

# PUMA regulates germ cell loss and primordial follicle endowment in mice

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## Abstract

The number of primordial follicles initially established within the ovary is influenced by the extent of germ cell death during foetal ovarian development, but the mechanisms that mediate this death have not been fully uncovered. In this study, we identified BBC3 (PUMA) (p53 upregulated modulator of apoptosis, also known as BCL2-binding component 3), a pro-apoptotic BH3-only protein belonging to the BCL2 family, as a critical determinant of the number of germ cells during ovarian development. Targeted disruption of the *Bbc3* gene revealed a significant increase in the number of germ cells as early as embryonic day 13.5. The number of germ cells remained elevated in *Bbc3*<sup>-/-</sup> female mice compared with WT female mice throughout the remainder of embryonic and early postnatal life, resulting in a 1.9-fold increase in the number of primordial follicles in the ovary on postnatal day 10. The increase in the number of germ cells observed in the ovaries of *Bbc3*<sup>-/-</sup> mice could not be attributed to the altered proliferative activity of germ cells within the ovaries. Furthermore, BBC3 was found to be not required for the massive germ cell loss that occurs during germ cell nest breakdown. Our data indicate that BBC3 is a critical regulator of germ cell death that acts during the migratory phase of oogenesis or very soon after the arrival of germ cells in the gonad and that BBC3-mediated cell death limits the number of primordial follicles established in the initial ovarian reserve.

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## Introduction

Primordial follicles, comprising an immature oocyte surrounded by a single layer of squamous granulosa cells, are the storage unit for the female germ line (Hutt *et al.* 2006). Despite recent controversy (Byсков *et al.* 2005, Telfer *et al.* 2005, Skaznik-Wikiel *et al.* 2007, Kerr *et al.* 2012a), it is widely believed that the pool of primordial follicles present in the ovary at birth represents the entire complement of oocytes available to females throughout their reproductive life, with all oocytes for subsequent ovulation being derived from this initial stockpile. From birth onwards, the number of primordial follicles steadily declines until the pool becomes exhausted, leading to the end of fertility, followed by the beginning of menopause in women (Faddy & Gosden 1995). The mechanisms that control the size of the primordial follicle pool at birth are poorly understood. However, it is essential to understand this process because the duration of a woman's reproductive

life is influenced by the number of primordial follicles with which she is endowed (de Bruin & te Velde 2004).

The primordial follicle population is established by a series of events that occur during embryonic and neonatal development (Pepling 2006). In mice, the process of oogenesis begins at embryonic (E) day 7.5 with the allocation of the embryonic precursors of adult gametes, known as primordial germ cells (Tam & Snow 1981, Lawson & Hage 1994). The germ cells migrate from the base of the allantois through the hindgut and begin to colonise the genital ridge at ~E11.5 (McLaren 1988, 2000, Molyneaux & Wylie 2004). Following their arrival in the developing gonad, the germ cells lose their motility and become oogonia. Primordial germ cells proliferate during their migration and continue to do so as oogonia after colonisation of the gonads, leading to a dramatic increase in the number of germ cells (Tam & Snow 1981, Molyneaux & Wylie 2004). The dividing oogonia are found clustered in germ cell nests, arising

from incomplete cytokinesis and cell aggregation (Pepling & Spradling 1998, Lei & Spradling 2013). At E13.5, the first oogonia cease to proliferate, begin to enter the early stages of meiosis and differentiate into primary oocytes (Borum 1961). The transition from mitosis to meiosis, occurring between E13.5 and E15.5 in mice, corresponds to the peak in germ cell number. Around the time of birth, and coincident with a massive wave of germ cell loss, the nests break down. The individual oocytes then arrest in meiosis I at diplotene and are enclosed in a layer of granulosa cells, becoming primordial follicles. In mice, the entire process of follicle formation is complete around postnatal (PN) day 5 (Pepling 2006). In humans, the process of primordial follicle endowment is analogous, with the peak in germ cell number occurring at mid-gestation and the completion of primordial follicle assembly occurring before birth (Motta *et al.* 1997).

Apoptosis probably occurs at all stages of oogenesis, but is currently best characterised during the mitotic–meiotic transition (mouse, E13.5–E15.5) and nest breakdown (mouse, E17.5–PN3) (Coucovanis *et al.* 1993, Pepling & Spradling 2001, McClellan *et al.* 2003), when the number of germ cells declines dramatically. The reason behind the massive depletion is not known, though it has been suggested that apoptosis may be required for nest breakdown to enable individual primordial follicles to assemble (Pepling & Spradling 2001) and/or to ensure that defective or low-quality oocytes (e.g. those with DNA damage) are eliminated (Pepling & Spradling 2001, Tilly 2001). It is likely that germ cells express a number of apoptotic regulators, providing them with flexibility to respond to a wide variety of death signals, ensuring that only the ‘best’ oocytes survive. However, the physiological triggers of germ cell death and the pathways mediating their demise have not been fully characterised.

BBC3 (PUMA) (p53 upregulated modulator of apoptosis, also known as BCL2-binding component 3) is a pro-apoptotic protein that belongs to the BH3-only subgroup of the BCL2 protein family. Interactions between the BH3-only proteins (BBC3, BCL2L11 (BIM), BID, BFL, PMAIP1 (NOXA), BAD, BIK and HRK), the BAX/BAK pro-apoptotic subgroup and the pro-survival BCL2 family members (BCL2, BCL2L1 (BCL-XL), BCL2L2 (BCL-W), MCL1 and A1) control the intrinsic (also called ‘mitochondrial’, ‘BCL2-regulated’ or ‘stress’) apoptosis pathway (Happo *et al.* 2012). BBC3 contains a small BH3 domain, which is essential for its apoptosis-initiating function. BH3-only proteins are activated transcriptionally and post-transcriptionally by developmental cues and a broad range of cytotoxic insults (Strasser *et al.* 2000). The BH3-only proteins initiate apoptosis by binding to the pro-survival BCL2 family members (Happo *et al.* 2012). This interaction relieves the inhibition of ‘primed’ pro-apoptotic BAX/BAK at the mitochondrial membrane, which results in

cytochrome *c* release, apoptosome formation, caspase activation and consequent cellular demolition. BBC3 and certain other BH3-only proteins (BCL2L11 and tBID) may also initiate apoptosis through direct binding of BAX/BAK (Merino *et al.* 2009, Happo *et al.* 2012). BBC3 (alongside BCL2L11 and tBID) is a particularly potent inducer of apoptosis because it can bind with high affinity to all pro-survival BCL2 family members, whereas other BH3-only proteins (e.g. BAD and PMAIP1) have a restricted binding profile (Willis *et al.* 2007).

We have previously shown that the DNA damage-induced apoptosis of primordial follicle oocytes is mediated by the transcriptional activation of BBC3 by Tap63 (Kerr *et al.* 2012b), highlighting an essential role for BBC3 in the elimination of oocytes following genotoxic stress during meiotic arrest. We also found that the initial pool of primordial follicles established within the ovary of PN10 *Bbc3*<sup>-/-</sup> mice was significantly larger than that of WT controls (Kerr *et al.* 2012b), indicating that BBC3 is required for developmentally regulated apoptosis within the female germ line. In the present study, we determined the role of BBC3-mediated cell death during the migratory and/or mitotic germ cell stage. The results indicate that a significant number of germ cells are lost early in development and these early germ cell losses significantly affect the number of primordial follicles established in the initial ovarian reserve.

## Materials and methods

### Mice

*Bbc3*<sup>-/-</sup> mice on a C57BL/6 background were generated and genotyped as described previously (Villunger *et al.* 2003). Mice were maintained under a 12 h light:12 h darkness cycle in a temperature-controlled pathogen-free facility with free access to mouse chow and water. Timed matings were set up to allow the collection of embryos, and the morning a vaginal plug was detected was designated as E0.5. Animal procedures were approved by the Animal Ethics Committee of Monash University and the Walter and Eliza Hall Institute and were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

### Germ cell stereology

Ovaries from E13.5, E15.5, E17.5, PN3, PN5 and PN10 WT and *Bbc3*<sup>-/-</sup> embryos ( $n=3-7/\text{genotype}$ ) to be used for stereology were fixed in Bouin’s solution and processed into hydroxyethyl methacrylate resin (Technovit 7100; Kulzer & Co., Friedrichsdorf, Germany). Ovaries were serially sectioned (20  $\mu\text{m}$ ) with a Leica RM2165 microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) and stained with periodic acid–Schiff and counterstained with haematoxylin. Stereological data were obtained using the optical disector/fractionator technique as described previously (Myers *et al.* 2004, Kerr *et al.* 2006) using a  $\times 100$  oil-immersion objective on an Olympus

BX50 microscope equipped with an Autoscan stage (Autoscan Systems Pty Ltd, Melbourne, VIC, Australia) in conjunction with the CASTGRID stereological system (CAST 2002; Olympus). CAST is a Computer Assisted Stereological Toolbox that enables the automatic sampling of ovarian tissue sections for unbiased and efficient stereological estimation of germ cell number. Every third section, beginning from a random start, was evaluated and the number of follicles in the ovary was determined by multiplying the raw count of oocytes sampled ( $Q^-$ ) with all the three sampling fractions ( $1/f_1$ ,  $1/f_2$  and  $1/f_3$ ) as described previously (Myers *et al.* 2004). This is achieved through sampling of three parameters: sections, depth and field.

### TUNEL assay

Ovaries were fixed in 4% paraformaldehyde or formalin, dehydrated, embedded in paraffin, serially sectioned (5  $\mu$ m) and mounted on glass slides. The Apop Tag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Temecula, CA, USA) was used to detect apoptosis according to the manufacturer's instructions. The sections were counterstained with haematoxylin. To compare the numbers of apoptotic cells between WT and *Bbc3*<sup>-/-</sup> ovaries, three to five sections per ovary per animal ( $n=3-5$  animals/age per genotype) were analysed using the TUNEL assay. The number of TUNEL-positive cells (mean  $\pm$  s.e.m.) is expressed per  $10^4 \mu\text{m}^2$  ovarian tissue area using a methodology described previously (Kerr *et al.* 2012b).

### Immunofluorescence and immunohistochemistry

As has been described above, 5  $\mu$ m sections were cut for immunohistochemistry ( $n=3-5$  animals/age per genotype). Antigen retrieval was performed by microwaving the sections for 10 min in 0.01 M citrate buffer (pH 6). Endogenous peroxidases were quenched for 30 min in 0.3%  $\text{H}_2\text{O}_2$ , and non-specific binding of antibodies was blocked by incubating the sections for 1 h with 10% normal goat serum. The sections were then incubated with primary antibody against BBC3 (1:200; Abcam) (previously validated by us in mouse ovaries and shown to specifically recognise BBC3 (Kerr *et al.* 2012b)) for 1 h at 37 °C or overnight at 4 °C and later with biotinylated goat antibody against rabbit IgG (1:400; Zymed (San Francisco, CA, USA); secondary antibody) for 1 h at room temperature. The sections were incubated for a further 30 min with the avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA), and peroxidase activity was visualised using 3,3'-diaminobenzidine as the substrate. The sections were counterstained with haematoxylin. Cells stained with Ig isotype-matched control antibodies or with the primary antibody omitted were used as negative controls. Additionally, the specificity of the antibody against BBC3 was confirmed using *Bbc3*<sup>-/-</sup> ovarian tissue sections as negative controls; no staining was observed.

The primary antibodies used for immunofluorescent staining were specific to DDX4 (MVH) (1:800; Abcam), H2AFX ( $\gamma$ H2AX) (1:500; Abcam), phosphohistone-3 (PH3; Ser28) (1:200; Upstate, Lake Placid, NY, USA) and TRP63 (TAP63) (gift from Dr F McKeon (Suh *et al.* 2006)). Antigen retrieval and blocking were performed as described above. After incubation with primary antibodies overnight at 4 °C, the sections were incubated at room

temperature for 1 h with Alexa568-conjugated goat antibodies against mouse IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa488-conjugated goat antibodies against rabbit IgG (1:1000; Molecular Probes, Mulgrave, VIC, Australia). The sections were counterstained with TOPRO3 (Molecular Probes). Slides were mounted with Fluorsave (Millipore, Temecula, CA, USA) and analysed by fluorescent confocal microscopy using an Olympus IX81 microscope.

The proportions of mitotic germ cells were estimated by dividing the number of germ cells positive for both PH3 and DDX4 by the total number of DDX4-positive germ cells in three to five sections per ovary per animal ( $n=3-5$  animals/age per genotype). Care was taken that a middle cross-section and at least one section on either side were analysed. Similar sampling was performed to determine the timing of meiotic progress (i.e.  $\gamma$ H2AX staining) and meiotic arrest (i.e. TAP63 staining).

### Statistical analysis

Data are presented as means  $\pm$  s.e.m., and statistical analysis was carried out using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA, USA). Two-tailed unpaired *t*-tests were used for single comparisons of WT and *Bbc3*<sup>-/-</sup> ovaries for each age. For each age, three to seven ovaries were assessed, and  $P < 0.05$  was considered statistically significant.

## Results

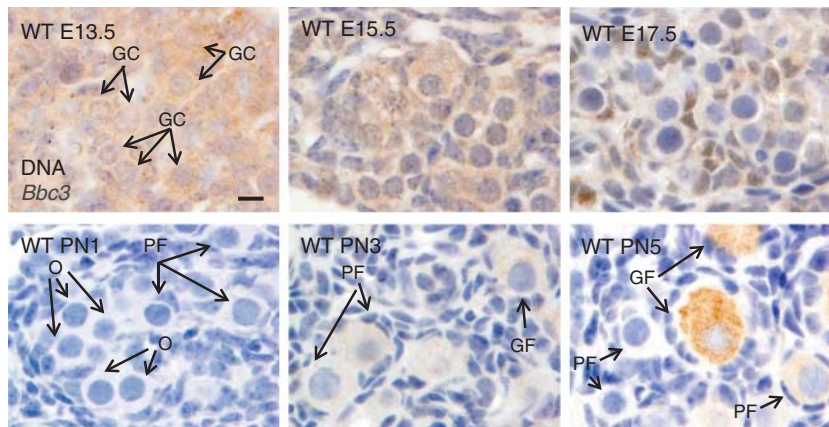
### BBC3 expression during ovarian development

The expression of BBC3 protein was assessed by immunohistochemistry in the ovaries of WT mice (Fig. 1). BBC3 was weakly detected, represented by diffuse staining, throughout the ovaries at E13.5 and E15.5. At E17.5, BBC3 was undetectable in germ cells, though some weak somatic cell staining was observed. BBC3 was undetectable in somatic cells, naked oocytes and primordial follicles at PN1, PN3 and PN5. However, the oocytes of some growing follicles, present in the ovary as early as PN3, expressed BBC3.

### Germ cell and follicle numbers are elevated in embryonic and early postnatal *Bbc3*<sup>-/-</sup> ovaries

Germ cells and follicles were enumerated in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice during embryonic and early postnatal development. The number of germ cells per ovary was elevated in *Bbc3*<sup>-/-</sup> embryos compared with their WT counterparts as early as E13.5 ( $2850 \pm 354$  WT vs  $6408 \pm 738$  *Bbc3*<sup>-/-</sup>,  $P < 0.05$ ; Fig. 2), which corresponds to the period when germ cells undergo proliferation to form germ cell nests. The number of germ cells was still elevated in the ovaries of *Bbc3*<sup>-/-</sup> mice at E15.5, though the difference when compared with WT controls was not significant ( $14\,587 \pm 1280$  WT vs  $21\,059 \pm 3440$  *Bbc3*<sup>-/-</sup>,  $P = 0.45$ ). At E17.5, the ovaries of WT mice contained  $13\,981 \pm 4052$  germ cells,





**Figure 1** BBC3 expression during ovarian development. BBC3 expression was examined in the ovaries of E13.5–PN5 WT mice by immunohistochemistry (brown). Data are representative of  $n=3-5$  animals/age. GC, germ cell; O, oocyte; PF, primordial follicle; GF, growing follicle. Scale bar: 10  $\mu\text{m}$ .

whereas those of  $Bbc3^{-/-}$  mice contained  $24\,984 \pm 4052$  germ cells ( $P < 0.05$ ). Between E17.5 and PN10, the period when germ cell nests break down and primordial follicles assemble, there was a reduction in the number of germ cells in the ovaries of both WT and  $Bbc3^{-/-}$  mice. However, the number of germ cells remained significantly higher in the ovaries of  $Bbc3^{-/-}$  mice than in those of WT mice at PN3 ( $7111 \pm 978$  WT vs  $10\,923 \pm 966$   $Bbc3^{-/-}$ ,  $P < 0.05$ ) and PN10 ( $4534 \pm 396$  WT vs  $8488 \pm 1233$   $Bbc3^{-/-}$ ,  $P < 0.05$ ) (Fig. 2). Thus, the 2.2-fold increase in the number of germ cells in  $Bbc3^{-/-}$  mice at E13.5 was maintained throughout ovarian development and resulted in a 1.9-fold increase in the number of primordial follicles initially established in the ovary.

### Apoptosis in the developing ovary

The TUNEL assay was used to assess apoptosis in the ovaries of WT and  $Bbc3^{-/-}$  mice throughout ovarian development (Fig. 3). Apoptotic cells were detected at all embryonic stages analysed (E13.5–E17.5), but there was no significant difference in the numbers of TUNEL-positive cells detected in the ovaries of WT and  $Bbc3^{-/-}$  mice. Apoptotic cells were also observed in similar numbers in the ovaries of both WT and  $Bbc3^{-/-}$  mice at PN1, the period when nest breakdown and follicle formation occur. Very low-to-negligible numbers of apoptotic cells were detected between PN3 and PN10.

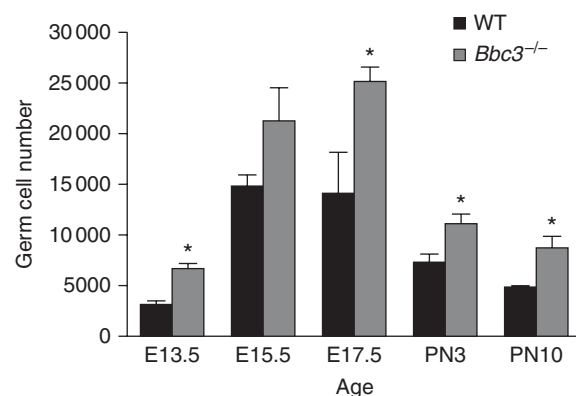
### Proliferation is normal in $Bbc3^{-/-}$ ovaries

As increased numbers of cells can result from increased proliferation, mitosis was investigated by immunofluorescent staining for PH3 (a marker of mitotic cells) and DDX4 (a marker of germ cells) (Fig. 4A). The ovaries of E13.5 mice were selected for this analysis because germ cell proliferation occurs at E13.5. No significant difference was observed in the percentages of germ cells labelled with PH3 in the ovaries of  $Bbc3^{-/-}$  and

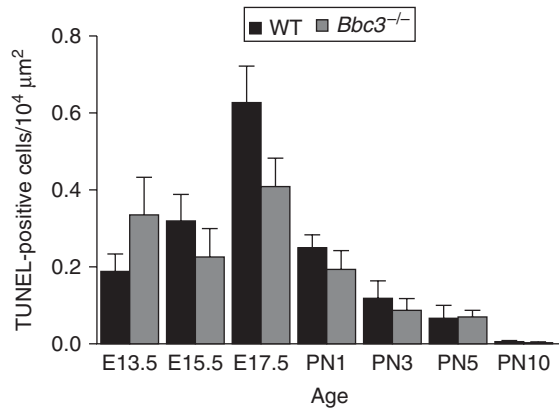
WT mice (Fig. 4B; WT =  $7.2 \pm 1.0\%$ ;  $Bbc3^{-/-}$  =  $7.9 \pm 0.6\%$ ).

### Timing of the mitotic–meiotic transition is normal in $Bbc3^{-/-}$ ovaries

Increased numbers of germ cells may also result if the proliferative period is prolonged. Therefore, the timing of meiotic progression was determined by immunostaining for the meiotic prophase marker H2AFX, which is expressed from leptotene to pachytene. The first H2AFX-positive oocytes were detected at E13.5 in the ovaries of both WT and  $Bbc3^{-/-}$  mice, but these meiotic cells were rare (Fig. 5A and B). In contrast, by E15.5, the majority of oocytes were labelled with H2AFX, but again no differences were observed between the ovaries of WT embryos and those of  $Bbc3^{-/-}$  embryos (Fig. 5B; E15.5: WT =  $93 \pm 2\%$ ;  $Bbc3^{-/-}$  =  $96 \pm 2\%$ ).  $\gamma$ H2AFX staining was no longer detected at PN3 in the oocytes of WT mice, although a very small number of oocytes in the ovaries of  $Bbc3^{-/-}$  mice exhibited persistent H2AFX expression (Fig. 5A, white arrowhead). However, the



**Figure 2** Germ cell numbers are abnormally elevated in  $Bbc3^{-/-}$  ovaries compared with WT ovaries. Germ cells were counted in ovaries from E13.5 to PN10 ( $n=3-7$  animals/age per genotype). Data are expressed as means  $\pm$  S.E.M. \* $P < 0.05$  for comparison of  $Bbc3^{-/-}$  vs WT mice at each age.



**Figure 3** Germ cell apoptosis in *Bbc3*<sup>-/-</sup> and WT ovaries during ovarian development. The number of apoptotic cells was determined by TUNEL staining in the ovaries of E13.5–PN10 WT and *Bbc3*<sup>-/-</sup> mice. Data are expressed as means  $\pm$  S.E.M.

expression of H2AFX was no longer detected in the ovaries of either WT or *Bbc3*<sup>-/-</sup> mice at PN5 (Fig. 5A and B), the period when oocytes progress into diplotene arrest and follicle formation is complete.

#### Entry into meiotic arrest is delayed in *Bbc3*<sup>-/-</sup> ovaries

Entry into meiotic arrest was monitored by staining for TRP63 (Fig. 6A and B), which has previously been shown to be expressed by oocytes as they enter diplotene (Suh *et al.* 2006). At E17.5, oocytes from the ovaries of both WT and *Bbc3*<sup>-/-</sup> mice did not express TRP63 (Fig. 6A and B). At PN1, 33% of the WT oocytes expressed TRP63, whereas TRP63 expression was not detected in any of the *Bbc3*<sup>-/-</sup> oocytes, indicating a slight delay in meiotic arrest in the ovaries of *Bbc3*<sup>-/-</sup> mice (Fig. 6A and B). No differences were detected between the ovaries of WT mice and those of *Bbc3*<sup>-/-</sup> mice at PN3 and PN5, with the expression of TRP63 being detected in nearly all WT and *Bbc3*<sup>-/-</sup> oocytes (Fig. 6A and B).

#### BBC3 is not required for nest breakdown

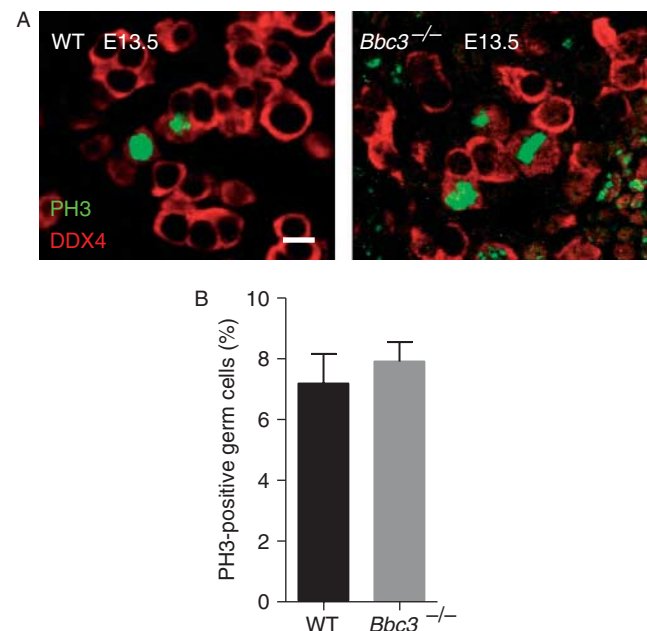
It has previously been hypothesised that apoptosis is required for germ cell nest breakdown (Pepling & Spradling 2001). Therefore, we investigated the role of BBC3-mediated apoptosis in germ cell nest breakdown by staining the ovaries of E13.5, E15.5, E17.5, PN1, PN3 and PN5 WT and *Bbc3*<sup>-/-</sup> mice for the cytoplasmic germ cell marker DDX4 to clearly visualise nest morphology (Fig. 7). Between E13.5 and E17.5, the formation of germ cell nests was evident by the appearance of germ cell clusters. No morphological differences were observed in the progression of nest development between the ovaries of WT mice and those of *Bbc3*<sup>-/-</sup> mice during this period (Fig. 7). Furthermore, nest breakdown progressed normally in the ovaries of *Bbc3*<sup>-/-</sup> mice between E17.5 and PN3 and

was complete by PN5 in the ovaries of both WT and *Bbc3*<sup>-/-</sup> mice, as indicated by the formation of individual follicle-enclosed oocytes (Fig. 7).

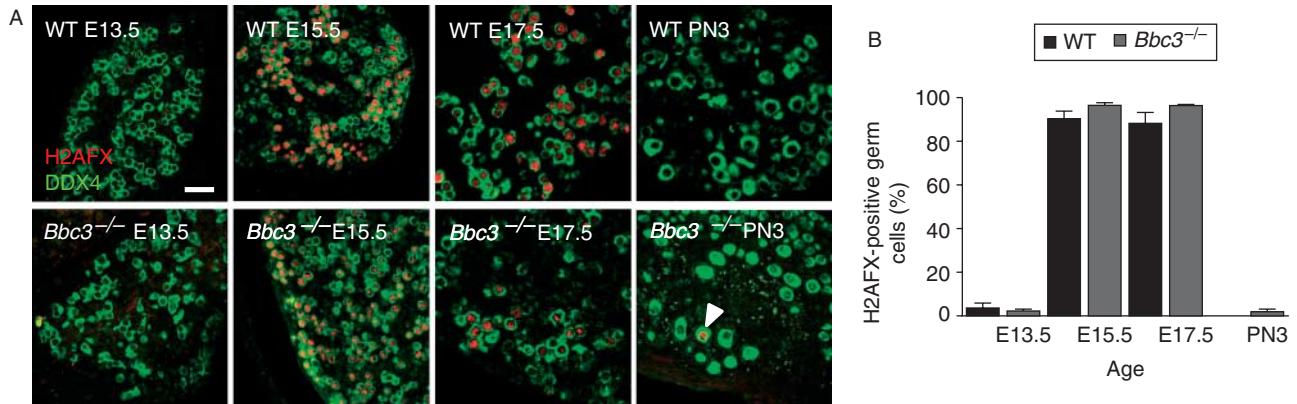
#### Discussion

The number of oocytes stored in the ovary as primordial follicles influences female fertility and reproductive life duration. The size of the initial primordial follicle reserve at birth is limited by germ cell apoptosis during embryonic ovarian development, with more than two-thirds of the germ cells being lost prior to and around the time of primordial follicle formation. Despite playing an essential role in ovarian development and female fertility, only a limited number of apoptotic regulators have been functionally characterised. We identified BBC3, a potent cell killer belonging to the BH3-only subgroup of the BCL2 family, as a key regulator of the germ cell number and primordial follicle endowment in mice. This finding, coupled with our recent investigations identifying BBC3 as a key initiator of DNA damage (elicited by  $\gamma$ -irradiation)-induced killing of primordial follicles during early postnatal development (PN5) (Kerr *et al.* 2012b), places BBC3 amongst the most important cell death regulators thus far identified in the female germ line.

Our data indicate that BBC3-mediated death has a marked effect on the number of germ cells retained at the



**Figure 4** Proliferation is normal in *Bbc3*<sup>-/-</sup> ovaries. (A) Proliferation was assessed in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice at E13.5 using immunostaining for the mitotic marker phosphohistone-3 (PH3) (red) and the germ cell marker DDX4 (green). Scale bar: 10  $\mu$ m. (B) The proportions of germ cells (DDX4 positive) that were also positive for PH3 were determined in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice at E13.5. Data are expressed as means  $\pm$  S.E.M.

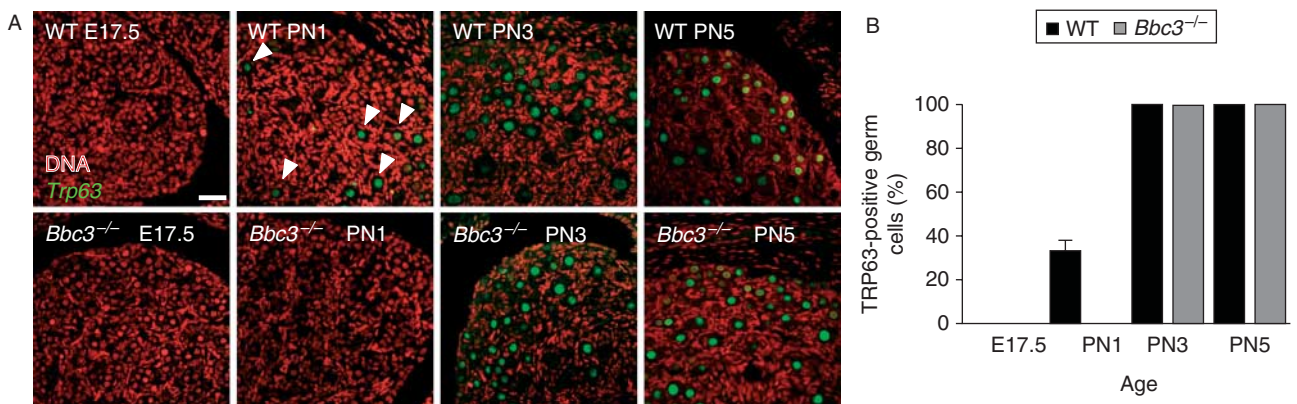


**Figure 5** *Bbc3*<sup>-/-</sup> oocytes enter meiosis at the appropriate time. (A) The timing of the mitotic–meiotic transition was assessed in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice from E13.5 to PN3 by immunostaining for the meiotic marker H2AFX (red) and the germ cell marker DDX4 (green). Arrowhead indicates the persistence of H2AFX in a *Bbc3*<sup>-/-</sup> oocyte at PN3. Scale bar: 50  $\mu$ m. Data are representative of  $n=3-5$  animals/age per genotype. (B) The proportions of germ cells (DDX4 positive) that were also positive for H2AFX were determined in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice. Data are expressed as means  $\pm$  S.E.M.

earliest stages of ovarian development. The loss of *Bbc3* was found to result in 2.2-fold more germ cells by E13.5, indicating that BBC3 acts prior to this period when proliferating germ cells are migrating or very soon after their arrival in the developing ovary. An increased number of oocytes were sustained throughout embryonic and early postnatal life to result in an overabundance of primordial follicles at PN10. These data highlight the importance of apoptosis in the regulation of the number of germ cells during migration or soon after their arrival in the gonad and indicate that BBC3-mediated actions are key determinants of the size of the ovarian reserve.

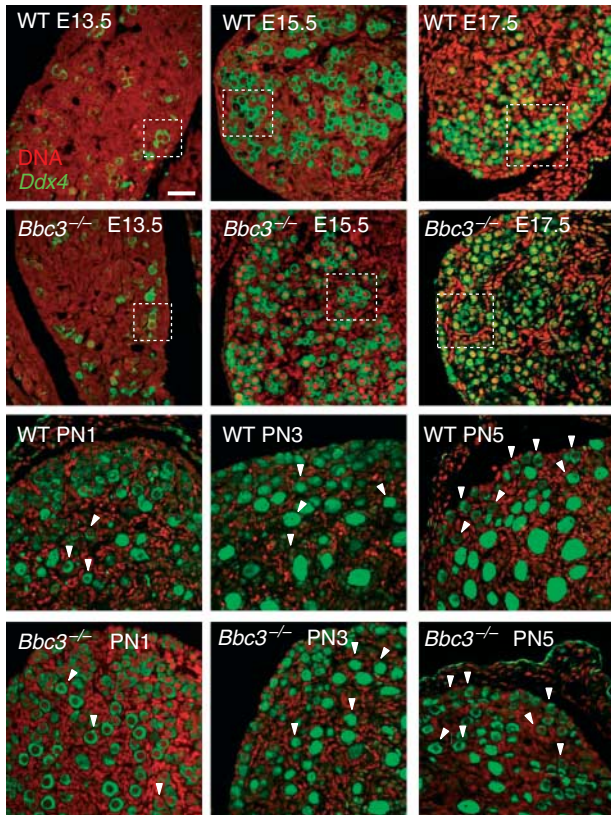
To rule out the possibility that the increased numbers of germ cells in the ovaries of *Bbc3*<sup>-/-</sup> mice were due to abnormally increased proliferation within the ovary, we examined germ cell mitosis at E13.5 using the mitotic marker PH3. We found no evidence for increased

proliferation that might explain the increase in the number of germ cells observed in *Bbc3*<sup>-/-</sup> mice. We also investigated the possibility that germ cells undergo prolonged proliferation in *Bbc3*<sup>-/-</sup> females by staining for the meiotic marker H2AFX to determine the onset of meiosis. Our data indicate that germ cells in the ovaries of both WT and *Bbc3*<sup>-/-</sup> mice enter meiosis between E13.5 and E15.5, which is consistent with previous reports (Greenfeld *et al.* 2007). Interestingly, although  $\gamma$ H2AX staining was no longer evident in WT oocytes at PN3, a small number of *Bbc3*<sup>-/-</sup> oocytes continued to express H2AFX during this stage. We also observed a slight delay in the expression of TRP63, a marker of diplotene arrest, in the ovaries of *Bbc3*<sup>-/-</sup> mice. These data indicate that *Bbc3*<sup>-/-</sup> oocytes enter meiosis at the appropriate time, but that a slight prolongation of the early meiotic prophase occurs in the absence of *Bbc3*. As all *Bbc3*<sup>-/-</sup> oocytes appeared to have progressed to meiotic arrest by PN5 and



**Figure 6** Meiotic arrest is slightly delayed in *Bbc3*<sup>-/-</sup> oocytes. (A) The timing of the meiotic arrest was assessed in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice from E17.5 to PN5 by immunostaining for the diplotene marker TRP63 (green). DNA was counterstained with TOPRO3 (red). Arrowheads indicate TAp63 staining at PN1 in WT oocytes but not in *Bbc3*<sup>-/-</sup> oocytes. Scale bar: 50  $\mu$ m. Data are representative of  $n=3-5$  animals/age per genotype. (B) The proportions of germ cells that were positive for TRP63 were determined in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice. Data are expressed as means  $\pm$  S.E.M.





**Figure 7** Germ cell nest assembly and breakdown proceed normally in *Bbc3*<sup>-/-</sup> ovaries. Germ cell nest formation and breakdown were assessed in the ovaries of WT and *Bbc3*<sup>-/-</sup> mice from E13.5 to PN5 by immunostaining for the cytoplasmic germ cell marker DDX4 (green). DNA was counterstained with TOPRO3 (red). Germ cell nests are indicated by clusters of germ cells that appear to be connected with continuously stained cytoplasm. Germ cell nests are enclosed with boxes (white dashed lines). Nest breakdown and follicle formation are indicated by the appearance of individual oocytes. Individual oocytes are indicated with white arrowheads. Scale bar: 50  $\mu$ m. Data are representative of  $n=3-5$  animals/age per genotype.

H2AFX could no longer be detected during this stage, the significance of this brief delay is not known.

Notably, we observed very little BBC3 expression within ovaries between E13.5 and PN5. We also did not observe a significant decrease in the number of TUNEL-positive cells in the absence of *Bbc3* between E13.5 and PN5. Furthermore, our data indicate that germ cell nest breakdown (i.e. between E17.5 and PN10 in the mouse) and follicle assembly occur normally in the absence of *Bbc3*. These data indicate that BBC3 does not play a significant role in cell apoptosis within the ovary during this period. Although it has been suggested that apoptosis may be required to facilitate nest breakdown and follicle assembly (Coucovanis *et al.* 1993), work carried out by Lei & Spradling (2013) has demonstrated that nest fragmentation actually precedes germ cell apoptosis in the ovary of mice, indicating that they are independent events. Thus, the key triggers and mediators of apoptosis responsible for massive germ cell death

during this period are not known and the underlying physiological reasons for this loss are still unclear.

Although we established an essential role for BBC3 in the regulation of germ cell number, acting prior to E13.5 in mice, it is not yet clear whether BBC3 directly mediates apoptosis of germ cells or whether BBC3 acts in somatic cells, with their loss negatively affecting germ cell survival. It is also not yet understood why BBC3 is activated to eliminate germ cells during the migratory and/or mitotic phase of development. We have previously shown that BBC3 is critical for the elimination of primordial follicle oocytes that have sustained DNA damage (Kerr *et al.* 2012b). Thus, it is possible that germ cells with genetic damage arising from rapid and successive rounds of mitosis are also eliminated by BBC3. Consequently, future studies will exactly pinpoint when, between germ cell specification and arrival in the gonad, BBC3 mediates apoptosis and determine precisely which cells are targeted for demolition by BBC3 and will also evaluate the quality of oocytes in *Bbc3*<sup>-/-</sup> mice. The intrinsic apoptosis pathway has previously been shown to regulate primordial germ cell death during the migratory phase, with *Bax* being identified as important for the elimination of germ cells lagging in development or those that migrate to ectopic sites (Stallock *et al.* 2003, Runyan *et al.* 2006). However, a role for BBC3 in the mediation of germ cell loss during this period has never been investigated.

The findings of the present study are consistent with those of previous gene overexpression and knockout studies in mice demonstrating the importance of the BCL2-regulated apoptosis pathway in the regulation of the death of germ cells and determination of the number of follicles making up the ovarian reserve. For example, a reduction in the function of the anti-apoptotic protein BCL2L1 was found to lead to a reduced number of primordial follicles in neonatal ovaries (Rucker *et al.* 2000). It was demonstrated that BCL2L1 is particularly important for the survival of germ cells between E12.5 and E15.5. Similarly, deletion of the anti-apoptotic protein BCL2 was also found to reduce follicle endowment (Ratts *et al.* 1995), whereas the overexpression of BCL2 was found to increase initial follicle number by 1.7-fold, though the number of primordial follicles did not remain elevated in adult life (Flaws *et al.* 2001, 2006). Finally, deletion of the pro-apoptotic multi-BH domain protein BAX was found to result in increased germ cell survival during embryonic development, with studies indicating that BAX mediates germ cell death prior to E13.5, similar to our findings for BBC3 (Alton & Taketo 2007, Greenfeld *et al.* 2007). Furthermore, deletion of *Bax* was found to result in a 1.4-fold increase in the number of primordial follicles at birth (Greenfeld *et al.* 2007), and another study indicated that the loss of *Bax* resulted in an elevated number of follicles throughout reproductive life, possibly due to reduced follicular atresia (Perez *et al.* 1999). In these mice,

fertility was not prolonged, but age-related health problems, including bone and muscle loss, were reduced as a consequence of prolonged ovarian function (Perez *et al.* 1999, 2007). These studies support the notion that ovarian function, and possibly fertility, may be prolonged by even a modest increase in the number of initial primordial follicles, conferring significant benefits to health and fertility. However, it is important to note that contrary to the study carried out by Perez *et al.* (1999), a recent study has found no difference in the number of primordial follicles in *Bax*<sup>-/-</sup> mice compared with WT females at 1 year of age (Ke *et al.* 2013). The disparity between these two studies may be due to differences in the method of follicle counting used. For example, Perez *et al.* (1999) reported follicle numbers per ovary, whereas Ke *et al.* (2013) reported follicle numbers per standard unit of area.

In conclusion, we have shown that BBC3 is an important regulator of the number of embryonic germ cells, which translates into an increased number of primordial follicles established in the initial ovarian reserve. Given the established role of BBC3 in apoptosis, our data indicate that BBC3 initiates (directly or indirectly) the death of germ cells prior to meiotic entry, either during migration to the gonad or soon after their arrival. Future studies will characterise the role of BBC3 during this period and will also determine whether the initial over-endowment of primordial follicles conferred by the loss of *Bbc3* persists throughout reproductive life and results in prolonged fertility.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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