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

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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 and Wilson, 2006; Gorjánácz et al., 2007; Lancaster et al., 2007; Nichols et al., 2006), which is involved in the assembly of the nuclear lamina in Caenorhabditis elegans (Gorjánácz 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 larva, 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...
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 ball mutant ovaries, however, we found fused structures in VASA expressing cells, which resembled fusomes of germline cysts (Fig. 1K,L). This result suggests that ball mutant PGCs differentiated prematurely. ball mutants 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^{-4}). 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, ball 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 ball mutant germline cells. At later stages of larval development, massive apoptosis in ball 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 oriented 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 ball mutant cell clones that derive from GSCs and CBs by the absence of an ovo^{B1} expressing transgene. ovo^{B1} expression induces apoptosis in germline cysts (Perrimon, 1998). Thus, only cysts that derive from a ball mutant GSC or CB lack the ovo^{B1} expressing transgene and hence, can develop into eggs. We generated ball 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 ball mutant eggs were first deposited six days AHT (Fig. 2B). This indicates that ball mutant germline clones can differentiate into mature eggs. However, ball mutant egg deposition vanished after three days (Fig. 2B). Germline...
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 germine 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 germine clones could be explained by a defect in germine 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 germine 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±0.017 (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±0.011 and 0.39±0.011 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 ball2 mutant GSCs had lost their capacity to self-renew and left the niche.

To analyse the differentiation of ball2 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 ball2 mutant and wild type cysts (Fig. 3D–F), indicating that oocyte specification was normal. However, we noted that in about half of the ball2 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 mutant cysts continued normally beyond the mitotic stages.
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 gonialblasts, carry a spectosome with a random orientation. GSCs can be unambiguously distinguished from gonialblasts 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±0.15 (n=93 testes) and 1.9±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±0.17 (n=99 testes) and 0.32±0.11 (n=85 testes), respectively, whereas the wild type control GSCs were maintained at the originally observed high frequency, i.e. 1.85±0.4 (n=35 testes) and 1.86±0.56 (n=75 testes) GSCs, respectively. No cell death was found in ball mutant GSCs between day two and four AHT (n≥50 testes per day). These results establish that BALL has a...
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 *ball*2 versus wild type twin clone pairs was 1.06±0.45 (n=31 pairs), indicating that wild type and *ball*2 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 *ball*2 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 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 *ball*2 mutant GSCs, pMAD was stained in GSCs (Fig. 6A,B). Consistent with functional BMP signalling and MAD phosphorylation, *bam* was properly repressed in *ball*2 mutant GSCs (Fig. 6C,D) and *bam* expression was normally upregulated in differentiating cells of *ball*2 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...
and McKearin, 2003; Song et al., 2004). bam\textsuperscript{186} single mutant GSCs generate daughter cells that fail to differentiate but instead develop stem cell-like tumours in the anterior region of the gerarium (Fig. 7A). We generated genetic mosaics with ball\textsuperscript{2} bam\textsuperscript{186} double mutant GSCs and asked whether their differentiation into cysts is a prerequisite for the elimination of ball mutant GSCs from the niche. ball\textsuperscript{2} bam\textsuperscript{186} double mutant GSCs, were lost from the stem cell niche (Fig. 7B) as described for ball\textsuperscript{2} single mutant GSCs (Fig. 2C). Moreover, in a time course experiment, we could not detect apoptotic ball\textsuperscript{2} bam\textsuperscript{186} double mutant GSCs in the niche (supplementary material Fig. S3D). Importantly, niche-detached ball\textsuperscript{2} bam\textsuperscript{186} 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\textsuperscript{186} 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\textsuperscript{186} mutant GSCs is abrogated by the loss of ball in ball\textsuperscript{2} bam\textsuperscript{186} 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\textsuperscript{2} bam\textsuperscript{186} double mutant cells did not form stem cell tumours after leaving the niche but eventually degenerated (Fig. 7B). This finding is different from ball\textsuperscript{2} bam\textsuperscript{186} 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\textsuperscript{2} bam\textsuperscript{186} double mutant germline cells outside the niche, larval PGCs lack BAM expression and ball\textsuperscript{2} 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\textsuperscript{2} 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\textsuperscript{2} 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. 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 Fibrillarin 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). bamD86 mutant tumourous germline cells maintained a GSC-like nucleolar size irrespective of the distance to the stem cell niche (Fig. 8I,J), consistent with the fact that these cells proliferate like GSCs outside the niche. Hence, we used the bamD86 mutant cells to address whether BALL is required for a GSC-like organization of the nucleolus. In ballD bamD86 double mutant mosaics, the nucleoli of about half of the mutant cells were only slightly smaller (53%, n = 110 cells) than the nucleoli of bamD86 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 ballD bamD86 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 ballD mutant cells (Fig. 8L,M) nor ballD bamD86 double mutant cells were affected in differentiating follicle cells (Fig. 8N,O).
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 ovariase, 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 Drosphila 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 snoRNP 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 provides a plausible link between ribosome biogenesis and BALL-dependent competitiveness of GSCs.

Once displaced from the niche, ball2 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 ball2 bamash 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 ruca e ball2 chromosome was generated by imprecise excision of P[EP]{P{GFP}arm-lacZ.V}82B/86 (ball1). The chromosomes P{neoFRT}82B, P{neoFRT}82B e ball2, P{neoFRT}82B e bamash and P{neoFRT}82B e bamash ball2 were constructed by meiotic recombination. The transgene P{w;wmc UASp-ball.T:Avic/EGFP=pballE}2.1 was used for BALL-EGFP expression.

Clones with ovoD1 transgenes were induced in 1–3 (ball): (1) y1 w+ P{ry+, hs-FLP}/1 w+; P{neoFRT}82B P{ovoDI-18}3R/P{neoFRT}82B e ball2 (2) y1 w+ P{ry+, hs-FLP}/1 w+; P{pballE}2.1/P{GALA-nos.NGT}40; P{neoFRT}82B P{ovoDI-18}3R/P{neoFRT}82B e ball2 (3) y1 w+ P{ry+, hs-FLP}/1 w+; P{pballE}2.1/wash61; P{neoFRT}82B P{ovoDI-18}3R/P{neoFRT}82B e ball2

GFP-marked clones were induced in: (4) y1 w+ P{ry+, hs-FLP}/1 w+; P{neoFRT}82B P{Ubi-GFP}/83 P{neoFRT}82B e ball2 (males: w+ X-chromosome replaced for Y chromosome) (5) y1 w+ P{ry+, hs-FLP}/1 w+; P{neoFRT}82B P{Ubi-GFP}/83 P{neoFRT}82B e bamash (6) y1 w+ P{ry+, hs-FLP}/1 w+; P{neoFRT}82B P{Ubi-GFP}/83 P{neoFRT}82B e bamash ball2 (7) y1 w+ P{ry+, hs-FLP}/1 Y; P{neoFRT}82B P{Ubi-GFP}/83 P{neoFRT}82B (8) y1 w+ P{ry+, hs-FLP}/1 P{bam-P-GFP}, w+; P{neoFRT}82B P{arm-lacZ.Y}/83B/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 ball2 mutant clones in germinaria 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 bam2 mutant and double mutant clones, the loss of function allele bamash was used in (5) and (6), respectively.

To obtain ball2 mutant larvae, this allele was balanced with TM3, Ser1, P{act-GFP}: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.OVA16d 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 SHGC DACD2 (1:10), mouse anti Fibrillin 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 PBTrx and washed 3 times for 20 min in PBTrx at RT. To visualize actin, staining with Phalloidin coupled to Alexa 568 (Life Technologies) was done at 2 U/ml in PBTrx for 30 min at RT. For staining DNA, tissue was treated with RNaseA at 2 mg/ml in PBTrx for 30 min, followed by staining with 10 μM draq5 (Biostatus, Shepshed, UK) or 1 μg/ml propidium iodide (Life Technologies) in PBTrx for 10 min, respectively. Before mounting in Prolong Gold antifade medium (Invitrogen). DNA was counterstained with 10 μg/ml propidium iodide.

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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


