Bällchen is required for self-renewal of germline stem cells in Drosophila melanogaster

Bettina Herzig*, Toma A. Yakulov†*, Kathrin Klinge, Ufuk Günesdogan‡, Herbert Jäckle and Alf Herzig§**

ABSTRACT

Self-renewing stem cells are pools of undifferentiated cells, which are maintained in cellular niche environments by distinct tissue-specific signalling pathways. In Drosophila melanogaster, female germline stem cells (GSCs) are maintained in a somatic niche of the gonads by BMP signalling. Here we report a novel function of the Drosophila kinase Bällchen (BALL), showing that its cell autonomous role is to maintain the self-renewing capacity of female GSCs independent of BMP signalling. ball mutant GSCs are eliminated from the niche and subsequently differentiate into mature eggs, indicating that BALL is largely dispensable for differentiation. Similar to female GSCs, BALL is required to maintain self-renewal of male GSCs, suggesting a tissue independent requirement of BALL for self-renewal of germline stem cells.

KEY WORDS: Bällchen, Drosophila, Germline stem cells

INTRODUCTION

Tissue specific stem cell populations are actively maintained as a source for distinct cell types required for growth, development and regeneration in the adult organism (Kohlmaier and Edgar, 2008; Weissman et al., 2001). To suppress their differentiation, stem cells require extracellular signalling cues which derive from a distinct cellular microenvironment called the stem cell niche (Losick et al., 2011). In the female ovary of Drosophila melanogaster, germline stem cells (GSCs) generate asymmetric cell fates with each cell division (Li and Xie, 2005; Morrison and Spradling, 2008). One daughter cell maintains the stem cell properties of the parent GSC, whereas the other daughter cell differentiates into a cystoblast. The cystoblast then develops into a germline cyst that includes a single cell that later becomes the oocyte. The germline cyst is surrounded by follicular epithelial cells, which are derived from follicle stem cells (FSCs). Similarly, the GSCs of the male germline also self-renew and differentiate into gonialblasts, which then give rise to sperm.

The stem cell niche of the ovary contains cap cells that secrete the decapentaplegic (DPP) ligand that activates the BMP pathway in GSCs. The resultant BMP signalling leads to phosphorylation of the transcription factor Mothers against Dpp (MAD), which represses the expression of the differentiation factor bag of marbles (BAM) (Chen and McKearin, 2003; Song et al., 2004). In the male germline, the Jak/Stat signalling pathway plays a major role for GSC self-renewal (Kiger et al., 2001). However, its cell autonomous function in GSCs is restricted to orienting the division plane of GSCs relative to the niche cells (Leatherman and Dinardo, 2010). The function of Jak/Stat signalling for GSC self-renewal primarily derives from activating the secretion of the BMP ligand glass bottom boat (GBB) in male somatic stem cells, which in turn activates the BMP pathway in GSCs (Kawase et al., 2004).

The progenitors of GSCs, which are called primordial germ cells (PGCs), also utilise BMP signalling through DPP to repress BAM in the larval ovary (Gilboa and Lehmann, 2004). Maintenance of FSCs in the ovary not only requires DPP signalling but also hedgehog and wingless pathway activity (for a review, see Kirilly and Xie, 2007). Thus, BMP signalling contributes both directly and indirectly to stem cell maintenance in various stem cell populations, but it acts in conjunction with other different external signals to suppress stem cell differentiation. However, common stem cell autonomous components that maintain stem cell properties and prevent differentiation have remained unknown.

Here we report that the protein kinase encoded by the Drosophila melanogaster gene bällchen (ball, also known as Nhk-1) has a common role in maintaining self-renewal of stem cell populations. ball protein (BALL) is orthologous to the Vaccinia-related Kinases (VRKs) of vertebrates and most closely related to VRK-1 (Aihara et al., 2004). VRKs are found in all metazoan species ranging from worms to humans. VRKs of different species were found to phosphorylate the Barrier-to-Autointegration Factor (BAF) (Bengtsson et al., 2006; Gorjánáczi et al., 2007; Lancaster et al., 2007; Nichols et al., 2006), which is involved in the assembly of the nuclear lamina in Caenorhabditis elegans (Gorjánáczi et al., 2007) and the organisation of chromatin in the nucleus (Margalit et al., 2007). Moreover, hypomorphic mutations in Drosophila ball cause aberrant chromatin organisation in the oocyte nucleus and an altered pattern of histone modifications (Ivanovska et al., 2005). The analysis of ball null mutants revealed defects in proliferating tissues of the larvae, including the brain and imaginal discs (Cullen et al., 2005).

We have used systemic null mutants and mosaic analyses to characterise the function of BALL in both progenitor cells and niche-dependent stem cells. We found that BALL is required to maintain self-renewal of stem cells, which suggests that the previously described defects in proliferating tissues of ball
mutant animals is caused by the premature or unscheduled differentiation of progenitor cells rather than a general function of BALL for cellular proliferation.

RESULTS

BALL is essential to maintain the larval germline

In order to assess the function of ball in proliferating tissue and in stem cells, we generated a null allele of ball (ballΔ; supplementary material Fig. S1). ballΔ homozygotes (hereafter referred to as ballΔ mutants) die during the pupal stage, confirming previous results described for other ball null alleles (Cullen et al., 2005). ballΔ mutants already show severe morphological defects by the end of larval development which include considerably reduced gonads in both sexes, the absence of imaginal discs and severely diminished larval brains. This mutant phenotype is solely due to the ballΔ mutation, as a genomic ball transgene rescued the mutants to produce viable and fertile adults (supplementary material Fig. S1).

We explored the function of BALL in developing male and female gonads. Growth of larval testes relies on asymmetric, niche-supported divisions of GSCs, whereas growth of larval ovaries relies on symmetric divisions of primordial germ cells (PGCs) (Dansereau and Lasko, 2008). Both larval cell types are derived from embryonic PGCs, which reside in the primitive gonads of embryos. In order to establish whether the initial number of PGCs was affected in ballΔ mutant embryos, we counted their number in embryonic gonads. ballΔ mutants contained on average 10.2 PGCs (SD=1.8, n=25 gonads) which was not significantly different from the number of PGCs observed in wild type control embryos (11.0 PGCs; SD=1.4, n=27 gonads). Therefore, the smaller size of larval gonads in ballΔ mutants is not caused by a reduced number of embryonic PGCs.

We next asked when the size reduction of the male gonads occurs during larval development. In early larval testes, about 8–12 PGCs adopt GSC fate after their recruitment to the somatic hub cells (Fig. 1A). GSCs then divide and give rise to self-renewed GSCs and gonialblasts, respectively. The gonialblasts undergo four incomplete cell divisions and form a 16-cell germline cyst. GSCs and differentiating cysts can be distinguished by their position in the developing testis and by the expression of the adducin-related protein, HTS (Fig. 1A). During mid larval development of wild type testes (48 h after larval hatching, ALH), HTS localises to a spectrosome in GSCs and a branched fusome in cysts, respectively (Fig. 1B,C). In ballΔ mutant testes 48 h ALH, differentiating germline cysts were formed, but the number of GSCs was reduced (Fig. 1D,E). By the end of larval development (96 h ALH), GSCs were completely depleted from ballΔ mutant testes (n=21 testes). The depletion of GSCs in mutant testes was not caused by apoptosis as shown by staining for activated Caspase 3 (supplementary material Fig. S2), suggesting that ball is required for the maintenance of GSCs. At late larval development, ballΔ mutant testes contained cysts at progressive stages of proliferation, including mature 16 cell cysts as in the case of wild type larval testes. Based on these observations, we cannot conclude that all the ballΔ mutant cysts would eventually complete their differentiation program and give rise to 16 cell cysts. However, the presence of germline cysts in ballΔ mutant testes, which are formed by the proliferation of gonialblasts indicates that BALL is not essential for cell cycle progression and suggests that its primary function is to maintain the self-renewing capability of GSCs.

We next asked whether the absence of BALL has the same effect in the female germline. Larval ovary development depends on symmetric divisions of PGCs (Fig. 1H) (Dansereau and Lasko, 2008). Wild type ovaries during midstage of larval development contain PGCs with spectrosomes, but no differentiating cysts with HTS stained fusomes (Fig. 1J). In ballΔ mutant ovaries, however, we found fused structures in VASA expressing cells, which resembled fusomes of germline cysts (Fig. 1K,L). This result suggests that ballΔ mutant PGCs differentiated prematurely. ballΔ mutant ovaries also contained fewer PGCs (26.7±3.6, n=20 ovaries) than wild type ovaries (35.2±6.6, n=23 ovaries; P=6*10^{-6}). The reduced number of PGCs does not result from apoptosis as staining for activated Caspase 3 showed that these differentiating germline cells were not apoptotic at mid larval development (supplementary material Fig. S2). Therefore, as observed with testes, ballΔ mutant germline cells in ovaries were able to proliferate. However, they proliferated at a slightly reduced rate compared to wild type. This reduction could either be due to a direct function of BALL in modulating cell cycle progression or reflect a difference in the cell cycle length between symmetrically dividing wild type PGCs and prematurely differentiating ballΔ mutant germline cells. At later stages of larval development, massive apoptosis in ballΔ mutant PGCs was observed (Fig. 1M,N). This observation is reminiscent of previous studies on the function of nanos and pumilio, showing that mutant PGCs enter apoptosis after their premature differentiation (Gilboa and Lehmann, 2004; Wang and Lin, 2004).

BALL is essential for self-renewal of female GSCs

Our results suggest that the primary function of BALL is to maintain the undifferentiated state of male and female germline progenitor cells. To discriminate between a cell autonomous function of BALL in stem cells and a systemic non-autonomous function of BALL, we generated genetic mosaics in adult ovaries. GSCs and early germline cysts of adult ovaries are located at the anterior tip of the ovariole within a stem cell niche formed by somatic cap cells and terminal filament cells (Fig. 2A). In wild type, GSC divisions typically result in a self-renewed GSC and a cystoblast (CB). The CB divides four times into a 16-cell germline cyst in which cells remain interconnected due to incomplete cytokinesis. The most posterior cell in the cyst adopts an oocyte fate, whereas the remaining 15 cells differentiate into nurse cells. GSCs and CBs both carry a spherical spectrosome, whereas differentiating cysts contain a branched fusome. GSCs remain in direct contact with the stem cell niche with their spectrosome always orientated towards the niche and thus, they can be unambiguously distinguished from CBs (Fig. 2A). In order to generate genetic mosaics, we used the FLP/FRT system (Xu and Rubin, 1993) to functionally identify ballΔ mutant cell clones that derive from GSCs and CBs by the absence of an ovoΔ expression transgene. ovoΔ expression induces apoptosis in germline cysts (Perrimon, 1998). Thus, only cysts that derive from a ballΔ mutant GSC or CB lack the ovoΔ expressing transgene and hence, can develop into eggs. We generated ballΔ mutant germline clones by heatshock-induced flipase expression (Xu and Rubin, 1993) and monitored the number of eggs deposited at various time points after heatshock treatment (AHT).

The results show that ballΔ mutant eggs were first deposited six days AHT (Fig. 2B). This indicates that ballΔ mutant germline clones can differentiate into mature eggs. However, ballΔ mutant egg deposition vanished after three days (Fig. 2B). Germine
specific expression of ball, achieved by combining a UASp-ball-EGFP transgene with a nos-GAL4 driver transgene, restored the capacity of ball2 mutant GSCs to sustain continuous egg production (Fig. 2B). In conclusion, these results show that germline autonomous expression of ball is sufficient to allow continuous egg production and, therefore, also to maintain self-renewing GSCs.

The lack of continuous egg production from ball mutant germline clones could be explained by a defect in germline differentiation which, however, was masked by the perdurance of BALL protein after clone induction. In order to directly address the function of ball for GSC self-renewal and germline differentiation, we generated genetic mosaics identifying ball2 mutant cell clones by the absence of GFP expression (Fig. 2C). In three replicate time course experiments, we found 0.32±0.017 ball2 mutant GSCs per germarium (n=102) at two days AHT. This number decreased to 0.13±0.021 (n=94 germaria) and 0.03±0.021 (n=125 germaria) three and four days AHT, respectively. To distinguish the loss of ball2 mutant GSCs from the regular turnover of GSCs, we counted the number of wild type ‘twin clone’ GSCs in the same set of germaria. These GSCs are initially generated at the same frequency as mutant GSCs in genetic mosaics and can be identified by their increased GFP fluorescence intensity (Fig. 2D). The number of twin clone GSCs remained constant over the time course (0.38±0.049, 0.39±0.11 and 0.39±0.11 after two, three and four days, respectively), showing that ball2 mutant GSCs were not lost due to regular turnover. Furthermore, ball2 mutant cells did not undergo cell death during the time period as shown by staining for activated Caspase 3 (supplementary material Fig. S3).
results show that $ball^2$ mutant GSCs had lost their capacity to self-renew and left the niche.

To analyse the differentiation of $ball^2$ mutant germline clones, we stained for the ORB protein, which becomes progressively enriched in the oocyte during cyst differentiation (Christerson and McKearin, 1994) (Fig. 3A–C). The distribution of ORB was similar in $ball^2$ mutant and wild type cysts (Fig. 3D–F), indicating that oocyte specification was normal. However, we noted that in about half of the $ball^2$ mutant germline cysts a single nurse cell degenerated and that all oocytes displayed the characteristic defect in chromatin organisation that has been previously reported (Ivanovska et al., 2005) (supplementary material Fig. S4). Nevertheless, the overall differentiation of $ball^2$ mutant cysts continued normally beyond the mitotic stages.

Fig. 2. Self renewal of female GSCs depends on BALL. (A) Schematic drawing of the germarium of an adult ovary that illustrates the positions of the stem cell niche formed by the terminal filament cells (orange) and cap cells (red), the GSCs (green) and the differentiating germline cells (blue). Also indicated are follicle stem cells (FSCs) and follicle cells (grey). Spectrosomes in GSCs and cystoblasts as well as fusomes of cysts are illustrated in yellow. (B) Deposition of $ball^2$ mutant eggs was scored per indicated day and per female after induction of germline clones. Egg deposition was not sustained beyond day nine. Egg deposition continued if $ball$ was expressed from a germline specific transgene ($ball^{2/2}$, UASp-ball + nos-Gal4) but not by the transgene without specific expression ($ball^{2/2}$, UASp-ball). (C) Germarium at two days AHT with $ball^2$ mutant cells (dashed circles, GFP absent) and twin clone cells (solid circles, elevated GFP) counterstained for HTS and DNA. The arrowhead marks a $ball^2$ mutant GSC, which lies next to a $ball^2$ mutant cystoblast. The graph shows averaged numbers of $ball^2$ mutant GSCs per germarium from three independent time course experiments. (D) Germarium at four days AHT with a $ball^2$ germline cyst (dashed circles, GFP absent) and twin clone GSC (arrowhead) that generated multiple differentiating cells (solid circles, elevated GFP). The graph shows quantification of twin clone GSCs from the same set of germaria analysed in panel C. Anterior is to the left in the micrographs. Scale bars: 10 μm. The total number of germaria is given by n.

Fig. 3. ORB localisation is not affected in $ball^2$ mutant egg chambers. (A–C) In wild type egg chambers (GFP positive), ORB is expressed in all germline cells and the protein is enriched in the oocyte located at the posterior of the egg chamber. Staining for ORB and detection of GFP are shown in separate panels for clarity, cells were counterstained for DNA. (D–F) $ball^2$ mutant egg chambers are identified by the absence of GFP detection. ORB distribution is indistinguishable from wild type, indicating normal specification of the oocyte. Cells were counterstained for DNA. Anterior is to the left in the micrographs. Scale bars: 5 μm.
This finding is consistent with the formation of mature eggs that were observed with the germline clone experiments (see above).

Using anti-BALL antibodies, we did not detect residual BALL protein in differentiating ball mutant germline cysts (data not shown). Germline cyst formation requires four cell divisions during which residual BALL protein present in a ball mutant CB would be diluted. Residual BALL protein in GSCs would similarly be diluted by cell divisions, i.e. ball mutant GSCs were lost within 4 days, which allows about four divisions of GSCs. Although we can formally not rule out residual BALL protein below the detection limit in differentiating cysts, the data strongly support that the requirement of BALL for GSC self-renewal is at least by far more critical than for germline differentiation.

BALL is essential for self-renewal of male GSCs

To assess whether the function of BALL for self-renewal of GSC is specific for the female germline, we examined the function of BALL in GSCs of adult testes. In adult testes, about nine GSCs are in direct contact with the stem cell niche formed by hub cells at their anterior tips (Fig. 4A). Both, GSCs and the differentiating daughter cells, called goniaiblasts, carry a spectrosome with a random orientation. GSCs can be unambiguously distinguished from goniaiblasts by their position adjacent to the niche. We generated genetic mosaics with ball mutant cells visualised by the absence of GFP expression (Fig. 4B,C). Instead of a twin-clone analysis, we generated GFP marked wild type cells, since twin clone analysis was not reliable in testes GSCs (Fig. 4D,E). We counted the number of marked GSCs per testis, showing that two days AHT, both ball mutant GSCs and wild type GSCs were equally frequent, with $2.04 \pm 0.15$ ($n=93$ testes) and $1.9 \pm 0.42$ ($n=79$ testes) GSCs per testis, respectively. After three and four days AHT, however, the frequency of ball mutant GSCs decreased to $0.95 \pm 0.17$ ($n=99$ testes) and $0.32 \pm 0.11$ ($n=85$ testes), respectively, whereas the wild type control GSCs were maintained at the originally observed high frequency, i.e. $1.85 \pm 0.4$ ($n=35$ testes) and $1.86 \pm 0.56$ ($n=75$ testes) GSCs, respectively. No cell death was found in ball mutant GSCs between day two and four AHT ($n \geq 50$ testes per day). These results establish that BALL has a

![Fig. 4. Self-renewal of male GSCs depends on BALL.](image-url)
critical function in maintaining self-renewing GSCs both in the male and female germline.

**BALL is essential for functional somatic FSCs in the ovary**

We next asked whether the function of BALL is limited to stem cells of the germline and thus, we extended our analysis on the somatic cell lineage in ovaries. During egg development, germline cysts become encapsulated by epithelial follicle cells (FCs), which are continuously generated by somatic follicle stem cells (FSCs) in the germarium (Fig. 2A).

Before FCs terminally differentiate, they undergo multiple cell divisions in the follicle epithelium around the egg chambers. To assess the proliferation of wild type and ball mutant FCs, we compared the sizes of corresponding mutant and wild type twin clones (Fig. 5A–D). The average ratio of cell numbers in ball2 versus wild type twin clone pairs was 1.06±0.45 (n=31 pairs), indicating that wild type and ball2 mutant FCs proliferated at a similar rate.

Since FCs are continuously displaced from the germarium together with the developing egg chambers, the functional availability of FSCs can be monitored by the appearance of newly generated FCs in the germarium. Two days after induction of clones, 45% of the germaria (n=33) contained ball2 mutant FCs (Fig. 5E,F). However, ten days after induction of clones, no ball mutant FCs were detected (n=35), but 40% of the germaria (n=30) contained wild type twin clone FCs and non-mutant control clones were also still present (Fig. 5G,H). Because we did not directly identify FSCs as we did in the case of the GSCs, we could not discriminate whether ball mutant FSCs died, stopped proliferation or have been dislocated from the niche and differentiated. The ball loss of function phenotype in the somatic lineage of the ovary however shares extensive similarity with the germline phenotype in a sense that BALL is dispensable for the proliferation of differentiating cells but required for the continuous supply of cells that maintains tissue homeostasis through self-renewing stem cells.

**BALL is not required for BMP signalling in female GSCs**

The stem cell populations in both male and female gonads rely directly or indirectly on BMP signalling. We used the female GSC system, in which the requirement of BMP signalling is most prominent, to address the relevance of BALL to BMP signalling. We assayed two hallmarks of BMP signalling in GSCs by immunostainings, i.e. the phosphorylation of the transcription factor MAD (pMAD) and the repression of the bag of marbles (bam) gene (Song et al., 2004). In wild type as well as in ball2 mutant GSCs, pMAD was stained in GSCs (Fig. 6A,B). Consistent with functional BMP signalling and MAD phosphorylation, bam was properly repressed in ball2 mutant GSCs (Fig. 6C,D) and bam expression was normally upregulated in differentiating cells of ball2 mutant cysts (Fig. 6E,F). In conclusion, these observations indicate that BALL acts independently or downstream of the BMP signalling pathway in female GSCs.

**Loss of ball mutant GSCs from the niche does not dependent on their differentiation**

Upregulation of the differentiation factor BAM is critically required for the differentiation of CBs into germline cysts (Chen

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Fig. 5. Proliferation of ball2 mutant follicle stem cells and follicle cells. (A,B) Two days after induction of genetic mosaics, germaria still contained early follicle cells that were mutant for ball2 (arrowheads, absent GFP signal). This indicates the presence of functional ball2 mutant FSCs. Germaria were counterstained stained for DNA and for HTS, which marks the circumferences of somatic cells. (C,D) Ten days after induction of genetic mosaics that contained wild type control GFP negative cells, we detected functional FSCs by the presence of large GFP negative cell clones of early follicle cells (in between arrowheads). We did no longer find ball2 mutant early follicle cells ten days after induction of respective genetic mosaics, indicating that ball2 mutant FSCs were not maintained. (E–H) Two days after induction of ball2 mutant genetic mosaics we stained differentiating follicle cells that surround egg chambers for BALL and for HTS. Cell clones in these cells do not derive from recombinant FSCs but from a recombination event in a proliferating follicle cell. Therefore we found ball2 mutant cell clones (GFP absent, dashed circle) associated with wild type twin clones (GFP increased, solid circle). Cell numbers in ball2 mutant clones were indistinguishable from cell numbers in twin clones, indicating that ball2 mutant follicle cells proliferate at wild type rate. Anterior is to the left in the micrographs. Scale bars: 5 µm.
ball differentiation into cysts is a prerequisite for the elimination of GSCs, were lost from the stem cell niche (Fig. 7B) as described not eliminated from the niche because they ectopically initiate differentiation. These results indicate that of BALL does not bypass the requirement of BAM for cells did not differentiate into germline cysts (Fig. 7B). Thus, loss of BALL mutant GSCs from the niche. (A) Germarium at two days AHT with a ball mutant GSC (dashed circle, GFP absent) and a wild type GSC (solid circle) counterstained for HTS and phosphorylated MAD protein (pMAD). The pMAD staining is shown separately to illustrate that loss of BALL does not interfere with MAD phosphorylation. (C,D) Germarium at two days AHT with a ball mutant GSC (dashed circle) marked by the absence of β-Galactosidase (β-Gal) expression and counterstained for HTS. Expression of bam-GFP, which is not detectable in the ball mutant GSC, is shown as a separate channel. (E,F) Germarium at four days AHT with multiple, differentiating ball mutant germline cells (dashed circle, absent β-Gal) counterstained for HTS. Expression of bam-GFP is upregulated in the differentiating ball mutant germline cysts. Anterior is to the left in the micrographs. Scale bars: 10 μm.

and McKearin, 2003; Song et al., 2004). bam single mutant GSCs generate daughter cells that fail to differentiate but instead develop stem cell-like tumours in the anterior region of the germarium (Fig. 7A). We generated genetic mosaics with ball bam double mutant GSCs and asked whether their differentiation into cysts is a prerequisite for the elimination of ball mutant GSCs from the niche. ball bam double mutant GSCs, were lost from the stem cell niche (Fig. 7B) as described for ball single mutant GSCs (Fig. 2C). Moreover, in a time course experiment, we could not detect apoptotic ball bam double mutant GSCs in the niche (supplementary material Fig. S3D). Importantly, niche-detached ball bam double mutant cells did not differentiate into germline cysts (Fig. 7B). Thus, loss of BALL does not bypass the requirement of BAM for differentiation. These results indicate that ball mutant GSCs are not eliminated from the niche because they ectopically initiate differentiation, but through a differentiation-independent process.

In addition to producing differentiation defective CBs, bam mutant GSCs become “super-competitive stem cells”, i.e. they displace wild type GSCs from the stem cell niche (Fig. 7A) through cellular competition (Jin et al., 2008; Rhiner et al., 2009). The fact that this increased competitiveness of bam mutant GSCs is abrogated by the loss of ball in ball bam double mutant GSCs suggests that BALL is a factor that mediates cellular competition and that ball mutant GSCs are lost from the stem cell niche due to reduced competitiveness and independent of their ability to differentiate.

We noticed that ball bam double mutant cells did not form stem cell tumours after leaving the niche but eventually degenerated (Fig. 7B). This finding is different from ball bam double mutant GSCs that were not degenerating while they resided in the niche (supplementary material Fig. S3D), i.e. in an environment that promotes the undifferentiated state of GSCs. Similarly to ball bam double mutant germline cells outside the niche, larval PGCs lack BAM expression and ball mutant PGCs undergo apoptosis (Fig. 1M,N). Therefore, we asked whether there is a general synergistic requirement of BAM and BALL for the survival of differentiating germline cells. Ectopic expression of bam from a transgene was sufficient to induce differentiation of wild type PGCs (Fig. 7C–E), but did not maintain the survival of differentiating ball mutant PGCs (Fig. 7D–F). These observations argue against an interdependent requirement of BAM and BALL for the survival of differentiating germline cells.

**BALL activity maintains the stem cell specific organization of the nucleolus**

Loss of BALL-dependent phosphorylation of BAF was identified as the primary cause for the chromatin organization defects in ball mutant oocytes (Lancaster et al., 2007). However, we did not observe severe chromatin organization defects in ball mutant GSCs or cells other than the oocyte (supplementary material Fig. S5). This observation suggests that BALL is not generally involved in global chromatin organization, but might affect specific aspects of it in specific cells or organs. While reinvestigating the subcellular localization of BALL, we found that BALL not only covers the entire chromatin (Aihara et al., 2004), but is also highly enriched in the nucleolus (Fig. 8A–E). The nucleolus is assembled around the chromatin region that harbours the rDNA repeats and can be visualized with antibodies against Fibrillarin that marks the granular zone of the nucleolus. The nucleolus is assembled around the chromatin region that harbours the rDNA repeats and can be visualized with antibodies against Fibrillarin that marks the granular zone of the nucleolus. Compared to GSCs, the size of the nucleolus becomes significantly decreased during the differentiation of germline cysts, which suggests a reorganization of the nucleolar content, and it increases again when postmitotic germline cyst cells start to polyploidize (Neumüller et al., 2008). To see whether this change of nucleolar organization includes a reorganization of the chromatin that carries the rDNA genes, we performed DNA-FISH
experiments in combination with high-resolution STED microscopy. Comparing GSCs to differentiating germline cells revealed that the rDNA genes in the nucleolus become significantly compacted during differentiation (Fig. 8G,H).

The volume of nucleoli, as assayed by Fibrillarina staining, continuously decreased with the distance of germline cells from the stem cell niche, which reflects the differentiation process of wild type germline cysts (Fig. 8I). bam\textsuperscript{D86} mutant tumorous germline cells maintained a GSC-like nucleolar size irrespective of the distance to the stem cell niche (Fig. 8J), consistent with the fact that these cells proliferate like GSCs outside the niche. Hence, we used the bam\textsuperscript{D86} mutant cells to address whether BALL is required for a GSC-like organization of the nucleolus. In ball\textsuperscript{D86} bam\textsuperscript{D86} double mutant mosaics, the nucleoli of about half of the mutant cells were only slightly smaller (53%, \( n = 110 \) cells) than the nucleoli of bam\textsuperscript{D86} mutant cells (Fig. 8I). This observation suggests that the mechanisms that induce the expansion of nucleoli in GSCs are still functional in the absence of BALL. Maintenance of this nucleolar expansion is however dependent on BALL activity, since in the other half of the mutant cells the nucleoli were either fragmented or completely disintegrated (47%, \( n = 110 \) cells) (Fig. 8K).

Interestingly, we did not observe degeneration of nucleoli in those ball\textsuperscript{D86} bam\textsuperscript{D86} double mutant cells that resided in the stem cell niche where self-renewal is supported by BMP signalling. This result suggested that BALL is either directly or indirectly required to maintain the nucleolus of GSC-like tumorous cells that cannot differentiate due to the absence of BAM, but no longer receive the niche derived maintenance signals. To see whether BALL is required for maintenance of expanded nucleoli in cells that normally differentiate, we asked whether the nucleoli of ball\textsuperscript{D86} mutant germline cells (H) that was observed without bam overexpression (compare to Fig. 1M,N). Anterior is to the left in the micrographs. Scale bars: 10 \( \mu \)m. The total number of germaria is given by \( n \).
These observations suggest that BALL is not generally required for the generation or maintenance of an expanded nucleolus but has a specific function in tumorous stem cells.

**DISCUSSION**

Our study shows that the VRK-1 kinase BALL is required for self-renewal of germline stem cells in *Drosophila*, including the symmetrically amplifying PGCs of larvae and both male and female GSCs. These stem cells are actively maintained undifferentiated and they require BMP signalling for self-renewal that emanates from their cellular niche environments (Losick et al., 2011). In *ball* mutant female GSCs, where the requirement of BMP signalling for self-renewal is most pronounced, known targets of BMP signalling are regulated as in wild type GSCs. This indicates that BALL participates neither in the transmission nor the regulation of BMP signalling, and that it is needed to maintain stem cell character in a cell autonomous manner, irrespective of the tissue-specific maintenance signals that emanate from the niches.

The loss of self-renewing stem cells could be caused by the induction of ectopic differentiation in these cells. We blocked the differentiation pathway in *ball* mutant female GSCs by removing also the central differentiation factor BAM. The results suggest...
that ball mutant GSCs are not eliminated from the stem cell niche because they initiate germline differentiation but that the GSCs differentiate because they lost the capacity for self-renewal.

It is unclear by which mechanism BALL mediates the ability for GSC self-renewal. In ovaries, GSCs and FSCs undergo a regular turnover and are continuously replaced in the niche either by their own daughter cells or by symmetric divisions of the neighbouring stem cells (Nystul and Spradling, 2007; Xie and Spradling, 2000). The replacement of GSCs involves competition between stem cells. Cells lacking BAM for instance, successfully displace less competitive wild type stem cells in the niche (Jin et al., 2008). However, if BALL is additionally removed from bam mutant cells, they appear to loose their competitive advantage. The molecular basis of stem cell competitiveness is still poorly understood. However, it has been shown that overexpression of the Drosophila dMyc transcription factor diminutive enhances the competitiveness of GSCs (Rhiner et al., 2009) and causes significantly enlarged nucleoli and increased RNA expression in epithelial cells (Grewal et al., 2005). These observations suggest a correlation between ribosome biogenesis and GSC competitiveness. Downregulation of ribosome biogenesis appears in fact to be directly required for germ cell differentiation, since BAM activates the Mei-P26 protein which downregulates the expression of dMyc. Furthermore, when overexpressed from a transgene, dMyc abrogates the tumour growth phenotype and the size increase of nucleoli in bam mutant cells (Neumüller et al., 2008). Additional support for the proposal that increased ribosome biogenesis in stem cells is crucial for their competitiveness and for maintaining their undifferentiated state derives from studies on wicked. Wicked is an essential component of the U3 snRNP pre-rRNA processing, which is required to maintain the self-renewal of GSC (Fichelson et al., 2009). Our findings that BALL is enriched in stem cell nucleoli and required for the structural integrity of nucleoli in tumourous GSCs provide a plausible link between ribosome biogenesis and BALL-dependent competitiveness of GSCs.

Once displaced from the niche, ball mutant female GSCs differentiate according to their germline fate with only minor defects. We did not address whether the differentiation of ball mutant cells is fully completed like in the respective wild type lineages in systems other than the adult female germline, but we found, irrespective of the system we looked at, that BALL is not strictly required as proliferation factor. Especially the analysis of dividing follicle cells showed that BALL is not a cell cycle regulator. With two remarkable exceptions, BALL is also not essential for cellular survival. These exceptions, i.e. ball mutant PGCs at late larval stages and ball bam double mutant germline cells outside the ovarian niche, represent conditions in which differentiation is either not supported by the tissue or not possible due to the lack of a differentiation factor. Therefore, it is tempting to speculate that BALL becomes only essential for cellular survival, when the ball mutant stem cells are unable to ‘escape’ from self-renewal into differentiation. Although ball clearly has multiple functions, e.g. oocyte chromatin organization or modulation of female PGC proliferation rate, the common defect observed in all systems examined so far is a failure in maintaining pools of undifferentiated cells. Since BALL is not an essential proliferation factor, our data suggest that also the additional defects in ball mutant larvae, i.e. lacking imaginal discs and degenerate brains, could be due to premature loss of undifferentiated progenitor or stem cells. Analysis of these systems will eventually show whether BALL is broadly required to maintain the undifferentiated state of cells during development.

MATERIALS AND METHODS

Fly strains

The rescue allele ball2 chromosome was generated by imprecise excision of P{EP}15956 (ball1). The chromosomes P{neoFRT}82B, P{neoFRT}82B e ball2, P{neoFRT}82B e bam86, and P{neoFRT}82B e bam86 ball2 were constructed by meiotic recombination. The transgene P[w+mc UASp-ball:Gal4/EGFP=phalle]2.1 was used for BALL-EGFP expression.

Clones with ovoD1 transgenes were induced in 1–3 (ball):

1. y* w* P{ry+, hs-FLP}/1 w*; P{neoFRT}82B P{ovoDI-18}3R/P{neoFRT}82B e ball2
2. y* w* P{ry+, hs-FLP}/1 w*; P{phalle}2.1/P{GALA-nos. NGT}40; P{neoFRT}82B P{ovoDI-18}3R/P{neoFRT}82B e ball2
3. y* w* P{ry+, hs-FLP}/1 w*; P{phalle}2.1/wg861; P{neoFRT}82B P{owoDI-18}3R/P{neoFRT}82B e ball2

GFP-marked clones were induced in:

1. y* w* P{ry+, hs-FLP}/1 w*; P{neoFRT}82B P{Ubi-GFP}/3 P{neoFRT}82B e ball2 (males: w* X-chromosome replaced for Y chromosome)
2. y* w* P{ry+, hs-FLP}/1 w*; P{neoFRT}82B P{Ubi-GFP}/3 P{neoFRT}82B e bam86
3. y* w* P{ry+, hs-FLP}/1 w*; P{neoFRT}82B P{Ubi-GFP}/3 P{neoFRT}82B e bam86 ball2
4. y* w* P{ry+, hs-FLP}/1 Y; P{neoFRT}82B P{Ubi-GFP}/3 P{neoFRT}82B
5. y* w* P{ry+, hs-FLP}/1 P{bam-P-GFP}, w*; P{neoFRT}82B P{arm-lacZ.Y}/3B P{neoFRT}82B e ball2

Female flies were used (1) to assay the ball requirement in egg production, (2) for germline specific rescue and (3) to serve as control to show that rescue was not caused by basal expression of the ball transgene. Female and male flies (4) were used to generate marked ball mutant clones in germlia or testis. Control non-mutant clones were induced in male flies (7). Repression of a bam-GFP reporter construct was assayed in (8). To generate ball mutant and double mutant clones, the loss of function allele bam86 was used in (5) and (6), respectively.

To obtain ball mutant larvae, this allele was balanced with TM3, Ser1, P{ActGFP}:JMIR2 and newly hatched GFP negative larvae were collected over a two hours interval. Controls and were treated identically. Larvae were subsequently reared at a controlled density before dissection. Induced expression of bam through the P{hs-bam.O}186d integration was accomplished by heat shocks (45 min, 37.5°C) every 24 hours starting from 12 h after larval hatching.

Induction of clones

Flies aged 0–2 d were fed on yeast for 1 d at 25°C. Subsequently, three heat shocks (1 h, 38°C) were applied in 12 h intervals by placing flies in empty vials with moist foam stoppers in a water bath. In between heat shocks flies were kept on yeast at 25°C. Following heat shock treatments flies were mated to wild type flies and kept well fed at 25°C for a time course starting with the last heat shock.

Dissection and staining

All dissections were done in Schneider’s cell culture medium (Life Technologies, Paisley, UK) at room temperature (RT) for no longer than 15 min before fixation in 4% (w/v) Paraformaldehyde/PBS/50 mM EDTA, pH 7.0 for 10 min. After fixation tissue was at RT rinsed in PBS, 0.1% (w/v) Triton X-100 (PBTx), extracted with 1% (w/v) Triton X-100 for 30 min and blocked with PBTx, 10% (v/v) goat serum for at least 30 min. Staining was done in blocking solution at 4°C over night (primary antibodies) or at RT for 2 h (secondary antibodies). Primary antibodies were affinity purified rabbit anti BALL (1:400, generated
against residues 1–352 of BALL, A.H.), rabbit anti VASA (1:2500, generated against full length Vasa, A.H.), rabbit anti Cleaved Caspase3 Asp175 (Xu et al., 2006) (1:150, Cell Signaling Technologies, Boston, MA), rabbit anti pSMAD1 PS1 (1:500, gift from C. H. Heldin, Uppsala, Sweden), rabbit anti GFP (1:500; Synaptic Systems, Göttingen, Germany), mouse anti HTS 1B1 (1:10, DSHB, Iowa, USA), mouse anti ORB (4H8/6H4 1:1 mix, diluted 1:30, DSHB), mouse anti SHG DCAD2 (1:10), mouse anti Fibrillarin 38F3 (1:1000, Abcam, Cambridge, UK), rat anti BAM-C (1:500) (McKearin and Ohlstein, 1995), chicken anti beta-Galactosidase (1:1000, Abcam), Secondary antibodies against mouse and rabbit IgGs were coupled to Alexa-488, –568, –633 (1:400, Life Technologies). Secondary antibodies against chicken IgG were coupled to Cy5 (1:400, Abcam). After antibody incubations tissue was rinsed twice in PBTx and washed 3 times for 20 min in PBTx at RT. To visualize actin, staining with Phalloidin coupled to Alexa 568 (Life Technologies) was done at 2 U/ml in PBTx for 30 min at RT. For staining DNA, tissue was treated with RNaseA at 2 mg/ml in PBTx for 30 min, followed by staining by 10 μM draq5 (Biostatus, Shepshed, UK) or 1 μg/ml propidium iodide (Life Technologies) in PBTx for 10 min, respectively. Before mounting in Prolong Gold antifade medium (Life Technologies) tissue was rinsed and washed for 10 min in PBTx. For identification of GFP-marked clones direct GFP fluorescence was assayed. For analysis of the bam-GFP reporter expression germlia were stained with anti GFP primary and Alexa 488 coupled secondary antibodies.

rDNA FISH for STEM microscopy
Germlia were fixed 4 min at 37° C in 4% paraformaldehyde, 15 mM PIPES, 80 mM KCl, 20 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM DTT pH 7.2, rinsed 4 times with 2× SSC, 0.1% Tween-20 (WB), washed 10 min at RT in WB and incubated 30 min at RT in 1 μg/ml RNAse A (DNase free, Sigma). After two washes with WB for 10 min at RT, germlia were rinsed and washed once in 2× SSC, 30% formamide (deionized, Sigma), 0.1% Tween-20 (PHB) at RT. After incubation with new PHB at 37° C for 30 min PHB was removed and 60 μl of probe solution was added that contained a 1 μM mix of 5‘ biotinylated oligonucleotides in 3x SSC, 30% formamide, 0.1% Tween-20 (HYB). Oligonucleotides were (5‘ to 3‘): taagaaatttataacat, caaacaacctgcttcaatca, agaggtgtggcaggcact, atatgctcaaggctagtt, catatgaggtggcaggttcatatagtt, aatatatacatatcatatagta, atattattatgatacgtagttgcatatcactcatccatctgtatgataagttcatatgta. Germlia were incubated with the probe solution for 15 min at 37° C, heated for 2 min to 91° C and incubated over night at 37° C. Then 500 μl of PHB were added at 37° C and germlia were washed in PHB 4 times for 10 min at 37° C.

For detection germlia were washed once in EB and twice in WB for 20 min at RT each and then for 20 min in blocking reagent/WB (Roche). Then Streptavidin-dye conjugate SA-Atto647N (ATTO-TEC, Siegen, Germany) diluted 1:250 in 250 μl new blocking solution was added. Incubation was for 2 h at RT in a thermomixer at 900 rpm. Germlia were then rinsed once and washed three times for 20 min at RT in WB. DNA was counterstained with 10 μM draq5 (Biostatus) in WB and samples were mounted in Prolong Gold antifade medium (Invitrogen). Images were acquired on a Leica TCS SPS STEM microscope.

Image data collection and processing
For nucleolar size measurements z-stacks were collected at 0.1 μm z intervals and analysed by a modified Connected Threshold Grower plugin for Image J. Details on the modified plugin are available on request.

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Competing interests
The authors have no competing interests to declare.

Author contributions
B.H., T.A.Y. and A.H. designed the experiments; B.H., T.A.Y., K.K. and A.H. performed experiments and analyzed data; H.J. and A.H. supervised the study; H.J., U.G. and A.H. wrote the manuscript.

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References


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Fig. S1. Schematics of the ball locus, the ball2 mutation and ball transgenes used in this study. Schematic illustration of the ball locus, and the adjacent genes His2Av and CG5491, which are transcribed in opposite orientation relative to ball. Transcription start sites are indicated by orthogonal arrows, introns by angled lines, coding sequences by boxes and dashed line arrows indicate that not the entire sequence is displayed. Two alternatively spliced transcripts are transcribed from the ball locus which both code for an identical protein. We originally identified the ball gene based on the male sterile phenotype associated with the EP(3)0863 P-element integration (ball1). By mobilization of EP(3)0863 we could revert male sterility and recovered the wild type allele ballrev. Imprecise excision of EP(3)0863 led to partial deletion of the P-element and an associated 152 base pair deletion in the ball 5’UTR and coding sequence (ball2). The deletion associated with the ball2 allele removes the initiation codon and parts of the BALL kinase domain. The ball2 allele is lethal either homozygous or if hemizygous over the deficiencies Df(3R)ro80b and Df(3R)Tl-l but not over the deficiency Df(3R)Tl-X or the amorphic His2Av810 allele. To rescue the ball2 mutant phenotype we constructed a GAL4 inducible transgene for expression of a BALL-EGFP fusion protein (P{UASp-ball-EGFP}). In addition we constructed a 3.5 kb genomic transgene including 1.5 kb of sequence 5’ to the ball initiation codon and 218 bp of sequence 3’ to the termination codon (P{gball}). The intron within the ball coding sequence was omitted. Within P{gball} the initiation codon of His2Av was changed from ATG to CTC, replacing the only Met in His2Av by Leu. The P{gball} transgene rescued homozygous ball2 mutants to viability and fertility. Based on these observations we concluded that the ball2 allele does not affect other essential genes.
Fig. S2. Staining for apoptotic cells in ball² mutant larval gonads. Gonads of ball² mutant larvae were stained for the apoptosis marker activated Caspase 3 (aCasp3), HTS and DNA. (A–C) Mutant testis at mid larval stage (48 h ALH) did not show an increase in apoptotic cells. (D–F) Also at late larval stage (96 h ALH), no increase in apoptosis was detectable. (G–I) Wild type testis at late larval stage (96 h ALH) shown for comparison and to illustrate that our stainings were able to detect occasionally appearing apoptotic cells in wild type. (J–L) Mutant ovaries at mid larval stage (48 h ALH) show no apoptotic cells. Anterior is to the left in the micrographs. Scale bars: 15 µm (A–C), 25 µm (D–F), 50 µm (G–I), 10 µm (J–L).

Fig. S3. Staining for apoptotic cells in ball² mutant adult gonads. Germaria and testis were stained for HTS and with an antibody detecting cleaved, activated Caspase 3. (A,B) Confocal sections are shown to illustrate that apoptotic cells were detectable in our stainings of wild type germaria (A) or testis (B). (C) Upon induction of ball² mutant clones, germaria were analyzed after two days (n=86), three days (n=115) and four days (n=70) for activated Caspase 3 staining. Neither mutant GSCs nor mutant CBs were found to be apoptotic, although we could reproduce the loss of GSCs over the time-course. (D) Upon induction of ball² bam¹⁸⁸ mutant clones, germaria were analyzed after two days (n=92), three days (n=87) and four days (n=78) for activated Caspase 3 staining. No mutant cells were found in the stem cell niche. Therefore the loss of niche associated GSCs that we observed during the time-course is not caused by apoptotic cell death. Anterior is to the left in the micrographs. Scale bars: 10 µm.
Fig. S4. Proliferation of ball2 mutant germline cyst cells. (A–I) Marked ball2 mutant germline clones were identified by reduced GFP signal (note the GFP signal in follicle cells). Egg chambers were counterstained for DNA and Actin. All images represent confocal sections. Examples are shown for stage 4 (A,D,G), stage 5 (B,E,H) and stage 10A (C,F,I) egg chambers. Most aspects of egg chamber development, including polypliodization of nurse cells, appeared to be normal in ball2 mutant egg chambers. We never observed degenerating mutant egg chambers. In all mutant egg chambers we found a defect in oocyte nuclear organization (white arrowheads, inset in panels D and G shows wild type oocyte nucleus). (J,K) Two confocal sections of a stage 7 egg chamber with a ball2 mutant germline and a defective oocyte nucleus (J, white arrowhead). In a different section a nurse cell nucleus that failed to endoreduplicate is indicated (K, yellow arrowhead). Anterior is to the left in the micrographs. Scale bars: 10 μm (A,B,D,E,G,H,J,K), 40 μm (C,F,I).
Fig. S5. Chromatin organization defects in ball² mutant GSCs. Marked ball² mutant cells were identified by reduced GFP fluorescence. Preparations were counterstained for DNA and either HTS to visualize spectrosomes, or for the B-type Lamin Dm0 (Lamin). Images represent confocal sections. (A–D) Example of a ball² mutant GSC (dashed outline) indicating that loss of BALL activity leads to mild defects in nuclear organization in GSCs. In this mutant GSC chromatin is preferentially localized at the nuclear periphery leading to depletion of chromatin from the central nucleus. (E–H) Germarial tip showing two heterozygous GSCs (solid outlines). (I–L) Germarial tip showing two ball² mutant GSCs of which one shows abnormal nuclear organization (arrowhead). In the second GSC, the effect is less pronounced. Counterstaining for Lamin revealed that assembly of the nuclear Lamina is not perturbed in ball² mutant GSCs. (M) Oocyte nuclei from heterozygous (ball²/+) or ball² mutant (ball²/²) germline clones. Defective karyosome formation in ball² mutant oocytes is accompanied by extensive re-localization of chromatin to the nuclear periphery, which is marked by Lamin staining. Anterior is to the left in the micrographs. Scale bars: 2.5 μm.