

# Analysis of TATA-binding protein 2 (TBP2) and TBP expression suggests different roles for the two proteins in regulation of gene expression during oogenesis and early mouse development

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## Abstract

Gametogenesis, the process during which germ cells are generated is essential for reproduction. In mammals, maternal mRNA and proteins present in the oocyte are required to ensure the progression of development until the embryo activates its genome after fertilisation. It is well established that the oocyte synthesises these maternal factors during oocyte growth and then undergoes a quiescent transcriptional period that will be resumed only after fertilisation. However, the mechanisms that govern transcriptional regulation and subsequent silencing during oogenesis are not well understood. Here, we have examined the expression and localisation of the TATA-binding protein (TBP) and the related protein TBP2 (also called TRF3, TBP-related factor 3) during oogenesis and in early mouse embryos. We show that TBP is expressed in the oocytes at the beginning of folliculogenesis, but it is undetectable during further stages of oocyte development, and becomes abundant again only after fertilisation. In contrast to TBP, we found that TBP2 is highly expressed in growing oocytes during folliculogenesis, declines upon ovulation, and is almost undetectable after fertilisation by the two-cell stage. The mirroring localisation profile of TBP and TBP2 suggests different roles for the two proteins in establishing specialised programs of gene expression during oocyte development and in early mouse embryos. Analysis of mutant mouse ovaries in which oocyte-specific factors have been knocked-out suggests that TBP2 is a potential candidate for regulating transcriptional control of oogenesis. Moreover, our results obtained with oocytes lacking the oocyte-specific nuclear chaperone nucleoplasm 2 suggest that TBP2 function may be related to non-condensed chromatin conformation.

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## Introduction

Oogenesis is the process whereby the female haploid reproductive cells are formed through meiosis. In mammals, it comprises a long and complex series of events within the ovarian follicles. Oocytes are generated from oogonia and then they undergo the early stages of meiotic prophase I. During reproductive life, oocytes are continuously selected from a pool of primordial follicles to develop further and undertake a growth phase during which they are arrested at diplotene of the prophase I. This process of preantral folliculogenesis ensures a source of fully grown oocytes for further maturation. Oocyte growth is accompanied by an extreme increase in volume that leads to one of the

largest cells in the body, which has an overall unique cellular metabolism (Wassarman & Josefowicz 1978). Proteins essential for the growing oocyte and for the proliferating surrounding follicular or granulosa cells, including the zona pellucida proteins (ZP1, ZP2, ZP3), the bone morphogenic protein 15 and the growth differentiation factor 9 (GDF9), are translated from some of the most abundant oocyte-specific mRNAs (Pangas & Rajkovic 2006). When the appropriate hormonal stimulus is provided, responsive early antral follicles increase their size and reach the preovulatory stage. At this phase, the fully grown germinal vesicle (GV) stage oocyte is ready for ovulation and resuming meiosis. After GV breakdown (GVBD), the oocyte

completes meiotic maturation until the metaphase II at which stage it awaits for fertilisation (Eppig *et al.* 2004).

The exclusive cell features of the growing diplotene oocyte allow the production and storage of RNAs that include messages for housekeeping components and proteins involved in meiotic maturation and early embryonic development (Bachvarova 1985b). The relatively high level of transcriptional activity in the oogonia falls to an undetectable level in primordial follicle oocytes at the beginning of the prophase I. Then, RNA synthesis rises again up to a significant level and increases during oocyte growth from the primary follicle to the preantral stage. Once the oocyte reaches its full size, RNA synthesis diminishes and there is essentially no transcription at the GVBD (Bachvarova 1985a). Large-scale changes in chromatin structure coincide temporally with changes in levels of transcriptional activity. During the phase of oocyte growth, the chromatin is in a decondensed configuration referred to as non-surrounded nucleolus (NSN). From the early antral stage to the preovulatory follicular stage, the chromatin gradually condenses and forms a compact, perinucleolar rim around the nucleolus (surrounded nucleolus (SN)); Mattson & Albertini 1990, Debey *et al.* 1993, Zuccotti *et al.* 1995). The role of this spatial arrangement of nuclear structure in mammalian oocyte has not been clarified yet. Among the oocyte-specific proteins, nucleoplasm 2 (NPM2) has been shown to be essential for compaction of oocyte chromatin and deacetylation of histone H3 during the final stages of oocyte development. NPM2-deficient GV oocytes undergo transcriptional repression at the preovulatory follicle stage in spite of the lack of DNA condensation, and development is arrested at the zygote to two-cell stage transition (Burns *et al.* 2003, De La Fuente *et al.* 2004). Nevertheless, processes involved in regulation of changes in chromatin structure, activation of oocyte-specific genes and their subsequent transcriptional repression are not well understood.

So far, only a few transcription factors, such as FIGLA (Soyal *et al.* 2000) and NOBOX (Rajkovic *et al.* 2004), have been revealed as crucial transcriptional regulators in oocyte development and early follicle formation. However, the complete repertoire of oocyte-specific transcriptional regulators required for oogenesis has not been identified. Transcription factors regulate gene expression profile, and in this way, generate cell-specific fates by communicating signals to the general transcription machinery. RNA polymerase II (Pol II) with a host of other factors, including the general transcription factors (TFIIA, -B, -D, -E, -F, -H), act together to form a preinitiation complex (PIC) and to allow subsequent transcription initiation (Orphanides *et al.* 1996). The binding of the general transcription factor TFIID, composed of TATA-binding protein (TBP) and 14 TBP-associated factors (TAFs), to the promoter represents a critical rate limiting step at which regulators can control transcription

(Tora 2002, Muller & Tora 2004). TBP has a conserved C-terminal DNA-binding core domain responsible for recognising the TATA-box and a variable N-terminal domain. In vertebrates, cells lacking TBP show detectable levels of Pol II transcription, providing evidence for TBP-independent Pol II transcription (Muller *et al.* 2001, Martianov *et al.* 2002, Davidson *et al.* 2004). A number of TBP homologues have been described in metazoans as possible candidates for substituting TBP function. TBP-like factors (TLFs, also called TRF2/TRP/TLP) were found to be present in all metazoans (Rabenstein *et al.* 1999, Teichmann *et al.* 1999). In contrast, other evolutionary conserved TBP-like factors, the TBP2s (also called TRF3), were found only in vertebrates (Persengiev *et al.* 2003, Muller & Tora 2004). The N-terminal domains of TBP2s are divergent amongst themselves and different from those of TBPs, however, the core domain of TBP2s and TBPs are almost identical. Similar to TBP, TBP2 binds the TATA box, interacts with TFIIA and TFIIB and can mediate Pol II transcription initiation *in vitro* (Bartfai *et al.* 2004). The zebrafish homologue of TBP2 has been partially characterised and shown to be preferentially expressed in adult gonads. Similarly, expression of murine *Tbp2* is primarily detected in the ovary (Bartfai *et al.* 2004). Thus, it is conceivable that various TBP- and/or TBP2-containing complexes having specific roles in recognition of different promoters will interact with distinct sets of general transcriptional factors and activators to regulate distinct developmental pathways. Recently, it has been shown that expression of *Tbp2* mRNA in the mouse ovary is restricted to the oocyte (Xiao *et al.* 2006), suggesting a role for *Tbp2* gene during female gametogenesis.

The storage of dormant maternal mRNAs and the selective translational repression in the oocyte are controlled, at least partially, by cycles of cytoplasmic polyadenylation and deadenylation (Bachvarova 1992, Richter 1996). In the frog, TBP mRNA is present in oocytes, but maternally stored for later use during early embryogenesis (Veenstra *et al.* 1999, Jallow *et al.* 2004). Because of the key role of translational control during growth and maturation of the oocyte and in early development, and due to the suggestion of a possible role for *Tbp2* during gametogenesis, it is essential to determine whether accumulation of TBP2 protein follows that of its mRNA. We have developed a specific TBP2 antibody and analysed the localisation of TBP2 during oogenesis and compared it with that of TBP. We found that TBP2 is abundant in the nuclei of the oocyte from the primordial follicle stages and until the completion of oocyte growth. TBP2 expression then declines concomitant with ovulation and becomes almost undetectable after fertilisation. In contrast, TBP is under the threshold of detection in the oocyte during growth and only begins to accumulate after fertilisation in the zygote. Our data provide the first detailed report

on the localisation of TBP2 protein during the various stages of folliculogenesis and suggests a potential role for TBP2 in the regulation of the transcriptional events that accompany oocyte growth and maturation.

## Materials and Methods

### Cell culture and transfection

NIH 3T3 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. The cDNA fragment corresponding to full-length mouse TBP2 open reading frame (ORF) was cloned into the pSG5-Flag expression vector (Nielsen *et al.* 2001). Cells were transfected with Flag-TBP2 pSG5 expression vector using JetPEI (Polyplus-transfection SA, France). The generation of cell lines stably expressing TBP2 will be described elsewhere.

### Antibody production and western blot analysis

To generate the anti-TBP2 monoclonal antibody, the pH91 peptide corresponding to amino acids 78 to 97 of mTBP2 (RDQTVTGNKLASEESCRTRD) was used for immunisation of mice as described (Brou *et al.* 1993b). The anti-TBP2 mouse monoclonal antibody (2B12) was specific for TBP2 and did not crossreact with TBP as it only recognised a single band corresponding to the size of TBP2 as detected by western blot analysis. The anti-TBP mouse monoclonal 3G3 antibody recognising the first 16 amino acids of human TBP was previously characterised (Brou *et al.* 1993b). Extracts from NIH 3T3 and TBP2-overexpressing NIH 3T3 cells were prepared by lysis in 20 mM Tris Cl pH 7.5, 2 mM dithiothreitol 400 mM KCl, 20% glycerol supplemented with protease inhibitors (2.5 mg/ml leupeptin, pepstatin, aprotinin, antipain and chymostatin) and three cycles of freezing and thawing. Protein extract (10 µg) or oocytes (50 GV) were denatured and separated by SDS-PAGE (10%). After blocking, membranes were incubated subsequently with the anti-TBP2 2B12 (1:1000), anti-TBP 3G3 (1:1000) and anti-actin (1:5000, Sigma) antibodies as indicated in the figure legends.

### Immunohistochemistry

Ovaries were dissected from 4, 6, 10 or 12 weeks old and newborn C57Bl6 mice, fixed in 4% paraformaldehyde overnight at 4 °C, washed in PBS, dehydrated in ethanol and embedded in paraffin. Sections of 5 µm thick were heated, immersed in Histolemon (Carlo Erba Reagenti, Limite, Milano, Italy), rehydrated and washed in PBS. Heat-induced antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). After further washes in PBST (0.1% Tween20 in PBS), sections were blocked for 1 h at room temperature with 5% normal goat serum (NGS), 0.5% BSA, 0.3% Triton100X, 0.1% Tween20, in PBS. The sections were then incubated

overnight at 4 °C with the anti-TBP2 2B12 mouse monoclonal antibody alone (1:500) or in combination with rabbit polyclonal anti-TBP antibody (1:2000) in 3% NGS in PBST. The polyclonal anti-TBP antibody was a kind gift from N Hernandez. The rabbit polyclonal anti-VASA antibody (1:500; Kotaja *et al.* 2006) was a kind gift from P Sassone-Corsi. Secondary antibodies (1:200) were CY3- and Oregon Green-conjugated goat anti-rabbit and anti-mouse IgG (Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK). Sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and nuclei were counterstained with DAPI. For the competition tests, sections were incubated with the primary antibodies in the presence of pH91 peptide or full-length recombinant hTBP expressed, then purified from *Escherichia coli* (Brou *et al.* 1993a). Sections from ovaries deficient in NOBOX (Rajkovic *et al.* 2004) were processed as above. Ovary sections from *Npm2*<sup>-/-</sup> (Burns *et al.* 2003) and *Gdf9*<sup>-/-</sup> mutants (Dong *et al.* 1996) were kindly provided by M Matzuk.

### Embryo and oocyte collection

GV stage oocytes were collected by puncturing the follicles of ovaries from 6 weeks old F1 (C57BL/6 × CBA/H) females (Hogan *et al.* 1994). Metaphase I oocytes were obtained from GV oocytes undergoing spontaneous maturation in M16 medium supplemented with 5% foetal calf serum at 37 °C under 5% CO<sub>2</sub>. Metaphase II oocytes were collected from ampullae of mice superovulated with i.p. injection of pregnant mare serum gonadotrophin and human chorionic gonadotrophin (hCG). Embryos were collected from F1 6 to 7 weeks old superovulated females mated with F1 males. Zygotes and two-cell stage embryos were collected at 24 and 48 h post-hCG (h phCG) respectively and processed immediately for immunostaining. All experiments were carried out according to Home Office legislation.

### Immunostaining of GV oocytes and early embryos

After removal of the zona pellucida with acid Tyrode's solution (Sigma), embryos were washed and fixed as described (Torres-Padilla *et al.* 2006). Embryos were incubated with the TBP2 antibody (1:200) for ~12 h at 4 °C and mounted in Vectashield (Vector Laboratories). Confocal microscopy was performed using a 60× oil objective in an Upright Confocal Laser Microscope. All the stainings were repeated independently twice with at least 10 oocytes/embryos.

### mRNA microinjections

Two-cell stage embryos were collected at ~46 h phCG and microinjected with 1–2 pl of a mixture containing 300 ng/µl mRNA for mouse TBP2 and 200 ng/µl mRNA

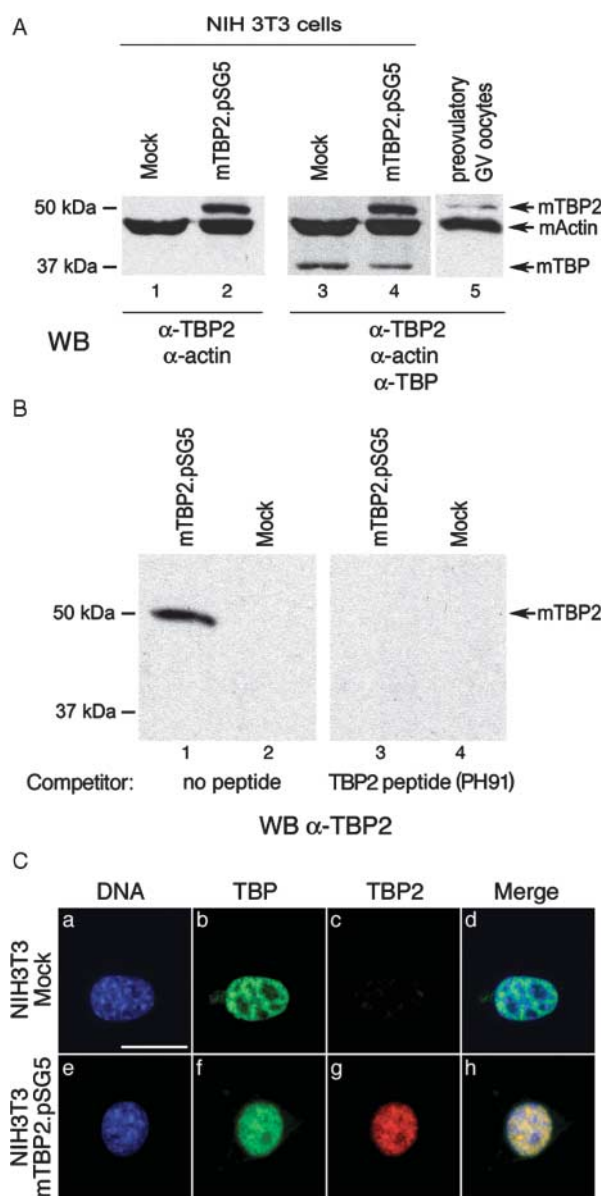


for DsRed, which were capped and transcribed *in vitro* from the pRN3P plasmid. Embryos were then cultured in KSOM medium under a 5% CO<sub>2</sub> atmosphere at 37 °C, fixed 24 h after mRNA injection and processed for immunofluorescence analysis with the TBP2 antibody.

## Results

In order to analyse the expression and distribution of mouse (m)TBP2 protein, we first developed and characterised a monoclonal antibody raised against a specific mTBP2 peptide. To test this new anti-TBP2 2B12 antibody, we first performed western blot analysis of whole cell extracts from NIH 3T3 cells transfected with the mTBP2.pSG5 expression vector. The anti-TBP2 2B12 antibody recognised a single polypeptide migrating at about the expected molecular weight (49 kDa) demonstrating that this antibody is specific and does not crossreact with TBP (Fig. 1A, lanes 1–2). To confirm this, the same membrane was then subsequently probed with an anti-TBP antibody, which revealed that TBP was present in these cell extracts and that mouse TBP migrates at the expected (~37 kDa) lower molecular weight than mTBP2. These experiments rule out the possibility that the anti-TBP2 antibody recognises TBP (Fig. 1A, lanes 3–4). Second, we performed competition assays with the corresponding TBP2 peptide against which the antibody was raised. The peptide blocked the recognition of mTBP2, indicating that the anti-TBP2 2B12 antibody is specific (Fig. 1B). Third, we performed immunofluorescence analysis of NIH 3T3 cells stably overexpressing TBP2 using the anti-TBP2 antibody. These analyses showed that the TBP2 antibody stains only the cells that overexpress TBP2, but not those cells that do not (Fig. 1C, panels c and g), in spite of TBP being present in both cell types (Fig. 1C, panels b and f). We also performed immunoprecipitation analysis of extracts from NIH 3T3 cells stably overexpressing TBP2 using the anti-TBP2 antibody. The anti-TBP2 antibody immunoprecipitated only TBP2, but not TBP (data not shown), further confirming that the TBP2 antibody is specific and does not crossreact with TBP. All these experiments demonstrate that the TBP2 and TBP antibodies used in this study are specific and they can discern between these two closely related proteins. Note that we did not detect TBP2 in any other cell types or tissue sections that we analysed (mouse embryonic fibroblasts, NIH 3T3, F9, testis derived TM3 and TM4 cell lines, lung, pancreas; Fig. 1 and data not shown).

Because of the technical difficulty of collecting enough oocytes from earlier follicular stages, we performed western blot analysis of GV stage oocytes collected from preovulatory follicles. This analysis demonstrated that the TBP2 antibody recognises the endogenous TBP2 protein and indicated that TBP2 is present in GV oocytes (Fig. 1A, lane 5). We also found



**Figure 1** Western blot analysis confirms the specificity of the anti-TBP2 antibody and reveals the presence of TBP2 in GV stage oocytes. (A) Whole cell extracts from NIH 3T3 cells expressing TBP2 (lanes 2 and 4) and GV stage oocytes (lane 5) were resolved by SDS-PAGE, blotted onto nitrocellulose membranes and probed first with anti-TBP2 and anti-actin antibodies (lanes 1–2). The membrane was then subsequently probed with anti-TBP antibody (lanes 3–5). Protein extract from NIH 3T3 cells transfected with empty pSG5 vector (Mock) was included as negative control (lanes 1 and 3). TBP2 migrates at ~49 kDa and TBP at ~37 kDa. The anti-TBP2 antibody does not recognise TBP. (B) Specific competition of the anti-TBP2 antibody with the TBP2-specific peptide. Extracts from NIH 3T3 cells expressing TBP2 (lanes 1 and 3) and Mock cells (lanes 2 and 4) were processed for western blot analysis. The anti-TBP2 antibody was incubated in the presence (right panel) or absence (left panel) of the TBP2 peptide (pH91) against which the antibody was raised. (C) NIH 3T3 cells stably overexpressing TBP2 or stably transfected with the empty pSG5 vector (Mock) were processed for immunofluorescence with the anti-TBP (green) and the anti-TBP2 (red) antibodies. DNA was stained with DAPI (blue). The anti-TBP2 antibody only stains the cells in which TBP2 is stably expressed. Scale bar is 10 μm.

that levels of TBP are below the detection limits in GV oocytes (see also below).

We next examined TBP2 distribution throughout various stages of oogenesis. We prepared ovary sections and immunostained them with a combination of rabbit polyclonal anti-TBP antibody and mouse monoclonal anti-TBP2 antibody. Both TBP and TBP2 were expressed in the nuclei of oocytes in the primordial follicles, which are the first follicles to form during folliculogenesis (Fig. 2A, panels b and c). Interestingly, TBP expression in the growing oocyte gradually decreased at the primary follicle stage (Fig. 2A, panel f), and the protein was undetectable by the preantral stage (Fig. 2A, panels j, n and r). In contrast to TBP, TBP2 persisted after the follicles enter the growth phase at the primary follicle, preantral secondary and antral secondary follicle stages (Fig. 2A, panels g, k and o). Note that TBP2 is excluded from the nucleoli and localises to regions of non-condensed chromatin in oocytes with a NSN (Fig. 2A, panels c and g) and partly NSN configuration (Fig. 2A, panels k and o). These results indicate that TBP2 is present in the oocyte throughout the period in which the oocyte increases its volume. Thus, contrary to TBP, we observed TBP2 in all preantral stages (Fig. 2A, panels c, g and k) and also in antral secondary follicles (Fig. 2A, panel o). However, TBP2 expression in the oocytes declines after the antral secondary follicle stage and it is almost undetectable in the preovulatory follicles before ovulation (Fig. 2A, compare panels o and s). Surprisingly, only TBP, but not TBP2, was detected in the somatic follicular cells surrounding the oocyte (Fig. 2A, panels f, j, n and r). Our data indicate that TBP2 and TBP display different distribution patterns within the ovary. TBP2 is the predominant form in the growing oocyte and hence TBP2 expression is restricted to specific stages of folliculogenesis.

In order to determine when TBP2 expression starts during oogenesis, we performed double immunostaining of TBP2 and VASA (also known as *Mouse vasa homolog*, *Mvh*) in ovary sections. Expression of VASA is first detected in primordial germ cells and maintained until meiosis is undertaken (Fujiwara *et al.* 1994, Tanaka *et al.* 2000). Thus, VASA expression helped us to identify early primary oocytes, even when they are not yet completely surrounded by follicular cells (Fig. 2B, panel b). Double immunostaining with anti-TBP2 and anti-VASA antibodies revealed that not all of the oocytes in primordial follicles express TBP2 (Fig. 2B, panel c). This finding suggests that TBP2 expression begins concomitant with initiation of oocyte growth.

To demonstrate that the staining for TBP and TBP2 was specific also in immunohistology, we performed competition assays on ovary sections. Results of this competition at the primary follicular stage are shown in Fig. 2C. TBP2 staining was lost upon incubation with the peptide against which the TBP2 antibody was raised (Fig. 2C panel c), but the signal in the oocyte nucleus remained

unchanged when the antibody was incubated with recombinant hTBP (Fig. 2C panel g). Similarly, the TBP-specific signal was abolished when the TBP antibody was challenged with recombinant full-length TBP protein (Fig. 2C panel f), but not when incubated with the peptide against which the TBP2 antibody was raised and whose sequence is specific to mTBP2 (Fig. 2C panel b). Similar results were observed at all stages of follicular development analysed (data not shown). Thus, our results confirm previous observations on the oocyte-specific detection of *Tbp2* mRNA (Xiao *et al.* 2006) and extend it to the protein level.

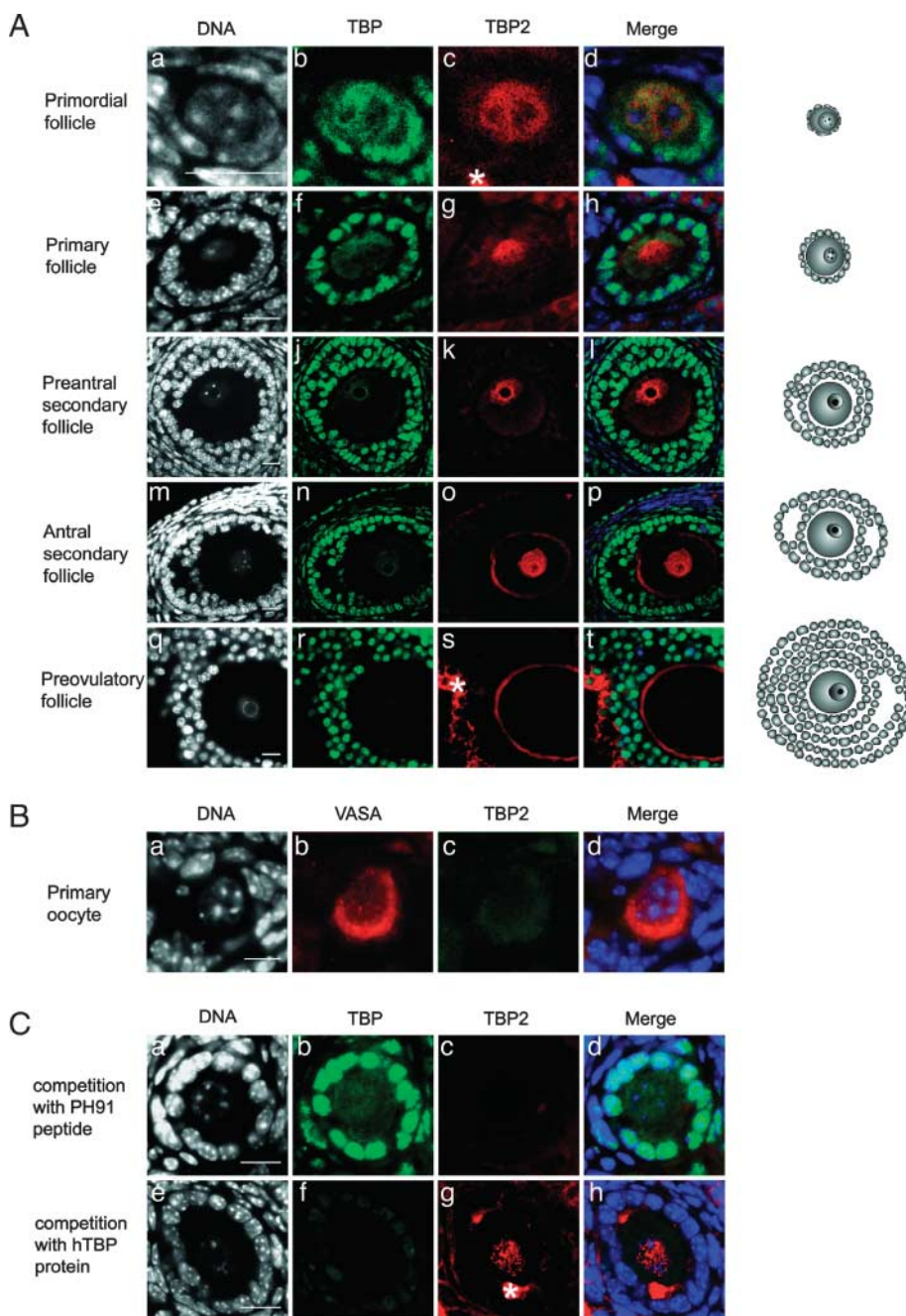
Taking advantage of the fact that fully grown oocytes at the GV stage removed from late antral follicles spontaneously resume meiosis when maintained in culture (Pincus & Enzmann 1934, Edwards 1965), we isolated GV oocytes and further examined the distribution of TBP and TBP2 in GV stage, metaphase I and metaphase II arrested oocytes developed in culture. We also collected zygotes and two-cell stage embryos. Confirming our earlier observations (Figs 1 and 2), TBP appeared below the threshold of detection in GV stage oocytes, and we detected no TBP signal in metaphase I or metaphase II oocytes (Fig. 3A, panels b, f and j). However, TBP accumulation was evident after fertilisation in both pronuclei of the zygote and in the nuclei of two-cell stage embryos (Fig. 3A, panels n and r). TBP2 was found to be present at low levels in the GV of preovulatory oocytes (panel d), but then decreased further upon progression through meiosis and was almost undetectable in zygotes and in two-cell stage embryos (panels h, l, p and t). As a positive control to confirm that the TBP2 antibody could recognise TBP2 in embryos, we overexpressed mTBP2 in the embryos by injection of mRNA. As shown in Fig. 3B, the anti-TBP2 antibody readily detects mTBP2 only in the blastomeres that had been injected with mRNA for TBP2. These experiments further confirm that the TBP2 antibody specifically recognises TBP2. Thus, while TBP2 is present in oocytes during folliculogenesis until the preovulatory stages, it is absent after ovulation. In contrast, TBP becomes abundant after fertilisation.

The proteins that are synthesised in the oocyte can be divided into two major groups. The first contains factors, which are essential for controlling oocyte growth and follicular development, and the second contains regulators of oocyte-specific transcription including transcription factors and chromatin remodellers (Andreu-Vieyra *et al.* 2006). Since many features of oocyte development cannot be studied *in vitro* without the natural environment provided by the surrounding follicular cells, genetically modified mouse models are used to analyse the function of oocyte-specific factors. Transgenic models in which oocyte-specific factors have been knocked-out could provide insights into the genetic pathways that may regulate TBP2 expression. Thus, we

analysed TBP2 expression in mutants of each representative class of oocyte-specific proteins.

GDF9 is a growth factor expressed in oocytes that is crucial for the proliferation of the surrounding granulosa cells and the growth of antral follicles and subsequent ovulation (McGrath *et al.* 1995, Elvin *et al.* 1999a, 1999b, 2000, Vitt *et al.* 2000, Vitt & Hsueh 2001). *Gdf9*<sup>-/-</sup> deficient follicles do not develop normally beyond the primary follicular stage, instead, oocytes undergo accelerated growth followed by degeneration

within the follicle (Dong *et al.* 1996, Carabatsos *et al.* 2000, Elvin *et al.* 2000). To analyse whether GDF9 controls TBP2 expression, we examined the localisation of TBP2 in *Gdf9*<sup>-/-</sup> ovaries. We found that TBP2 accumulation is lost at the primary follicle stage at which stage the loss of GDF9 leads to oocyte degeneration (Fig. 4A panel f). Thus, the onset of TBP2 accumulation takes place in these mutants (panel b), but TBP2 expression is not maintained in the absence of GDF9 (panels f and i), suggesting that only the maintenance of





TBP2 expression depends, directly or indirectly, on GDF9 (Supplementary Fig. 1 which can be viewed online at [www.reproduction-online.org/supplemental/](http://www.reproduction-online.org/supplemental/)).

Changes in chromatin condensation coincide with changes in global levels of transcriptional activity in the oocyte. Oocyte-specific ablation of NPM2, a chaperon involved in heterochromatin organisation, results in the absence of a prominent nucleolus, the absence of chromatin condensation in the oocyte, and the subsequent abnormal embryonic development (Burns *et al.* 2003, De La Fuente *et al.* 2004). However, the global transcriptional repression achieved at the end of the growth phase remains unchanged in *Npm2*<sup>-/-</sup> mutants (Burns *et al.* 2003, De La Fuente *et al.* 2004). This suggested that transcriptional repression occurs independently of the chromatin condensation events (De La Fuente *et al.* 2004). To investigate whether TBP2 plays a role in chromatin condensation and/or transcription, we examined TBP2 expression in *Npm2*<sup>-/-</sup> mutants. We found that TBP2 was present in the nuclei of the oocyte within the primordial follicles and displayed a similar distribution to the wild type until the antral secondary follicle stage (Fig. 4A panels c, g, j and l). The finding that TBP2 expression remains unchanged until this stage when compared with the wild type, suggests that NPM2 does not influence the localisation of TBP2 on the non-condensed chromatin. However, in contrast to the wild type, TBP2 remained present at a high level in the antral preovulatory stage and remained associated to the non-condensed chromatin at the latest preovulatory stages even when global transcriptional repression occurs (compare panels m and n). This result suggests that TBP2 function is linked to an open chromatin configuration.

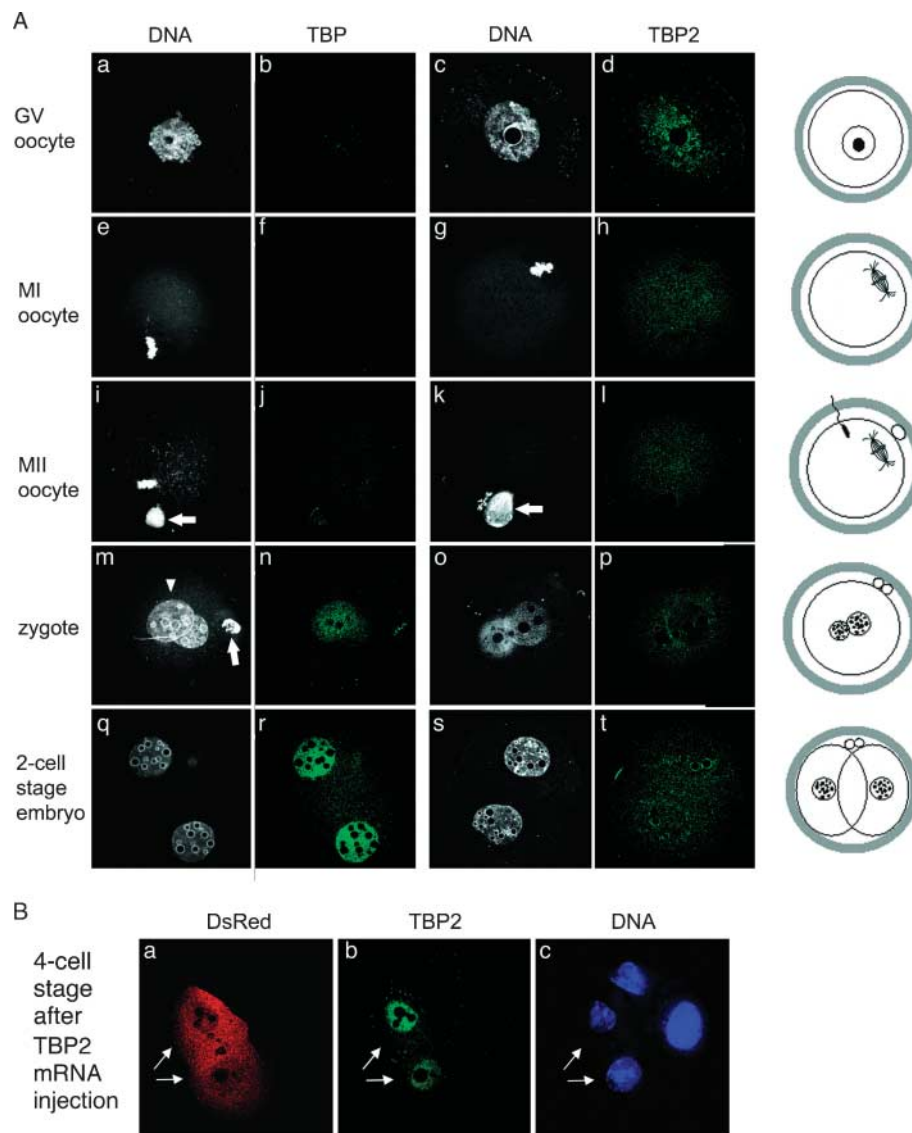
Among the transcription factors known to be involved in gene regulation during folliculogenesis, NOBOX was shown to be required for regulating a subset of genes

specific to oocytes, although expression of housekeeping genes does not require NOBOX (Rajkovic *et al.* 2004). Its mRNA and protein are expressed in the oocyte throughout folliculogenesis. To determine whether NOBOX regulates TBP2 expression, we examined TBP2 localisation in *Nobox*<sup>-/-</sup> ovaries. NOBOX deficiency results in abnormal oocytes in the primordial follicles and in the absence of further stages of folliculogenesis (Rajkovic *et al.* 2004). Thus, we could only analyse TBP2 expression at the primordial follicle stage in these mutants. We observed that oocytes lacking NOBOX do express TBP2, but the protein is abnormally diffused into the ooplasm, probably reflecting the defective primordial follicle formation due to loss of NOBOX (Fig. 4A panel d). We also found that loss of NOBOX induced an early onset of TBP2 accumulation, since, in contrast to the wild type, early primary oocytes from *Nobox*<sup>-/-</sup> newborn mice express TBP2 prior to follicle formation (Fig. 4B, panel b). Thus, NOBOX seems to regulate the onset of TBP2 expression and influence its subcellular localisation.

## Discussion

We have analysed the expression and localisation of TBP and TBP2 at the protein level during oocyte development throughout folliculogenesis. Our results indicate that while TBP protein levels decrease with the formation of the first follicles, those of TBP2 increase concomitantly. The mirroring localisation profile of TBP and TBP2 suggests different roles for the two proteins in regulating gene expression during oocyte growth and in the early mouse embryo. We found that the TBP2 protein is specifically expressed in the developing oocyte after primordial follicle formation until the oocyte completes its growth phase within the follicle through

**Figure 2** Localisation of TBP and TBP2 during mouse folliculogenesis. (A) TBP and TBP2 exhibit a different localisation pattern, while TBP expression is absent in the oocyte but present in surrounding follicular cells, TBP2 is detected exclusively in nuclei of oocytes. Sections from ovaries of 12 weeks old mice were immunostained with anti-TBP (green), anti-TBP2 (red) antibodies and nuclei were counterstained with DAPI (blue). Panels from the top to the bottom represent the sequential stages of follicular development as it is indicated on the left. Schematic drawings of the corresponding stages of folliculogenesis are shown on the right. At the primordial follicle stage, the two proteins are present in the non-condensed chromatin of the oocyte (panels a–d). Note that at this stage, the oocyte displays a NSN-configuration. At the primary follicle stage, as the oocyte enters the growth phase, TBP expression decreases, while TBP2 remains expressed (panels e–h). TBP2 remains also at preantral and antral secondary follicle stages, where the partly NSN stage of the chromatin turns into the SN configuration (panels k and o). At preovulatory follicle stage, when the chromatin is completely condensed in the SN configuration, TBP2 becomes barely detectable (panel s). Higher magnifications of all the oocyte nuclei shown in this figure are shown in supplementary Fig. S1. (B) TBP2 expression starts at the primordial follicle stage of folliculogenesis. Not all primary oocytes express TBP2 during primordial follicle formation (panel c). Ovary sections from 4 weeks old wild-type mice were immunostained with antibodies against TBP2 (green) and VASA (red). The VASA (red) staining allowed us to identify all of the primary oocytes (panel b). Note that the incomplete primordial follicle formation at this age, e.g. the presence of primary oocytes without organised surrounding follicular cells may be strain specific. (C) Competition assay demonstrates the specificity of the TBP and TBP2 antibodies. Ovary sections from 12 weeks old mice were incubated with anti-TBP and anti-TBP2 antibodies in the presence of either the pH91 peptide against which the anti-TBP2 antibody was raised (upper panels) or recombinant full-length hTBP protein against which the anti-TBP antibody was raised (lower panels). Only TBP2 labelling is abolished upon incubation with the pH91 peptide confirming the specificity of the anti-TBP2 antibody (panel c). Only the anti-TBP antibody labelling is abolished upon incubation with the hTBP protein (panel f), but not that of the anti-TBP2 labelling (panel g). Sections shown in panels A and C were not analysed in parallel and therefore the intensity of the fluorescence levels are not directly comparable. Scale bars are 10 µm. The stars \* indicate non-specific signals outside of the cells. Note also the background staining known to appear in the mammalian ovary due to secondary antibody binding to the zona pellucida.



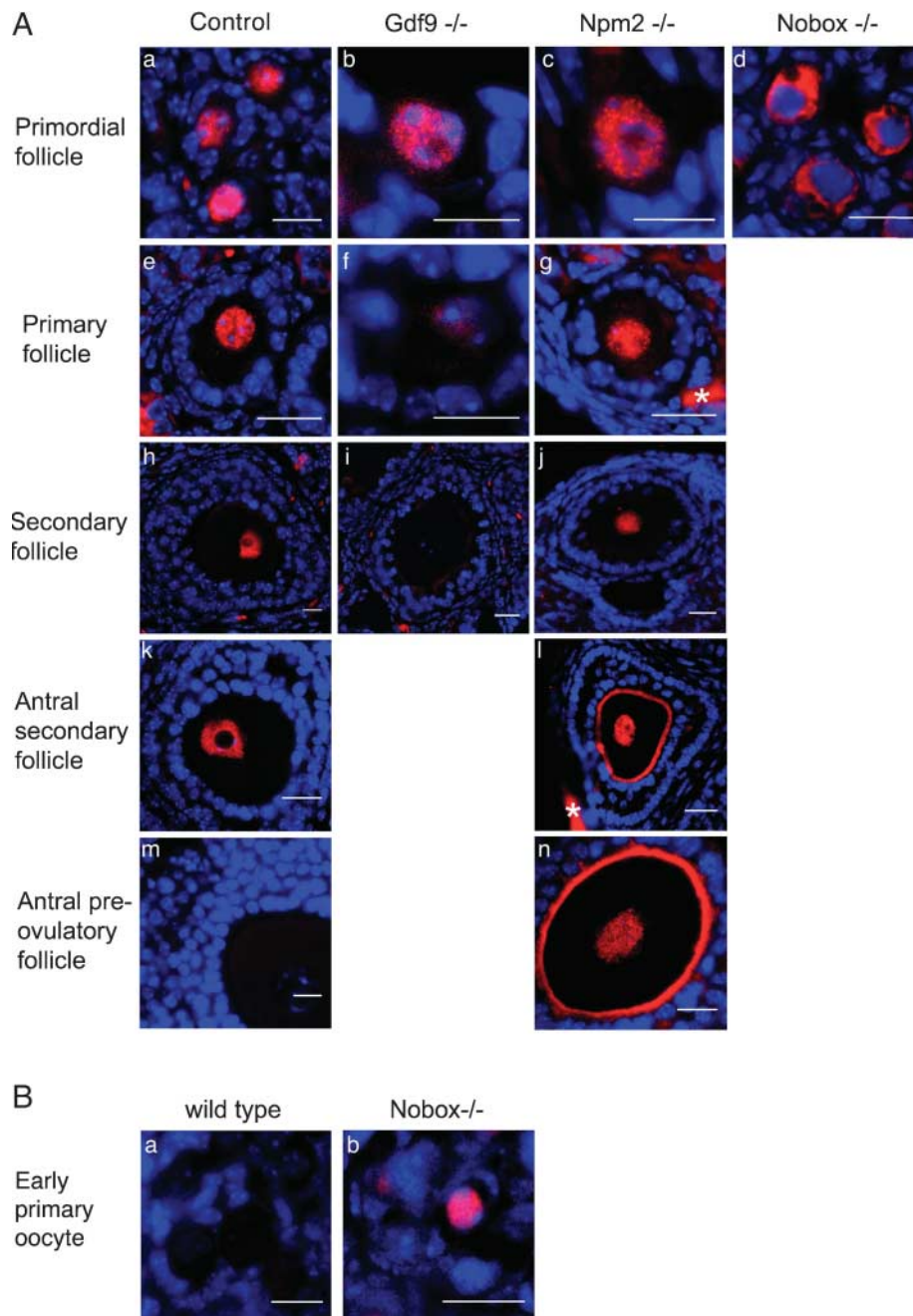
**Figure 3** Distribution of TBP and TBP2 in mature oocytes and early mouse embryos. (A) TBP2 accumulation gradually decreases after ovulation, while TBP is undetectable in mature oocytes, but accumulates progressively in the pronuclei of the zygote. Germinal vesicle (GV), metaphase I or metaphase II arrested oocytes, zygotes and two-cell stage embryos were processed for immunostaining with anti-TBP and anti-TBP2 antibodies separately. Samples were processed in parallel and are comparable between stages, but not between antibodies. In MI oocytes, the homologous chromosomes are aligned on the metaphase plate (panels e and g). In MII oocytes, the haploid number of chromosomes and the contents of the first polar body (arrows) are also seen (panels i and k). In the zygote, the male pronucleus (arrowhead) displays higher levels of TBP accumulation than the female one (panel n). This is in line with the male pronucleus undergoing transcriptional activation before the female pronucleus. The second polar body is not visible in the two-cell stage embryos shown (panels q and s). Pictures shown are stack projections of confocal sections taken every 1.5  $\mu\text{m}$ . Schematic drawings of the corresponding stages of oocyte maturation and embryonic development are shown on the right. (B) One of the blastomeres from two-cell stage embryos was injected with mRNA for mTBP2 and for DsRed. Embryos were cultured to the four-cell stage and stained with the anti-TBP2 antibody. The progeny of the injected blastomere are depicted by arrows as indicated by the presence of DsRed. Note that the TBP2 antibody only stains the blastomeres injected with mRNA for mTBP2, further indicating that the anti-TBP2 antibody is specific.

the antral stages. The finding that not all the oocytes express TBP2 at the primordial follicle stage suggests that TBP2 expression occurs only in those primordial follicles, which have been selected to develop further to the preovulatory follicle stage in which the oocytes reach their maximum size. Moreover, TBP2 localisation to the nuclei of growing oocytes suggests that TBP2 has a specialised role in transcriptional initiation

and/or regulation in the oocyte during its growth phase.

Although TBP is initially present in the nuclei of primordial follicle oocytes, it is rapidly downregulated in the following follicular stages. The levels of TBP transcripts in the oocyte were shown to be high during folliculogenesis, they decrease in metaphase II arrested oocytes, followed by a further reduction in the zygote





**Figure 4** TBP2 localisation in ovaries of *Gdf9*<sup>-/-</sup>, *Npm2*<sup>-/-</sup> and *Nobox*<sup>-/-</sup> mutant mice. (A) TBP2 shows different localisation pattern depending on the phenotype of the mutant mice analysed. In the control (*Gdf9*<sup>+/-</sup>), TBP2 is expressed in the nuclei of the oocytes within primordial follicles and during the growth phase. In *Gdf9*<sup>-/-</sup> mice, TBP2 levels are comparable with the control at the primordial follicle stage, but it decreases and becomes undetectable at the primary follicle stage, in agreement with the loss of GDF9 causing defects in oocyte growth at this stage. In *Npm2*<sup>-/-</sup> mutants, TBP2 expression is unaffected until the late antral stage of folliculogenesis. In contrast to the control, in *Npm2*<sup>-/-</sup> mutants, TBP2 expression remains high at the preovulatory follicle stage, where it colocalises with the abnormally diffuse chromatin of the oocyte. In primordial *Nobox*<sup>-/-</sup> follicles, accumulation of TBP2 strikingly differs from the control in that it is excluded from the nucleus and distributed into the ooplasm. Sections from ovaries of control (*Gdf9*<sup>+/-</sup>), *Gdf9*<sup>-/-</sup>, *Npm2*<sup>-/-</sup> and *Nobox*<sup>-/-</sup> mice were immunostained with anti-TBP2 (red) antibodies and nuclei were counterstained with DAPI (blue). All sections derived from 10 weeks old mice, except for the *Npm2*<sup>-/-</sup> mutants, which derive from 6 weeks old females. Panels from the top to the bottom represent successively the stages of folliculogenesis that take place in the mutant mice analysed. (B) Early onset of TBP2 accumulation in *Nobox*<sup>-/-</sup> ovary. TBP2 is absent before the formation of the follicles in the control, wild-type ovary. In contrast, in *Nobox*<sup>-/-</sup> mice TBP2 is highly expressed in nuclei of early primary oocytes. Ovary sections from 1-day-old wild type and *Nobox*<sup>-/-</sup> mice were immunostained with the anti-TBP2 (red) antibody. Scale bars are 10 μm. The stars \* indicate non-specific signal (note that the signal is located outside of the cells). Note the background staining known to appear due to secondary antibody binding to the zona pellucida.

(Xiao *et al.* 2006). This finding together with our results suggest that TBP mRNA accumulates, but is not translated in the growing oocyte. Indeed, we found that TBP presence becomes evident again only after fertilisation, coincident with the two waves of transcriptional activation of the genome in the late zygote and the two-cell stage embryos (Schultz 2002). Our observation that TBP accumulation peaks around the time of genome activation is in line with previous findings (Worrad *et al.* 1994, Martianov *et al.* 2002). Although it is unclear whether the antibodies used by Worrad *et al.* (1994) would discriminate between TBP and TBP2. We found that TBP is abundant in the nuclei of mitotic follicular cells suggesting that TBP expression is confined to cells with proliferation activity. We can hypothesise that the TBP detected in the early primary oocyte was translated before the last mitotic division upon formation of the oocyte from oogonia, and that TBP is involved in regulating transcription initiation only when the first mitotic division will be resumed after fertilisation. Indeed, Pol II transcription is severely affected in early embryos where TBP has been invalidated (Muller *et al.* 2001, Martianov *et al.* 2002, Davidson *et al.* 2004). However, not all Pol II-dependent transcription is affected in these embryos suggesting that there may be genes with distinct requirements for TBP-type factors during genome activation.

In this work, we have shown the oocyte-specific expression of a TBP-type factor, TBP2, extending previous studies on *Tbp2* mRNA to the protein level (Xiao *et al.* 2006). We found that TBP2 is undetectable in the oocyte upon maturation. However, it has been shown that TBP2 mRNA is present in mature oocyte and also in zygote suggesting that the mRNA is degraded only after fertilisation (Xiao *et al.* 2006 and this study). It is well established that early embryonic events are orchestrated through post-transcriptional control of maternal mRNA, including regulation of translation and mRNA degradation (Schultz 2002). Indeed, the observation that we do not detect TBP2 protein, but the mRNA is present in the mature oocyte, suggests that TBP2 is also subject to translational regulation of maternal TBP2 mRNA.

Another recent report documented ubiquitous expression of TBP2 at the protein level different from our observations (Yang *et al.* 2006). However, that study also contrasts with previous RT-PCR and *in situ* hybridisation analyses showing that TBP2 mRNA is expressed exclusively in the ovary (Bartfai *et al.* 2004, Xiao *et al.* 2006). Given the highly similar structures of TBP2 and TBP, it is possible that previous results were misinterpreted because of crossreactivity of antibodies. We use in our study a new antibody for TBP2 that we have developed and thoroughly characterised. Importantly, this antibody does not crossreact with TBP.

Since the core DNA-binding domain of TBP2 and TBP share the same structure and since TBP2 can form Pol II

PICs and thus mediate Pol II transcription (Bartfai *et al.* 2004), our data suggest that TBP2 may be involved in establishing a specialised program of gene expression in the female germ line when TBP is absent. The fact that TBP is not expressed in the developing oocyte, together with the observation that the N-terminal amino acid tail of TBP2 is very different from TBP, suggest that this domain could establish contacts to form specific regulatory complexes in the oocyte, which may be different from those functioning with TBP. These complexes may be involved in both transcriptional repression as well as initiation of transcription of specific genes. Moreover, the pattern of localisation of TBP2 indicates that TBP2 is associated with non-condensed chromatin in the oocyte nucleus, and suggests that this association may be linked to its function. This is supported by the localisation of TBP2 in the *Npm2*<sup>-/-</sup> mutants, where TBP2 accumulation does not decrease, but remains associated with the abnormally non-condensed chromatin at the preovulatory stage.

Similar to TBP2, NPM2 localises to the oocyte nuclei and is excluded from the nucleolus before GVBD, but after GVBD, it is distributed and diluted in the cytoplasm. Contrary to TBP2, NPM2 becomes abundant again after fertilisation in the pronuclei of the zygote, where it plays a role in chromatin remodelling (Burns *et al.* 2003, De La Fuente *et al.* 2004). The observation that chromatin condensation towards the SN configuration does not take place in *Npm2*<sup>-/-</sup> oocytes, but that TBP2 expression remains unaffected in these mutants suggests that TBP2 may not be directly involved in these global chromatin rearrangements. Indeed, global transcriptional repression occurs without the chromatin condensation events in *Npm2*<sup>-/-</sup> mutants (De La Fuente *et al.* 2004). However, TBP2 accumulation in oocytes lacking NPM2 persists at high levels in late follicular stages suggesting that appropriate chromatin condensation during the prophase of the meiosis is necessary for the decrease in TBP2 accumulation that we observed in wild-type oocytes.

The notion of TBP2 being involved in oocyte-specific pathway(s) is further supported by our analysis of TBP2 localisation in mutants for a major oocyte-specific transcription factor, NOBOX, where the onset of TBP2 expression is misregulated. Unfortunately, these mutants cannot be analysed through further stages of oocyte development because of their early phenotypic defects. It is noteworthy though, that the localisation of TBP2 in NOBOX mutants is cytoplasmic, suggesting that the subcellular localisation of TBP2 is related to NOBOX function. Moreover, a crucial controlling factor for follicular development, GDF9, is necessary for maintaining TBP2 expression in the oocyte. The absence of TBP2 in primary and secondary *Gdf9*<sup>-/-</sup> oocytes may reflect the general defect in oocyte development resulting from GDF9 loss or a direct role of GDF9 in controlling TBP2 expression. Whether TBP2 is involved

in the initiation of transcription and consequent activation of particular oocyte-specific genes or has a different role in regulation of Pol II transcription remains to be determined. The possible involvement of TBP2 in regulating transcription in the maternal germ line, in the progression through meiosis or in early mouse development deserves further investigation.

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