Tob1 is expressed in developing and adult gonads and is associated with the P-body marker, Dcp2

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Short Title

Expression of Tob1 in germ cells

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Abbreviations
CCR4: Carbon catabolite repression 4
CAF1: Ccr4p associated factor
Cpeb1: Cytoplasmic polyadenylation binding protein 1
PABP: Poly(A)binding protein:
PAM2: Poly(A)-binding protein interacting motif 2
NOT: Negative on TATA-less

ABSTRACT

Tob1 is a member of the BTG/TOB family of proteins with established anti-proliferative function. In *D. rerio* and *X. laevis, Tob1* gene is expressed from the one-cell stage through to early gastrula stages, followed in later development by discrete expression in multiple tissues including the notochord and somites. In both mouse and human, *Tob1* is expressed in multiple adult tissues including the testis and ovary; however the specific cell types are unknown. In this study we examined *Tob1* gene expression in mouse in developing germ cells and in sorted male germ cells (gonocytes, spermatogonia, pachytene spermatocytes and round spermatids) by droplet digital RT-PCR, as well as anti-Tob1 protein staining in adult ovary and testis by immunofluorescence. By digital RT-PCR *Tob1* expression was very low in developing male germ cells but was highly expressed in round spermatids. In developing female germ cells, undergoing entry into meiosis, it increased 10-fold. Tob1 was also highly expressed in round spermatids and in oocytes in all stages of folliculogenesis. Notably, a marker for P-bodies, Dcp-2, was also highly expressed in round spermatids and all oocyte stages examined. The cytoplasmic presence of the Tob1 protein in round spermatids and oocytes, and association with Dcp2, in both cell types, suggests Tob1 protein may play a role in post-transcriptional mechanisms.

INTRODUCTION

Mammalian gametogenesis relies on a complex program of mitotic, meiotic and differentiation processes that are strictly regulated by stage- and germ-cell specific gene expression. We previously identified *Tob1* in an expression screen directed at identifying oocyte-specific genes in a marsupial species, *Sminthopsis macroura* (Au, et al., 2008).

Tob1 is a member of the B cell translocation gene/transducer of ErbB-2 (BTG/TOB) family of proteins, which have two highly conserved regions (defined as Box A & B) within the N-terminal that mediate protein-protein interactions (Jia and Meng, 2007). This family of proteins predates the evolution of vertebrates as, although most vertebrates contain all six BTG/TOB members, two isoforms are present in *Drosophila melanogaster*. Tob proteins also contain a conserved C-terminal amino acid domain, designated as a Poly(A) binding protein interacting motif 2 (PAM), which is also involved in protein-protein interactions. All BTG/Tob proteins have well-established roles as negative regulators of the cell cycle (Jia and Meng, 2007, Winkler, 2010).

Invertebrates including *D. melanogaster* [*Tob*, (Bourbon, et al., 2002)] and *C. elegans* [*Fog-3*, (Chen, et al., 2000)] have one *Tob* gene, whereas vertebrate species examined to date including amphibians, zebrafish and mammals have two genes -*Tob1* and *Tob2*. During development in *D. rerio* [*zTob1*, (Shi, et al., 2004)] and *X. laevis* [*xTob1* (Yoshida, et al., 2003)] the *Tob1* gene is expressed at all stages up to gastrula, but following gastrulation, expression becomes more restricted, with notable expression detected in mesodermal tissues such as the notochord and somites. *xTob1* plays an important role in ventral patterning by inhibiting transcriptional stimulation by β-catenin (Xiong, et al., 2006). Intriguingly these authors also showed mouse *Tob1* had similar biological activity in *Xenopus* embryos suggesting that *Tob1* function in dorsal-ventral patterning may be conserved.

Expression of *Tob* in *Drosophila* has not been examined but deletion of *Tob* results in recessive embryonic lethality (Bourbon et al., 2002). In *C. elegans*, *Fog3* is highly expressed during sperm formation in larval stages but is attenuated in early adult stages when eggs are being produced (Chen and Ellis, 2000). When *Fog-3* is deleted in XX hermaphrodites, oocytes rather than sperm are produced during larval stages (Chen, et al., 2001). Similarly oocytes are produced in XO males that carry *Fog-3* mutations suggesting an essential function for this gene in sperm determination.

Very little is known about the function of *Tob1* in the mammalian ovary or testis. Previous studies have demonstrated *Tob1* gene expression by Northern blot in human adult ovary and testis (Matsuda, et al., 1996) and murine ovary (Yoshida, et al., 1997). While *Tob2* transcripts have been detected in oocytes (Ikematsu, et al., 1999), spatiotemporal expression data and identification of the cell types that express Tob genes are lacking.

Recently, Ogami and colleagues demonstrated that Tob1 forms a complex with cytoplasmic polyadenylation binding protein one (Cpeb1) and CAF1 deadenylase to negatively regulate the expression of *c-myc* mRNA in quiescent cells (Ogami, et al., 2014). When these same cells were stimulated to re-enter the cell cycle, the Cpeb-Tob1-CAF1 complex was destabilised, leading to c-myc translation suggesting that translational regulation of key genes contributes to cell-cycle control. The CAF1-CCR4-CNOT complex has a well-known role in post-transcriptional mRNA processing, particularly in deadenylation of transcripts. These studies suggest that Tob1 may be involved in post-transcriptional regulation.

Other Tob1 protein interactions with members of the CCR4-NOT complex have been reported. Tob1 can interact with Cpeb or Poly(A) binding protein (PABP) and recruit the CCR4-NOT complex to initiate deadenylation (Shirai, et al., 2014). However, it is possible that the Tob1-CCR4-NOT interaction can also lead to suppression of deadenylase activity (Miyasaka, et al., 2008). Interestingly,

Ezzeddine and colleagues demonstrated human TOB11 not only regulates deadenylation and decay of target mRNAs, but importantly, is localised in processing bodies (P-bodies) (Ezzeddine, et al., 2007).

P-bodies are self-assembling, membrane-less, cytoplasmic structures that are one type of messenger ribonucleoprotein (mRNP) granules that regulate mRNA translation, storage, localization and stability (Buchan, 2014). P-bodies contain a large number of factors including many mRNAs, deadenylases including CCR4 and CAF1, XRN1 5'-3' exoribonuclease, Dcp2 decapping factor, and Cpeb1 (Eulalio, et al., 2007). P-bodies are found in most tissues, including oocytes in both *Drosophila* (Fan, et al., 2011) and mouse (Flemr, et al., 2010) but disaggregate as mouse oocytes mature (Flemr, et al., 2010). Further, an interaction of Tob1 with the mRNA binding protein, Cpeb1, that directs the formation of deadenylation complexes, has been demonstrated (Hosoda, et al., 2011, Ogami, et al., 2012). This suggests that Tob1 has a role in translational regulation. Importantly, Cpeb1 controls oocyte maturation in mouse (Racki and Richter, 2006), and *orb* (fly homologue of mammalian Cpeb1) plays a critical role in regulating *oskar* protein expression in *Drosophila* oocytes (Chang, et al., 1999).

The assembly of mRNP granules is dynamic and reversible. Many of the proteins involved are conserved across species and the role of RNA binding proteins in storage and translational repression of specific mRNA and their effects on gametogenesis, particularly meiotic arrest, are well known in *C. elegans* (McCarter, et al., 1999), *Drosophila* (de Moor, et al., 2005) and mouse (Ivshina, et al., 2014). Timing of translational activation of specific mRNAs is carefully controlled in germ cells and by storing mRNAs within mRNP granules translational competence is conferred by recruited protein partners that determine the balance between deadenylation (degradation) and polyadenylation (stability) and allowing the germ cell to respond rapidly to changes in its environmental conditions (Voronina, et al., 2011).

Tob1 is an intriguing protein that moves between the cytoplasm and the nucleus, and has been implicated in cell differentiation, growth and apoptosis via regulation of transcriptional and post-transcriptional mechanisms. In this study we investigated the expression of *Tob1* in the developing and adult ovary and testis, and its protein localization in adult mouse gonads and show that Tob1 associates with the P-body protein, Dcp2, in the developing oocyte and in round spermatids.

MATERIALS AND METHODS

Animal Ethics

All animal procedures were carried out in accordance with the guidelines stipulated by the Animal Care and Ethics Committees of the Murdoch Children's Research Institute, the University of Newcastle and the University of Melbourne.

Embryonic gonad collection, RNA Isolation and Amplification

C57Bl6 transgenic males carrying the OG2 (*Oct4* [*Pou5f1*]-eGFP) were crossed with CD1 females and successful mating was confirmed by observation of vaginal plugs. Germ cells (positive for GFP fluorescence) and somatic cells (negative for GFP fluorescence) were isolated from dissociated gonads by fluorescence activated cell sorting (FACS). Cell type identity was confirmed by the presence of *Oct4(Ddx4)* and *Mvh* germ cell-specific markers and Mvh protein in GFP positive cells, while GFP negative cells showed no expression of these markers. In addition, GFP negative cells expressed the gonad somatic cell-specific marker, *Sox9* but this marker was not expressed in GFP positive cells. These results were reported previously by van den Bergen, et al., 2009. Total RNA was isolated from embryonic day (E) 12 – E15 male and female gonads (germ cells and somatic cells), amplified and reverse transcribed as previously described (van den Bergen, et al., 2009). Final cDNA was diluted 1:10, and 1ul of cDNA was used in each ddPCR reaction.

Sorted Germ Cell Collection, Isolation and RNA extraction

Gonocytes were isolated from testes of 1 day old male mice, spermatogonia from 8-10 day old testis, pachytene spermatocytes and round spermatids were isolated from 8 week old dissociated mouse testes using a combination of collagenase and trypsin. Samples were loaded onto a 2–4% continuous bovine serum albumin (BSA) gradient, as described previously (Sutherland, et al., 2014). Cells sediment over time by gravity and are separated on the basis of size (Baleato, et al., 2005, McIver, et

al., 2012). The identities of the cell populations obtained were confirmed previously using RT-PCR (McIver, et al., 2012).

RNA extraction, cDNA synthesis and Droplet Digital PCR (ddPCR)

Cells were homogenized in TRI Reagent (Sigma, USA). RNA was extracted using RNeasy Micro Kit (Qiagen, Australia) according to the manufacturer's instructions. For evaluation of RNA, fragment size and quantification of samples were analyzed using the Agilent 2200 Tape Station (Agilent Technologies, Germany). RIN numbers were routinely >7. Total RNA (100ng) was reverse transcribed into cDNA using iScript™ Advanced cDNA Synthesis Kit (Bio-Rad, USA) according to manufacturer's instructions. For ddPCR, cDNA samples were diluted with RNAse-free water 1:10 to 1:1000 for expression analysis.

ddPCR was performed using a BioRad QX100 system. A two-step thermocycling protocol [95°C, 10 minutes; 40 x [(94°C, 30s, 60°C, 60s); 98°C, 10min, ramp rate set at 2.5°C/s)] was carried out in a BioRad C1000 Touch thermal cycler. Analysis of the ddPCR data was performed with QuantaSoft analysis software (BioRad).

ddPCR data were normalized to gene controls: Mitogen-activated protein kinase-1(*Mapk1)*, Calnexin *(Canx)*, or Peptidylprolyl Isomerase A (*Ppia or Cyclophilin A*). Each ddPCR was performed in triplicate (unless otherwise stated) on at least three separate cell or tissue isolations, and reported as mean ± standard error of the mean (S.E.M.). *Canx* Forward Primer: 5'-CACATAGGAGGTCTGACAGC-3'; *Canx* Reverse Primer: 5'-AATTATCTACCCAGGCACCAC-3'; *Mapk1* Forward Primer: 5'-CCTTCAGAGCACTCCAGAAA-3' and *Mapk1* Reverse Primer: 5' -AATCTATGCAGTTTGGGATACAAC-3'. Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA). *Canx*-HEX 5'-/5HEX/TCGGGTCCT/ZEN/CTGGAGCACAAGGCTTT/3IABKFQ/-3'and *Mapk1*-HEX 5'-/5HEX/TCTGCTCTG/ZEN/TACTGTGGATGCCTT/3IABKFQ/-3' probes were also purchased from IDT. Tob1 PrimeTime qPCR Assay Kit was from IDT. *Tob1* Forward Primer: 5'-

CTTCAGGAGGTCGTTCACAT-3; *Tob1* Reverse Primer: 5'-AACCTTTACCACTGCCACTT-3; 5'-/56-FAM/AGCTCGCAC/ZEN/TTCTCCGATCAACC/3IABkFQ/-3', and the *Cyclophilin-A* Taqman Real-Time Assay Kit (Mm02342429_g1) were from Life Technologies.

Histology and Immunofluorescence

Ovaries and testes from 12-week-old, wild-type mice, were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight and processed for paraffin embedding. Immunofluorescence, without acidic citrate antigen retrieval, was performed on paraffin sections (7 µm) as previously described (Cain, et al., 2008). Briefly, following dewaxing, sections were blocked with 5% normal horse serum in 0.1% BSA/PBS (blocking solution) for 30 minutes before they were incubated overnight at 4°C degrees with primary antibodies to Tob1 (SC-133095, Santa Cruz Biotechnology, Dallas TX) and Dcp2 (ab28658, AbCam, Cambridge UK) diluted 1:200 in blocking solution. Control sections were incubated in the absence of the primary antibody or with a non-immune rabbit or mouse IgG to control for non-specific reactivity.

Reactivity was visualized using a secondary antibody (anti-mouse or anti-rabbit IgG) conjugated to Alexafluor-488 dye or Alexafluor-594 dye (Invitrogen, Australia), diluted 1:500 in PBS/0.1% BSA for 60 min at room temperature. After rinsing in PBS/BSA, sections were stained with 1µg/ml Hoechst dye (Sigma, Castle Hill, Australia) for 10 min to label nuclei. Images were captured using either a Zeiss Axioskop II microscope, equipped with epifluorescence and a Zeiss HC camera linked to Axioviosion Imaging software (Carl Zeiss, Melbourne, VIC, Australia), or an LSM510 Meta confocal microscope (Carl Zeiss).

RESULTS

Developmental expression male and female germ cells

To investigate the developmental expression of *Tob1* during male and female embryonic gonadal development, dissociated gonadal cells from *Pou5f1-GFP* transgenic mice (E12.5-E15.5) were sorted by FACS to isolate somatic and germ cells (van den Bergen, et al., 2009). *Tob1* expression was very low in developing male germ cells that were undergoing mitotic arrest from E12.5 – E15.5. However, in the female gonads, expression increased significantly (10-fold) in developing female germ cells undergoing entry into meiosis from E12.5 – E15.5 (Fig 1). Tob1 was also expressed in somatic cells of the developing ovary but expression levels were unchanged during this period (Fig 1). The temporal increase in *Tob1* expression during early female gonadal development, suggests this factor may play a role during early meiosis in oocytes as they begin to undergo differentiation.

Tob1 gene expression in sorted germ cells taken from neonatal and adult testis

Tob1 expression was low in gonocytes, spermatogonia and pachytene spermatocytes but appeared to be increased in the radially symmetrical round spermatids (Fig 2), which are at the initial stage of spermiogenesis, and have not yet undergone differentiation. Consistent with the RT-ddPCR and immunofluorescence data (see below), *in situ* hybridisation with riboprobes generated from the PCR fragments showed similar patterns of expression in the round spermatids (Fig. S1c-d). The higher level of expression in this cell type suggests *Tob1* may also play a role in spermiogenesis or differentiation. As far as we are aware this is the first report of *Tob1* gene expression in germ cells in the developing and adult mouse testis.

Tob1 protein expression in the adult ovary and testis associates with Dcp2, a specific P-body marker

To examine the spatial patterns of Tob1 in the ovary and testis we conducted immunofluorescence experiments with a Tob1 antibody. Strong immunoreactivity for Tob1 was detected in oocytes of early antral follicles (Fig. 3c, arrowhead) but very little reactivity was detected in surrounding granulosa cells. Comparison with control sections, incubated with non-immune mouse IgG, indicated that non-specific reactivity was detected in the stromal cells surrounding the oocytes but not within the oocytes

or granulosa cells (Fig. 3f, g). Consistent with this in situ hybridisation showed specific localization of Tob1 mRNA in the oocyte, with background levels of signal in the follicular cells (Fig. S1a,b). To determine if Tob1 was associated with P-body proteins, we conducted a co-labelling experiment with an antibody to the de-capping protein, Dcp2. Tob1 and Dcp2 were both strongly expressed in the antral follicle oocyte cytoplasm (Fig. 3b-d, arrowhead). Consistent with previous findings that P-bodies disaggregate in maturing oocytes (Flemr *et al*. 2010), both Tob1 and Dcp2 were predominantly co-localised diffusely throughout the oocyte cytoplasm as shown by confocal microscopy (Fig. 3n, o). However, the localizations were heterogeneous, with some areas of cortical cytosol enriched in Tob1, but not Dcp2, and in some regions distinct granular structures of colocalization were observed (Fig. 3p). In early primordial oocytes discrete P-bodies were evident (Fig. 3j).

In the adult testis, Tob1 immunoreactivity was evident in the cytoplasm of round spermatids (Fig 4 c) where it co-localized with Dcp2 (Fig 4d arrowheads), and in a few pachytene spermatocytes (Fig. 4d asterisks) and elongating spermatids (Fig 4d, open arrowheads) in a Stage VII seminiferous tubule. Spermatogonia and later stage round spermatids (Fig 4d arrows) are negative for both Tob1 and Dcp2 staining. Higher resolution confocal microscopy showed diffuse staining for Tob1 and Dcp2 in the cytoplasm of round spermatids but also showed co-localization in distinct puncta that resembled chromatoid bodies (Fig. 4f; arrowheads). Only weak non-specific immunoreactivity was detected in Leydig cells, when the sections were incubated with non-immune mouse and rabbit IgG (Fig. 3e). The strong immunoreactivity for Tob1, predominantly in round spermatids is consistent with the ddPCR data (Fig. 2) and mRNA expression detected by *in situ* hybridisation (Fig. S1).

DISCUSSION

In a previous PCR-based subtractive-hybridization screen, directed at identifying oocyte-specific genes in a marsupial species, *Sminthopsis macroura* (Au et al., 2008) using a technique known as cDNA Representational Difference Analysis [RDA, (Hubank and Schatz, 1994)], we isolated 70 oocyte-specific genes from primary ovarian follicles including *Tob1*, *zona pellucida 2* and *c-mos* genes. Consistent with this finding we now report that *Tob1* is developmentally regulated during development of female germ cells. In addition, *Tob1* is expressed in round spermatids in the adult gonad, prior to onset of sperm differentiation.

Our study describing *Tob1* gene expression in developing and adult mouse male germ cells extends and confirms the previous finding that *Tob1* was expressed in the human testis by Northern analysis (Matsuda, et al., 1996). In the developing testis germ cells undergo rounds of mitotic division then arrest (Western, 2009). Male germ cells become committed to spermatogenesis and undergo mitotic arrest by E13.5 -14.5 depending upon whether inbred or outbred strains are used for study (Western, et al., 2011). Analysis by ddPCR, showed *Tob1* expression was relatively low in male germ cells during this period, suggesting *Tob1* may not be involved in mitotic arrest or commitment to differentiation. As Tob1 has well-established antiproliferative activity in other tissues and cell lines (Jia and Meng, 2007, Winkler, 2010), it is plausible that Tob1 may be involved in the transition from the highly proliferative state to mitotic arrest in the developing male germ cells. However, this remains to be demonstrated.

Intriguingly, our expression data show that *Tob1* mRNA and protein are highly expressed in round spermatids of the adult testis. These radially symmetric cells are the products of meiosis and are at the onset of spermiogenesis. However, Tob1 protein expression is rapidly down-regulated as the cells complete differentiation and become sperm. Together with its co-localization with the P-body protein, Dcp2, these data suggests that Tob1 may play a role in regulating post-transcriptional processes in the differentiation of spermatids to sperm. The RNA targets and the mode of regulation (promotion of translation or deadenylation or degradation) remain to be determined.

Round spermatids are transcriptionally active and it is not until the elongated spermatid stage that transcription is silenced. During meiotic cell divisions in spermatocytes, abundant RNA binding proteins are synthesized and untranslated transcripts are stored into mRNP granules, particularly the chromatoid body which is later found in sperm (Schisa, 2012). Consistent with the finding of Dcp1 in the chromatoid body (Kotaja, et al., 2006), we found evidence for Dcp2 and Tob1 in structures that resemble chromatoid bodies in the round spermatids. However, much of the reactivity for these proteins was also found in the cytosol of these spermatids. It is well-known that the final stages of spermiogenesis rely on stored mRNAs and translational regulation. For example, protamine expression is known to be regulated at the level of translation and is among the last transcripts to be translated during differentiation of spermatozoa (Balhorn, et al., 1984). Moreover protamine transcripts are found in mRNPs during spermiogenesis (Kleene, et al., 1984). Thus it is tempting to speculate that in round spermatids, Tob1 may function to suppress translation of protamine transcripts until needed at later stages of spermiogenesis.

The temporal increase in expression during female germ cell development suggests *Tob1* plays a role in early meiosis. Female germ cells in the mouse initiate meiosis at E13.5 to 14.5. Coincident with this, the level of *Tob1* gene expression in female germ cells increased rapidly and reached a 10-fold significant increase in expression by E15.5. At these stages of development female germ cells are transcriptionally active (van den Bergen, et al., 2009) and a number of well-known oocyte-specific factors are expressed during this time including *Pou5f1, Nanog, Foxo3a, Nobox, Foxl2, and Figla* (Jagarlamudi and Rajkovic, 2012). Of these, null mutations in *Nobox and Figla* led to loss of oocytes at or before birth (Adhikari, et al., 2009). Whether these factors are upstream regulators of *Tob1* expression or indeed whether these are targets of *Tob1* is unknown.

Our findings confirm adult ovarian expression of *Tob1* reported in previous studies (Matsuda, et al., 1996, Yoshida, et al., 1997). Interestingly, *Tob1* was recently identified as an oocyte-specific gene in human primordial follicles and metaphase II oocytes as determined by transcriptome profiling (Gröndahl, et al., 2013), suggesting expression of *Tob1* in oocytes may be conserved in mammals. The

expression of Tob1 in oocytes beyond the early antral stage were not evident in this study so we do not know whether Tob1 is expressed and co-localised in the oocyte at later stages of egg maturation.

Loss of *Tob1* in mice does not appear to affect viability and leads to an increase in bone mass and larger numbers of osteoblast (Yoshida, et al., 2000). However, the effect of this mutation on other tissues or organs has not been reported. The cytoplasmic presence of the Tob1 protein in round spermatids and oocytes, and co-localization with a marker of P-bodies, Dcp2, in both cell types, suggests Tob1 may play a role in post-transcriptional mechanisms in gametogenesis. While further studies of fertility and gametogenesis in *Tob1* mutant mice are required, there is some supporting evidence from studies of mice that lack other processing granule components such as Cpeb1 (Ivshina, et al., 2014, Ogami, et al., 2014) or CCR4-NOT complex (Doidge, et al., 2012). Importantly, Cpeb1 controls oocyte maturation in mouse (Tay and Richter, 2001). Both male and female *Cpeb1* knockout mice are sterile, and interestingly in both sexes, germ cell development arrests at pachytene due to a lack of polyadenylation and translation of the synaptonemal complex. And Cpeb1 is also required for the G2 to M transition to complete meiosis in oocytes. The role of translation regulation in these processes in germ cell development is well-known (Ivshina, et al., 2014).

Notably the CCR4-NOT complex has been implicated in multiple processes including initiation of transcription and post-transcriptional functions, and depletion of individual CNOT proteins leads to male sterility, cardiac dysfunction and osteopenia (Shirai, et al., 2014). The many functions of the CCR4-NOT complex has led to speculation that it acts as a ‘chaperone platform’ and specificity of function is provided by other docking proteins (Collart and Panasenko, 2012). Tob1 may therefore be important for guiding specific proteins or transcripts to the chaperone platform.

Clearly, RNA-binding proteins and associated mRNP granule complexes are important in the oocyte and male germ cells. The expression of the *Tob1* and its protein association with the P-body protein, Dcp2, within male and female germ cells, suggests Tob1 plays a role in translational regulation. The

identity of Tob1-mRNP granule target transcripts will lead to a better understanding of the cellular processes regulated by the transcripts sequestered into Tob1 containing P-bodies.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest in presenting the material, information or techniques described in this paper.

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FIGURE LEGENDS

Figure 1. *Tob1* gene expression increases during female germ cell development but expression in male germ cell development is low and unchanging. These germ cells carry an Oct4 (*Pou5f1*)-GFP expressing transgene which was exploited in FACS sorting to enrich for these cells. Expression is low in male developing germ cells but Tob1 expression increases during female germ cell development. Tob1 is also expressed in male and female gonadal somatic cells but expression is unchanging. Gene expression is expressed as copies/µl and normalised against *Mapk1* expression in germ cells and *Canx* in somatic cells. n=3 ± S.E.M. One-way ANOVA using Graph Prism was undertaken on all samples. * $p < 0.05$ E12.5 *vs* E15.5, ** $p < 0.05$ E13.5 *vs* E15.5. Abbreviations: GC – Germ cells, SC – Somatic cells

Figure 2: *Tob1* is highly expressed in round spermatids in the adult testis.

Tob1 is highly expressed in round spermatids; however *Tob1* has low expression in other cell types in the neonatal and adult testis. Highest expression for *Tob1* is seen in round spermatids and very low gene expression in other cell types. Expression is expressed as copies/µl normalised against *Cyclophilin A* expression. n=3 ± S.E.M. (except for Round Spermatids n=2). Abbreviations: **G** – gonocytes, **S** – spermatogonia, **P** – pachytene spermatocytes, **RS** – round spermatids

Figure 3: Tob1 and Dcp2 are co-expressed in the oocyte in all stages of folliculogenesis.

a-h: Representative sections of early antral follicles fluorescently labelled with Hoechst nuclear stain (**a, e**), anti-Dcp2 rabbit IgG (**b**) anti-Tob1 mouse IgG (**c**), non-immune rabbit IgG (**f**), non-immune mouse IgG (**g**) with merged images (**d, h**) showing all channels. Co-staining of Dcp2 and Tob1 in the oocyte cytoplasm (arrowheads, **b-d**) is indicated by yellow-orange staining, whereas the control non-immune IgG show no reactivity in oocytes (**f-h**). **j-m:** Representative sections of higher magnification merged images of follicles at various stages showing co-localization of Tob1 and Dcp2 in a primordial follicle (**j**), an early primary follicle (**k**), a primary follicle (**l**) and a secondary follicle (**m**). **n-p:**

Confocal images through oocytes of secondary (**n,o,p**), showing heterogeneity of Tob1 and Dcp2 colocalizations. The Z planes are shown as panels above and to the left of each X-Y plane image. In mature late secondary follicles, reactivity for both proteins is predominantly fine granular and some regions, particularly sub-cortical, show stronger reactivity for Tob1 (asterisks, **n, o**), whereas other more central regions in the same oocyte show intense co-localization of Tob1 and Dcp2 (dagger, **n, o**). In another oocyte, colocalization of Dcp2 and Tob1 often occurred in distinct granules, particularly in the cortical oocyte cytoplasm (arrows, **p**). Scale bar: **a-h**, 50 µm; **j-l**, 8 µm; **m-o**, 20 µm, **p**, 10 µm.

Figure 4: Tob1 and Dcp2 are co-expressed in early round spermatids during spermatogenesis. Representative sections of seminiferous tubules fluorescently labelled with Hoechst nuclear stain (**a, d**), anti-Dcp2 rabbit IgG (**b, d**) anti-Tob1 mouse IgG (**c, d**), non-immune rabbit and mouse IgG (**e**). **a-c:** At low power distinct labelling for Dcp2 (**b**) and Tob1 (**c**) is found within round spermatids within the seminiferous tubule. **d:** Merged image showing higher magnification view of the boxed region in **c**. Tob1 and Dcp2 are strongly co-localised in early round spermatids, which have large round nuclei, diffusely labelled by Hoechst dye (arrowheads). Apart from co-labelling in occasional pachytene spermatids (*), both proteins are largely absent from spermatogonia and pachytene spermatids in the basal layers of the tubules. Later stage round spermatids, which contain the characteristic chromocentre (intensely labelled with Hoechst dye), are also negative for Dcp2 and Tob1 (small arrow). Occasional elongating spermatids show diffuse labelling for Tob1, but not for Dcp2, in residual cytoplasm (open arrowheads). The approximate position of the basal lamina is indicated by the white dashed line in **d. f:** High magnification confocal microscopy, including the Z planes (top and side panels), showed distinct co-localization of Tob1 and Dcp2 in bright puncta near the nucleus that resemble chromatoid bodies (arrowheads). No specific labelling was seen if antibodies were replaced with non-immune IgG from rabbit and mouse (**e**). Scale bar: **a-c**: 75 µm; **d**: 40 µm; **e**: 100 µm; f, 20 µm.

SUPPLEMENTAL DATA

MATERIALS AND METHODS

In situ hybridisation

Digoxigenin (DIG) labelled riboprobes for *in situ* hybridization, were generated from PCR templates that incorporated T7 RNA polymerase binding sequences (Table S1) or from pGEM-T vectors (Promega, Madison WI) into which the 348 bp Tob1 PCR fragment had been cloned. The T7-PCR templates were amplified using Phusion DNA polymerase (New England BioLabs, Ipswich MA) to generate blunt-ended templates. The resulting PCR fragments were isolated from 1% agarose gels (Qiagen agarose gel extraction kit) and re-suspended in RNase-free water (200 ng/μl) for transcription of DIG-labelled sense and antisense RNA probes, using T7 RNA polymerase (Promega), according to manufacturer's instructions.

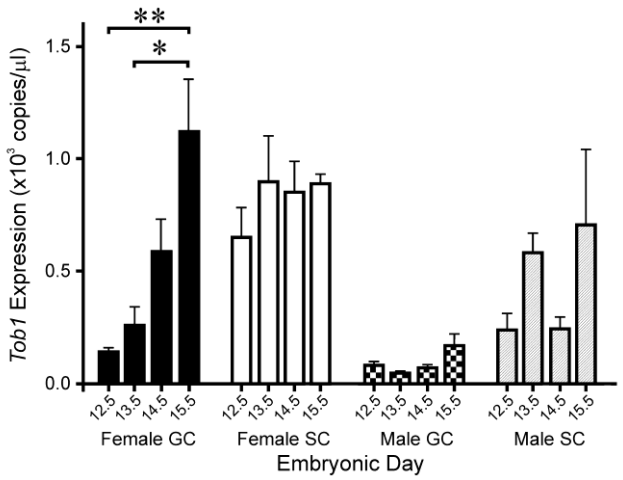
To generate riboprobes from the pGEM-T vectors, the 323 bp PCR fragment was cloned into pGEM-T according to manufacturer's instructions and both sense and antisense orientations identified by restriction digest (Pst1/HindIII) analysis. Antisense and sense plasmids were linearized with PstI, purified and re-suspended in RNAse-free water (1 μg/μl) to generate DIG-labelled sense and antisense RNA probes, using T7 RNA polymerase (Promega). *In situ* hybridization was performed on 4% paraformaldehyde-fixed frozen sections or paraffin-embedded sections. For frozen tissues, cryostat sections were fixed for 20 minutes with 4% paraformaldehyde in PBS (PFA/PBS), rinsed in PBS and digested with proteinase K (20 ug/ml in 10 mM Tris pH8, 1 mM EDTA) for 7 minutes at room temperature. Paraffin sections were de-waxed, re-hydrated and treated with proteinase-K as described above. After post-fixing in PFA/PBS, all sections were rinsed in PBS and acetylated in 0.0025% acetic anhydride in 0.1 M triethanolamine pH8 for 10 minutes at room temperature. Following treatment with 2X SSC and dehydration, sections were hybridized (55-58°C) overnight with DIG-labelled sense or antisense transcripts (>500 ng/ml) and washed with increasing stringency (4X to 0.1X SSC at 58°C) over a period of 1.5 h. Hybridization signal was visualized by incubation with anti-DIG antibody

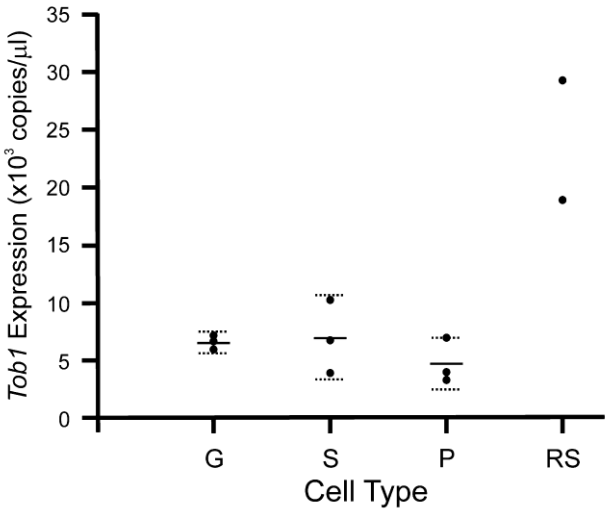
(1:1000) conjugated to alkaline phosphatase (AP) and histochemical detection with NBT/BCIP (Roche Diagnostics, Castle Hill, NSW Australia), according to manufacturer's instructions.

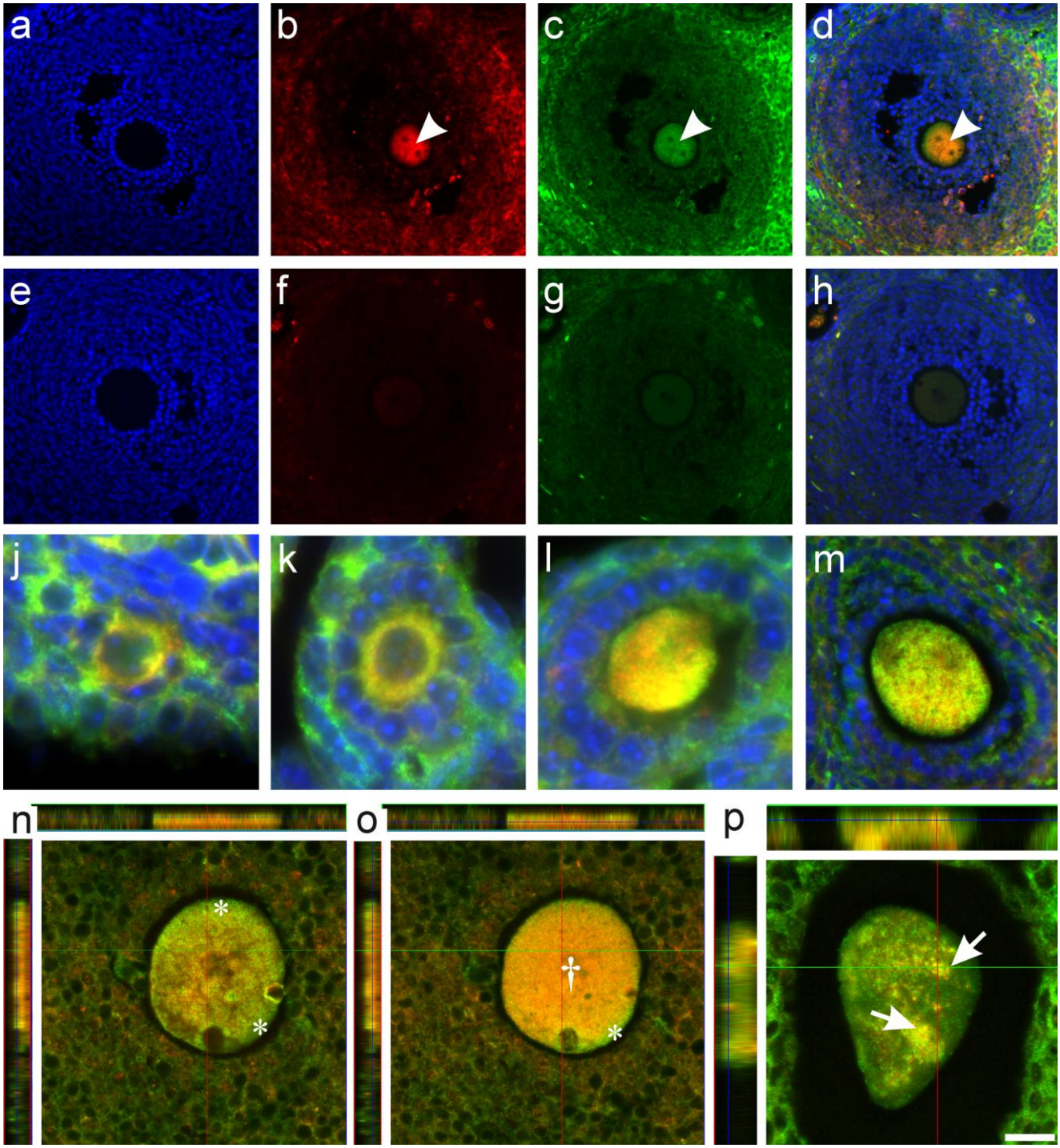
Table S1 – T7 primers used for *in situ* hybridisation probes

Mm Tob1-ISH probe	mTob1B-T7F SENSE	5'-GAAATTAATACGACTCACTATAGGGCAGCTTGAACAAGACCACG-3' 5'-ACCCTGAGCCTTTGTATGGC-3'	324 or 348 bp	62°C
	mTob1B-T7R ANTISENSE	5'- GCAGCTTGAACAAGACCACG-3' 5-GAAATTAATACGACTCACTATAGGACCCTGAGCCTTTGTATGGC-3'		

Figure S1: *In situ* hybridisation with Tob1 sense (**a, c**) and antisense (**b, d**) riboprobes in mouse ovary (**a, b**) and mouse testis (**c, d**). **a**. In frozen sections of the ovary, hybridisation signal with the sense probe is present in the follicular granulosa cells of an antral follicle but not in the oocyte cytoplasm (*).**b**. The antisense probe shows intense labelling in follicular cells, but specific staining is present in the oocyte cytoplasm (*). **c**. In paraffin sections of the testis, no specific staining was observed with the sense probe in the seminiferous tubules **d**. Intense specific labelling was detected with the antisense probe in the round spermatids (solid arrowheads) with a weaker signal detected in elongating spermatids (open arrowheads). Inset in **d** shows higher magnification of labelled round spermatids (solid arrowhead) and elongating spermatids (open arrowhead). Scale bar: A-D, 100 µm; inset 50 µm.


**
*
1.5
1.0
0.5
0.0
Tob1 Expression (x10³ copies/μl)
12.5
13.5
14.5
15.5
Female GC
Female SC
Male GC
Male SC
Embryonic Day


35
30
25
20
15
10
5
0
Tob1 Expression (x10^3 copies/µl)
G
S
P
RS
Cell Type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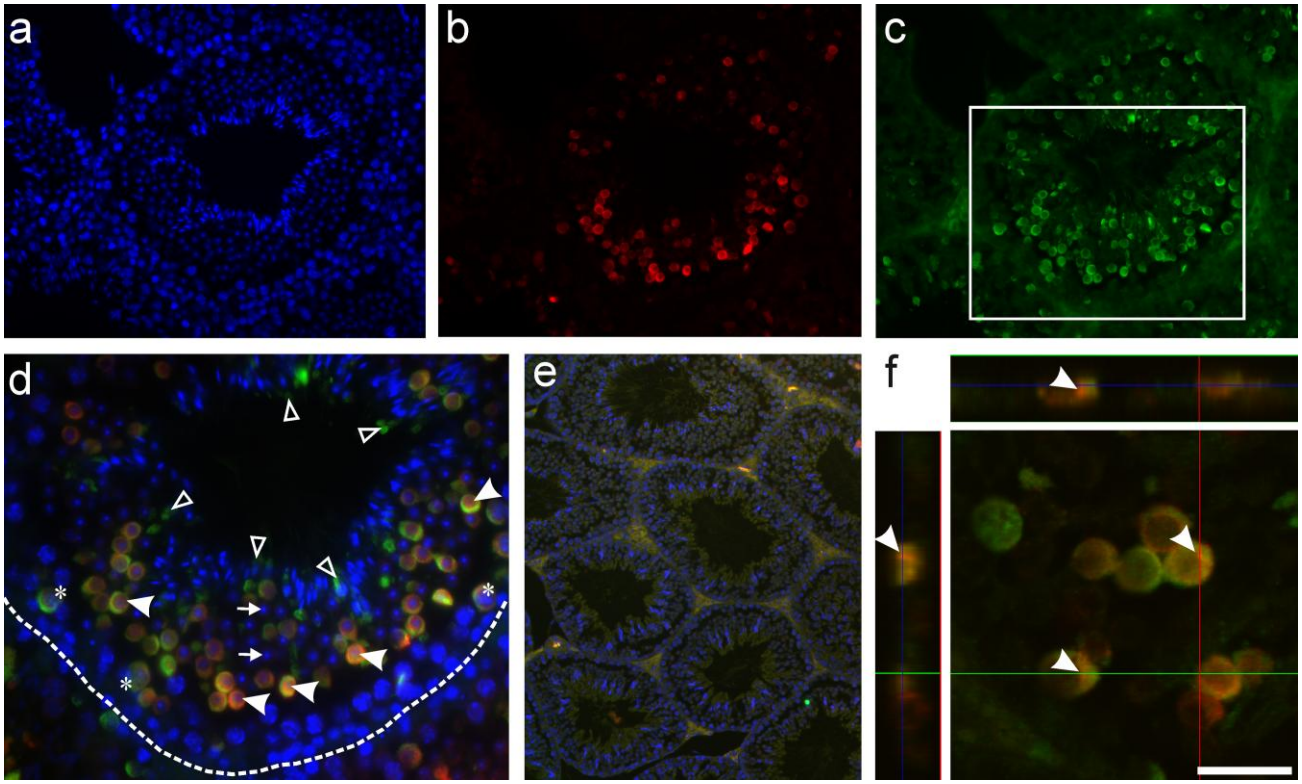
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