-219

129. Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 2474-2479

130. Kumar TR, Wang Y, Lu N, Matzuk MM (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nature genetics* **15:** 201-204

131. Lai D, Ho KC, Hao Y, Yang X (2011) Taxol resistance in breast cancer cells is mediated by the hippo pathway component TAZ and its downstream transcriptional targets Cyr61 and CTGF. *Cancer research* **71:** 2728-2738

132. Lai ZC, Wei X, Shimizu T, Ramos E, Rohrbaugh M, Nikolaidis N, Ho LL, Li Y (2005) Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120:** 675-685

133. Law NC, Weck J, Kyriss B, Nilson JH, Hunzicker-Dunn M (2013) Lhcgr expression in granulosa cells: roles for PKA-phosphorylated beta-catenin, TCF3, and FOXO1. *Molecular endocrinology (Baltimore, Md)* **27:** 1295-1310

134. Leask A, Abraham DJ (2006) All in the CCN family: essential matricellular signaling modulators emerge from the bunker. *Journal of cell science* **119:** 4803-4810

135. Lee DH, Park JO, Kim TS, Kim SK, Kim TH, Kim MC, Park GS, Kim JH, Kuninaka S, Olson EN, Saya H, Kim SY, Lee H, Lim DS (2016) LATS-YAP/TAZ controls lineage specification by regulating TGFbeta signaling and Hnf4alpha expression during liver development. *Nature communications* **7:** 11961

136. Lee JH, Kim TS, Yang TH, Koo BK, Oh SP, Lee KP, Oh HJ, Lee SH, Kong YY, Kim JM, Lim DS (2008) A crucial role of WW45 in developing epithelial tissues in the mouse. *The EMBO journal* **27:** 1231-1242

137. Lee KP, Lee JH, Kim TS, Kim TH, Park HD, Byun JS, Kim MC, Jeong WI, Calvisi DF, Kim JM, Lim DS (2010) The Hippo-Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 8248-8253

138. Lei QY, Zhang H, Zhao B, Zha ZY, Bai F, Pei XH, Zhao S, Xiong Y, Guan KL (2008) TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. *Molecular and cellular biology* **28:** 2426-2436

139. Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV (2001) Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Molecular endocrinology (Baltimore, Md)* **15:** 184-200

140. Levasseur A, Paquet M, Boerboom D, Boyer A (2017) Yes-associated protein and WW-containing transcription regulator 1 regulate the expression of sex-determining genes in Sertoli cells, but their inactivation does not cause sex reversal. *Biology of reproduction* **97:** 162-175

141. Li Q, Pangas SA, Jorgez CJ, Graff JM, Weinstein M, Matzuk MM (2008) Redundant roles of SMAD2 and SMAD3 in ovarian granulosa cells in vivo. *Molecular and cellular biology* **28:** 7001-7011

142. Li Y, Pei J, Xia H, Ke H, Wang H, Tao W (2003) Lats2, a putative tumor suppressor, inhibits G1/S transition. *Oncogene* **22:** 4398-4405

143. Li Y, Zhang L, Hu Y, Chen M, Han F, Qin Y, Chen M, Cui X, Duo S, Tang F, Gao F (2017) beta-Catenin directs the transformation of testis Sertoli cells to ovarian granulosa-like cells by inducing Foxl2 expression. *The Journal of biological chemistry* **292:** 17577-17586

144. Li Z, Zhao B, Wang P, Chen F, Dong Z, Yang H, Guan KL, Xu Y (2010) Structural insights into the YAP and TEAD complex. *Genes & development* **24:** 235-240

145. Lian I, Kim J, Okazawa H, Zhao J, Zhao B, Yu J, Chinnaiyan A, Israel MA, Goldstein LS, Abujarour R, Ding S, Guan KL (2010) The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. *Genes & development* **24:** 1106-1118

146. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91:** 197-208

147. Lin CW, Chang YL, Chang YC, Lin JC, Chen CC, Pan SH, Wu CT, Chen HY, Yang SC, Hong TM, Yang PC (2013) MicroRNA-135b promotes lung cancer metastasis by regulating multiple targets in the Hippo pathway and LZTS1. *Nature communications* **4:** 1877

148. Liu-Chittenden Y, Huang B, Shim JS, Chen Q, Lee SJ, Anders RA, Liu JO, Pan D (2012) Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes & development* **26:** 1300-1305

149. Liu CY, Lv X, Li T, Xu Y, Zhou X, Zhao S, Xiong Y, Lei QY, Guan KL (2011) PP1 cooperates with ASPP2 to dephosphorylate and activate TAZ. *The Journal of biological chemistry* **286:** 5558-5566

150. Liu CY, Zha ZY, Zhou X, Zhang H, Huang W, Zhao D, Li T, Chan SW, Lim CJ, Hong W, Zhao S, Xiong Y, Lei QY, Guan KL (2010) The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. *The Journal of biological chemistry* **285:** 37159-37169

151. Liu Z, de Matos DG, Fan HY, Shimada M, Palmer S, Richards JS (2009a) Interleukin-6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. *Endocrinology* **150:** 3360-3368

152. Liu Z, Rudd MD, Hernandez-Gonzalez I, Gonzalez-Robayna I, Fan HY, Zeleznik AJ, Richards JS (2009b) FSH and FOXO1 regulate genes in the sterol/steroid

and lipid biosynthetic pathways in granulosa cells. *Molecular endocrinology (Baltimore, Md)* **23:** 649-661

153. Lu L, Li Y, Kim SM, Bossuyt W, Liu P, Qiu Q, Wang Y, Halder G, Finegold MJ, Lee JS, Johnson RL (2010) Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 1437-1442

154. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM, O'Malley BW (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & development* **9:** 2266-2278

155. Ma X, Dong Y, Matzuk MM, Kumar TR (2004) Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proceedings of the National Academy of Sciences of the United States of America* **101:** 17294-17299

156. Maatouk DM, DiNapoli L, Alvers A, Parker KL, Taketo MM, Capel B (2008) Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal. *Human molecular genetics* **17:** 2949-2955

157. Makita R, Uchijima Y, Nishiyama K, Amano T, Chen Q, Takeuchi T, Mitani A, Nagase T, Yatomi Y, Aburatani H, Nakagawa O, Small EV, Cobo-Stark P, Igarashi P, Murakami M, Tominaga J, Sato T, Asano T, Kurihara Y, Kurihara H (2008) Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ. *American journal of physiology Renal physiology* **294:** F542-553

158. Malik AR, Liszewska E, Jaworski J (2015) Matricellular proteins of the Cyr61/CTGF/NOV (CCN) family and the nervous system. *Front Cell Neurosci* **9:** 237

159. Mattioli M, Gloria A, Turriani M, Berardinelli P, Russo V, Nardinocchi D, Curini V, Baratta M, Martignani E, Barboni B (2012) Osteo-regenerative potential of ovarian granulosa cells: an in vitro and in vivo study. *Theriogenology* **77:** 1425-1437

160. Matzuk MM, Burns KH (2012) Genetics of mammalian reproduction: modeling the end of the germline. *Annual review of physiology* **74:** 503-528

161. Mauviel A, Nallet-Staub F, Varelas X (2012) Integrating developmental signals: a Hippo in the (path)way. *Oncogene* **31:** 1743-1756

162. McArdle CA, Roberson MS (2015) Gonadotropes and Gonadotropin-Releasing Hormone Signaling. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik AJ (eds), Vol. 1, Fourth Ed. edn, 10, pp 335-397. London, U.K.: Academic Press

163. McClatchey AI, Saotome I, Ramesh V, Gusella JF, Jacks T (1997) The Nf2 tumor suppressor gene product is essential for extraembryonic development immediately prior to gastrulation. *Genes & development* **11:** 1253-1265

164. McGee EA, Hsueh AJ (2000) Initial and cyclic recruitment of ovarian follicles. *Endocrine reviews* **21:** 200-214

165. McKenna NJ (2015) Gonadal Steroid Action. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik A.J. (ed), Vol. 1, 4th edn, 9, pp 313-333. London, UK: Academic Press

166. McMullen ML, Cho BN, Yates CJ, Mayo KE (2001) Gonadal pathologies in transgenic mice expressing the rat inhibin alpha-subunit. *Endocrinology* **142:** 5005-5014

167. McNeill H, Reginensi A (2017) Lats1/2 Regulate Yap/Taz to Control Nephron Progenitor Epithelialization and Inhibit Myofibroblast Formation. *J Am Soc Nephrol* **28:** 852-861

168. McPherson JP, Tamblyn L, Elia A, Migon E, Shehabeldin A, Matysiak-Zablocki E, Lemmers B, Salmena L, Hakem A, Fish J, Kassam F, Squire J, Bruneau BG, Hande MP, Hakem R (2004) Lats2/Kpm is required for embryonic development, proliferation control and genomic integrity. *The EMBO journal* **23:** 3677-3688

169. Menon KM, Menon B (2014) Regulation of luteinizing hormone receptor expression by an RNA binding protein: role of ERK signaling. *Indian J Med Res* **140 Suppl:** S112-119

170. Menon KM, Menon B, Wang L, Gulappa T, Harada M (2010) Molecular regulation of gonadotropin receptor expression: relationship to sterol metabolism. *Molecular and cellular endocrinology* **329:** 26-32

171. Messinis IE (2006) Ovarian feedback, mechanism of action and possible clinical implications. *Human reproduction update* **12:** 557-571

172. Miesfeld JB, Gestri G, Clark BS, Flinn MA, Poole RJ, Bader JR, Besharse JC, Wilson SW, Link BA (2015) Yap and Taz regulate retinal pigment epithelial cell fate. *Development (Cambridge, England)* **142:** 3021-3032

173. Miller E, Yang J, DeRan M, Wu C, Su AI, Bonamy GM, Liu J, Peters EC, Wu X (2012) Identification of serum-derived sphingosine-1-phosphate as a small molecule regulator of YAP. *Chem Biol* **19:** 955-962

174. Miller WL, Auchus RJ (2011) The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine reviews* **32:** 81-151

175. Mittaz L, Russell DL, Wilson T, Brasted M, Tkalcevic J, Salamonsen LA, Hertzog PJ, Pritchard MA (2004) Adamts-1 is essential for the development and function of the urogenital system. *Biology of reproduction* **70:** 1096-1105

176. Mizuno T, Murakami H, Fujii M, Ishiguro F, Tanaka I, Kondo Y, Akatsuka S, Toyokuni S, Yokoi K, Osada H, Sekido Y (2012) YAP induces malignant mesothelioma cell proliferation by upregulating transcription of cell cycle-promoting genes. *Oncogene* **31:** 5117-5122

177. Monniaux D (2016) Driving folliculogenesis by the oocyte-somatic cell dialog: Lessons from genetic models. *Theriogenology* **86:** 41-53

178. Morin-Kensicki EM, Boone BN, Howell M, Stonebraker JR, Teed J, Alb JG, Magnuson TR, O'Neal W, Milgram SL (2006) Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Molecular and cellular biology* **26:** 77-87

179. Myers M, Middlebrook BS, Matzuk MM, Pangas SA (2009) Loss of inhibin alpha uncouples oocyte-granulosa cell dynamics and disrupts postnatal folliculogenesis. *Developmental biology* **334:** 458-467

180. Nagashima T, Kim J, Li Q, Lydon JP, DeMayo FJ, Lyons KM, Matzuk MM (2011) Connective tissue growth factor is required for normal follicle development and ovulation. *Molecular endocrinology (Baltimore, Md)* **25:** 1740-1759

181. Nautiyal J, Steel JH, Rosell MM, Nikolopoulou E, Lee K, Demayo FJ, White R, Richards JS, Parker MG (2010) The nuclear receptor cofactor receptor-interacting protein 140 is a positive regulator of amphiregulin expression and cumulus cell-oocyte complex expansion in the mouse ovary. *Endocrinology* **151:** 2923-2932

182. Nguyen Q, Anders RA, Alpini G, Bai H (2015) Yes-associated protein in the liver: Regulation of hepatic development, repair, cell fate determination and tumorigenesis. *Dig Liver Dis* **47:** 826-835

183. Nishio M, Hamada K, Kawahara K, Sasaki M, Noguchi F, Chiba S, Mizuno K, Suzuki SO, Dong Y, Tokuda M, Morikawa T, Hikasa H, Eggenschwiler J, Yabuta N, Nojima H, Nakagawa K, Hata Y, Nishina H, Mimori K, Mori M, Sasaki T, Mak TW, Nakano T, Itami S, Suzuki A (2012) Cancer susceptibility and embryonic lethality in Mob1a/1b double-mutant mice. *The Journal of clinical investigation* **122:** 4505-4518

184. Nishioka N, Inoue K, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N, Makita R, Kurihara H, Morin-Kensicki EM, Nojima H, Rossant J, Nakao K, Niwa H, Sasaki H (2009) The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Developmental cell* **16:** 398-410

185. Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H (2008) Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mechanisms of development* **125:** 270-283

186. Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ, Wiltbank MC (1994) Luteal function: the estrous cycle and early pregnancy. *Biology of reproduction* **50:** 239-247

187. Ochsner SA, Day AJ, Rugg MS, Breyer RM, Gomer RH, Richards JS (2003a) Disrupted function of tumor necrosis factor-alpha-stimulated gene 6 blocks cumulus cell-oocyte complex expansion. *Endocrinology* **144:** 4376-4384

188. Ochsner SA, Russell DL, Day AJ, Breyer RM, Richards JS (2003b) Decreased expression of tumor necrosis factor-alpha-stimulated gene 6 in cumulus cells of the cyclooxygenase-2 and EP2 null mice. *Endocrinology* **144:** 1008-1019

189. Oh JS, Han SJ, Conti M (2010) Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption. *The Journal of cell biology* **188:** 199-207

190. Oki Y, Ono H, Motohashi T, Sugiura N, Nobusue H, Kano K (2012) Dedifferentiated follicular granulosa cells derived from pig ovary can transdifferentiate into osteoblasts. *The Biochemical journal* **447:** 239-248

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

192. Ottolenghi C, Omari S, Garcia-Ortiz JE, Uda M, Crisponi L, Forabosco A, Pilia G, Schlessinger D (2005) Foxl2 is required for commitment to ovary differentiation. *Human molecular genetics* **14:** 2053-2062

193. Ottolenghi C, Pelosi E, Tran J, Colombino M, Douglass E, Nedorezov T, Cao A, Forabosco A, Schlessinger D (2007) Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. *Human molecular genetics* **16:** 2795-2804

194. Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS, Haber DA (2006) Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 12405-12410

195. Pan D (2010) The hippo signaling pathway in development and cancer. *Developmental cell* **19:** 491-505

196. Pangas SA (2012) Bone morphogenetic protein signaling transcription factor (SMAD) function in granulosa cells. *Molecular and cellular endocrinology* **356:** 40-47

197. Pangas SA, Choi Y, Ballow DJ, Zhao Y, Westphal H, Matzuk MM, Rajkovic A (2006a) Oogenesis requires germ cell-specific transcriptional regulators Sohlh1 and Lhx8. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 8090-8095

198. Pangas SA, Jorgez CJ, Tran M, Agno J, Li X, Brown CW, Kumar TR, Matzuk MM (2007) Intraovarian activins are required for female fertility. *Molecular endocrinology (Baltimore, Md)* **21:** 2458-2471

199. Pangas SA, Li X, Robertson EJ, Matzuk MM (2006b) Premature luteinization and cumulus cell defects in ovarian-specific Smad4 knockout mice. *Molecular endocrinology (Baltimore, Md)* **20:** 1406-1422

200. Pangas SA, Rajkovic A (2015) Follicular Development: Mouse, Sheep, and Human Models. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik, A.J. (ed), Vol. 1, 4th edn, 21. Academic Press

201. Paramasivam M, Sarkeshik A, Yates JR, 3rd, Fernandes MJ, McCollum D (2011) Angiomotin family proteins are novel activators of the LATS2 kinase tumor suppressor. *Molecular biology of the cell* **22:** 3725-3733

202. Park GS, Oh H, Kim M, Kim T, Johnson RL, Irvine KD, Lim DS (2016) An evolutionarily conserved negative feedback mechanism in the Hippo pathway reflects functional difference between LATS1 and LATS2. *Oncotarget*

203. Park HW, Guan KL (2013) Regulation of the Hippo pathway and implications for anticancer drug development. *Trends in pharmacological sciences* **34:** 581-589

204. Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M (2004) EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science (New York, NY)* **303:** 682-684

205. Park OK, Mayo KE (1991) Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. *Molecular endocrinology (Baltimore, Md)* **5:** 967-978

206. Park Y, Maizels ET, Feiger ZJ, Alam H, Peters CA, Woodruff TK, Unterman TG, Lee EJ, Jameson JL, Hunzicker-Dunn M (2005) Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. *The Journal of biological chemistry* **280:** 9135-9148

207. Pasquini C, Spurgeon T, Pasquini S (2007) *Anatomy of Domestic Animals*, 11th edn. Texas, USA: Sudz Publishing.

208. Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. *Nature reviews Molecular cell biology* **11:** 9-22

209. Pei L, Dodson R, Schoderbek WE, Maurer RA, Mayo KE (1991) Regulation of the alpha inhibin gene by cyclic adenosine 3',5'-monophosphate after transfection into rat granulosa cells. *Molecular endocrinology (Baltimore, Md)* **5:** 521-534

210. Pepling ME, Spradling AC (2001) Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Developmental biology* **234:** 339-351

211. Pesce M, Klinger FG, De Felici M (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:** 15-24

212. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* **29:** e45

213. Piccolo S, Dupont S, Cordenonsi M (2014) The Biology of YAP/TAZ: Hippo Signaling and Beyond. *Physiological reviews* **94:** 1287-1312

214. Pisarska MD, Kuo FT, Bentsi-Barnes IK, Khan S, Barlow GM (2010) LATS1 phosphorylates forkhead L2 and regulates its transcriptional activity. *American journal of physiology Endocrinology and metabolism* **299:** E101-109

215. Pobbati AV, Hong W (2013) Emerging roles of TEAD transcription factors and its coactivators in cancers. *Cancer biology & therapy* **14:** 390-398

216. Poon CL, Mitchell KA, Kondo S, Cheng LY, Harvey KF (2016) The Hippo Pathway Regulates Neuroblasts and Brain Size in Drosophila melanogaster. *Current biology : CB* **26:** 1034-1042

217. Praskova M, Khoklatchev A, Ortiz-Vega S, Avruch J (2004) Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *The Biochemical journal* **381:** 453-462

218. Praskova M, Xia F, Avruch J (2008) MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. *Current biology : CB* **18:** 311-321

219. Qin H, Blaschke K, Wei G, Ohi Y, Blouin L, Qi Z, Yu J, Yeh RF, Hebrok M, Ramalho-Santos M (2012) Transcriptional analysis of pluripotency reveals the Hippo pathway as a barrier to reprogramming. *Human molecular genetics* **21:** 2054-2067

220. Rajah R, Glaser EM, Hirshfield AN (1992) The changing architecture of the neonatal rat ovary during histogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists* **194:** 177-192

221. Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM (2004) NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science (New York, NY)* **305:** 1157-1159

222. Rankin T, Soyal S, Dean J (2000) The mouse zona pellucida: folliculogenesis, fertility and pre-implantation development. *Molecular and cellular endocrinology* **163:** 21-25

223. Rao R, Salloum R, Xin M, Lu QR (2016) The G protein Galphas acts as a tumor suppressor in sonic hedgehog signaling-driven tumorigenesis. *Cell cycle (Georgetown, Tex)* **15:** 1325-1330

224. Reddy P, Liu L, Adhikari D, Jagarlamudi K, Rajareddy S, Shen Y, Du C, Tang W, Hamalainen T, Peng SL, Lan ZJ, Cooney AJ, Huhtaniemi I, Liu K (2008) Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science (New York, NY)* **319:** 611-613

225. Reddy P, Zheng W, Liu K (2010) Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends in endocrinology and metabolism: TEM* **21:** 96-103

226. Rendi MH, Muehlenbachs A, Garcia RL, Boyd KL (2012) Female Reproductive System. In *Comparative Anatomy and Histology*, Treuting PM DS (ed), 1 edn, 17, pp 253-270. London, UK: Academic Press

227. Richards AJ, Enders GC, Resnick JL (1999) Activin and TGFbeta limit murine primordial germ cell proliferation. *Developmental biology* **207:** 470-475

228. Richards JS (1980) Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiological reviews* **60:** 51-89

229. Richards JS (1994) Hormonal control of gene expression in the ovary. *Endocrine reviews* **15:** 725-751

230. Richards JS (2005) Ovulation: new factors that prepare the oocyte for fertilization. *Molecular and cellular endocrinology* **234:** 75-79

231. Richards JS, Liu Z, Shimada M (2008) Immune-like mechanisms in ovulation. *Trends in endocrinology and metabolism: TEM* **19:** 191-196

232. Richards JS, Liu Z, Shimada M (2015a) Ovulation. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik, A.J. (ed), Vol. 1, 4th edn, 22. Academic Press

233. Richards JS, Liu Z, Shimada M (2015b) Ovulation. In *Knobil and Neill's Physiology of Reproduction*, Plant TM, Zeleznik A.J. (ed), Vol. 1, 4th edn, 22, pp 997-1021. London, UK: Academic Press

234. Richards JS, Midgley AR, Jr. (1976) Protein hormone action: a key to understanding ovarian follicular and luteal cell development. *Biology of reproduction* **14:** 82-94

235. Richards JS, Pangas SA (2010a) New insights into ovarian function. *Handbook of experimental pharmacology***:** 3-27

236. Richards JS, Pangas SA (2010b) The ovary: basic biology and clinical implications. *The Journal of clinical investigation* **120:** 963-972

237. Richards JS, Russell DL, Robker RL, Dajee M, Alliston TN (1998) Molecular mechanisms of ovulation and luteinization. *Molecular and cellular endocrinology* **145:** 47-54

238. Robker RL, Russell DL, Yoshioka S, Sharma SC, Lydon JP, O'Malley BW, Espey LL, Richards JS (2000) Ovulation: a multi-gene, multi-step process. *Steroids* **65:** 559-570

239. Rose UM, Hanssen RG, Kloosterboer HJ (1999) Development and characterization of an in vitro ovulation model using mouse ovarian follicles. *Biology of reproduction* **61:** 503-511

240. Rosenbluh J, Nijhawan D, Cox AG, Li X, Neal JT, Schafer EJ, Zack TI, Wang X, Tsherniak A, Schinzel AC, Shao DD, Schumacher SE, Weir BA, Vazquez F, Cowley GS, Root DE, Mesirov JP, Beroukhim R, Kuo CJ, Goessling W, Hahn WC (2012) beta-Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell* **151:** 1457-1473

241. Ross A, Munger S, Capel B (2007) Bmp7 regulates germ cell proliferation in mouse fetal gonads. *Sexual development : genetics, molecular biology, evolution, endocrinology, embryology, and pathology of sex determination and differentiation* **1:** 127-137

242. Russell DL, Gilchrist RB, Brown HM, Thompson JG (2016) Bidirectional communication between cumulus cells and the oocyte: Old hands and new players? *Theriogenology* **86:** 62-68

243. Salah Z, Cohen S, Itzhaki E, Aqeilan RI (2013) NEDD4 E3 ligase inhibits the activity of the Hippo pathway by targeting LATS1 for degradation. *Cell cycle (Georgetown, Tex)* **12:** 3817-3823

244. Sasaki H (2017) Roles and regulations of Hippo signaling during preimplantation mouse development. *Dev Growth Differ* **59:** 12-20

245. Sawada A, Kiyonari H, Ukita K, Nishioka N, Imuta Y, Sasaki H (2008) Redundant roles of Tead1 and Tead2 in notochord development and the regulation of cell proliferation and survival. *Molecular and cellular biology* **28:** 3177-3189

246. Schlegelmilch K, Mohseni M, Kirak O, Pruszak J, Rodriguez JR, Zhou D, Kreger BT, Vasioukhin V, Avruch J, Brummelkamp TR, Camargo FD (2011) Yap1 acts downstream of alpha-catenin to control epidermal proliferation. *Cell* **144:** 782-795

247. Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, Treier M (2004) The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development (Cambridge, England)* **131:** 933-942

248. Sela-Abramovich S, Chorev E, Galiani D, Dekel N (2005) Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology* **146:** 1236-1244

249. Sforza C, Vizzotto L, Ferrario VF, Forabosco A (2003) Position of follicles in normal human ovary during definitive histogenesis. *Early Hum Dev* **74:** 27-35

250. Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS (2006) Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Molecular endocrinology (Baltimore, Md)* **20:** 1352-1365

251. Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ, Weinberg RA (1996) Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* **384:** 470-474

252. Simon AM, Goodenough DA, Li E, Paul DL (1997) Female infertility in mice lacking connexin 37. *Nature* **385:** 525-529

253. Simoni M, Gromoll J, Nieschlag E (1997) The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocrine reviews* **18:** 739-773

254. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, et al. (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine reviews* **15:** 342-355

255. Song H, Mak KK, Topol L, Yun K, Hu J, Garrett L, Chen Y, Park O, Chang J, Simpson RM, Wang CY, Gao B, Jiang J, Yang Y (2010) Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 1431-1436

256. Song S, Ajani JA, Honjo S, Maru DM, Chen Q, Scott AW, Heallen TR, Xiao L, Hofstetter WL, Weston B, Lee JH, Wadhwa R, Sudo K, Stroehlein JR, Martin JF, Hung MC, Johnson RL (2014) Hippo coactivator YAP1 upregulates SOX9 and endows esophageal cancer cells with stem-like properties. *Cancer research* **74:** 4170-4182

257. Soyal SM, Amleh A, Dean J (2000) FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development (Cambridge, England)* **127:** 4645-4654

258. Soyal SM, Mukherjee A, Lee KY, Li J, Li H, DeMayo FJ, Lydon JP (2005) Cre-mediated recombination in cell lineages that express the progesterone receptor. *Genesis (New York, NY : 2000)* **41:** 58-66

259. St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, Parlow AF, McGrath J, Xu T (1999) Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nature genetics* **21:** 182-186

260. Sterneck E, Tessarollo L, Johnson PF (1997) An essential role for C/EBPbeta in female reproduction. *Genes & development* **11:** 2153-2162

261. Stouffer RL, Hennebold JD (2015) Structure, Function, and Regulation of the Corpus Luteum. In *Knobil and Neill's Physiolog of Reproduction*, Plant TM, Zeleznik, A.J. (ed), Vol. 1, 4th edn, 23. Academic Press

262. Strano S, Munarriz E, Rossi M, Castagnoli L, Shaul Y, Sacchi A, Oren M, Sudol M, Cesareni G, Blandino G (2001) Physical interaction with Yes-associated protein enhances p73 transcriptional activity. *The Journal of biological chemistry* **276:** 15164-15173

263. Su T, Bondar T, Zhou X, Zhang C, He H, Medzhitov R (2015) Two-signal requirement for growth-promoting function of Yap in hepatocytes. *eLife* **4**

264. Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA, Matzuk MM, Eppig JJ (2008) Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development (Cambridge, England)* **135:** 111-121

265. Su YQ, Wu X, O'Brien MJ, Pendola FL, Denegre JN, Matzuk MM, Eppig JJ (2004) Synergistic roles of BMP15 and GDF9 in the development and function of the

oocyte-cumulus cell complex in mice: genetic evidence for an oocyte-granulosa cell regulatory loop. *Developmental biology* **276:** 64-73

266. Sudol M (1994) Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene* **9:** 2145-2152

267. Sugiura K, Su YQ, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ (2007) Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development (Cambridge, England)* **134:** 2593-2603

268. Sugiura K, Su YQ, Eppig JJ (2009) Targeted suppression of Has2 mRNA in mouse cumulus cell-oocyte complexes by adenovirus-mediated short-hairpin RNA expression. *Molecular reproduction and development* **76:** 537-547

269. Sun T, Pepling ME, Diaz FJ (2015) Lats1 Deletion Causes Increased Germ Cell Apoptosis and Follicular Cysts in Mouse Ovaries. *Biology of reproduction* **93:** 22

270. Takahashi Y, Miyoshi Y, Takahata C, Irahara N, Taguchi T, Tamaki Y, Noguchi S (2005) Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11:** 1380-1385

271. Tamm C, Bower N, Anneren C (2011) Regulation of mouse embryonic stem cell self-renewal by a Yes-YAP-TEAD2 signaling pathway downstream of LIF. *Journal of cell science* **124:** 1136-1144

272. Tanaka SS, Nishinakamura R (2014) Regulation of male sex determination: genital ridge formation and Sry activation in mice. *Cellular and molecular life sciences : CMLS* **71:** 4781-4802

273. Tang Y, Feinberg T, Keller ET, Li XY, Weiss SJ (2016) Snail/Slug binding interactions with YAP/TAZ control skeletal stem cell self-renewal and differentiation. *Nature cell biology* **18:** 917-929

274. Tao W, Zhang S, Turenchalk GS, Stewart RA, St John MA, Chen W, Xu T (1999) Human homologue of the Drosophila melanogaster lats tumour suppressor modulates CDC2 activity. *Nature genetics* **21:** 177-181

275. Tapon N, Harvey KF, Bell DW, Wahrer DC, Schiripo TA, Haber D, Hariharan IK (2002) salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110:** 467-478

276. Taylor SS (1989) cAMP-dependent protein kinase. Model for an enzyme family. *The Journal of biological chemistry* **264:** 8443-8446

277. Teplyuk NM, Galindo M, Teplyuk VI, Pratap J, Young DW, Lapointe D, Javed A, Stein JL, Lian JB, Stein GS, van Wijnen AJ (2008) Runx2 regulates G protein-coupled signaling pathways to control growth of osteoblast progenitors. *The Journal of biological chemistry* **283:** 27585-27597

278. Thomas FH, Vanderhyden BC (2006) Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. *Reproductive biology and endocrinology : RB&E* **4:** 19

279. Tingen C, Kim A, Woodruff TK (2009) The primordial pool of follicles and nest breakdown in mammalian ovaries. *Molecular human reproduction* **15:** 795-803

280. Tiwari M, Prasad S, Tripathi A, Pandey AN, Ali I, Singh AK, Shrivastav TG, Chaube SK (2015) Apoptosis in mammalian oocytes: a review. *Apoptosis* **20:** 1019-1025

281. Tomizuka K, Horikoshi K, Kitada R, Sugawara Y, Iba Y, Kojima A, Yoshitome A, Yamawaki K, Amagai M, Inoue A, Oshima T, Kakitani M (2008) R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Human molecular genetics* **17:** 1278-1291

282. Tullet JM, Pocock V, Steel JH, White R, Milligan S, Parker MG (2005) Multiple signaling defects in the absence of RIP140 impair both cumulus expansion and follicle rupture. *Endocrinology* **146:** 4127-4137

283. Uda M, Ottolenghi C, Crisponi L, Garcia JE, Deiana M, Kimber W, Forabosco A, Cao A, Schlessinger D, Pilia G (2004) Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Human molecular genetics* **13:** 1171-1181

284. Udan RS, Kango-Singh M, Nolo R, Tao C, Halder G (2003) Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nature cell biology* **5:** 914-920

285. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, Ponten F (2015) Proteomics. Tissue-based map of the human proteome. *Science (New York, NY)* **347:** 1260419

286. Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, Treier AC, Klugmann C, Klasen C, Holter NI, Riethmacher D, Schutz G, Cooney AJ, Lovell-Badge R, Treier M (2009) Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* **139:** 1130-1142

287. Urtasun R, Latasa MU, Demartis MI, Balzani S, Goni S, Garcia-Irigoyen O, Elizalde M, Azcona M, Pascale RM, Feo F, Bioulac-Sage P, Balabaud C, Muntane J, Prieto J, Berasain C, Avila MA (2011) Connective tissue growth factor autocriny in human hepatocellular carcinoma: oncogenic role and regulation by epidermal growth factor receptor/yes-associated protein-mediated activation. *Hepatology* **54:** 2149-2158

288. Vaccari S, Horner K, Mehlmann LM, Conti M (2008) Generation of mouse oocytes defective in cAMP synthesis and degradation: endogenous cyclic AMP is essential for meiotic arrest. *Developmental biology* **316:** 124-134

289. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP (1999) Female development in mammals is regulated by Wnt-4 signalling. *Nature* **397:** 405-409

290. Van Ebbenhorst Tengbergen WJ (1955) The morphology of the mouse anterior pituitary during the oestrous cycle. *Acta Endocrinol (Copenh)* **18:** 213-218

291. Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B (2010) The emerging mechanisms of isoform-specific PI3K signalling. *Nature reviews Molecular cell biology* **11:** 329-341

292. Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM (2002) Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Molecular endocrinology (Baltimore, Md)* **16:** 1154-1167

293. Varelas X, Miller BW, Sopko R, Song S, Gregorieff A, Fellouse FA, Sakuma R, Pawson T, Hunziker W, McNeill H, Wrana JL, Attisano L (2010a) The Hippo pathway regulates Wnt/beta-catenin signaling. *Developmental cell* **18:** 579-591

294. Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J, Yaffe MB, Zandstra PW, Wrana JL (2008) TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nature cell biology* **10:** 837-848

295. Varelas X, Samavarchi-Tehrani P, Narimatsu M, Weiss A, Cockburn K, Larsen BG, Rossant J, Wrana JL (2010b) The Crumbs complex couples cell density sensing to Hippo-dependent control of the TGF-beta-SMAD pathway. *Developmental cell* **19:** 831-844

296. Vassilev A, Kaneko KJ, Shu H, Zhao Y, DePamphilis ML (2001) TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes & development* **15:** 1229-1241

297. Vidal VP, Chaboissier MC, de Rooij DG, Schedl A (2001) Sox9 induces testis development in XX transgenic mice. *Nature genetics* **28:** 216-217

298. Visser S, Yang X (2010) LATS tumor suppressor: a new governor of cellular homeostasis. *Cell cycle (Georgetown, Tex)* **9:** 3892-3903

299. Vitolo MI, Anglin IE, Mahoney WM, Jr., Renoud KJ, Gartenhaus RB, Bachman KE, Passaniti A (2007) The RUNX2 transcription factor cooperates with the YES-associated protein, YAP65, to promote cell transformation. *Cancer biology & therapy* **6:** 856-863

300. von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, Ma Q, Ishiwata T, Zhou B, Camargo FD, Pu WT (2012) YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proceedings of the National Academy of Sciences of the United States of America* **109:** 2394-2399

301. Wang R, Li Y, Hu E, Kong F, Wang J, Liu J, Shao Q, Hao Y, He D, Xiao X (2017a) S100A7 promotes lung adenocarcinoma to squamous carcinoma transdifferentiation, and its expression is differentially regulated by the Hippo-YAP pathway in lung cancer cells. *Oncotarget* **8:** 24804-24814

302. Wang X, Freire Valls A, Schermann G, Shen Y, Moya IM, Castro L, Urban S, Solecki GM, Winkler F, Riedemann L, Jain RK, Mazzone M, Schmidt T, Fischer T, Halder G, Ruiz de Almodovar C (2017b) YAP/TAZ Orchestrate VEGF Signaling during Developmental Angiogenesis. *Developmental cell* **42:** 462-478 e467

303. Wang Y, Liu J, Ying X, Lin PC, Zhou BP (2016) Twist-mediated Epithelial-mesenchymal Transition Promotes Breast Tumor Cell Invasion via Inhibition of Hippo Pathway. *Scientific reports* **6:** 24606

304. Wayne CM, Fan HY, Cheng X, Richards JS (2007) Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. *Molecular endocrinology (Baltimore, Md)* **21:** 1940-1957

305. Wei X, Shimizu T, Lai ZC (2007) Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. *The EMBO journal* **26:** 1772-1781

306. Wilhelm D, Palmer S, Koopman P (2007) Sex determination and gonadal development in mammals. *Physiological reviews* **87:** 1-28

307. Wong WY, Richards JS (1991) Evidence for two antigenically distinct molecular weight variants of prostaglandin H synthase in the rat ovary. *Molecular endocrinology (Baltimore, Md)* **5:** 1269-1279

308. Wu J, Zhang L, Wang X (2000) Maturation and apoptosis of human oocytes in vitro are age-related. *Fertility and sterility* **74:** 1137-1141

309. Wu S, Huang J, Dong J, Pan D (2003) hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114:** 445-456

310. Wu S, Liu Y, Zheng Y, Dong J, Pan D (2008) The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. *Developmental cell* **14:** 388-398

311. Xia Y, Zhang YL, Yu C, Chang T, Fan HY (2014) YAP/TEAD co-activator regulated pluripotency and chemoresistance in ovarian cancer initiated cells. *PloS one* **9:** e109575

312. Xiao L, Chen Y, Ji M, Dong J (2011) KIBRA regulates Hippo signaling activity via interactions with large tumor suppressor kinases. *The Journal of biological chemistry* **286:** 7788-7796

313. Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, Porrello ER, Mahmoud AI, Tan W, Shelton JM, Richardson JA, Sadek HA, Bassel-Duby R, Olson EN (2013) Hippo pathway effector Yap promotes cardiac regeneration. *Proceedings of the National Academy of Sciences of the United States of America* **110:** 13839-13844

314. Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, Richardson JA, Bassel-Duby R, Olson EN (2011) Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Science signaling* **4:** ra70

315. Xu MZ, Chan SW, Liu AM, Wong KF, Fan ST, Chen J, Poon RT, Zender L, Lowe SW, Hong W, Luk JM (2011) AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. *Oncogene* **30:** 1229-1240

316. Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A (2007) Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. *Development (Cambridge, England)* **134:** 3827-3836

317. Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM (2001) Synergistic roles of

bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular endocrinology (Baltimore, Md)* **15:** 854-866

318. Yang CC, Graves HK, Moya IM, Tao C, Hamaratoglu F, Gladden AB, Halder G (2015) Differential regulation of the Hippo pathway by adherens junctions and apical-basal cell polarity modules. *Proceedings of the National Academy of Sciences of the United States of America* **112:** 1785-1790

319. Yang N, Morrison CD, Liu P, Miecznikowski J, Bshara W, Han S, Zhu Q, Omilian AR, Li X, Zhang J (2012) TAZ induces growth factor-independent proliferation through activation of EGFR ligand amphiregulin. *Cell cycle (Georgetown, Tex)* **11:** 2922-2930

320. Yang R, Wu Y, Zou J, Zhou J, Wang M, Hao X, Cui H (2016) The Hippo transducer TAZ promotes cell proliferation and tumor formation of glioblastoma cells through EGFR pathway. *Oncotarget* **7:** 36255-36265

321. Yao HH, Matzuk MM, Jorgez CJ, Menke DB, Page DC, Swain A, Capel B (2004) Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists* **230:** 210-215

322. Yeung B, Ho KC, Yang X (2013) WWP1 E3 ligase targets LATS1 for ubiquitin-mediated degradation in breast cancer cells. *PloS one* **8:** e61027

323. Yi BR, Kim TH, Kim YS, Choi KC (2015) Alteration of epithelial-mesenchymal transition markers in human normal ovaries and neoplastic ovarian cancers. *International journal of oncology* **46:** 272-280

324. Yi C, Troutman S, Fera D, Stemmer-Rachamimov A, Avila JL, Christian N, Persson NL, Shimono A, Speicher DW, Marmorstein R, Holmgren L, Kissil JL (2011) A tight junction-associated Merlin-angiomotin complex mediates Merlin's regulation of mitogenic signaling and tumor suppressive functions. *Cancer cell* **19:** 527-540

325. Yimlamai D, Christodoulou C, Galli GG, Yanger K, Pepe-Mooney B, Gurung B, Shrestha K, Cahan P, Stanger BZ, Camargo FD (2014) Hippo pathway activity influences liver cell fate. *Cell* **157:** 1324-1338

326. Yin F, Yu J, Zheng Y, Chen Q, Zhang N, Pan D (2013) Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. *Cell* **154:** 1342-1355

327. Yu FX, Zhao B, Panupinthu N, Jewell JL, Lian I, Wang LH, Zhao J, Yuan H, Tumaneng K, Li H, Fu XD, Mills GB, Guan KL (2012) Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. *Cell* **150:** 780-791

328. Yu J, Zheng Y, Dong J, Klusza S, Deng WM, Pan D (2010) Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. *Developmental cell* **18:** 288-299

329. Yu P, Shen X, Yang W, Zhang Y, Liu C, Huang T (2018) ZEB1 stimulates breast cancer growth by up-regulating hTERT expression. *Biochemical and biophysical research communications* **495:** 2505-2511

330. Zaidi SK, Sullivan AJ, Medina R, Ito Y, van Wijnen AJ, Stein JL, Lian JB, Stein GS (2004) Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. *The EMBO journal* **23:** 790-799

331. Zeleznik AJ, Midgley AR, Jr., Reichert LE, Jr. (1974) Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. *Endocrinology* **95:** 818-825

332. Zhang H, Liu CY, Zha ZY, Zhao B, Yao J, Zhao S, Xiong Y, Lei QY, Guan KL (2009a) TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. *The Journal of biological chemistry* **284:** 13355-13362

333. Zhang H, Risal S, Gorre N, Busayavalasa K, Li X, Shen Y, Bosbach B, Brannstrom M, Liu K (2014a) Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice. *Current biology : CB* **24:** 2501-2508

334. Zhang H, von Gise A, Liu Q, Hu T, Tian X, He L, Pu W, Huang X, He L, Cai CL, Camargo FD, Pu WT, Zhou B (2014b) Yap1 is required for endothelial to mesenchymal transition of the atrioventricular cushion. *The Journal of biological chemistry* **289:** 18681-18692

335. Zhang J, Ji JY, Yu M, Overholtzer M, Smolen GA, Wang R, Brugge JS, Dyson NJ, Haber DA (2009b) YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. *Nature cell biology* **11:** 1444-1450

336. Zhang N, Bai H, David KK, Dong J, Zheng Y, Cai J, Giovannini M, Liu P, Anders RA, Pan D (2010) The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. *Developmental cell* **19:** 27-38

337. Zhao B, Kim J, Ye X, Lai ZC, Guan KL (2009) Both TEAD-binding and WW domains are required for the growth stimulation and oncogenic transformation activity of yes-associated protein. *Cancer research* **69:** 1089-1098

338. Zhao B, Li L, Lei Q, Guan KL (2010a) The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes & development* **24:** 862-874

339. Zhao B, Li L, Lu Q, Wang LH, Liu CY, Lei Q, Guan KL (2011) Angiomotin is a novel Hippo pathway component that inhibits YAP oncoprotein. *Genes & development* **25:** 51-63

340. Zhao B, Li L, Tumaneng K, Wang CY, Guan KL (2010b) A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes & development* **24:** 72-85

341. Zhao B, Li L, Wang L, Wang CY, Yu J, Guan KL (2012) Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. *Genes & development* **26:** 54-68

342. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, Zheng P, Ye K, Chinnaiyan A, Halder G, Lai ZC, Guan KL (2007) Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & development* **21:** 2747-2761

343. Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang CY, Chinnaiyan AM, Lai ZC, Guan KL (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes & development* **22:** 1962-1971

344. Zhou D, Conrad C, Xia F, Park JS, Payer B, Yin Y, Lauwers GY, Thasler W, Lee JT, Avruch J, Bardeesy N (2009) Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer cell* **16:** 425-438

345. Zhou D, Zhang Y, Wu H, Barry E, Yin Y, Lawrence E, Dawson D, Willis JE, Markowitz SD, Camargo FD, Avruch J (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 of the United States of America* **108:** E1312-1320

346. Zhou J, Kumar TR, Matzuk MM, Bondy C (1997) Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Molecular endocrinology (Baltimore, Md)* **11:** 1924-1933

347. Zhou Q, Melton DA (2008) Extreme makeover: converting one cell into another. *Cell stem cell* **3:** 382-388