

# Stem-loop binding protein localization, expression patterns, and regulation of histone mRNA in wild-type and transgenic mouse oocytes

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## Abstract

Histone proteins are essential for the cell cycle and are massively accumulated during S-phase of cell division in somatic cells and during early oogenesis. Histone mRNA differs from the majority of metazoan mRNA by terminating with a highly conserved stem-loop in its 3'-untranslated region. The stem-loop binding protein (SLBP) is the only known protein specifically binding to histone mRNA stem-loops and is believed to be responsible for the regulation of histone translation. SLBP is thought to also control mRNA levels through stabilization and post-transcriptional processing. We studied the localization and expression pattern of SLBP in ovaries of wild-type and transgenic mice using immunohistochemistry and immunofluorescence techniques. Our data show that SLBP translocates from the cytoplasm of developing oocytes to the nucleus shortly after release from meiotic arrest. SLBP subsequently localizes back out into the cytoplasm as the oocyte continues its maturation. Our transgenic mice show a significant decrease in SLBP expression levels. The results present encouraging evidence for the role and localization of SLBP during oogenesis.

## Keywords

**Histone:** tetrameric proteins responsible for DNA packaging into condensed chromatin and gene regulation; **Stem-loop:** a DNA or RNA structure generally involving a single strand folding back onto and binding with itself to form a loop; **Stem-loop binding protein (SLBP):** a protein specifically binding to the stem-loop of histone mRNA; **Cell cycle:** the cycle which all somatic and germ cells undergo at some time in their lifespan. Mitosis and meiosis are both parts of the cell cycle; **Immunofluorescence:** using an antibody attached to a fluorescent molecule to specifically bind to a target to verify its location and quantify its expression in a cell.

## Introduction

The eukaryotic cell cycle is the result of an accumulation of complex mechanisms employed by the growing cell. The collaboration between these numerous and assorted pathways is crucial to the success of the cell in completing its designated roles. Histone proteins bind to eukaryotic DNA during nearly all phases of the cell cycle and play key roles in the formation of chromatin as a form of DNA packaging, chromatin stability and compaction, as well as gene regulation (Zhao et al., 2004).

During S-phase of the cell cycle, the concentration of histone proteins necessary for DNA condensation is dramatically increased. The abundance of histone proteins is critical to ensure proper DNA packaging of duplicated genetic material in daughter cells (Schumperli, 1988; Marzluff et al., 2002). As such, the transcription of histone mRNA and translation of histone proteins are tightly coupled to cellular proliferation and are amongst the mechanisms essential to cellular division (Whitfield et al., 2000; Zheng et al. 2003).

Whilst increased expression of histone mRNA is important in somatic cells, it is vital in oogenesis, which occurs in several steps. Multiplying primordial germ cells in the embryo and fetus give rise to millions of primary oocytes, which enter meiotic arrest at the first meiotic prophase. At puberty, the arrest is lifted from a small number of primary oocytes, which then continue development and meiotic division (Vanderhyden, 2002). The multiple stages of meiosis and cell division in early oocyte maturation require rapid production of considerable amounts of essential histone proteins. As such, mediation of histone mRNA translation is crucial to ensure proper gene activity.

Translation regulation in metazoan cells involves two classes of cellular mRNA that are differentiated by their 3'-untranslated region. The majority of metazoan protein mRNAs are polyadenylated at their 3' ends and are regulated by translation initiation factors such as eIF-4E and eIF-4G during protein synthesis. Eukaryotic histone protein mRNAs are not polyadenylated; instead, a highly conserved stem-loop is found at the 3'-untranslated region. The stem-loop is hypothesized to be responsible for the mediation of histone mRNA levels at the posttranscriptional stage through adjustments to mRNA processing and stability. The stem-loop structure is therefore, to a certain degree, functionally homologous to poly (A) tails of other metazoan mRNAs (Muller et al., 1997; Sanchez et al., 2002).

The stem-loop binding protein (SLBP) is a thirty-one kilodalton RNA-binding protein responsible for binding the stem-loop of the histone mRNA and promoting its translation (Muller et al., 1997; Sanchez et al., 2002). The specificity of SLBP binding to the stem-loop of histone mRNAs allows us to monitor the localization and expression level of SLBP as a measure of histone mRNA gene expression. Concurrently, the role of SLBP in translational regulation can be assessed.

Proper SLBP and histone mRNA and protein expression is critical to oogenesis and oocyte survival (Song et al., 2005). During early embryogenesis, control of gene expression is

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dependant on mRNAs that have already been synthesized and stored in the oocyte (Sanchez et al., 2002). Histone mRNAs are therefore accumulated in early oogenesis and translation is activated during oocyte maturation. Thus, as an oocyte moves past meiotic arrest, interacts, and develops alongside its follicle, histone mRNAs should localize from the nucleus to the cytoplasm where translation can take place. The role of SLBP suggests possible localization to the nucleus to bind histone mRNA and subsequent relocation to the cytoplasm. Given that SLBP is the only known protein specifically binding histone mRNA stem-loop, observation of its localization and expressions could prove invaluable to the understanding of replication-dependent histone transcription and translation.

## Materials and Methods

The localization and expression of SLBP and underlying processes of mediation of histone mRNA by SLBP were observed using immunofluorescence and immunohistochemistry techniques on sectioned mouse ovaries aged five days, ten days, fifteen days, and twenty days. Immunofluorescence is ideally used to localize proteins within cells, and sections of ovarian tissue serve as excellent templates for antibodies binding specifically to SLBP. Furthermore, the levels of fluorescence can be quantified to reveal the level of expression of a particular protein. All results were controlled for by replacing the primary antibody with PBS (Phosphate Buffered Saline), while the same materials and procedures were applied to the remainder of the experiment.

### Transgenic mice

A transgenic mouse line was created in Dr. Hugh Clarke's laboratory to study the effects of SLBP loss. A 700 base pair SLBP dsRNA cassette was inserted into mouse DNA next to the *Zona-pellucida-3* gene promoter. The promoter is responsible for gene expression during oocyte growth (Arnold and Clarke, unpublished data). Therefore, the dsRNA is only transcribed during oocyte growth. When it is expressed, the dsRNA specifically inhibits SLBP mRNA by RNA interference, resulting in SLBP knockdown during oogenesis after the first meiotic arrest.

### Histology

Ovaries dissected from CD-1 wild-type mice and SLBP-dsRNA-knockout transgenic mice were fixed overnight in 4% paraformaldehyde at 4°C with agitation. We then dehydrated the ovaries and removed the fixative by washing the tissues in a graded ethanol and xylene series. Tissues were then sent to the McGill Cancer Centre to be embedded in paraffin blocks and stored at -20°C. We cut 5mm thick sections of tissue from the paraffin block using a microtome and subsequently mounted them on slides, dried them overnight, and adhered them to the slides at 60°C for 20 minutes the next day. The slides can be stored at either 4°C or room temperature. Rehydration of the sectioned tissues through the ethanol and xylene series was followed by 20 minutes of antigen recovery using 0.1% sodium citrate antigen recovery buffer at 80°C to 90°C.

### Immunofluorescence and Immunohistochemistry

Once antigen recovery has been performed, we treated the slides for 30 minutes with a blocking solution composed of 5% goat serum, 5% bovine serum albumin, and 0.1% PBST

(Phosphate Buffered Saline and 0.1% Tween). We then incubated the tissues with a 1:100 blocking buffer dilution of anti-SLBP primary antibody, provided by the Clarke laboratory, overnight at 4°C with agitation. The slides were then washed with blocking buffer and incubated with a 1:100 PBST dilution of horseradish peroxidase-conjugated secondary antibody and YOYO-1 DNA stain for 60 minutes at room temperature. After several PBS washes, the sections were treated with tyramide signal amplification working solution. The slides were then washed and mounted with Mowiol. Storage was at 4°C and measurements were taken with a confocal microscope.

### Image analysis

Image analysis and fluorescence levels were determined using Image J and the colour histogram plug-in. When possible, 20 pixel by 20 pixel selections were made for analysis. Graphs created by the program separate red, green, and blue channels. The program also calculates the mean level of luminescence intensity for each. SLBP fluorescence is shown in the red channel and is of primary interest. DNA fluorescence is shown in the green channel.

### Results

Five-day-old ovaries generally had a high number of both dormant and developing primordial and primary follicles. Ten-day-old ovaries retained a considerable amount of primordial follicles; however, a small number of primordial follicles had developed into the primary and secondary stages. Both fifteen and twenty-day-old ovaries had large amounts of secondary follicles and tertiary follicles.

The granulosa cells of primordial follicles have a flat structure. As these primordial follicles develop into primary follicles, the granulosa cells assume a cuboidal configuration. The acquisition of a second granulosa layer marks the development of the follicle into the secondary stage. Maturation continues as the follicle continues to grow, gaining ever more layers of granulosa cells. Tertiary follicles can be identified by the formation of a fluid filled space adjacent to the oocyte.

Immunofluorescence results showed SLBP presence in the cytoplasm of primary oocytes surrounded by flat-structured granulosa cells of primary follicles (**Figure 1a**). No significant amount of fluorescence was found in the nucleus of these arrested oocytes (**Figure 1b**). However, in oocytes that have initiated growth and moved past the first meiotic arrest, fluorescence of the antibody is confined to the nucleus, with significantly less staining in the cytoplasm (**Figure 1c**). Indeed, image analysis shows that fluorescence in the nucleus is double the intensity of staining in the cytoplasm (**Figure 1d**). The low levels of staining in the cytoplasm indicate that oocytes in primary follicles have high concentrations of SLBP in their nucleus, and relatively low concentrations elsewhere. Furthermore, it is important to note that, in CD-1 wild-type mice, staining is two times stronger in primary follicles than in primordial follicles (**Figure 6**).

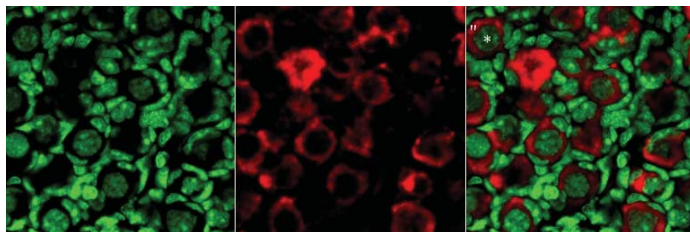
Slides containing transitional follicles caught between primordial and primary stages or primary and secondary stages appear to have equal staining in both the nucleus and the cytoplasm (**Figure 2a**; **Figure 2b**). However, as the follicle acquires its second layer of granulosa cells, fluorescence in

the cytoplasm of the oocyte is more than double the intensity of the nuclear staining, indicating relocation of SLBP from the nucleus back into the cytosol (**Figure 2a**; **Figure 2c**).

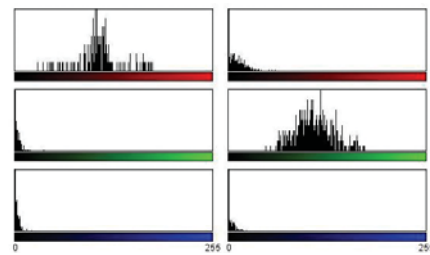
In later stages of oogenesis and folliculogenesis, as seen in the ten-day-old, fifteen-day-old, and twenty-day-old ovary sections, SLBP staining is limited to the cytoplasm of the oocytes, with no staining in the nucleus (**Figure 3**).

SLBP-knockout mice had strong SLBP staining in the cytoplasm of non-developing oocytes enclosed in primordial follicles (**Figure 4**). However, only extremely low levels of fluorescence were detected in developing oocytes enclosed in primary, secondary, and tertiary follicles (**Figure 5**). Wild-type oocytes exhibited more than five times stronger fluorescence than transgenic oocytes (**Figure 5b**).

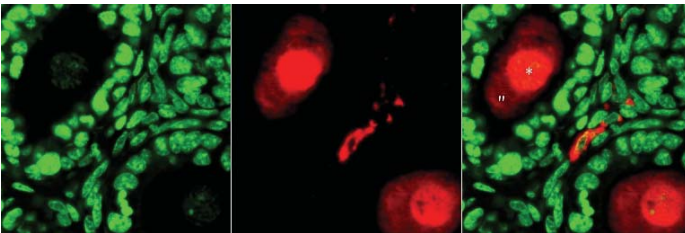
**Figure 1.** Primordial and primary follicles of CD-1 wild-type mouse ovaries. Left: YOYO-1 DNA staining in green. Middle: immunofluorescence SLBP staining in red. Right: combination overlap. (\* marks the oocyte nucleus; " marks the cytoplasm)



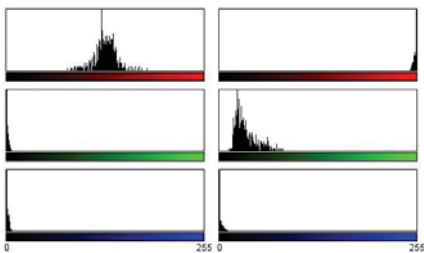
**Figure 1a.** Primordial follicles in 5-day-old CD-1 wild-type mouse ovaries.



**Figure 1b.** SLBP fluorescence RGB (colour intensity) histogram of primordial follicle and oocyte seen in **Figure 1a**. Left column: cytoplasm. Right column: nucleus.



**Figure 1c.** Primary follicles in 5-day-old CD-1 wild-type mouse ovaries.



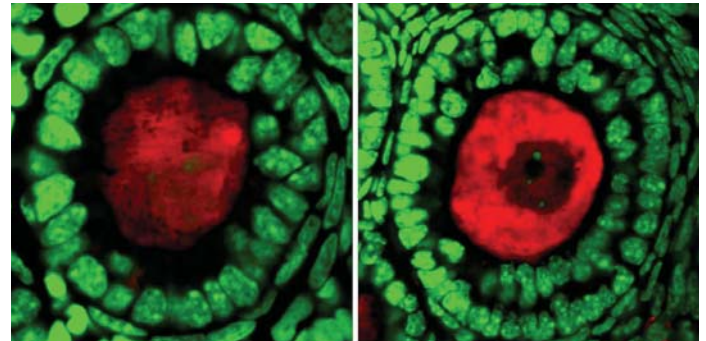
**Figure 1d.** SLBP fluorescence RGB (colour intensity) histograms of oocyte seen in **Figure 1c**. Left column: cytoplasm marked by " Right column: nucleus marked by \*

## Discussion

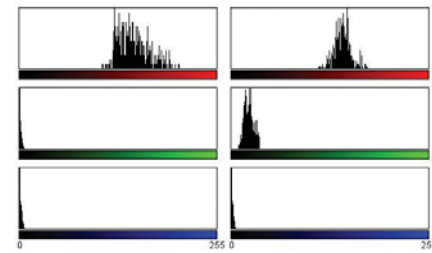
Our data from CD-1 wild-type mice suggests that in the early stages of oogenesis and folliculogenesis, SLBP is translated in and localized in moderate amounts to the cytoplasm of the primary oocyte (**Figure 1**). Indeed, oocytes in primordial follicles have no SLBP staining the nucleus (**Figure 1b**), indicating

that the protein is first stored in the cytosol after its initial translation. The development of the oocyte is then interrupted. However, when the oocyte is released from meiotic arrest, SLBP production is dramatically increased. In fact, primary follicle oocytes fluoresce with double the intensity of primordial follicle oocytes (**Figure 6**). As the primordial follicle surrounding the oocyte begins to develop into its primary stage, the increase in SLBP production is accompanied by SLBP localization from the cytoplasm of the oocyte to the nucleus, where it is sequestered until metaphase II (**Figure 1c**). At this phase, the antibody staining in the nucleus of the oocyte is twice as strong as the fluorescence of the cytoplasm (**Figure 1d**). Therefore, the data seem to indicate that SLBP expression is greatly enhanced after the commencement of oocyte development and reaches its peak at the primary follicle stage.

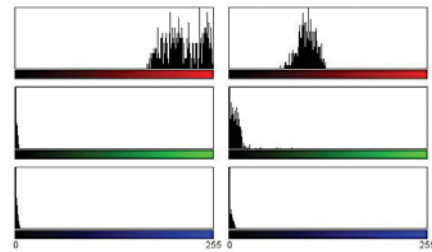
**Figure 2.** Primary follicles in transition to secondary follicle stage in CD-1 wild-type mouse ovaries. YOYO-1 DNA staining (green) and SLBP immunofluorescence staining (red) merged overlap. (\* marks the oocyte nucleus; " marks the cytoplasm)



**Figure 2a.** Follicles transitioning from primary to secondary stage. Left: oocyte at beginning of transition from primary to secondary stage. Right: oocyte at end of transition from primary to secondary stage.



**Figure 2b.** SLBP fluorescence RGB (colour intensity) histograms of oocyte on left side of **Figure 2a**. Left column: cytoplasm. Right column: nucleus.



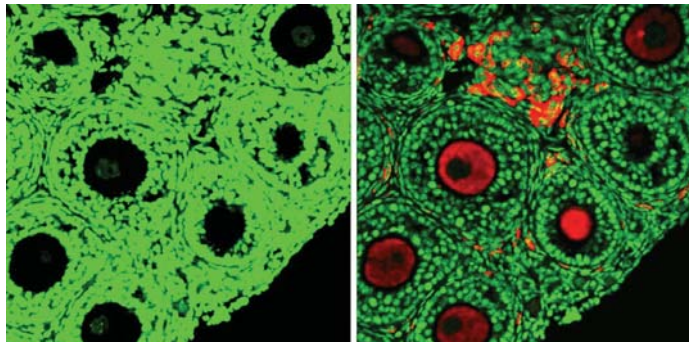
**Figure 2c.** SLBP fluorescence RGB (colour intensity) histograms of oocyte on right side of **Figure 2a**. Left column: cytoplasm. Right column: nucleus.

The movement of SLBP across the nuclear membrane is thus directly correlated with the growth of the oocyte and development of the follicle. The localization of SLBP into the nucleus is highly significant, as it suggests that histone mRNA stored in the nucleus of the oocyte requires binding with SLBP to exit the nucleus and commence translation.

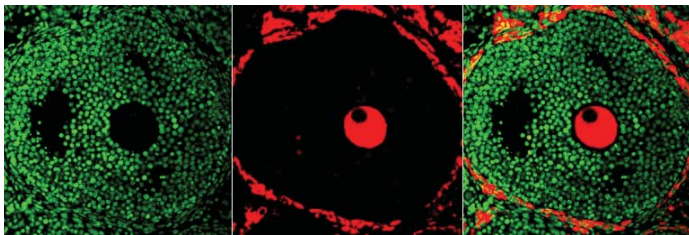
As the oocyte continues to develop past metaphase II of its first meiotic division, the follicle containing it moves past primary stage and becomes a secondary follicle (Allard et al.,

2002). As the transition takes place, the SLBP concentration in the cytoplasm becomes as high as the concentration in the nucleus and continues to increase until it is more than double the amount (**Figure 2b**; **Figure 2c**). Molecularly, it is expected that the translocated SLBP has bound specifically to the stem-loop of the histone mRNA stored in the nucleus. SLBP then appears to return to the cytoplasm as the nucleus is completely emptied of the protein (**Figure 3a**; **Figure 2**). As such, we can hypothesize that the translation of histone mRNA within the maturing oocyte occurs at the secondary follicle stage.

**Figure 3.** Secondary and tertiary follicles of CD-1 mouse ovaries. YOYO-1 DNA staining in green; immunofluorescence SLBP staining in red.



**Figure 3a.** Secondary follicles in 10-day-old CD-1 wild-type mouse ovaries. Left: YOYO-1 DNA staining. Right: merged overlap of YOYO-1 with immunofluorescence SLBP staining. Note that in oocytes appearing to have uniform staining, the nucleus is not included in the section, and only cytoplasmic staining is visible.

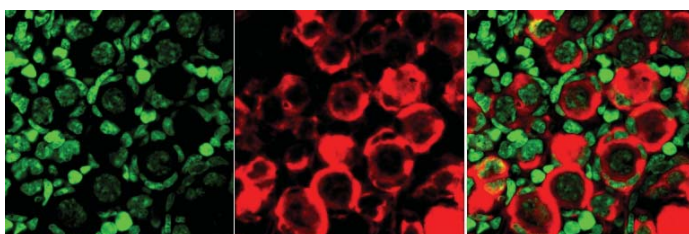


**Figure 3b.** Early tertiary follicle in 20-day-old CD-1 wild-type mouse ovaries. Left: YOYO-1 DNA staining. Middle: immunofluorescence SLBP staining. Right: merged overlap.

Further analysis shows persistently strong staining in the cytoplasm of maturing oocytes in early and late tertiary follicles (**Figure 3b**). Indeed, significant quantities of histone proteins may have already been synthesized at this point. Remaining histone mRNA will be regulated by SLBP in the cytoplasm until translation is complete. Thus, SLBP is accordingly kept in the cytoplasm instead of being degraded.

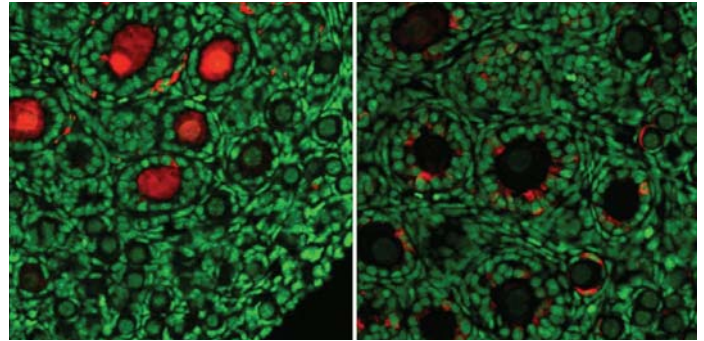
Although our data correlates the localization of SLBP with oocyte development, it is important to note certain limitations of the study. Previous research has determined the target of

**Figure 4.** Primordial follicles in five-day-old transgenic mouse ovaries. Left: YOYO-1 DNA staining in green. Middle: immunofluorescence SLBP staining in red. Right: merged overlap.

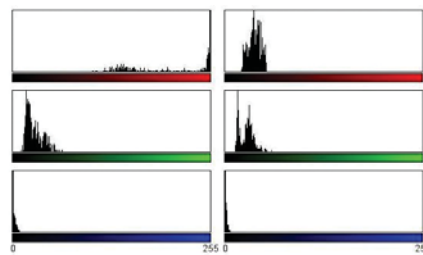


SLBP to be the stem-loop of the histone mRNA (Muller et al., 1997; Sanchez et al., 2002). However, detection of histone mRNA was not performed. Further research on this subject should focus on the visualization of histone mRNA through in situ hybridization or other mRNA detection techniques.

**Figure 5.** Primary follicles in transition to secondary follicle stage in CD-1 wild-type mouse ovaries. YOYO-1 DNA staining (green) and SLBP immunofluorescence staining (red) merged overlap. (\* marks the oocyte nucleus; \*\* marks the cytoplasm)

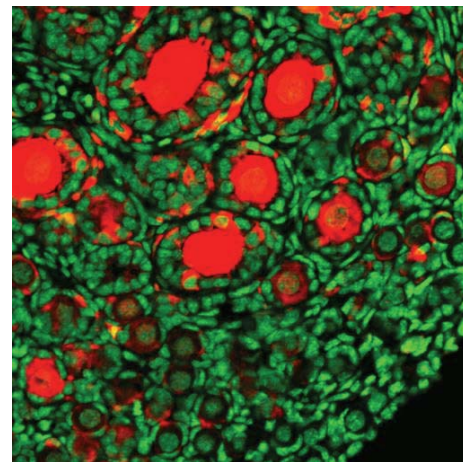


**Figure 5a.** Primordial and primary follicles in 5-day-old and 10-day-old ovaries. Left: CD-1 wild-type ovary section. Right: transgenic (right) ovary section.

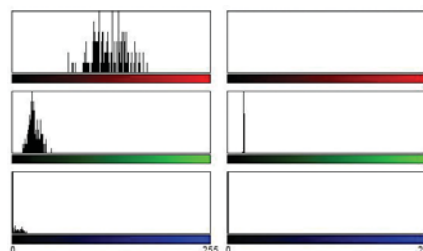


**Figure 5b.** SLBP fluorescence RGB (colour intensity) histograms of CD-1/transgenic comparison. Left column: CD-1 mouse SLBP immunofluorescence. Right column: transgenic mouse SLBP immunofluorescence.

**Figure 6.** SLBP fluorescence comparison in primordial and primary follicles of CD-1 mice. YOYO-1 DNA staining in green; immunofluorescence SLBP staining in red.



**Figure 6a.** Primordial and primary follicles in CD-1 mouse ovary.



**Figure 6b.** SLBP fluorescence RGB (colour intensity) histograms of primordial and primary follicles in **Figure 6a**. Left column: Oocyte contained in a primordial follicle. Right column: Oocyte contained in a primary follicle.

Transgenic SLBP-knockdown mice have been created using RNA interference techniques in the Clarke laboratory. The expression of dsRNA in these mutant mice is triggered only in the maturing oocyte. Therefore, oocytes that are in meiotic arrest are not affected by the induced mutation and are indistinguishable from wild-type oocytes. Immunohistochemistry and immunofluorescence performed on ovarian sections from these mutant mice showed identical results for SLBP staining in non-developing oocytes in primordial follicles (**Figure 4**). Since the expression of mutant phenotype is only present in maturing oocytes and follicles, the moderate levels of fluorescence in primordial follicles is expected. However, as oocytes in older ovaries matured, SLBP signal became very weak throughout all stages of folliculogenesis, implying successful SLBP knockout (**Figure 5**). Actually, SLBP antibody fluorescence in wild-type mice is five times stronger than fluorescence in transgenic mice. (**Figure 5b**). Data from other experiments performed by colleagues in the Clarke laboratory indicate that transgenic mouse oocytes abruptly stop development at the two-cell stage. Presumably, this is due to the lack of SLBP regulation of histone mRNA translation, resulting in a shortage of histone proteins necessary for continued meiotic division.

Development in both somatic and germ cells are undeniably critical to the growth of an organism. The exploration of underlying mechanisms is one of many current pursuits to further our knowledge and offers insight on the complexity of the cellular cycle. The regulation of histone mRNA is an important step for cellular replication in all eukaryotes, without which cell division would be difficult if not impossible. By ascertaining patterns of localization of SLBP and its role in the regulation of histone mRNA translation, we have increased our understanding of an important gateway affecting the proliferation of not only individual cells, but of the organism as well. Although still in its early stages, future research involving SLBP and histone mRNA pathways could lead to novel methods of controlling cell growth and development. Such progress has the potential to not only greatly advance our knowledge of the natural world, but may lead to breakthroughs in the treatment of tumours and infertility through inhibition or activation of somatic and germ cell growth.

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