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Multiple Mechanisms Cooperate to Constitutively Exclude the Transcriptional Co-Activator YAP from the Nucleus During Murine Oogenesis[1]

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ABSTRACT

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

fertility, Hippo, intracellular localization, oocyte, oogenesis, YAP

INTRODUCTION

Reproduction depends on the generation of healthy oocytes that are able to develop as embryos following fertilization. Identifying inter- and intracellular mechanisms that control and direct oocyte development has been a focus of intensive research, with the aim of applying this knowledge to design and improve recent therapeutic innovations, including activation of oocytes in primordial follicles to enter the growth pool [1–5], growth of oocytes in vitro [6–11], and generation of oocytes from pluripotent stem cells [12, 13], whose common goal is to preserve fertility. For example, after the key role of PTEN (phosphatase and tensin homolog deleted on chromosome 10) activity in the oocyte in maintaining its quiescent state in primordial follicles was uncovered [14, 15], pharmacological inhibitors of PTEN were successfully used to activate growth of primordial follicles [1–3], thereby generating a potential supply of oocytes that can be used for fertilization. The widespread introduction and efficient application of these new reproductive therapies, particularly in the context of increasingly common conditions such as primary ovarian insufficiency and reproductive aging, will depend crucially, however, on a more complete and comprehensive understanding of the function of different signaling pathways during oocyte differentiation, growth, and meiotic maturation.

The Hippo pathway, originally identified in Drosophila and named for the overgrowth phenotype induced by mutation in genes encoding its members, is an evolutionarily conserved regulator of a wide range of cellular functions, including growth and proliferation, stem cell activity, and tumorigenesis [16–20]. Three protein complexes make up the Hippo core in mammalian cells: 1) MST1/2 (mammalian STE20-like protein kinase) and SAV (Salvador family WW domain-containing protein), 2) their substrates LATS1/2 (large tumor suppressor) and MOB1A/B (MOB kinase activator), and 3) their substrates YAP (Yes-associated protein) and its paralogue WWTR1 (WW domain containing transcription regulator; also known as TAZ [transcriptional coactivator with a PDZ-binding domain]). In contrast to its conserved core components, a wide range of extra- and intracellular signals, including but not limited to G-protein coupled receptors, WNTs, and changes in the state of

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actin polymerization, can regulate the activity of the Hippo pathway.

YAP and WWTR1, the key effectors of Hippo signaling, are transcriptional co-activators. Each can be phosphorylated by the LATS kinases on multiple sites. In their nonphosphorylated form, YAP and WWTR1 are able to accumulate in the nucleus. Neither possesses a known DNA-binding domain, however, so their nuclear accumulation depends on physical association with DNA-binding proteins, principally members of the TEA domain (TEAD) family [21–25]. The YAP/WWTR1-TEAD complex is thought to activate transcription of target genes, although only a small number of such targets have so far been identified [19, 25]. In contrast, phosphorylation of YAP on serine (S) 127 (human)/S112 (mouse) or WWTR1 on S89 prevents their nuclear accumulation [18, 26, 27]. YAP and WWTR1 phosphorylated at these sites instead become associated with 14-3-3 proteins and thereby anchored in the cytoplasm [28–30]. Nonphosphorylated YAP and WWTR1 can also be anchored in the cytoplasm through interaction with the angiomotin (AMOT), a plasma membrane-associated protein [31, 32]. Cytoplasmic YAP and WWTR1 may serve specific functions, such as by binding to and sequestering β-catenin in the cytoplasm [33]; these functions have been little explored, however, and it is noteworthy that cytoplasmic YAP and WWTR1 can be phosphorylated via LATS1/2 at additional sites leading to their degradation [17, 34]. Thus, LATS1/2-dependent phosphorylation of YAP and WWTR1 plays a central role in regulating the Hippo pathway.

Recent studies have shown that subjecting ovarian fragments to mechanical or pharmacological interventions that inactivate the Hippo pathway can trigger human primordial follicles to enter the growth phase [35, 36]. Moreover, when the fragments were transplanted into patients, healthy live births were obtained, confirming that the experimental treatment induced normal oocyte development [36]. Immunohistochemical studies using mice revealed that YAP was localized in the nuclei of the growing oocytes, suggesting that inactivation of the Hippo pathway in the oocyte itself might be the mechanism by which growth was induced. However, the antibody employed to assess YAP expression in oocytes, although widely used, is not specific to YAP when used in immunohistochemistry or immunofluorescence [37, 38]. Notably, this antibody recognizes nuclear antigens in cells that lack YAP [38]. Moreover, YAP has also been reported to be restricted to the cytoplasm in oocytes [39]. Conversely, WWTR1, although thought to be coregulated with YAP ([17, 19] was localized in the nucleus of growing oocytes as well as granulosa cells [39]. Thus, the potential role of the Hippo pathway in regulating oocyte development remains uncertain. Focusing on YAP because the specificity of the available antibodies has been verified [37, 38], we used immunoblotting and immunohistochemistry to systematically investigate its expression, phosphorylation, and intracellular distribution during pre- and postnatal oocyte development. We find that phosphorylation-dependent and -independent mechanisms cooperate to ensure that YAP does not accumulate in the nuclei of oocytes at any stage of development, indicating that nuclear YAP does not play a significant physiological role during mammalian oogenesis.

MATERIALS AND METHODS

Ethical Approval

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

Animals

CD-1 mice were obtained from Charles River Canada. $Nf2^{+/+}$ and $Nf2^{-/-}$ mice were maintained and genotyped as described [40]. Bovine oocytes were collected from 2- to 6-mm follicles, and oocytes displaying homogenous cytoplasm, a complete cumulus cloud with no signs of atresia, and a diameter greater than 120 μm were selected. To obtain mouse fetal ovaries, male and 6- to 8-wk-old female 129/SvJae mice were caged as individual pairs and the female was examined daily for the presence of a vaginal plug in the morning. The day of the plug appearance was designated Embryonic Day 0.5 (E0.5).

Collection of Oocytes and Embryos

To obtain cumulus-oocyte complexes (COCs) containing immature fully grown oocytes arrested at prophase I of meiosis, ovaries were dissected from 19-day-old female CD-1 mice and transferred to Hepes-buffered minimum essential medium with Earle salts (MEM-H; pH 7.2) (Life Technologies) supplemented with sodium pyruvate (0.25 mM; Sigma Chemicals), penicillin G (63 mg/L) (Sigma), streptomycin (50 mg/L) (Sigma), and BSA (1 mg/ml) (Sigma) at 37°C. Dibutyryl cyclic AMP (dbcAMP) (0.1 mg/ml) (Sigma) was added to the medium to maintain the oocytes in meiotic arrest. The ovarian follicles were punctured using a 30-gauge needle to isolate the enclosed COCs. Granulosa-oocyte complexes (GOCs) containing growing oocytes were collected from 12-day-old female pups using enzymatic methods as previously described [41]. Where required, granulosa- or cumulus-free oocytes were obtained by mechanically stripping the granulosa cells from the GOC or COC [42, 43]. Embryos were produced and collected as described [44].

Cell Culture and Drug Treatment

Complexes, oocytes, and embryos were incubated at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2, 90% N_2 in bicarbonate-buffered MEM (complexes and oocytes) or KSOM (embryos) as described [41]. Dibutyryl cyclic AMP (D0627; Sigma) was prepared at 10 mg/ml in water and used at 0.2 mg/ml. Roscovitine (R7772; Sigma) was prepared at 40 mM in dimethyl sulfoxide and used at 100 μM. KT5720 (420320; Millipore) was prepared at 2 mM in dimethyl sulfoxide and used at 30 μM. Leptomycin B (L2913; Sigma) was prepared at 20 μM in ethanol and used at 20 nM.

Reverse Transcription and PCR

RNA purification from freshly collected oocytes, cDNA synthesis, and RT-PCR reaction were performed as described [45]. *Actb* was used as a positive control, and a reaction without template was used as the negative control. Primer pairs are listed as below (forward primer given first, followed by reverse primer): *Actb*: 5′-GGCTGTATTCCCCTCCATCG-3′; 5′-CCAGTTGGTAA CAATGCCATGT-3′. *Yap1*: 5′-CCGTTTCTCCTGGGACACTC-3′; 5′-TGCTCCAGTGTAGGCAACTG-3′.

Immunoblotting

Immunoblotting was performed as previously described [46]. The primary antibodies used were directed against YAP (4912; Cell Signaling Technologies), phospho-S127 YAP (113008, Cell Signaling Technologies), phospho-S133 CREB (9198; Cell Signaling Technologies), MAPK3/1 (sc-94; Santa Cruz Biotechnology), and tubulin (T8203; Sigma). All primary antibodies were used at 1:1000 dilution. Blots were scanned using a Storm phosphorimager (Amersham), and the intensity of the signals was quantified using Image J software (National Institutes of Health).

Immunofluorescence

Oocytes, GOCs, or COCs were fixed in freshly prepared 2% (w/v) paraformaldehyde (Fisher Scientific) in phosphate-buffered saline containing 0.1% Triton X-100 followed by washing and storing in blocking buffer (PBS containing 0.1% Triton X-100 and 3% bovine serum albumin). Samples were incubated overnight at 4°C in primary antibodies—mouse anti-YAP (H00010413-M01; Abnova) or (101199; Santa Cruz Biotechnology) and rabbit

histone H3-acetyl-K9 (9671; Cell Signaling Technologies)—diluted 1:100 in blocking buffer. The next day, samples were washed twice for 10 min each in blocking buffer at room temperature, then transferred to blocking buffer containing secondary antibody diluted 1:100 in blocking buffer that also contained 5 μM DRAQ5 (4084S; New England Biolabs). YAP was detected using Alexa546-conjugated donkey anti-mouse (A10036; Life Technologies). Acetylated histone H3 was detected using Alexa488-conjugated goat anti-rabbit (A11008; Life Technologies). To mount the samples, a 9 × 0.12 mm spacer (GBL654008; Sigma) was attached to a glass microscope slide. A 2 μl drop of PBS was placed in the center of the spacer and covered with 20 μl of mineral oil. Samples were then transferred into the drop of PBS and a cover slip was placed on top. Samples were imaged using a Zeiss LSM510 or Quorum spinning disk confocal microscope.

Immunohistochemistry

To prepare cryosections, ovaries were fixed in freshly prepared 2% (w/v) paraformaldehyde (EMS Biosciences) in PBS at 4°C for 2 h, then washed through a sucrose gradient (10%, 20%, 30% in PBS), embedded in Tissue-Tek OCT (Sakura Finetek), and stored at −80°C. Sections were cut at 8 μm thickness and rehydrated for 10 min in PBS. Immunostaining was performed using Vector Mouse on Mouse Immunodetection Kit (2201), following the manufacturer's directions except that the PBS was supplemented with 0.05% Triton X-100 and 0.15% glycine. Slides were counterstained using 4′,6-diamidino-2-phenylindole and covered with coverslips using Vectashield (Vector) as an antifading solution. Slides were examined using a TCS-SP5 laser-scanning confocal microscope (Leica), and images were analyzed using LAS AF (Leica Microsystems CMS GmbH) and Imaris (Bitplane). Primary antibodies used were rabbit polyclonal anti-MVH (1:1000 dilution, ab13840; Abcam) and mouse anti-YAP (1:100 dilution, 101199; Santa Cruz Biotechnology). Paraffin-embedded sections were prepared and used for immunohistochemistry as previously described [42], using the same primary antibodies and dilutions as for immunofluorescence. YAP was detected using Alexa488-conjugated rabbit anti-mouse (1:500 dilution, A11059; Life Technologies). Images were recorded using an LSM 510 confocal microscope (Zeiss).

Statistical Analysis

Quantitative data were analyzed using the Student *t*-test or ANOVA. A *P*-value < 0.05 was considered significant.

RESULTS

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

To determine whether YAP is expressed in oocytes, we used growing oocytes obtained from primary and secondary follicles and fully grown oocytes obtained from antral follicles. The granulosa cells can easily be removed from oocytes at these stages of development, allowing a purified cell population to be analyzed. Using RT-PCR with primers specific for *Yap,* we detected a product of the expected size in both the growing and the fully grown oocytes (Fig. 1A). We then used immunoblotting to test whether YAP protein was present. Because fully grown oocytes contain more total protein than partially grown oocytes, we loaded a larger number of growing oocytes into the gels so that we would obtain approximately equal amounts of total protein at the two stages. Equal loading was confirmed by the similar signal intensities observed for MAPK3/1, which is expressed throughout oocyte growth [47, 48] (Fig. 1B). We observed that YAP protein was expressed in both growing and fully grown oocytes (Fig. 1B). Moreover, the signal intensities at the two stages were similar, which suggests that the amount of YAP as a fraction of total cellular protein does not change substantially during oocyte growth (Fig. 1C).

We then examined whether YAP was present in the oocyte nucleus, as would be expected if it promotes oocyte growth via its canonical function as a transcriptional co-activator. The antibody we used for immunoblotting (4912; Cell Signaling), although very effective for that application, is not suitable for immunolocalization studies because it recognizes nuclear antigens in situ that are not YAP [37, 38]. We used instead an antibody (H00010413-M01; Abnova) whose specificity for YAP in immunofluorescence has been established [37, 40]. In preliminary experiments, we confirmed that this antibody recognized a single species in immunoblots and stained the nuclei of trophectodermal cells but not of the inner cell mass in mouse blastocysts (Supplemental Fig. S1, A and B; all Supplemental Data are available online at www.biolreprod.org), consistent with previous reports [37, 40]. Using an antibody against histone H3 acetylated on K9, we also verified that we could detect nuclear antigens in oocytes (Supplemental Fig. S1C). When we stained growing and fully grown oocytes using the YAP antibody, however, although we observed a strong fluorescent signal in the cytoplasm at both stages (Fig. 2A), little or no fluorescence was detectable in oocyte nuclei at either stage. We also observed the same staining pattern using a different YAP antibody (101199; Santa Cruz) (data not shown). This result suggested that YAP is largely excluded from the nuclei of growing and fully grown oocytes.

We were concerned that the experimental intervention of removing the granulosa cells surrounding the oocyte prior to fixation might have altered YAP localization. Therefore, we immunostained intact GOCs containing growing oocytes and COCs containing fully grown oocytes. As observed using the granulosa cell-free oocytes, fluorescence was detectable in the oocyte cytoplasm but not in the nucleus in both GOCs and COCs (Fig. 2B). It was also possible that removing the oocyte from the follicular environment might have altered YAP localization or that YAP was present in the nucleus at a stage of oocyte development not represented in the samples that we had collected. Therefore, we also immunostained tissue sections of paraffin-fixed ovaries after verifying that we could detect nuclear acetylated histone H3 in these sections (Supplemental Fig. S1D). Oocytes within primordial, primary, secondary, and antral follicles all displayed strong cytoplasmic YAP fluorescence. In contrast, nuclear YAP fluorescence was weak or undetectable at all stages (Fig. 2C). We conclude that YAP is mainly excluded from oocyte nuclei at all stages of postnatal development.

We then examined prenatal oogenesis. We obtained ovarian sections from mice at E13.5, E15.5, and E18.5 and from 2-day-old pups, and stained these for Mouse Vasa Homologue (MVH) to identify germ cells and YAP to assess its localization in these cells. YAP was barely detectable in the germ cells at E13.5. At later stages, including when primordial follicles were present, YAP was present in the cytoplasm but undetectable in the nucleus (Fig. 3). Hence, YAP appears to be predominantly localized in the cytoplasm throughout female germ cell development in the mouse.

YAP in Oocytes Is Phosphorylated at S112

The exclusion of YAP from the nucleus throughout oogenesis implies that a robust mechanism restricts it to the cytoplasm. In other cell types, the intracellular localization of YAP is regulated by phosphorylation. In particular, phosphorylation of S112 (S127 in human) has been identified as a critical determinant because this modification enables YAP to associate with 14-3-3 proteins that anchor it in the cytoplasm [28, 49]. To test whether YAP in oocytes is phosphorylated at S112, we obtained growing and fully grown oocytes free of granulosa cells and subjected them to immunoblotting using a well-characterized antibody that is specific for S112-phosphorylated YAP. We detected S112-phosphorylated YAP in both growing and fully grown oocytes (Fig. 4A). The phospho-specific antibody also detected a species of the appropriate

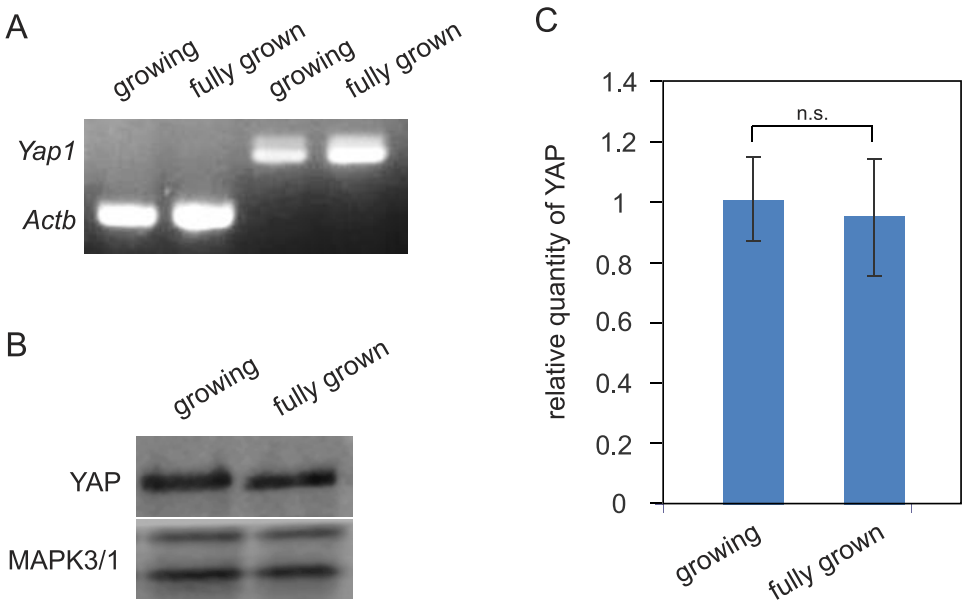

FIG. 1. Expression of YAP in oocytes. **A**) Messenger RNA was extracted from growing and fully grown oocytes. *Yap1* and *Actb* were detected using RT-PCR. **B**) Growing and fully grown oocytes were subjected to immunoblotting using antibodies against YAP and MAPK3/1; 150 growing oocytes and 80 fully grown oocytes were loaded. **C**) Quantification of immunoblots. YAP signal was normalized to MAPK3/1 signal. The ratio of YAP:MAPK3/1 in growing and fully grown oocytes did not significantly (n.s.) differ (Student *t*-test).

molecular weight in bovine oocytes (Fig. 4B), suggesting that phosphorylation of YAP on S112 (or its equivalent) is a conserved property of mammalian oocytes. Two immunoreactive bands were present in the blot of mouse fully grown oocytes, whereas only the faster-migrating band was detectable in growing oocytes. This may reflect phosphorylation of additional sites on YAP in fully grown oocytes. Unexpectedly, whereas growing and fully grown oocytes contain approximately the same amount of total YAP when equal amounts of cellular protein are analyzed (Fig. 1B), less S112-phosphorylated YAP was detectable in growing oocytes (Fig. 4A). This implies that growing oocytes contain both phosphorylated YAP and a subpopulation of YAP that is not phosphorylated on S112.

Protein Kinase A Regulates S112 Phosphorylation of YAP in Oocytes

We next sought to identify the mechanism responsible for YAP phosphorylation. S112 phosphorylation is typically regulated by the Hippo pathway, and the membrane-associated FERM-domain protein, neurofibromatosis-2 (NF2), is required for Hippo signaling in a broad range of cell types [22, 50]. Notably, in mouse blastocysts lacking *Nf2,* YAP accumulates in the nuclei of the inner cell mass whereas it is exclusively cytoplasmic in these cells in wild-type blastocysts [40], indicating that NF2 regulates YAP in the early embryo. When we examined oocytes in which *Nf2* had been deleted, however, YAP remained largely excluded from the nucleus (Fig. 2D). Crucially, we could detect no difference in the nucleocytoplasmic distribution of YAP in the presence or absence of *Nf2*. Although we did not directly examine phosphorylation in these experiments, this result indicates that, in contrast to the embryo, NF2 does not regulate YAP in oocytes.

The cAMP-dependent protein kinase A regulates YAP phosphorylation in a small number of cell types [51–53]. Because protein kinase A activity is high in growing and fully grown oocytes [54–58], we hypothesized that it might play a key role in regulating S112 phosphorylation of YAP. To test this, we first removed fully grown oocytes from the follicle, which causes protein kinase A activity in the oocyte to rapidly fall, and allowed them to undergo maturation in vitro. We found a dramatic reduction in the amount of S112-phosphorylated YAP in oocytes that had matured to metaphase II (Fig. 4C). The small amount that remained migrated more slowly than YAP in immature (germinal vesicle-stage) oocytes, consistent with the possibility that other sites on the protein became phosphorylated during maturation. Crucially, the loss of phosphorylated YAP was not due to degradation of the protein, whose quantity remained stable during maturation (Fig. 4C). In contrast, when we incubated fully grown oocytes overnight with dbcAMP, which maintains high protein kinase A activity, YAP remained phosphorylated on S112 (Fig. 4D).

The loss of S112-phosphorylated YAP in mature oocytes could be due to the decrease in protein kinase A activity or to the process of meiotic maturation. To resolve this point, we isolated fully grown oocytes and incubated them in the presence of an inhibitor of cyclin-dependent kinase (CDK) activity, roscovitine. These oocytes thus possessed low protein kinase A activity, as a result of being removed from the follicle, but were unable to mature. Strikingly, S112-phosphorylated YAP was substantially diminished in the roscovitine-treated oocytes (Fig. 4C), and this was not due to degradation of the protein (Fig. 4C). Taken together, these results indicate that protein kinase A activity regulates S112 phosphorylation of YAP in fully grown oocytes.

We then tested whether protein kinase A also regulates S112 phosphorylation of YAP in growing oocytes. We first isolated GOCs containing growing oocytes and incubated them overnight in the presence or absence of dbcAMP, after which the oocytes were removed from the GOC and immunoblotted. Unexpectedly, YAP remained phosphorylated on S112 even in the absence of dbcAMP (Fig. 5A). To understand this result, we considered prior observations that the granulosa cells supply the oocyte with cGMP, which inhibits phosphodiesterase (PDE) 3A and thereby helps to maintain high intra-oocyte cAMP and protein kinase A activity [59–61]. It was possible

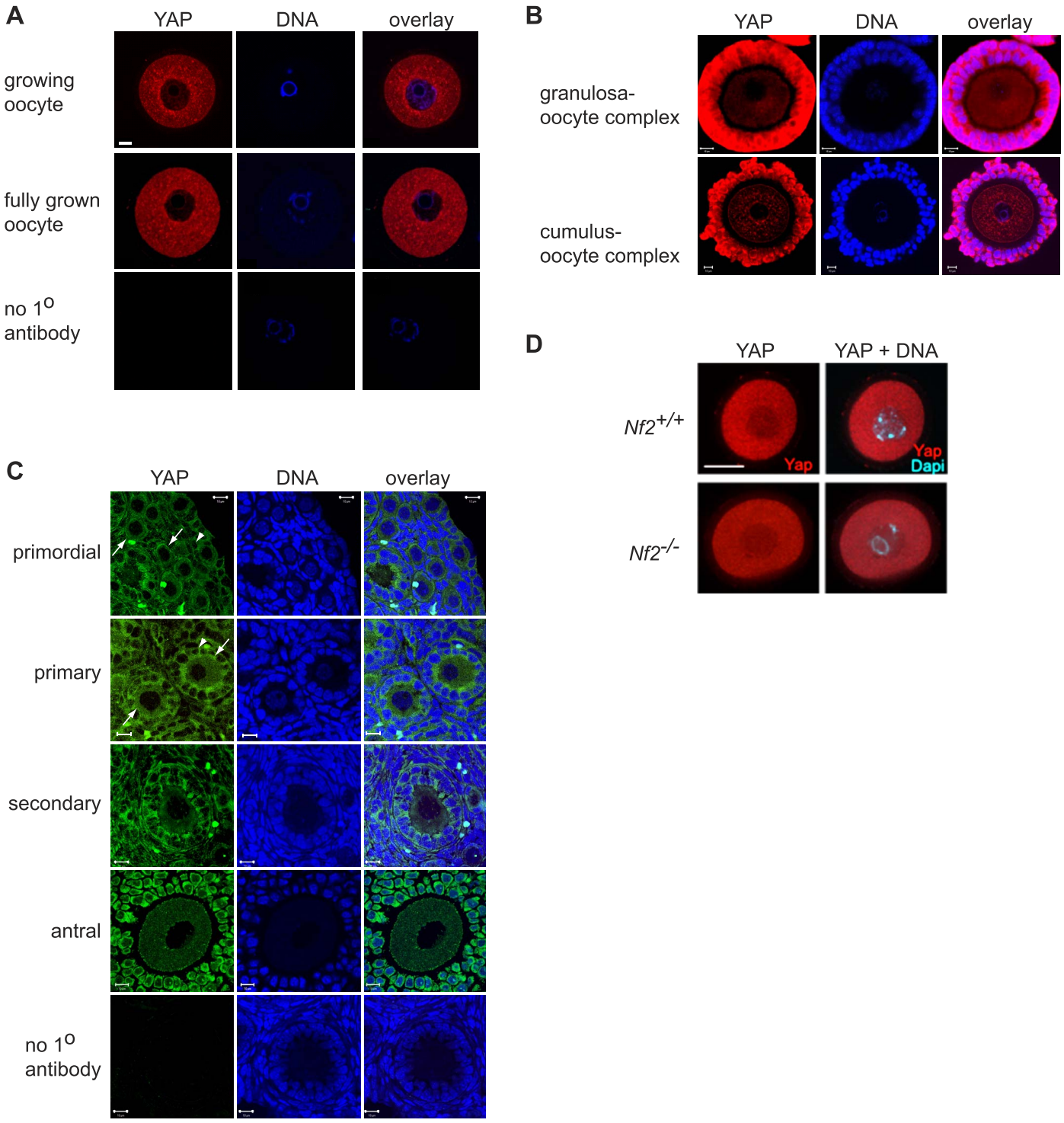

FIG. 2. Intracellular localization of YAP in oocytes. **A**, **B**) Growing and fully grown oocytes (**A**) or granulosa-oocyte complexes (GOCs) and cumulus-oocyte complexes (COCs) (**B**) were stained using anti-YAP. DNA was stained using DRAQ5. Bottom panel in **A** shows staining when primary antibody was omitted. YAP is present in the cytoplasm but is undetectable in the nucleus. The GOC in **B** is overexposed to reveal YAP in oocytes. Bar = 10 μm. **C**) Paraffin sections were stained using anti-YAP. DNA was counterstained using DRAQ5. Representative examples of oocytes in primordial, primary, secondary, and antral follicles are shown. Arrows indicate oocytes. Arrowheads indicate granulosa cells. YAP is present in the cytoplasm but is undetectable in the nucleus. Bar = 10 μm. **D**) Oocytes of $Nf2^{+/+}$ and $Nf2^{-/-}$ littermates were stained using anti-YAP. No difference in staining pattern is detectable between the genotypes. Bar = 50 μm.

that the granulosa cells in the cultured GOCs had supplied the oocyte with enough cGMP to maintain high protein kinase A activity in the absence of external dbcAMP. Therefore, we removed the granulosa cells from the growing oocytes prior to overnight incubation. Even in the absence of the granulosa cells, however, YAP remained phosphorylated on S112 in the growing oocytes (Fig. 5B).

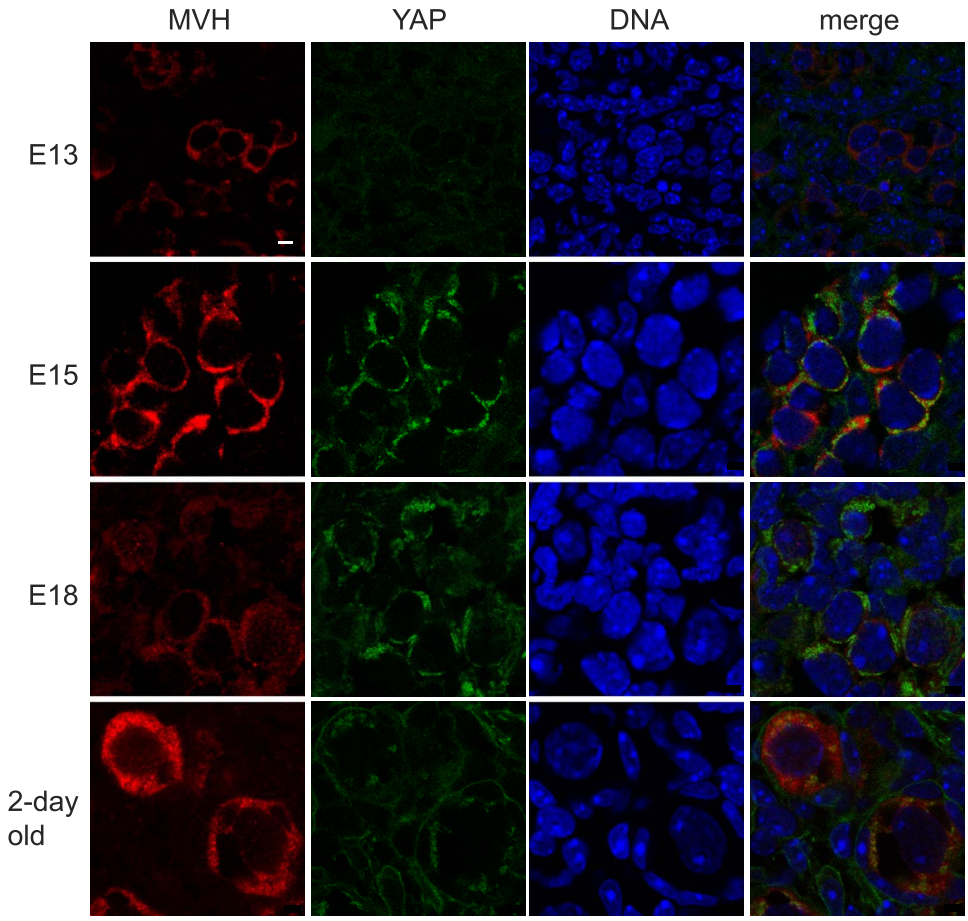

FIG. 3. Intracellular localization of YAP in pre- and perinatal oocytes. Frozen sections of ovary obtained at the indicated stages of embryonic (E) development or 2 days after birth were stained using anti-MVH to identify germ cells, anti-YAP, and DRAQ5. YAP is barely detectable at E13.5 but is present in oocyte cytoplasm at E15.5, E18.5, and newborns. YAP is not detectable in the oocyte nucleus at any stage. Bar = 5 μm.

These results could indicate that S112 phosphorylation of YAP in growing oocytes does not require protein kinase A activity or that, in contrast to fully grown oocytes, growing oocytes are able to maintain high protein kinase A activity in vitro in the absence of an extracellular source of cAMP (i.e., dbcAMP). To test the role of protein kinase A directly, we isolated GOCs containing growing oocytes and incubated them overnight in KT5720, a cell-permeable inhibitor of protein kinase A that acts by blocking its ATP-binding site. Incubation in the presence of KT5720 reduced the phosphorylation of CREB, a known substrate of protein kinase A, by about 50% (Fig. 5C). KT5720 also induced a quantitatively similar reduction in the amount of S112-phosphorylated YAP (Fig. 5D). These results imply that protein kinase A regulates S112 phosphorylation of YAP in growing oocytes as well as in fully grown oocytes.

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

Because a portion of the YAP in growing oocytes was not phosphorylated on S112, as discussed above, we were surprised that it was not detectable in the nuclei at this stage. Moreover, when we incubated either growing or fully grown oocytes under conditions that reduced S112 phosphorylation of YAP, we did not detect nuclear YAP at either stage (Fig. 6, A and B). These results suggested that, even when YAP was not phosphorylated on S112, it was unable to accumulate in the oocyte nucleus. To understand the basis for this nuclear exclusion, we incubated growing oocytes in the presence of leptomycin B, an inhibitor of nuclear export. Under these conditions, we observed a robust accumulation of YAP in the oocyte nuclei (Fig. 6A). These results confirm that our fixation and processing conditions permitted nuclear YAP to be detected when it was present. More importantly, they indicate that a portion of the oocyte YAP, likely that which is not phosphorylated on S112, is transported to the nuclei in growing oocytes. However, unless trapped there using an export inhibitor, the nonphosphorylated YAP is not retained and instead rapidly returns to the cytoplasm.

In striking contrast, when we treated fully grown oocytes with leptomycin B, together with roscovitine to prevent nuclear membrane breakdown, YAP did not accumulate in the nucleus (Fig. 6B). This was not due to an unanticipated effect of the roscovitine because the drug did not block nuclear accumulation of YAP in growing oocytes (data not shown). Rather it appears that nonphosphorylated YAP is not transported to the nucleus in fully grown oocytes. Thus, fully grown oocytes possess an additional mechanism not present in growing oocytes that prevents YAP from accumulating in the nucleus.

YAP Is Primarily Cytoplasmic in the Granulosa Cells

Although the primary focus of our study was the oocyte, we were also able to examine the intracellular distribution of YAP in the granulosa cells of the follicle. In intact GOCs and COCs

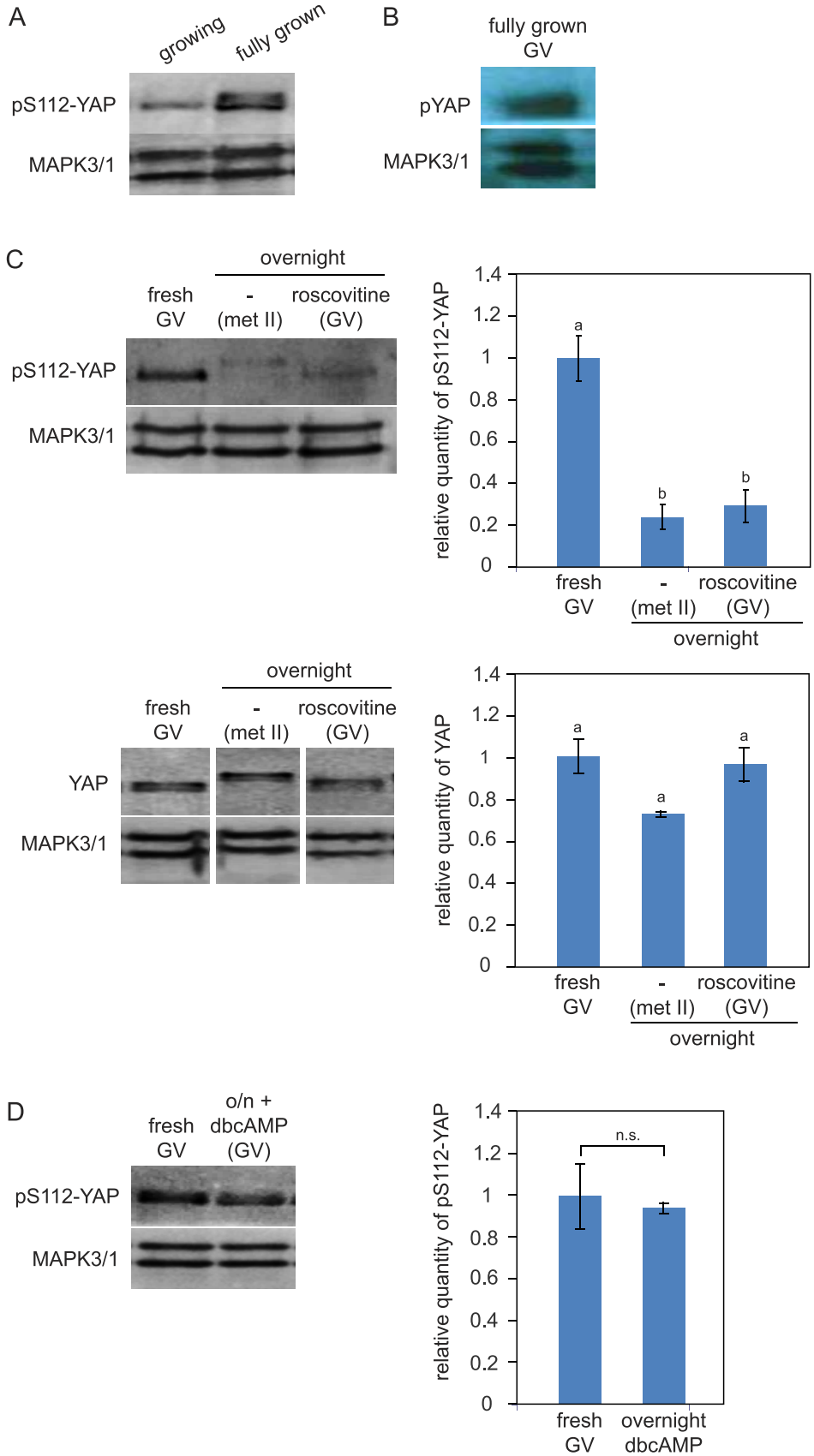

FIG. 4. Phosphorylation of YAP on S112 in oocytes. **A**) Growing (150) and fully grown (80) oocytes were subjected to immunoblotting using anti-phosphorylated S112-YAP antibody and anti-MAPK3/1. The slow-migrating band in fully grown oocytes may be phospho-S112 YAP carrying additional modifications or an unrelated protein. **B**) Bovine oocytes (40) were immunoblotted as in **A**. The position of the serine corresponding to S112 in mouse is not certain. The experiment was performed twice. **C**) Fully grown oocytes were collected and one portion (fresh GV) was reserved immediately for immunoblotting while the remaining oocytes were incubated overnight in the absence or presence of roscovitine. Oocytes that reached

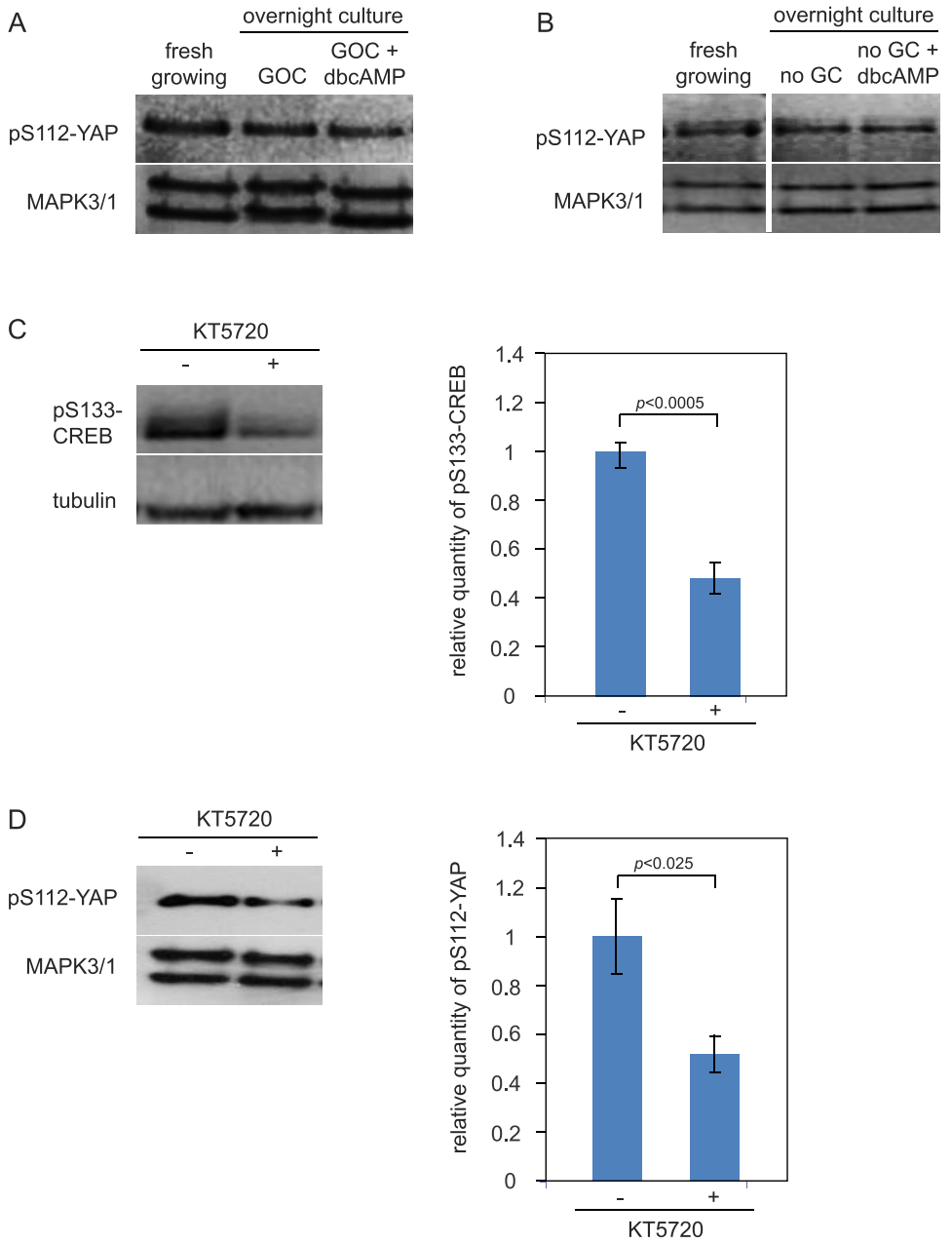

FIG. 5. Regulation of S112-YAP phosphorylation in growing oocytes. **A**, **B**) Granulosa-oocyte complexes (GOCs) were collected. The oocyte was isolated from one portion and reserved for immunoblotting. The remaining GOCs (**A**) or oocytes isolated from the GOCs (**B**) were incubated overnight in the absence or presence of dbcAMP, after which the oocyte used for immunoblotting. Both pS112-YAP and MAPK3/1 were detected. Dibutyryl cyclic AMP is not required to maintain pS112-YAP during overnight incubation. **C**, **D**) Growing oocytes were incubated overnight in the absence or presence of the protein kinase A inhibitor, KT5720, then subjected to immunoblotting to detect pS133-CREB (**C**) or pS112-YAP (**D**). Tubulin was used as a loading control in **C** because the molecular weights of CREB and MAPK3/1 are similar. Both pS133-CREB and pS112-YAP were decreased in the presence of KT5720 (Student *t*-test, $P < 0.05$).

(Fig. 2B) as well as in ovarian tissue sections (Fig. 2C), we consistently observed cytoplasmic staining of the granulosa cells. In contrast, we never detected nuclear granulosa cell staining in follicles at any stage. These results indicate that YAP is largely excluded from the nucleus of the granulosa cells throughout postnatal follicular growth.

metaphase II in the absence of roscovitine or remained at the GV stage in its presence were used. Phospho-S112-YAP (upper panel) or total YAP (lower panel) and MAPK3/1 were detected as in **A**. In the total YAP blot, intervening lanes present in the gel have been removed from the micrograph, as indicated by the white lines between lanes. Phospho-S112-YAP is lost during maturation and in GV oocytes incubated in roscovitine. **D**) Fully grown oocytes were collected and one portion (GV) was reserved immediately for immunoblotting while the remaining oocytes were incubated overnight in the presence of dbcAMP. Phospho-S112-YAP remains in oocytes incubated in the presence of dbcAMP. In all histograms, YAP signal was normalized to MAPK3/1 signal of the same sample and results were analyzed using the Student *t*-test (**A**, **D**) or ANOVA (**C**) where different superscripts indicate a statistically significant difference ($P < 0.05$); n.s., not significantly different.

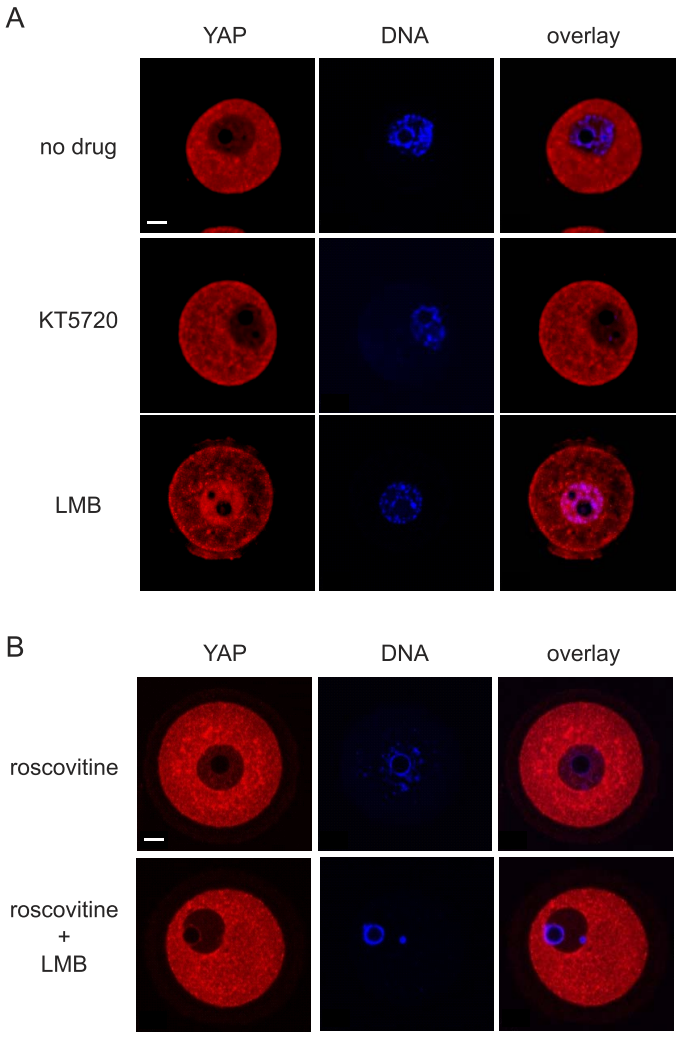

FIG. 6. Transient nuclear localization of YAP in growing oocytes. **A**) Growing oocytes were incubated overnight under control conditions or in the presence of the protein kinase A inhibitor, KT5720, or the nuclear expost inhitor, leptomycn B (LMB), then stained using anti-YAP. LMB induces accumulation of nuclear YAP. **B**) Fully grown oocytes were incubated overnight in the presence of the CDK1 inhibitor, roscovitine, or roscovitine and LMB. No nuclear accumulation of YAP is detectable. Bar = 10 μm.

DISCUSSION

We have investigated the potential role of YAP during oocyte development. We find that YAP is expressed as early as Day 15.5 of embryonic development and continues to be expressed during all stages of oocyte development up to and including meiotic maturation. Throughout all these stages, however, YAP is predominantly present in the cytoplasm and is largely excluded from the nucleus. These results identify and map for the first time the expression and intracellular localization of YAP during pre- and postnatal oogenesis in vertebrates. They also strongly suggest that nuclear YAP does not regulate mammalian oocyte development under physiological conditions.

Our results reveal that multiple mechanisms cooperate to prevent YAP accumulation in the oocyte nucleus. First, phosphorylation of S112 enables YAP to associate with 14-3-3 proteins, which in other cell types anchors it in the cytoplasm [28, 29]. Protein kinase A has recently been identified as an effector of S112 phosphorylation, through its activity to phosphorylate the LATS kinases [52, 53]. High protein kinase A activity is a characteristic property of growing and fully grown mammalian oocytes as well as those of nonmammalian species [54–58]. In rodents, this activity is maintained by cyclic AMP, whose synthesis is stimulated by a constitutively active G-protein coupled receptor (GPR3 in mice; GPR12 in rats) [62–64]. Although less is known of cAMP and protein kinase A levels at earlier stages of oogenesis, adenyl cyclase was recently detected in mouse oocytes as early as E15.5 [65]. This concords strikingly with our observation that YAP is cytoplasmic even in oocytes at this stage. Crucially, it also suggests that protein kinase A activity may be high throughout postmitotic oogenesis. Moreover, oocytes express numerous members of the 14-3-3 family of proteins [66]. Thus, it is likely that much of the YAP in growing and fully grown oocytes, because it is phosphorylated at S112, is associated with 14-3-3 proteins that anchor it in the cytoplasm.

Second, although phosphorylation at S112 would likely anchor YAP in the cytoplasm, we observed using the nuclear export inhibitor, leptomycin B, that some YAP can enter the nucleus in growing oocytes. Leptomycin B also promotes YAP nuclear localization in other cell types [26, 67]. Because a portion of the YAP in growing oocytes is not phosphorylated at S112, we speculate that this nonphosphorylated YAP can enter the nucleus. Yet this YAP is rapidly exported back to the cytoplasm. Therefore, oocytes are unable to retain YAP in the nucleus. YAP does not possess a known DNA-binding domain and relies on binding partners that possess DNA-binding activity to remain in the nucleus. YAP principally associates with the TEAD family of proteins [23], although other binding partners have been identified [19, 68]. Importantly, binding to TEAD is required to retain YAP in the nucleus [21]. Growing oocytes express mRNAs encoding several TEAD proteins but the expression of the encoded proteins has not been reported [69]. We suggest that these partners may be expressed too weakly to retain a detectable quantity of YAP in the nucleus or that posttranslational modifications of YAP prevent stable association with them [70]. As a result, YAP returns to the cytoplasm. Thus, in the absence of a mechanism to retain it in the nucleus, dephosphorylated YAP accumulates in the cytoplasm by default. It would be valuable to examine the localization of YAP in a recently described Yap^{S112A} mutant [27].

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

Multiple intracellular mechanisms thus cooperate to ensure that YAP does not accumulate in the oocyte nucleus (Fig. 7). What function might be served by nuclear exclusion? On the one hand, it may be that, if abundant in the nucleus, YAP

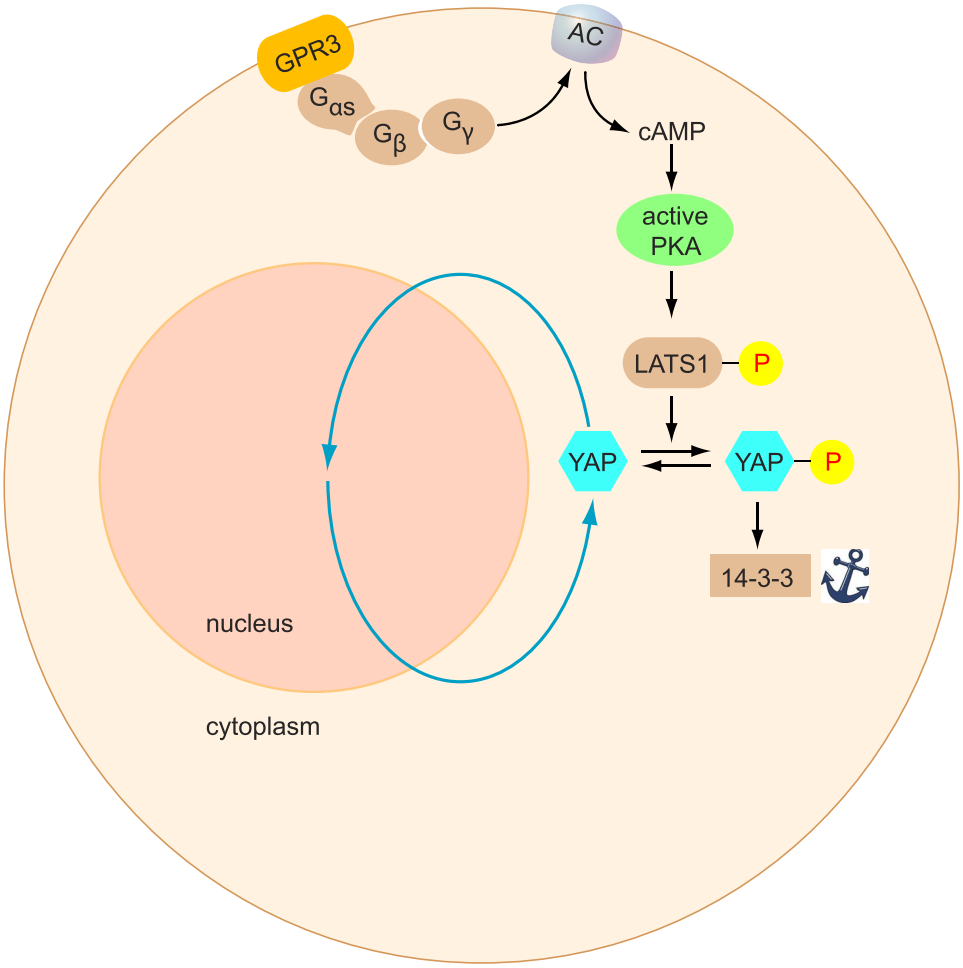

FIG. 7. Control of YAP intracellular localization in oocytes. The cAMP synthesized by oocytes via G-protein receptor (GPR)-coupled adenyl cyclase (AC) maintains high activity of protein kinase A (PKA), which in turn directly or indirectly phosphorylates LATS1, thereby increasing its kinase activity toward YAP. S112-phosphorylated YAP associates with 14-3-3 proteins that anchor it in the cytoplasm. A portion of the YAP in growing oocytes remains nonphosphorylated and enters the nucleus but is rapidly exported back to the cytoplasm.

would impair oocyte development. The link between inactivation of the Hippo pathway and tumorigenesis suggests that nuclear YAP can regulate cell-cycle progression [16, 17]. In oocytes, the cell cycle becomes arrested at late G2 before they are assembled into primordial follicles and does not resume until meiotic maturation. Nuclear exclusion of YAP might be important to ensure that the cell cycle does not resume precociously. Intriguingly, global deletion of LATS1 causes perinatal germ-cell apoptosis and precocious growth of the oocytes that remain [39]. As LATS1 deletion would be expected to favor nuclear localization of YAP, this result supports the notion that normal oocyte development depends on excluding YAP from the nucleus. It is also possible that cytoplasmic YAP serves a function during oocyte development, perhaps by sequestering molecules away from the nucleus [33]. The apparent abundance of YAP, as indicated by intense immunofluorescent signal, is consistent with a cytoplasmic function. Targeted deletion of *Yap1* in the oocyte could test its role directly.

As discussed in the *Introduction,* the YAP paralogue, WWTR1, has been detected using immunohistochemistry in the nuclei of oocytes at all stages of growth [39]. YAP and WWTR1 share substantial sequence identity, including the region that binds to 14-3-3 proteins, and are thought to be coregulated [17]. WWTR1 also contains a serine at position 89, corresponding to S112 in YAP. These structural similarities suggest that WWTR1 in oocytes is likely to be phosphorylated at S89 and anchored in the cytoplasm. Hence, its apparent nuclear localization is unanticipated. WWTR1 lacks certain domains that are present in YAP, however, including an N-terminal proline-rich region and an SH3 (Src homology 3)-binding motif. Although these domains have not been implicated in YAP intracellular localization, it is possible that their absence in WWTR1 permits nuclear accumulation even when the protein is phosphorylated. Alternatively, if a fraction of the WWTR1 in oocytes is nonphosphorylated, a WWTR1-specific mechanism might retain the nonphosphorylated form in the nucleus. Further studies of the phosphorylation state and localization of WWTR1 within the oocyte should help to resolve these apparently conflicting results.

Our results demonstrate that YAP is excluded from the nucleus throughout oocyte development beginning at prenatal stages. Moreover, although we did not systematically study YAP expression in the somatic compartment of the follicle, we found no stage at which it was nuclear in the granulosa cells. These results provide strong evidence that nuclear YAP does not play a physiological role in oocyte or follicular development. Yet, previous studies have unambiguously demonstrated that experimental interventions that repress Hippo signaling trigger oocyte and follicular growth [35, 36]. How may these

be reconciled? It was proposed that these interventions repressed the Hippo pathway by inducing actin rearrangements, which in other cell types can induce YAP nuclear localization [71]. Thus, experimental inactivation of the Hippo pathway in the oocyte or somatic cell compartment may trigger oocyte and follicular growth even if nuclear YAP does not regulate these processes in a physiological context. Our results nonetheless indicate that maintaining the normal intracellular distribution and function of YAP should be an important consideration during the development and evaluation of conditions designed to recapitulate normal oocyte and follicle development.

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