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An Important Role of Pumilio 1 in Regulating the Development of the Mammalian Female Germline[1]

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ABSTRACT

Pumilio/FBF (PUF) proteins are a highly conserved family of translational regulators. The *Drosophila* PUF protein, Pumilio, is crucial for germline establishment and fertility. In mammals, primordial folliculogenesis is a key process that establishes the initial cohort of female mammalian germ cells prior to birth, and this primordial follicle pool is a prerequisite for female reproductive competence. We sought to understand whether PUF proteins have a conserved role in mammals during primordial folliculogenesis and female reproductive competency. In mammals, two homologs of Pumilio exist: Pumilio 1 (Pum1) and Pum2. Here, we report that PUMILIO (PUM) 1 plays an important role in the establishment of the primordial follicle pool, meiosis, and female reproductive competency, whereas PUM2 does not have a detectable function in these processes. Furthermore, we show that PUM1 facilitates the transition of the late meiotic prophase I oocyte from pachytene to diplotene stage by regulating SYCP1 protein. Our study reveals an important role of translational regulation in mammalian female germ cell development.

meiosis, primordial folliculogenesis, Pumilio, RNA binding protein, subfertility, translational regulator

INTRODUCTION

Establishing the primordial follicle pool is a critically important event in a mammalian female's reproductive lifecycle, as her entire reproductive potential relies on the quantity and quality of these follicles. In humans, infertility and premature menopause are consequences of a reduced number and/or quality of primordial follicles. Studying this important process in humans, however, is virtually impossible, given that primordial folliculogenesis initiates during embryogenesis. Therefore, the mouse is a valuable genetic model for understanding the processes occurring in primordial folliculogenesis.

Primordial folliculogenesis in the mouse requires several steps, beginning at Embryonic Day (E) 10.5 and lasting until Postnatal Days (PNDs) 5–7. The sequence of events is as follows: proliferation of primordial germ cells (PGCs) and their migration to the gonadal ridges [1]; their acquisition of the female fate and entry of meiosis of oocytes [2]; and germ cell cyst breakdown into primordial follicles [3, 4]. Two distinct waves of germ cell death occur during the establishment of primordial follicles, one at the onset of meiosis in the PGCs (at E13.5) and the other as the oocytes begin to arrest at diplotene stage and form primordial follicles shortly before birth (E17.5). Prior elegant work in the mouse reveals the role of various cell adhesion molecules, cell signaling proteins, and transcription factors during this multistep process [reviewed in Ref. 5]. However, the role of translational regulators during folliculogenesis and subsequent steps of oogenesis has not been well explored.

The RNA-binding protein families have been implicated in posttranscriptional regulation of reproductive functions. They include cytoplasmic polyadenylation element binding (CPEB) protein family [6], polyA binding proteins (PABP) [6], Deleted in azoospermia (DAZ) family [7–9], and Pumilio (Pum)/FBF family (PUF) [10, 11]. The translational regulators, CPEB and DAZLA have been shown to regulate meiosis during mouse embryonic gonad development [12, 13]; however, the role of the PUF family during this process is unknown.

Our study focuses on the PUF family proteins, which have known functions in germline development in invertebrates. The founding member of the PUF protein family, the *Drosophila* Pum protein, was originally discovered to be a maternal effect gene required in establishing posterior patterning of the embryo [14]. Subsequently, germline functions of *Pum* were uncovered, and these include: PGC proliferation; germline stem cell maintenance during oogenesis; ovarian morphogenesis; and oviposition [10, 15, 16]. The PUF proteins bind to a consensus sequence called the Pum response element (PRE) in 3′ untranslated region (UTR) of their target mRNA and, following binding, will mediate translational repression and/or mRNA decay [17, 18] through

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either poly-(A)-dependent [19–21] or -independent mechanisms [22].

There are two mammalian PUF proteins, PUMILIO (PUM) 1 and 2, both of which are expressed in diverse tissues. They have overlapping mRNA targets, with PUM1 having a larger cohort of targets [23]. The role of PUM2 in mammalian reproduction was investigated by Xu et al. [24], who generated *Pum2*-deficient mice by a gene trap strategy. Surprisingly, both male and female mice had normal fertility. They noted that the male mice had smaller testes secondary to a defect in testicular and seminiferous tubule development, but not in spermatogenesis. Independently, we generated *Pum1* knockout mice using the cre/lox approach and found that $Pum1^{-/-}$ males display a 40% decrease in fertility [23]. This subfertility was attributed to an increase in apoptosis of spermatocytes through up-regulation of p53. However, the function of mammalian PUM1 in oogenesis remains unknown. Our study identifies a role of PUM1 in the mammalian female reproductive system and, more specifically, in controlling primordial folliculogenesis.

MATERIALS AND METHODS

Mouse Strains and Animal Care

All transgenic mice were on a mixed 129/B6 background. $Pum1^{-/-}$ (global Pum1 knockout) and $Pum1^{-/flox}$*;vasa cre* mice used in this study were previously characterized by Chen et al. [23]. Controls for $Pum1^{-/-}$ females were wild-type littermates. Controls for $Pum1^{-/flox}$*;vasa cre* females were $Pum1^{+/+}$*;vasa cre* females. *Pum2* knockout mice were generated at the University of Connecticut Gene Targeting and Transfer Facility (Farmington, CT). $Pum2^{flox/+}$ mice were mated with *EIIa-Cre* mice, which express Cre recombinase under the promoter of the adenovirus *EIIa* gene that is expressed early in development. Mice in which the floxed *Pum2* allele was excised were confirmed by genotyping, and all subsequent crosses were performed on global *Pum2* heterozygous mice in the absence of Cre to establish $Pum2^{-/-}$ mice. CD1 females were used for SSEA-1 FACS and PUM1/2 immunoblot of embryonic ovary lysates. Animals used in these studies were maintained and euthanized according to the principles and procedures described in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. These studies were approved by the Yale University Institutional Animal Care and Use Committee and conducted in accordance with the specific guidelines and standards of the Society for the Study of Reproduction.

Fertility Test

Females (6 wk old) were housed singly with wild-type stud males of proven fertility for at least 6 mo. The litter sizes were recorded continuously.

Oocyte and Embryo Collection

Females (6 wk old) were superovulated with 5 IU eCG followed 46 h later with 5 IU hCG. The oocytes were collected from the oviducts 15–16 h post-hCG and incubated with hyaluronidase (300 μg/ml) to remove the cumulus cells. The oocytes were then washed in M2 medium (Gibco) and categorized into nondegenerate metaphase II (MII) stage oocytes or degenerate oocytes. For two-cell embryo collections, 6-wk-old females were mated with wild-type males of proven fertility and vaginal plugs were checked daily. At 1 day after the vaginal plug (1.5 days postcoitum [dpc]) was observed, the oviduct was flushed with M2 medium (Gibco) to collect two-cell embryos.

SSEA-1 FACS

The method used to obtain a purified population of germ and somatic cells at 13.5 dpc was adapted from Liu et al. [25]. In brief, 20–40 female gonads were dissected from 13.5 dpc embryos in Dulbecco modified Eagle medium (DMEM) media and digested with 0.05% trypsin for 30 min at 4°C. The digested gonads were pipetted several times to obtain a single-cell suspension. The cells were washed with DMEM and resuspended in DMEM with 10% FBS. Alexa-Fluor 647-conjugated SSEA-1 antibody (1:50) was added to the cell suspension for at least 1 h. Subsequent washes were performed to remove excess antibody. Propidium iodide was added to the cell suspension to stain for live cells. The FACS sorting was carried out to obtain SSEA-1-positive germ cells and SSEA-1-negative somatic cells.

Immunoblot Analysis

Samples were prepared in M-Per Mammalian protein extraction reagent (ThermoScientific) according to manufacturer's instructions. For FACS-sorted somatic cells and oocytes, all cells obtained after each independent FACS experiment were processed separately. For embryonic gonads (13.5 dpc, 15.5 dpc, 18.5 dpc), for each independent experiment, 10–20 gonads were pooled to prepare the protein lysates. For postnatal ovaries, a pair of ovaries per animal was used to prepare each independent sample. Protein lysates were loaded onto 4%–15% SDS linear gradient gel (Bio-Rad). Standard blotting was performed using a nitrocellulose membrane with a semidry transfer machine at 16 V for 1 h (Bio-Rad). The membrane was blocked with 5% milk TBST solution for 30 min and then incubated with the primary antibody overnight at 4°C. The next day, three 10-min washes with TBST were performed. Incubation with secondary antibody (1:4000 anti-rabbit conjugated horseradish peroxidase; Life Technologies) diluted in 5% milk TBST solution was performed at room temperature for 1 h. Again, three 10-min washes with TBST were performed. Supersignal West Dura extended substrate (ThermoScientific) was used and chemiluminescent signals were detected using radiograph film. Antibodies used for immunoblot analyses are listed separately. Densitometry analysis was performed using ImageJ software where applicable.

Ovarian Histology and Follicle Counting

Ovaries were collected and examined at PND1, PND5, 4 wk, and 6 mo of age. Ovaries were fixed in buffered formalin, paraffin embedded, and serially sectioned at 5 μm (Yale Histology core). The sections were stained with hematoxylin/eosin (Yale Histology core). The total numbers of primordial, primary, and preantral follicles were counted in every fourth section for PND1 and every eighth section for PND5, 4 wk, and 6 mo of age. Follicles were classified as previously described by Morita et al. [26]. In brief, follicles were scored as primordial follicles if there was a compact oocyte surrounded by a single layer of squamous pregranulosa cells, as primary follicles if there was an enlarged oocyte surrounded by at least a single layer of cuboidal granulosa cells, and as small, preantral follicles if there was an enlarged oocyte surrounded by at least a partial or complete second layer of cuboidal granulosa cells and no more than four layers of cuboidal granulosa cells. Only follicles with an observed nucleus were scored in each section. Each ovary was given a numerical code and scored blindly without knowledge of the genotype. Two investigators scored each ovary independently. The total follicle counts per ovary were calculated by multiplying the raw counts with either a factor of 4 (PND1) or 8 (PND5, 4 wk, and 6 mo). If two or more germ cells were in contact and surrounded by pregranulosa cells, these germ cells were considered to be in germ cell nests. The number of oocytes present in germ cell nests was expressed as a percentage of total oocyte number.

Immunofluorescence and Germ Cell Counts

Gonads from female embryos (13.5, 15.5, and 18.5 dpc) were dissected and fixed with 2% paraformaldehyde, paraffin embedded, and serially sectioned at 5 μm. The sections were deparaffinized with xylene and rehydrated with 100%, 95%, and 70% ethanol washes and a final wash in PBS. The sections were processed for immunofluorescence staining as previously described by Mak et al. [27] Anti-VASA antibody was used as a germ cell marker. Omitting the primary antibody served as a negative control. Images were taken using a Zeiss Axio Imager Z1 fluorescence microscope, and the total number of VASA-positive germ cells was counted in every fourth section. The sum of the germ cell counts was multiplied by four. Similarly, if two or more germ cells were in contact, these germ cells were considered to be in germ cell nests. The number of oocytes present in germ cell nests was expressed as a percentage of the total number of oocytes.

Meiotic Spreads

Gonads were dissected from females at 15.5 dpc, 18.5 dpc, and PND1 (1 day after birth) and placed in 0.05% trypsin for 1 h at 4°C. DMEM with 10% fetal bovine serum was added to stop the trypsin activity. A 25-G needle was used to disaggregate the gonads into single cells. The single cells were washed with PBS and resuspended in hypotonic solution (30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate dehydrate, 5 mM ethylenediaminetetra-acetic acid, pH 8.2). The subsequent steps for preparing the meiotic spreads are detailed by Peters et al. [28]. After the meiotic spreads were obtained, permeabilization was carried with 0.5% Trition X-100/0.1% Tween 20 solution and the meiotic

spreads were incubated in primary antibodies, SYCP1 and SYCP3, for 1 h, and subsequently with the appropriate secondary antibody. Images were taken with a Zeiss Axio Imager Z1 fluorescence microscope. The stages of meiotic prophase I were identified according to chromosomal axial element staining by SYCP3 [29, 30]. SYCP1 staining was categorized as: linear SYCP1 (SYCP1 staining that was present throughout the synaptonemal complex [SC] and colocalized with SYCP3); punctate SYCP1 staining (SYCP1 staining that was dispersed along the SC with linear SYCP3 staining); or absent SYCP1 staining (only linear SYCP3 staining with no SYCP1 staining). SYCP1 disassembly was signified by the presence of punctate SYCP1 staining [30]. A minimum of 100 meiotic spreads was scored per animal.

VASA/TUNEL Costaining

PND1 ovaries were dissected and the ovaries were fixed and serially sectioned as described above for immunofluorescence. Antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6.0). The ApopTag Fluorescein Direct In situ Apoptosis Detection Kit (Millipore) was used according to the manufacturer's protocol. Prior to mounting the slides in 4 ,6-diamidino-2-phenylindole (DAPI), the slides were incubated with anti-Vasa antibody at 4°C overnight, followed by the appropriate Alex-fluor 568 secondary antibody at room temperature for 1 h. The slides were mounted in Vectashield with DAPI. Images were taken with the Zeiss Axio Imager Z1 fluorescence microscope. For each ovary, at a least five sections were counted documenting the total number of VASA-positive cells (germ cells) and total number of VASA/TUNEL-positive cells.

FSH Sera Levels

Serum was obtained from 6-wk-old females and submitted to the Center for Research in Reproduction Ligand Assay and Analysis Core at the University of Virginia (NCTRI P50-HD28934) for FSH measurement using the mouse FSH RIA assay.

AMH Sera Levels

Serum was obtained from 8- to 10-wk-old females and submitted to the Center for Research in Reproduction Ligand Assay and Analysis Core for mouse/rat AMH immunoassay (as described above).

Antibodies for Immunoblot and Immunofluorescence

PUM1 (1:1000; ab92545; Abcam); PUM2 (1:1000; ab92390; Abcam); VASA (1:1000 immunoblot; 1:100 immunofluorescence; ab13840; Abcam); SYCP3 (1:500; sc74569; Santa Cruz); SYCP1 (1:500; ab15090; Abcam); Alexa Fluor 647 SSEA-1 (1:500; MC-480; BioLegend).

Statistical Analysis

GraphPad Prism 6 software (GraphPad Software, Inc.) was used for all statistical analyses. The paired and unpaired Student *t*-test was used to calculate the difference of the means between groups of paired (sibling pairs) and unpaired (nonsibling pairs) datasets, respectively. $P < 0.05$ was considered statistically significant, and denoted by asterisks in the figures. The error bars denote mean ± SEM unless specifically noted in the text.

RESULTS

PUM1, but Not PUM2, Is Required for Mammalian Female Reproduction

To understand the function of PUM1 during female mammalian reproduction, we first tested the fertility of *Pum1*$^{-/-}$ global knockout females (n = 11) by mating them to wild-type males for at least 6 mo. *Pum1*$^{-/-}$ females lack PUM1 protein in all tissues [31], including the ovaries (Fig. 1A). PUM2 protein levels are unchanged in ovaries of *Pum1*$^{-/-}$ females (Fig. 1A). The *Pum1*$^{-/-}$ females had a significant decrease in litter size (6.4 ± 1.5, $P = 9 \times 10^{-5}$) compared to wild-type females (9.4 ± 0.94; Fig. 1B). Furthermore, the subfertility observed in the *Pum1*$^{-/-}$ females worsened with age (Fig. 1C), such that, by 6 mo, 63% of the *Pum1*$^{-/-}$ females had become sterile, suggesting a diminished ovarian reserve phenotype. Similarly to the results reported by Xu et al. [24], the *Pum2*$^{-/-}$ females showed apparently normal fertility (data not shown). These results indicate that PUM1 plays a more significant role in murine female reproductive competence than PUM2, and that PUM2 does not compensate for loss of PUM1.

Because the subfertility of *Pum1*$^{-/-}$ females could be related to a variety of defects, such as abnormality in folliculogenesis, a defect in ovulation, fertilization, and/or embryo development, we further dissected the function of PUM1, first by assessing the number of MII oocytes ovulated by *Pum1*$^{-/-}$ females. The females were superovulated and MII oocytes were counted. Overall, the total number of oocytes ovulated by the *Pum1*$^{-/-}$ females (19.5 ± 2.9 SEM) was not significantly different from the wild-type females (26.1 ± 3.0 SEM; Fig. 1D). However, *Pum1*$^{-/-}$ females had a greater number of the oocytes that were degenerate (6.4 ± 2.2 SEM) as compared to wild-type females (2.7 ± 0.65 SEM; Fig. 1, D and E). Therefore, after adjustment for the degenerate oocytes, the number of nondegenerate oocytes available for fertilization was significantly reduced by 44% in the *Pum1*$^{-/-}$ females. In support of this finding, the number of two-cell embryos obtained from *Pum1*$^{-/-}$ females after natural matings with wild-type stud males was also reduced by 50% when compared to wild-type females (Fig. 1F). This finding is consistent with the decrease in number of viable oocytes ovulated, and therefore available for fertilization. In addition, to exclude the possibility of dysfunction in the hypothalamic-pituitary-gonadal axis causing subfertility phenotype, the random FSH levels between 6-wk-old wild-type (n = 10) and *Pum1*$^{-/-}$ (n = 9) females were compared (6.15 ± 0.69 [SEM] ng/ml and 5.07 ± 0.57 ng/ml, respectively), and no significant differences were found. These findings show that the *Pum1*$^{-/-}$ females likely have a defect during folliculogenesis that leads to a reduced number of viable oocytes being ovulated, which is most likely the cause of the observed subfertility.

PUM1, but Not PUM2, Plays a Role in the Establishment of the Primordial Follicle Pool

To further investigate the specific role of PUM1 during folliculogenesis, we examined the ovarian phenotype of *Pum1*$^{-/-}$ females. Gross inspection of 6-wk-old *Pum1*$^{-/-}$ adult ovaries revealed that *Pum1*$^{-/-}$ ovaries were 40% (by weight) smaller than age-matched wild-type siblings (Fig. 2, A and B). However, *Pum1*$^{-/-}$ females were 36% smaller than their wild-type siblings by body weight. After adjusting for this difference in body weight, there was no difference in this ratio. In addition, in adult ovaries of both genotypes, primordial, primary, and preantral follicles were present (Fig. 2, A and B).

To ascertain whether lack of PUM1 had any effect on the primordial follicle pool size, we counted the number of primordial, primary, and preantral follicles in serial sections of prepubertal (4-wk-old) ovaries from *Pum1*$^{-/-}$ mice and wild-type littermates. *Pum1*$^{-/-}$ females had a 52% reduction in primordial follicle pool size when compared to controls (Fig. 2C), but there were no significant differences in the other follicle types. Furthermore, *Pum1*$^{-/-}$ females showed a consistent reduction (61%) in primordial follicle pool size at 6 mo as well (Fig. 2D). AMH levels were determined for 8- to 10-wk-old wild-type (n = 10) and *Pum1*$^{-/-}$ (n = 6) siblings; paired *t*-test did not show statistical significance (wild-type, 90.68 ± 9.5 ng/ml; *Pum1*$^{-/-}$, 72.08 ± 14.0 ng/ml).

To understand whether *Pum2* also had a role in primordial folliculogenesis, follicle counts were similarly performed with 4-wk-old *Pum2*$^{-/-}$ ovaries (Fig. 2E). There was no significant differences observed in the *Pum2*$^{-/-}$ females when compared

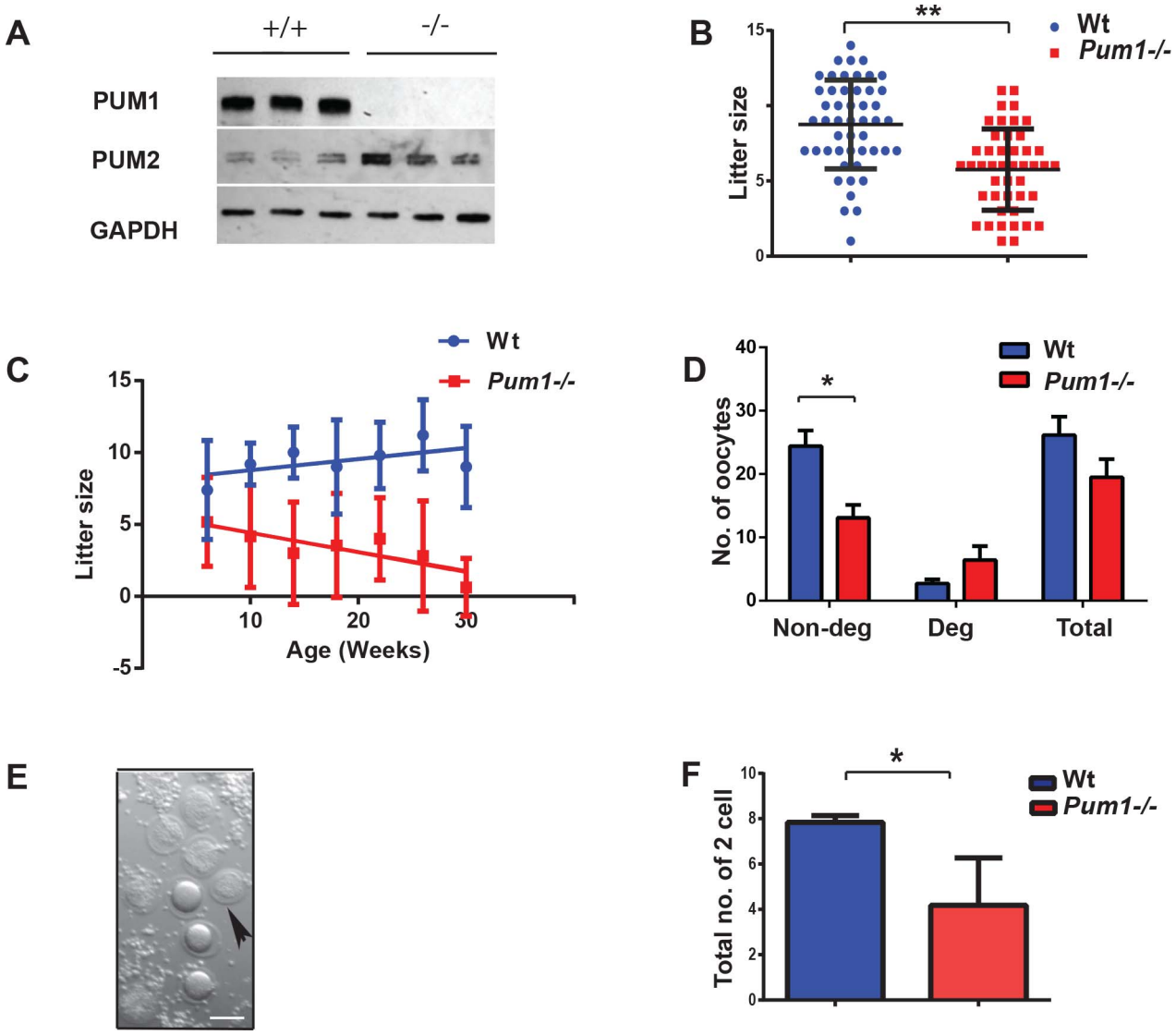

FIG. 1. *Pum1*$^{-/-}$ females have subfertility due to a lower number of viable oocytes that are ovulated. **A**) Immunoblot analysis showing PUM1 and PUM2 protein levels in wild-type mouse ovaries (+/+) and *Pum1*$^{-/-}$ ovaries (–/–) from 6- to 10-wk-old female mice. This immunoblot was performed three times with a total of 10 biological replicates. Glyceraldehyde phosphate dehydrogenase (GAPDH) used as loading control. Densitometric measurements showed no significant differences in PUM2 protein levels between the two genotypes. **B**) Litter sizes of wild-type (n = 5) and *Pum1*$^{-/-}$ (n = 11) females singly housed with a wild-type stud male continuously collected over a minimum of 6 mo, revealing a 32% reduction in average litter sizes. $^{**}P < 0.01$. Error bars represent SD. **C**) Litter size distribution of wild-type and *Pum1*$^{-/-}$ females with advancing age. Error bars represent SD. Using linear regression analysis, *Pum1*$^{-/-}$ litter size negatively correlated with age and significantly deviated from zero with a $P < 0.05$. No correlation was found with wild-type females. **D**) Total number of superovulated MII nondegenerate (Non-deg) and degenerate (Deg) oocytes per 6- to 7-wk-old wild-type (n = 14) and *Pum1*$^{-/-}$ (n = 15) females. $^{*}P < 0.05$. Total number of oocytes between the two groups was not statistically significant. **E**) Brightfield image of nondegenerate MII oocytes (no arrow) and degenerate MII oocytes (arrowhead). Bar = 50 μm. **F**) Total number of two-cell embryos obtained from wild-type (n = 6) and *Pum1*$^{-/-}$ (n = 11) females naturally mated with wild-type stud male. $^{*}P < 0.05$.

with their wild-type littermates. This result shows that PUM1 plays a predominant role in regulating primordial folliculogenesis, whereas PUM2 does not have a detectable role in the process.

PUM1 and PUM2 Are Present Throughout Mammalian Female Oogenesis and PUM1 Is Enriched in Oocytes

Primordial folliculogenesis is a multistep process initiated during embryogenesis with PGC proliferation, migration, and meiosis, culminating in the formation of primordial follicles, and shortly after birth in mice (but prior to birth in humans). Our phenotypic analysis indicates that PUM1 should be present during female embryonic ovarian development, yet PUM2 may not. Immunoblot analysis shows that PUM1 and PUM2 proteins are both present in embryonic mouse ovaries (Fig. 3A), implying that PUM2 is functionally redundant during this part of development.

To understand the distribution of PUM1 protein in somatic and germ cells in the mouse embryonic ovaries, we performed immunoblot analysis on SSEA-1 FACS-sorted female somatic and germ cells. This FACS protocol was highly efficient at sorting germ cells from somatic cells, as shown by the immunoblot analysis, which indicated that only the SSEA-1-positive germ cells have VASA protein (a germline marker), but SSEA-1-negative somatic cells do not have measurable VASA protein (Fig. 3B). PUM1 protein is present in both somatic and female germ cells, and is enriched in the latter

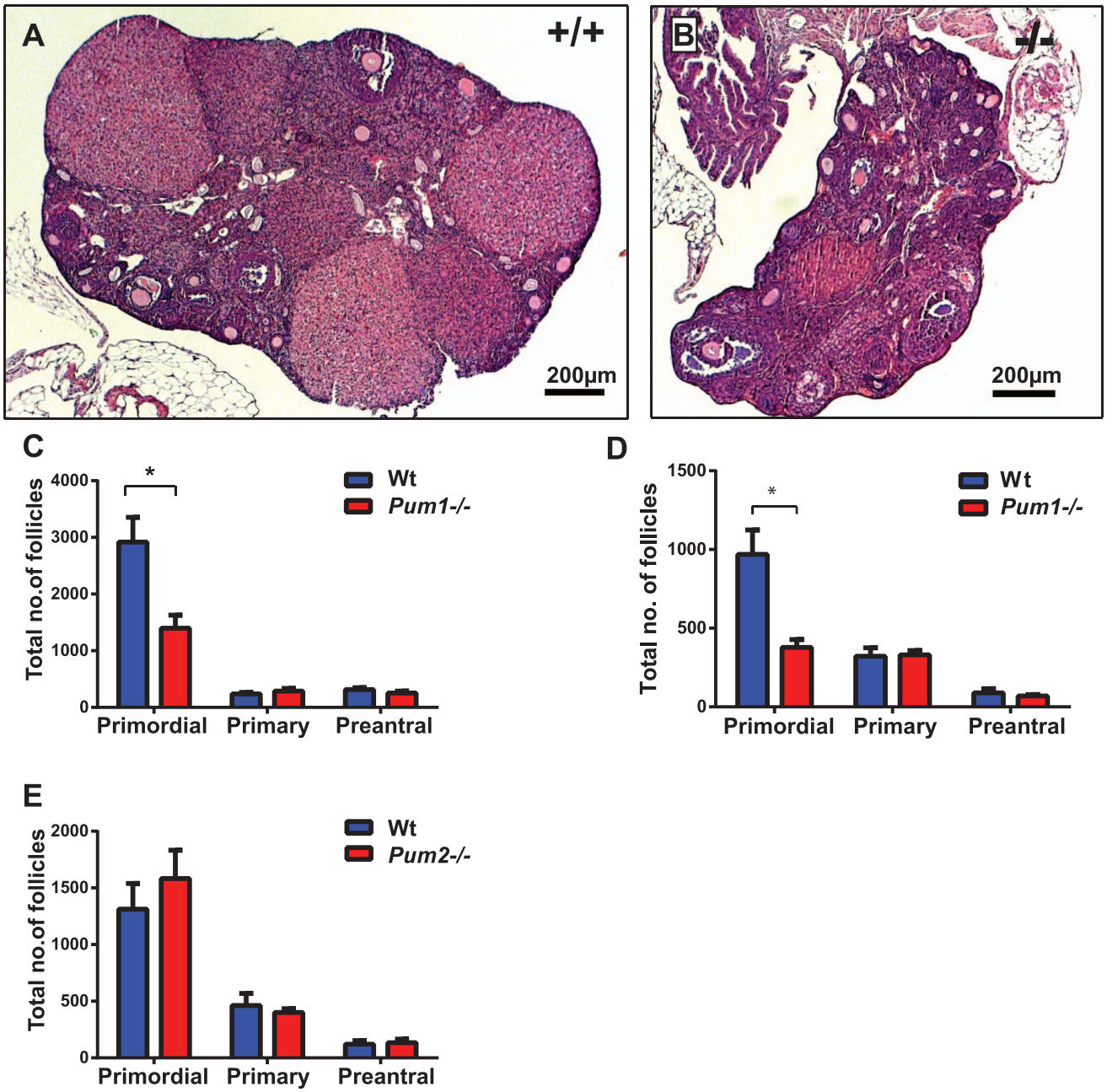

FIG. 2. PUM1 is involved in the establishment of the primordial follicle pool. **A** and **B**) Histological sections of 6-wk-old wild-type (+/+) and *Pum1*$^{-/-}$ (–/–) ovaries, respectively, stained with hematoxylin/eosin. **C**) Total number of primordial, primary, and preantral follicles in 4-wk-old wild-type (n = 6) and *Pum1*$^{-/-}$ (n = 6) littermate ovaries. *$P < 0.05$. **D**) Total number of primordial, primary, and preantral follicles in 6-mo-old wild-type (n = 5) and *Pum1*$^{-/-}$ (n = 6) littermate ovaries. *$P < 0.05$. **E**) Total number of primordial, primary, and preantral follicles in 4-wk-old wild-type (n = 4) and *Pum2*$^{-/-}$ (n = 5) littermate ovaries.

(Fig. 3, B–D). This is consistent with a prior report that PUM1 protein is present in both germ and somatic cells in human fetal and adult ovaries, whereas PUM2 protein was detectable only in germ cells in human and murine ovaries [31].

PUM1 Expression in Oocytes Appears to Be Dispensable for Oogenesis, but Leads to a Meiotic Defect During Primordial Folliculogenesis

We next investigated whether the function of PUM1 in primordial folliculogenesis was germline autonomous. We analyzed the follicle counts of prepubertal *Pum1*$^{-/flox}$*;vasa cre* females. These females have *Pum1* specifically knocked out in their oocytes, mostly from 15.5 dpc onwards, when *vasa cre* is maximally expressed. When compared with *Pum1*$^{+/+}$*;vasa cre* females (control), there was no significant difference found in the number of follicles (Fig. 3C). Thus, PUM1 is unlikely to have a cell-autonomous role in primordial folliculogenesis after 15.5 dpc, which is when *vasa cre* is maximally expressed and PUM1 protein will be depleted in the oocyte. To understand further the timing and distinct role of PUM1 during primordial folliculogenesis, we examined the role of PUM1 protein in the early events during primordial folliculogenesis. After arrival of the PGCs in the female gonadal ridges at 10.5 dpc, PGCs undergo mitosis until the onset of meiosis at 13.5 dpc. Total germ cell counts were performed using immunofluorescence

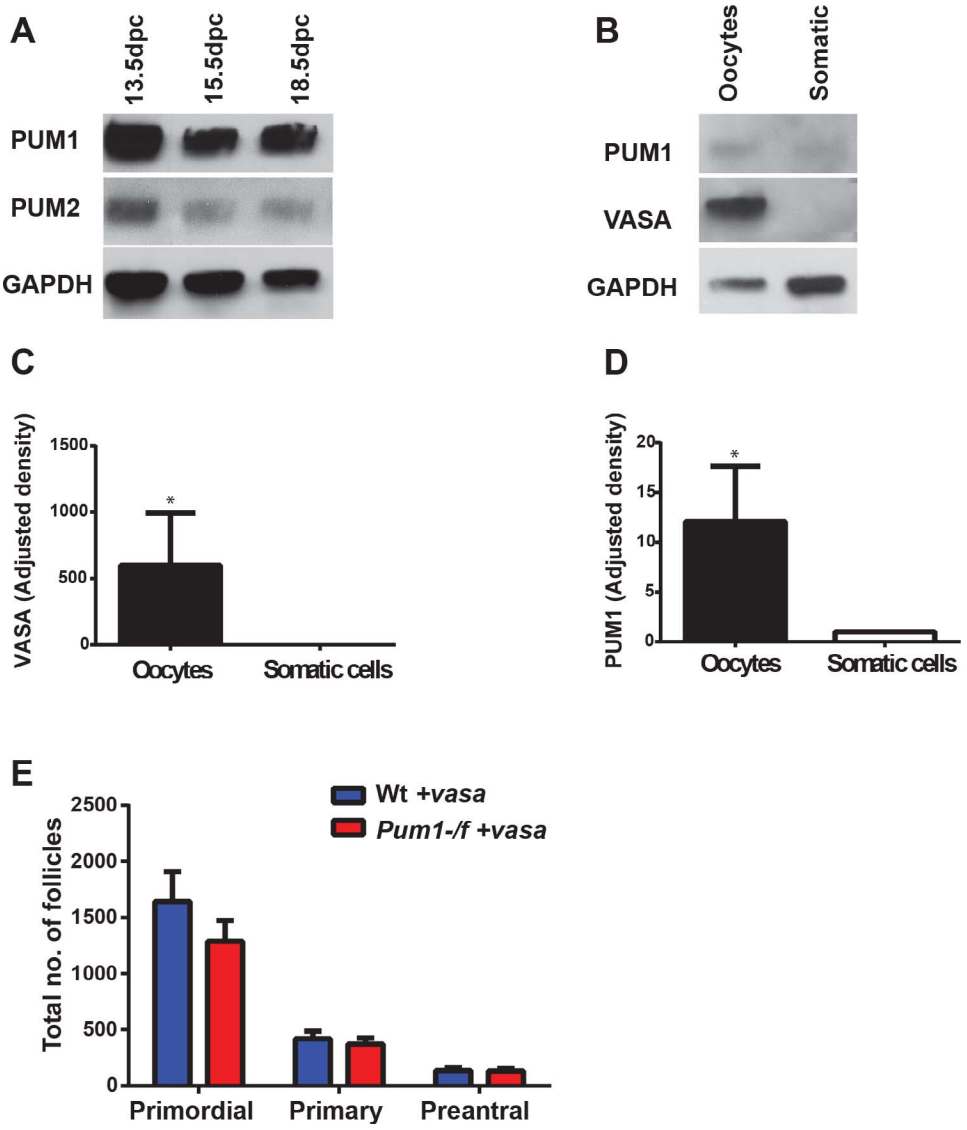

FIG. 3. PUM proteins are present during embryonic female gonadal development, and PUM1 is enriched, but not required, in oocytes for oogenesis. **A**) Immunoblot analysis of whole-ovary lysates from 13.5, 15.5, and 18.5 dpc female embryos (n = 10–15 per biological replicate) with anti-PUM1, anti-PUM2, and anti-GAPDH antibodies. This immunoblot was performed three times with new samples each time. Both PUM proteins are present in all developmental time points. **B**) Immunoblot analysis of lysates from 13.5-dpc FACS-sorted oocytes and somatic cells (n = 20–40) by anti-SSEA1 antibody. Per FACS sort, usually more than 20 000 oocytes and more than 60 000 somatic cells are sorted. Total protein lysate was loaded per experiment. The anti-SSEA-1 FACS-sorting was highly efficient at separating the oocyte and somatic cell compartment. GAPDH was used as loading control. **C** and **D**) The bar graph shows the summary of the densitometric measurements (mean ± SEM) of the immunoblots performed on four different FACS sorts done on four separate days. Paired Student *t*-test was performed. $*P < 0.05$. **E**) Total number of primordial, primary, and preantral follicles in ovaries of 4-wk-old-wild-type mice with *vasa Cre* (Wt + *vasa*); n = 6) and $Pum1^{-/flox}$ with *vasa cre* mice ($Pum1^{-/f}$; + *vasa cre*; n = 6). Error bars represent SD.

with anti-VASA antibody as a marker of germ cells in serial sections of embryonic ovaries of control and $Pum1^{-/-}$ gonads from 13.5-, 15.5-, and 18.5-dpc female embryos (Fig. 4A). The total germ cell count did not differ between the two genotypes at 13.5 dpc, therefore indicating normal mitosis of PGCs (Fig. 4B). From 13.5 dpc onward, continual apoptosis in the female germline occurs to similar extents in the $Pum1^{-/-}$ ovaries compared to control (Fig. 4B).

We then examined whether $Pum1^{-/-}$ mutants are defective in meiosis, which begins at 13.5 dpc and culminates in the majority of oocytes arresting in diplotene/dictyate around PND5. We performed meiotic spreads with double immunostaining of SYCP1 and SYCP3, two SC markers, at 15.5 dpc, 18.5 dpc, and PND1 with control (wild-type siblings) and $Pum1^{-/-}$ ovaries (Fig. 5A). Starting as early as 15.5 dpc, meiosis in the $Pum1^{-/-}$ ovaries was delayed, with a trend toward more leptotene-stage oocytes in $Pum1^{-/-}$ females (Fig. 5B). At 18.5 dpc, there was also a trend toward delay in meiosis, with an increase in pachytene-stage oocytes in $Pum1^{-/-}$ females compared to controls (Fig. 5B). At PND1, there was a significant accumulation of pachytene-stage oocytes (two-fold increase) in $Pum1^{-/-}$ females (Fig. 5B). There was also a concomitant decrease in diplotene-stage oocytes. These results indicate that the slower meiotic progression in the $Pum1^{-/-}$ ovaries might lead to a decrease in oocytes becoming arrested in diplotene/dictyate as the mutant females age. Consistent with this possibility, we found that $Pum1^{-/-}$ ovaries contain significantly fewer primordial follicles at PND1 and −5 (Fig. 6, A–F). The above results show that PUM1 protein is involved in the meiotic process as early as 15.5 dpc.

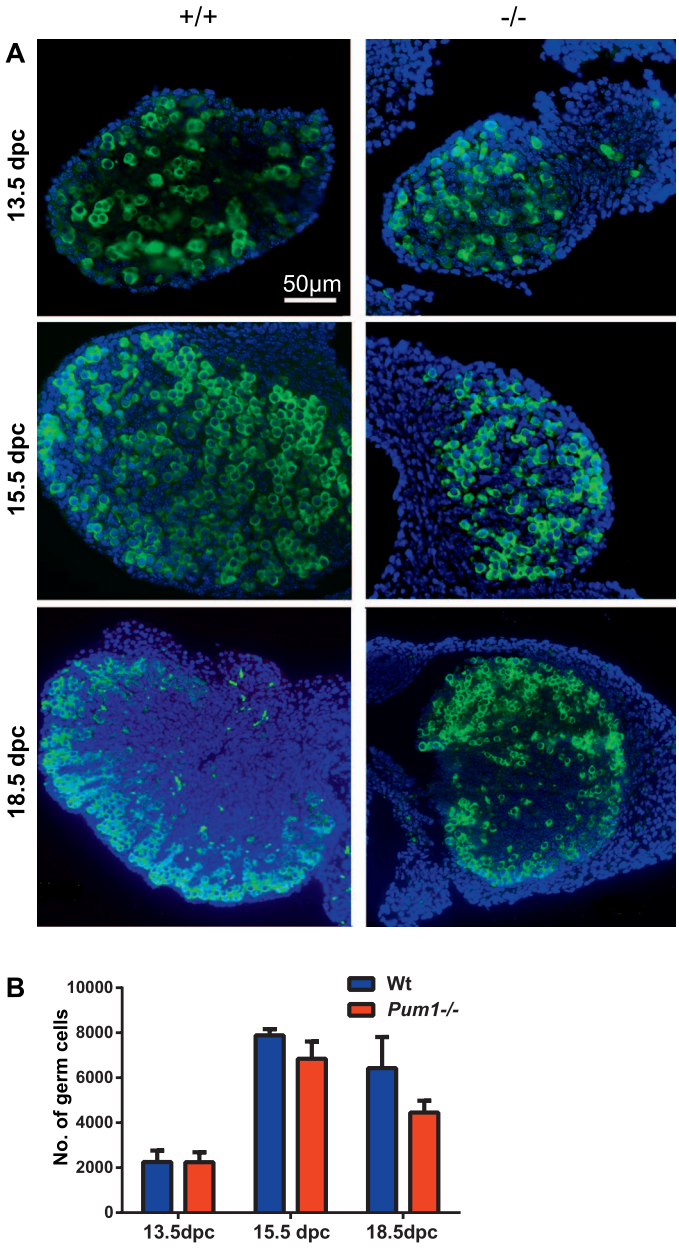

FIG. 4. PUM1 is not required for PGC mitosis or to prevent excessive female germline apoptosis. **A**) Images of immunofluorescence staining with the germ cell marker VASA (green) on sections of 13.5-, 15.5-, and 18.5-dpc ovaries of wild-type (+/+) and *Pum1*$^{-/-}$ (–/–) females. **B**) The total number of germ cells in 13.5-, 15.5-, and 18.5-dpc ovaries from sibling-matched wild-type (n = 5, 3, 3, respectively) and *Pum1*$^{-/-}$ females (n = 5, 4, 3, respectively).

We then examined *Pum1*$^{-/flox}$;*vasa cre* ovaries at PND1 and PND5, in which *Pum1* gene in the oocyte was deleted from 15.5 dpc onward. There was no difference in primordial follicle count at either time point (Fig. 6, C and F). This result was not surprising, given that PUM1 expression in the oocyte appears dispensable for folliculogenesis after 15.5 dpc.

We further investigated the likely defects associated with the meiotically arrested oocytes at PND1. To examine whether there is increased apoptosis in meiotically arrested oocytes, we conducted combined TUNEL and VASA immunostaining. We calculated the percentage of double-positive TUNEL/VASA cells, which reflect the apoptotic germ cells. At PND1, in wild-type ovaries (n = 4), 1.55 ± 0.63% (SEM) of the total germ cells were apoptotic, and in *Pum1*$^{-/-}$ ovaries (n = 4), 1.30 ± 0.41% (SEM) of the germ cell population were apoptotic. Therefore, we concluded that the abnormally meiotically arrested oocytes did not lead to an increase in apoptosis at PND1.

To address whether meiotic arrest of the oocytes leads to a failure of germline cyst breakdown, we examined wild-type and *Pum1*$^{-/-}$ for the presence of oocytes remaining in germline cyst during embryonic to postnatal life, and therefore, if differences were found, this would indicate failure of cyst formation and/or cyst breakdown (Fig. 6G). There was no significant difference in the number of oocytes remaining in germline cyst in wild-type and *Pum1*$^{-/-}$ females from 15.5 dpc to PND5. Therefore, PUM1 is unlikely to be involved in the process of germline cyst breakdown. Hence, our findings show that PUM1 plays a role in regulating meiosis by facilitating the transition from pachytene to diplotene, and ultimately dicytate, transition, although subtle changes occur as early as 15.5 dpc. In PND1 *Pum1*$^{-/-}$ ovaries, the oocytes delayed/arrested at the pachytene stage are unable to form primordial follicles, as they have not progressed to the diplotene stage; arrest in dicytate is a prerequisite for primordial follicle formation [30]. These pachytene-arrested oocytes do not contribute to the primordial follicle pool, and are lost prior to PND5. The mechanism for this loss is unlikely due to apoptosis or failure of germline cyst breakdown.

PUM1 Down-Regulates the SYCP1 Protein from the SC During Late Meiotic Prophase I

Meiosis requires the normal assembly and disassembly of the SCs that contain SYCP1, SYCP2, and SYCP3 proteins. The SC is composed of lateral elements (SYCP2 and SYCP3) that are connected by transverse filaments to the central element containing SYCP1 [32]. Transgenic knockout mouse studies show that any disturbance in these proteins can lead to abnormal meiosis and failure of germline development [33–35]. Disassembly/degradation of SYCP1 protein, a major constituent of the SC, is required for oocytes to proceed to the diplotene stage [30, 36]. Given that PUM1 has a role during the pachytene-to-diplotene transition, we considered the possibility that PUM1 is involved in the regulation of the degradation/disassembly of SYCP1 protein in the SC. To test this possibility, we investigated whether the SYCP1 protein dynamics during meiosis was affected by lack of PUM1. We scored the number of oocytes that had linear (intact), punctate, or absent SYCP1 staining at PND1 in both wild-type and *Pum1*$^{-/-}$ ovaries (Fig. 7). Normally, at PND1, the majority of oocytes is expected to be in the diplotene/dicytate stage, and therefore should no longer have linear SYCP1 staining. However, *Pum1*$^{-/-}$ ovaries at PND1 had a significant increase in the percentage of oocytes with linear SYCP1 staining (38 ± 4.6% SEM) compared to controls (21 ± 4.5% SEM; Fig. 7). This finding indicates that there is a significant proportion of *Pum1*$^{-/-}$ oocytes that fail to degrade/disassemble SYCP1 protein in the SC and cannot transition from pachytene to diplotene. This result demonstrates that PUM1 protein is involved in the dynamics of SYCP1 protein in the SC during late meiotic prophase I, possibly by regulating SYCP1 protein expression.

DISCUSSION

Although PUF proteins are well known for germline development in lower animals, little is known about their function in mammalian oogenesis. In *Drosophila*, *Pum* mutants

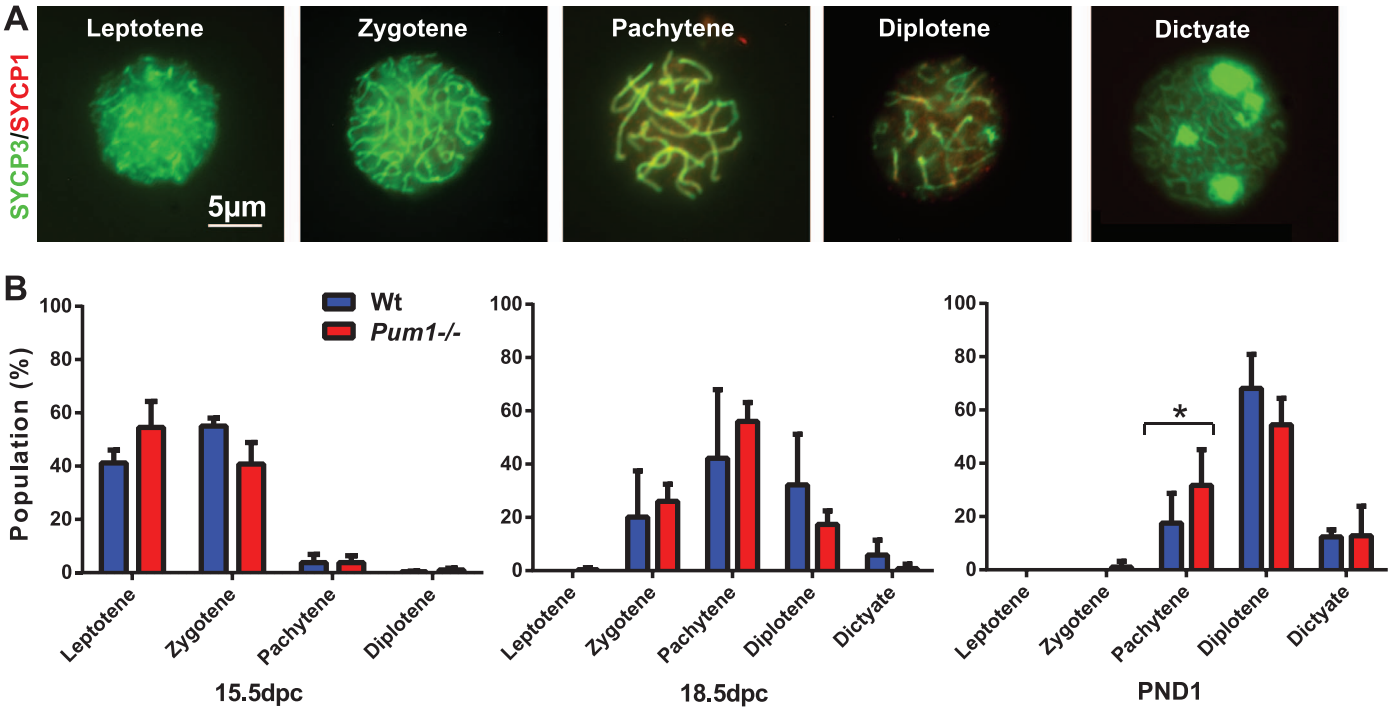

FIG. 5. PUM1 plays a role during female meiotic progression. **A**) Representative images of immunofluorescence staining of meiotic spreads with SYCP1 (red) and SYCP3 (green). Examples of each stage during mouse meiotic prophase I are shown: during the leptotene stage, the chromatin starts to condense into fine threads; at the zygotene stage, distinct chromosomes with tripartite SCs at homologous pairing sites are seen; at the pachytene stage, maximal shortening of the homologous paired chromosomes occurs; at the diplotene stage, homologous paired chromosomes start to separate; at the dictyate stage, decondensation of chromatin and formation of between two and four nucleoli occur. **B**) Distribution of meiotic prophase I stages in 15.5-dpc (n = 5), 18.5-dpc (n = 5), and PND1 (n = 4) ovaries in sibling-matched wild-type and *Pum1*$^{-/-}$ females. A paired Student *t*-test was used for this analysis. *$P < 0.05$.

show abnormalities throughout female germline development, starting with PGC defects and throughout oogenesis [10, 15, 16]. Here, we report a conserved role of a PUF protein, PUM1, in establishing the mammalian female germline and during folliculogenesis. We have shown that PUM1 is important in establishing the primordial follicle pool, for the pachytene-to-diplotene transition during meiosis, and, eventually, to supply a normal number of viable oocytes for mammalian female reproductive competence. Lack of PUM1 in mice leads to a diminished ovarian reserve and likely premature ovarian failure and infertility. Interestingly, a marker for the ovarian reserve, AMH, was not significantly decreased in the *Pum1*$^{-/-}$ females. One explanation is that the mice used for this analysis may have been too young to show a significant decrease. An alternative explanation is the limitation of AMH as a surrogate marker for primordial follicle reserve. AMH reflects the number of primary and preantral follicles, and neither was significantly changed in the *Pum1*$^{-/-}$ females compared to their wild-type siblings.

We cannot rule out other minor functions of PUM1 during oogenesis and folliculogenesis that might also contribute to female infertility, such as a decrease in viability of the oocytes or their surrounding granulosa cells during oocyte maturation. The normal fertility of the oocyte-specific conditional *Pum1* knockout suggests that the effect of PUM1 on the oocyte may stem from its function in surrounding somatic cells. This concept is consistent with a study by Chen et al. [37], showing that somatic signals can regulate maternal transcripts in the oocyte that are important for embryo development.

Although the two mouse PUF proteins, PUM1 and PUM2, share a high homology (91% identity and 97% similarity) [38] and expression pattern, our data show that PUM2 cannot compensate for the loss of PUM1 function during primordial folliculogenesis. A possible explanation is that there is a larger cohort of mRNA targets that are regulated by PUM1. For example, using RNA binding protein immunoprecipitation in HeLa cells, 1766 PUM1 and 751 PUM2 target genes have been identified [39]; 88% of PUM2 target genes are also found to be PUM1 target genes. Therefore, there may be redundancy in the function of the PUM proteins, such that some of the function of PUM may be partially compensated by PUM2 in its absence, but lack of PUM2 can be fully compensated by the presence of PUM1 [23, 39, 40].

Our findings contribute significantly to an understanding of primordial folliculogenesis, as prior studies have focused on transcriptional regulators in this process, and much less is known about the role of translational regulators. The best-characterized translational regulators in the mammalian female reproductive system are CPEB and DAZ [12, 13]. In comparison to *Dazl*$^{-/-}$ and *Cpeb*$^{-/-}$ females that are infertile, the *Pum1*$^{-/-}$ females are subfertile. One possible explanation is that these transgenic lines were backcrossed to B6 background, and the *Pum1*$^{-/-}$ females in our study are from a mixed background (129/B6). The severity of a transgenic phenotype usually increases as a strain becomes pure bred (more homozygous for the genome). Interestingly, both CPEB and DAZ are germ cell specific. In contrast, our study shows that the PUF proteins are present throughout mouse ovarian development, and that PUM1 is present in both somatic and germline cells. Based on our findings, a role for PUM1 could be cell autonomous early in oogenesis, prior to 15.5 dpc, as it is enriched in the germline during early oogenesis, and subtle abnormalities in meiosis occur as early as 15.5 dpc. Therefore, the lack of a phenotype in the oocyte-specific conditional *Pum1* knockout females could reflect that PUM1 is knocked out too late in development to have a significant phenotype. Future

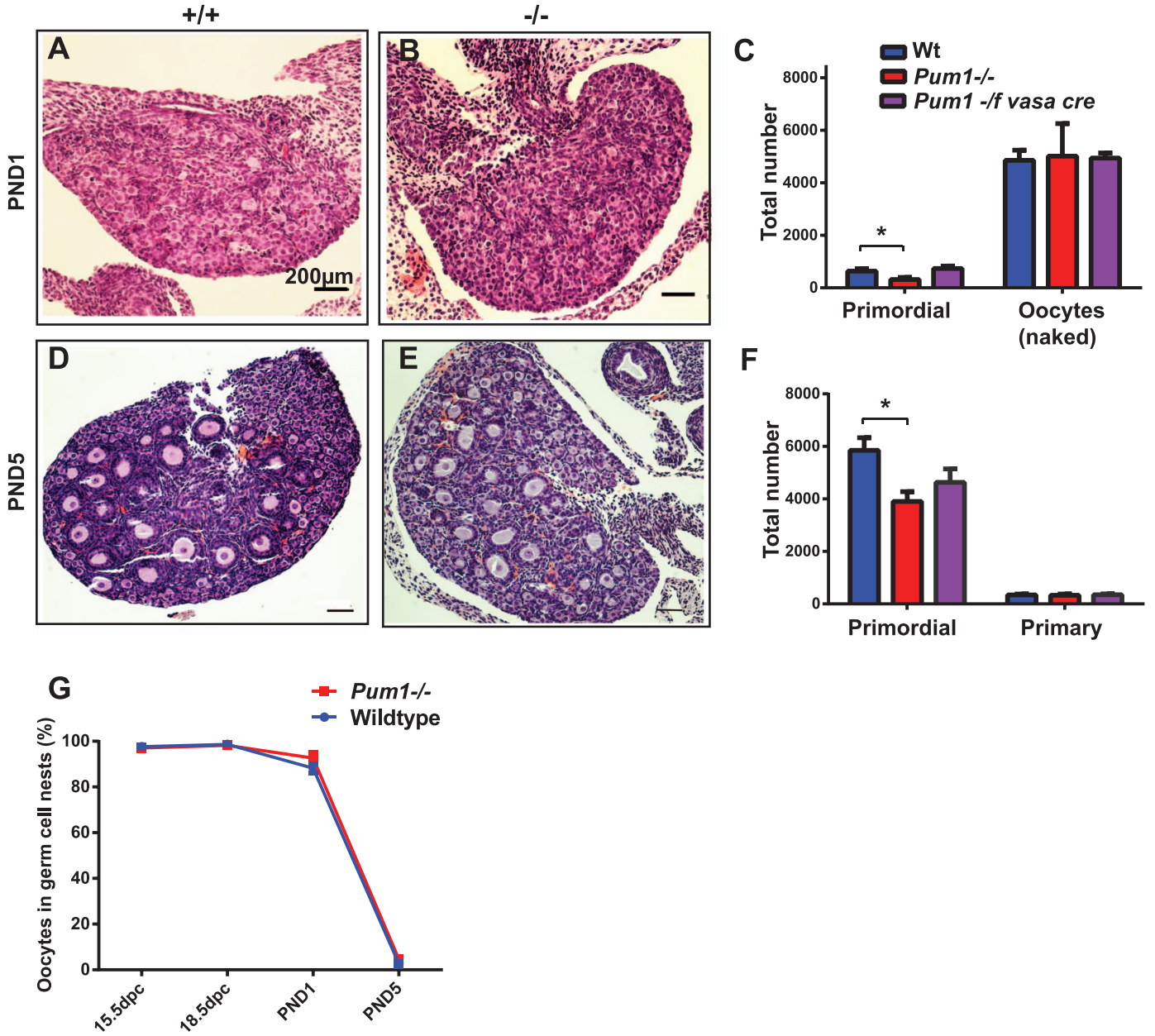

FIG. 6. Aberrant meiosis in *Pum1*$^{-/-}$ females leads to reduction in primordial follicle formation postnatally. **A** and **B**) Histological sections of PND1 wild-type (+/+) and *Pum1*$^{-/-}$ (–/–) ovaries, respectively, stained with hematoxylin/eosin. **C**) Total number of primordial follicles and oocytes without surrounding pregranulosa cells (naked oocytes) in wild-type (n = 5) and *Pum1*$^{-/-}$ (n = 5) littermate ovaries, and *Pum1*$^{-/flox}$*;vasa cre* (n = 5) PND1 ovaries. *$P < 0.05$. **D** and **E**) Histological sections of PND5 wild-type (+/+) and *Pum1*$^{-/-}$ (–/–) ovaries, respectively, stained with hematoxylin/eosin. *$P < 0.05$. **F**) Total number of primordial and primary follicles in wild-type (n = 8) and *Pum1*$^{-/-}$ (n = 6) littermate ovaries, and *Pum1*$^{-/flox}$*;vasa cre* (n = 6) PND5 ovaries. *$P < 0.05$. **G**) Percentage of oocytes at 15.5 dpc, 18.5 dpc, PND1, and PND5 present in germ cell nests in wild-type (n = 3, 3, 5, 8, respectively) and *Pum1*$^{-/-}$ (n = 4, 3, 5, 6, respectively) ovaries. At each developmental time point, Mann-Whitney test was performed, and no statistically significant difference was found.

studies to generate an oocyte conditional *Pum1* knockout with deletion occurring earlier in development (using *Blimp1* or *Oct-4* cre) will clarify whether the function of PUM1 is cell autonomous. Another intriguing explanation for our findings is that PUM1 in the surrounding somatic cells indirectly regulates oocyte development, yet germ cell-specific PUM1 has either no role or a nonessential role for oocyte meiosis.

Our current and previous studies together indicate that *Pum1* has important functions in both male and female gametogenesis [23]. This is similar to posttranscriptional regulators, *Cpeb* and *Dazla*, the knockout mice of which have both male and female germline defects. In both *Cpeb*$^{-/-}$ females [13] and *Dazla*$^{-/-}$ females [12], oocytes are present at 15.5 dpc. However, by 18.5 dpc, only a few oocytes remain, and only vestigial ovaries persist to adulthood. In these studies, CPEB was shown to be important in translational activation of both SYCP1 and SYCP3 proteins [13]. In *Pum1*$^{-/-}$ females, a delay in meiosis from 15.5 dpc was observed, and germ cell defect is more pronounced later in development at PND1. In addition, PUM1 is also involved in the regulation of SYCP1 in the SC. To assess whether SYCP1 is a direct target of PUM1, we examined its mRNA sequence. No PRE was found in the

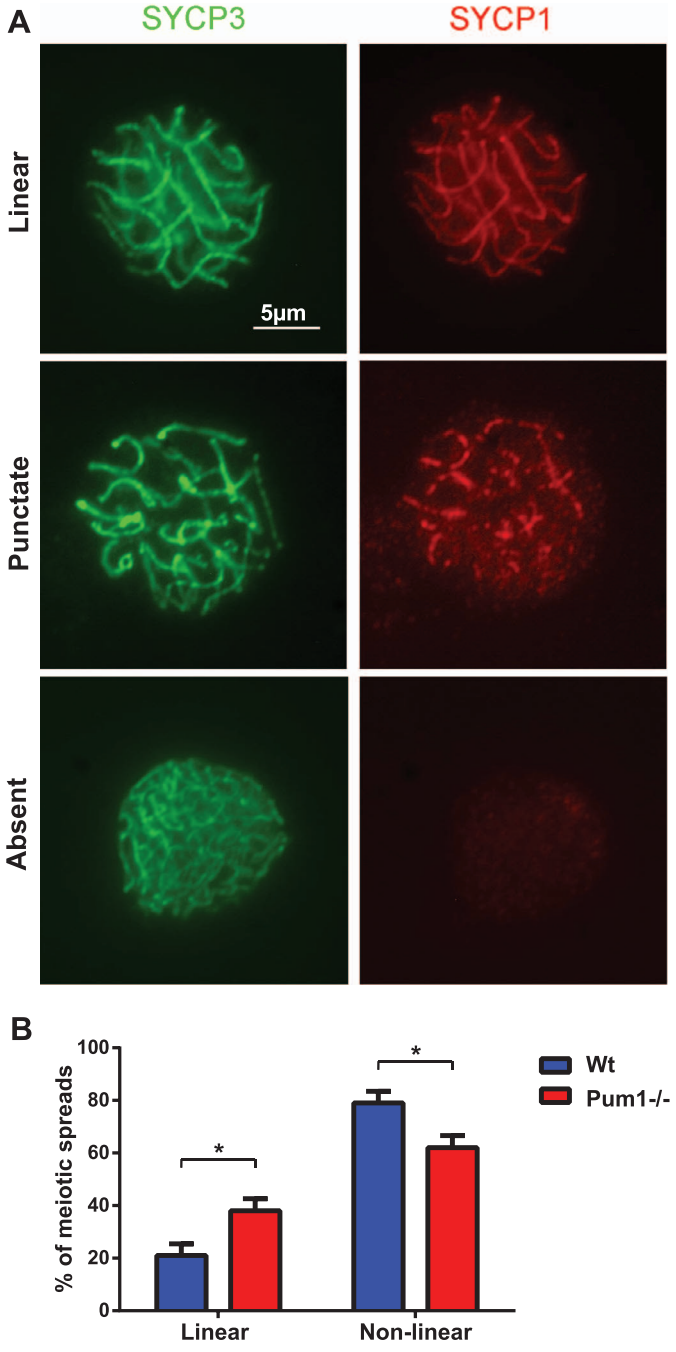

FIG. 7. PUM1 down-regulates SYCP1 during late meiotic prophase I in mouse oocytes. **A**) Representative images of immunofluorescence staining with SYCP1 (red) and SYCP3 (green); examples of linear, punctate, and absent SYCP1 protein are shown. **B**) Total number of meiotic spreads with linear and nonlinear (sum of punctate and absent SYCP1 spreads) SYCP1 immunostaining in PND1 wild-type and *Pum1*$^{-/-}$ littermate ovaries. *$P <$ 0.05.

mRNA; therefore, SYCP1 is unlikely to be a direct PUM1 target. PUM1 likely regulates the expression and localization of SYCP1 via its impact on other relevant cellular mechanisms. Interestingly, in *Xenopus* oocytes, CPEB and PUM1 form a protein complex that is involved in oocyte maturation [41]. Therefore, it is possible that PUM1 and CPEB form a protein complex during meiosis; lack of PUM1 would destabilize/inactivate CPEB function, impacting on the expression of SYCP1, SYCP3, and other molecules, leading to a meiotic defect. Furthermore, *Cpeb* mRNA has a predicted PRE in its 3′ UTR; therefore, it could be translationally regulated by the PUM proteins.

In summary, the translational regulators, *Pum1*, *Cpeb*, and *Dazla*, all play an important role during female meiotic prophase I by regulating the SYCP proteins in SCs. It is intriguing to hypothesize that the function of CPEB is to activate translation of SYCP1 and SYCP3 proteins early in meiotic prophase I in oocytes, which are required to establish a stable SC in pachytene. After pachytene formation, SYCP1 is required to be degraded/disassembled from the SC, and PUM1 could function to fine tune this meiotic progression through translational regulation and/or direct interaction with CPEB, resulting in repression of translation of SYCP1 protein and leading, ultimately, to the successful transition to diplotene. Finally, our study demonstrates that PUM1 has a conserved role in female mammalian germline development.

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