Bällchen participates in proliferation control and prevents the differentiation of Drosophila melanogaster neuronal stem cells

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ABSTRACT

Stem cells continuously generate differentiating daughter cells and are essential for tissue homeostasis and development. Their capacity to self-renew as undifferentiated and actively dividing cells is controlled by either external signals from a cellular environment, the stem cell niche, or asymmetric distribution of cell fate determinants during cell division. Here we report that the protein kinase Bällchen (BALL) is required to prevent differentiation as well as to maintain normal proliferation of neuronal stem cells of Drosophila melanogaster, called neuroblasts. Our results show that the brains of ball mutant larvae are severely reduced in size, which is caused by a reduced proliferation rate of the neuroblasts. Moreover, ball mutant neuroblasts gradually lose the expression of the neuroblast determinants Miranda and aPKC, suggesting their premature differentiation. Our results indicate that BALL represents a novel cell intrinsic factor with a dual function regulating the proliferative capacity and the differentiation status of neuronal stem cells during development.

KEY WORDS: Bällchen, Drosophila, Neuroblasts, Stem cells

INTRODUCTION

Multicellular organisms have to maintain a balance between cell proliferation and differentiation. Differentiation leads to mitotically quiescent cells, whereas development, growth, tissue homeostasis and regeneration require cellular proliferation (Buttitta and Edgar, 2007). One strategy to ensure a balance between these cellular processes is based on self-renewing stem cells. Stem cells are maintained as proliferative and undifferentiated cells, whereas their daughter cells initiate differentiation (Kim and Hirth, 2009). Therefore, stem cell divisions require repetitive cell fate decisions, which are either controlled by external signals that emanate from stem cell niches or dependent on asymmetrically distributed factors in the dividing stem cells (Kim and Hirth, 2009).

Neuronal stem cells of Drosophila melanogaster, called neuroblasts (NBs), represent a well-studied stem cell system, which depends on asymmetric distribution of cell fate determinants (Chia et al., 2008; Kim and Hirth, 2009; Knoblich, 2010). During embryogenesis, NBs delaminate from a neuroepithelium and maintain the apical-basal polarity of this epithelium. This inherent asymmetry is then used to localize cell fate determinants either at the apical or basal cell cortex of the NB, which subsequently leads to an asymmetric partitioning of these determinants between the two daughter cells during cell division (Knoblich, 2010). Self-renewed NBs inherit the apical cortical proteins such as the atypical protein kinase C, Par6, Bazooka/Par3, Insuteable, Partner of Insuteable and Gz1, whereas the basal cortical proteins, that include cell fate determinants such as Prospero and Brat, are inherited by the differentiating ganglion mother cell (GMC). Their localized retention requires the coiled-coil adaptor protein Miranda (MIRA), which is subsequently degraded in the differentiating GMC (Shen et al., 1997). NB divisions are also characterized by a morphological asymmetry, since the differentiating ganglion mother cell (GMC) is much smaller than the self-renewed NB. In many NB cell lineages GMCs undergo only one further division to either generate a pair of neurons or glial cells that undergo terminal differentiation.

In other Drosophila stem cell systems, like the germline stem cells (GSCs), cell fate distinction is mainly mediated by extracellular signaling from a stem cell niche (Morrison and Spradling, 2008). We have recently found that GSC self-renewal requires the activity of the gene bällchen (ball) (Herzig et al., 2014), which encodes a member of the metazoan specific VRK-1 protein kinase family (Aihara et al., 2004). ball orthologous from vertebrates and invertebrates encode proteins that phosphorylate the Barrier-to-Autointegration Factor protein (BAF), which is proposed to participate in the establishment of higher order chromatin structures (Gorjánicz et al., 2007; Nichols et al., 2006; Bengtsson and Wilson, 2006; Lancaster et al., 2007). However, phenotypic analyses of Drosophila ball mutants showed that severe chromatin defects are restricted to the oocyte nucleus (Ivanovska et al., 2005). Notably, ball mutants show extensive degeneration of tissues that rely on the proliferation of undifferentiated progenitor cells or stem cells, such as the nervous system, the imaginal discs as well as the gonads (Cullen et al., 2005; Herzig et al., 2014), which suggests that ball has a role in the maintenance of progenitor and stem cells.

A central question in stem cell biology is whether mechanisms exist that maintain the undifferentiated state of cells irrespective of the mode by which these different stem cell populations establish their cell fate decisions during self-renewal. There is mounting evidence that the differentiation of stem cell descendants requires a lowering of their capacity to proliferate through down-regulation of growth related processes (Chia et al., 2008; Knoblich, 2010). Consistently, recent work comparing the transcriptomes of purified pNBs and differentiated neurons revealed that genes coding for components of metabolic
pathways and ribosome biosynthesis were up-regulated in pNBs (Berger et al., 2012). To restrict proliferation and to allow differentiation of GMCs, ribosome biogenesis needs to be down-regulated in GMCs through the expression of the Brat protein (Bowman et al., 2008). However, it remained unclear to what extent the proliferative potential of stem cells is a prerequisite to maintain their undifferentiated state and thereby their capacity to self-renew. Here we report that the BALL kinase is required to maintain the proliferative potential of NBs and that this function of BALL is a prerequisite for self-renewal. Our results show that ball mutant NBs proliferate at a reduced speed and progressively lose stem cell markers and differentiate untimely during development. Recently, we reported that BALL is crucial to maintain the undifferentiated state of niche supported germline stem cells (Herzig et al., 2014). Therefore our results on neuronal stem cells indicate that distinct stem cell populations employ a common factor, BALL, to remain undifferentiated.

MATERIALS AND METHODS

Fly strains

Unless otherwise stated, all chromosomes and insertions are described in the Flybase database (http://flybase.org). The ru ca e ball2 chromosome was generated by imprecise excision of the P(EP)ball82B P-element integration after recombination to the recessive markers. The deletion associated with the ball2 allele removes the ball initiation codon and parts of the kinase domain coding sequence. The chromosomes P(neoFRT)82B and P(neoFRT)82B e ball2 were constructed by meiotic recombination. The transgene P[w+]; UAS-ball: T-Avic/EGFP=PballIE)2.1 contains the complete ball coding sequence for GAL4 dependent expression of a BALL-EFPP fusion protein with the P[w+GAL4.A] neuroblast driver line (gift from J. Knoblich). Strains to identify ball2 mutant animals were w; ru ca e ball2/TM3, Ser1, P[35UZ2](embryos) and w; ru ca e ball2/TM3, Ser1, P[Avic](JMR2), P[35UZ2](mitotic) clones (embryos) and w; ru ca e ball2/TM3, Ser1, P[35UZ2](embryos) and w; ru ca e ball2/TM3, Ser1, P[Avic](JMR2), P[35UZ2](mitotic). We used y; 70FLP3[F]/w; P[UAS-lacZp]; P[tubP-Gal4]/+; P(neoFRT)82B e ball2 (ball2 mutant clones), y; w1118 P[70FLP3[F]/w; P[UAS-lacZp]; P[tubP-Gal4]/+; P(neoFRT)82B e ball2 (ball2 mutant clones).

Larval brain preparation

Staged larvae were obtained by collecting newly hatched larvae over 2 h intervals and placing them into food vials at controlled density. Optionally, placing vials in a 38°C water bath for 1 h induced flipase expression for generation of genetic mosaics (Lee and Luo, 2001). At indicated time points, the larval tissue was dissected from larvae in Schneider’s cell culture medium (Life Technologies, Paisley, UK) within a 30 min interval before fixation.

Antibody staining

Antibody incubations were done in PBS, 0.1% Triton X-100, 10% goat serum (PBTS) either over night at 4°C or for 2 h at room temperature. Washings between the incubations were two rinses in PBS, 0.1% Triton X-100 (PBTS), followed by three changes in PBTS for 20 min each. For immunofluorescence, embryos were fixed 20 min in 4% paraformaldehyde, PBS, 50 mM EGTA, pH 7.0, deoxidized, dehydrated in methanol and rehydrated (Rothwell and Sullivan, 2000). All other tissue was fixed for 10 min in the same solution. Blocking was done by a 20 min incubation in PBTS. Primary antibodies and dilutions were: affinity purified rabbit anti-BALL (1:400), rabbit anti-histone H3 S10ph (1:1000, Upstate Biotechnology, Lake Placid, NY), rabbit anti-apPKC (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Cleaved Caspase 3 (1:500, Cell Signaling Technologies, Boston, MA), rabbit anti-MIRA (1:1000, gift from J. Knoblich, IMBA, Vienna, Austria), chicken anti-beta Galactosidase (1:1000, Abcam, Cambridge, UK), mouse anti-PROS (1:25, DSHB, University of Iowa, Iowa City, USA), mouse anti-ELAV (1: 1:25, DSHB), mouse anti-REPO (1:25, DSHB), mouse anti-GRH (1:2, gift from Sarah Bray, University of Cambridge, Cambridge, UK). Secondary antibodies were: goat anti-rabbit or goat anti-mouse coupled to Alexa488, Alexa568 or Alexa635 (Life Technologies) and goat anti-chicken coupled to Cy2 (Jackson ImmunoResearch, Newmarket, UK). All secondary antibodies were used at a 1:500 dilution. For DNA staining, samples were incubated in PBT with 1 mg/ml RNase A for 10 min and stained for 10 min in PBT with 1 µg/ml Propidium iodide (Life Technologies) or 5 µM DRAQ5 (Biostatus, Shepshed, UK). After a single wash in PBT samples were embedded in ProLong Gold (Life Technologies).

In situ hybridization

Embryos were fixed with 7.4% paraformaldehyde for 20 min and hybridized at 57°C using standard protocols. Digoxegenin (DIG) labeled RNA probes for in situ hybridizations were obtained from ball cDNA LD27410 (Source BioScience, Nottingham, UK). For non-fluorescent detection sheep anti-DIG-AP Fab (Roche, Mannheim, Germany) was used at 1:2000 in combination with the NBT/BCIP detection reagent (Roche) at 1:100 following manufacturer’s instructions. For fluorescent detection, primary incubation with 1:2000 sheep anti-DIG (Roche) was followed by incubation with 1:1000 donkey anti-Sheep Biotin (Jackson ImmunoResearch). For signal amplification, embryos were incubated for 45 min with ABC reagents (Vector Laboratories, Peterborough, UK), followed by 5 minutes incubation with TSA Cyanine3 reagents (Perkin Elmer, Waltham, MA) diluted 1:50 following the manufacturer’s instructions. Fluorescent RNA detection was then followed by antibody staining to detect proteins.

Image analysis

Images were acquired on a Leica SP2 LSM or a Leica SP5 LSM. For quantitative analyses, z-stacks were sampled at 0.1 µm z intervals. Cell numbers were analyzed in Image J by manual markup of individual cells in the stacks (Cell Counter plugin). Volume analysis was carried out with a modified Connected Threshold Grower plugin and manual thresholding. Details on the modified plugin are available on request.

RESULTS

ball expression is enriched in neuronal stem cells

We analyzed the expression of ball by RNA in situ hybridization in embryos. During early syncytial cleavage divisions of the embryo and up to stage 10 of embryogenesis when most cells of the embryo are mitotically active, ball is expressed ubiquitously (Fig. 1A,B). From stage 11 onwards, however, ball transcripts become enriched in the nervous system, which is at this stage the major site of cell proliferation in the embryo (Fig. 1C). By the end of embryogenesis, ball transcripts fade from the mitotically quiescent nervous system and become enriched in the developing embryonic gonads, which will resume proliferation at larval hatching (Fig. 1D).

We identified embryonic neuroblasts (eNBs) by morphological criteria (Doe, 1992) and found that ball transcripts were enriched in most if not all eNBs that are arranged in a highly stereotyped pattern (Fig. 1E). Transcripts were less abundant in GMCs as shown by fluorescence in situ hybridization of ball transcripts combined with antibody staining for the GMC marker protein Prospero (PROS) (Fig. 1F–H). Anti-BALL antibody staining showed that also BALL protein was enriched in NBs (supplementary material Fig. S1). BALL protein is also present in GMCs (Fig. 11–K), either due to expression of ball in GMCs or due to segregation of BALL to GMCs during eNB division, since BALL was associated with chromatin during mitosis (supplementary material Fig. S2). In summary, ball transcripts and protein are enriched but not exclusively present in embryonic neuroblasts.
In order to address the function of BALL for neuronal development, we used the ball mutant allele (Herzig et al., 2014). Anti-BALL antibody staining revealed that ball mutant eNBs have greatly reduced BALL levels due to the lack of zygotic ball expression (supplementary material Fig. S3). Such ball mutant embryos were viable and hatched (98% of expected; n = 3037 embryos), and we detected no defects in their nervous systems based on staining with the 22C10 monoclonal antibody recognizing the Futsch protein (Fujita et al., 1982), which marks neurons of the central and peripheral nervous system (data not shown). Thus, BALL has either no critical function in eNBs or the level of maternally derived BALL protein in such embryos is sufficient to drive the apparently normal early embryonic nervous system formation in the absence of zygotic BALL expression.

**BALL is essential for larval brain development**

The lethal phase of homozygous ball mutants is the pupal stage (Herzig et al., 2014) (supplementary material Fig. S4). Prior to pupariation, mitotically active tissues of ball mutant larvae, including the brain, were severely reduced in size, whereas no defects were observed in postmitotic endoreduplicating tissue of larvae (supplementary material Fig. S4). In the larval brains, mitotic proliferation depends on postembryonic neuroblasts (pNBs), which represent eNBs that re-entered proliferation after a phase of mitotic quiescence (Sousa-Nunes et al., 2010). Before the pNBs resume proliferation, they increase in cell size and express the NB marker protein Miranda (MIRA). In wild type larvae, large MIRA expressing pNBs are maintained until the end of larval development (Fig. 2A) and continue to express high levels of BALL (supplementary material Fig. S5). In ball mutant brains, however, MIRA expressing pNBs were present in early but not in late stage larvae (Fig. 2B,C). pNBs of early ball mutant larvae were dividing, as shown by the asymmetric distribution of MIRA during pNB division and by the presence of PROS expressing GMCs next to the pNBs (Fig. 2D). These observations suggest that BALL is not strictly required for cell cycle progression of pNBs but for their maintenance.

We therefore asked whether pNBs were lost from ball mutant brains or failed to express MIRA at later stages. To address this question, we used the MARCM system in order to label pNBs independently of neuroblast markers (Lee and Luo, 2001). With this approach, individual ball mutant pNBs were generated that express a tub-GAL4-driven UAS-lacZ (β-Galactosidase, β-Gal) reporter gene irrespective of their stem cell identity. This experimental design allowed us to find out whether ball mutant pNBs were maintained at later stages of larval development and also whether they generated complete cell lineages. By focusing on thoracic pNBs of the ventral ganglion (Fig. 2), we determined the cell number in distinct cell lineages and the proliferation rate of a pNB.

Thoracic pNBs resume proliferation at about 36 h after larval hatching (ALH) (Maurange and Gould, 2005). To visualize entire cell lineages that derived from wild type and ball mutant NBs, we induced MARCM clones at 24 h ALH, dissected the brains at 96 h ALH and stained them with antibodies against β-Gal and the neuronal marker protein ELAV. Both wild type and ball mutant lineages contained multiple ELAV positive neurons, small ELAV negative GMCs and one large ELAV negative pNB (Fig. 3), which was confirmed by antibody staining to visualize additional NB markers such as MIRA and αPKC (see below). The observation that ball mutant pNBs were able to generate cell lineages including differentiating neurons demonstrates that BALL is dispensable for the differentiation of both GMCs and neurons.
BALL regulates the rate of larval NB proliferation

Although BALL is not strictly required for cellular proliferation, we noticed a significant difference in the total volume of wild type (1,423.5 μm³, SD=391.6 μm³, n=30) and ball² mutant cell lineages (862 μm³, SD=254.2 μm³, n=18) at 96 h ALH in the MARCM experiments. Anti-ELAV antibody staining revealed that the ball² mutant cell lineages contained only about half the number of neurons (22.0 neurons, SD=4.5, n=21 lineages) than observed with the wild type controls (56.8 neurons, SD=14.3, n=21 lineages; Fig. 3E). This reduction of ball² mutant neurons was rescued by re-expression of BALL through a UASp-ball-EGFP transgene in ball² mutant lineages (40.2 neurons; SD=6.9, n=12 lineages), indicating that the ball² mutant phenotype is indeed caused by the lack of BALL. It is important to note that 96% of the ball² mutant lineages at 72 h ALH (n=227 lineages) contained a morphologically distinct pNB, although the lineages already were clearly reduced in size (Fig. 3E). This finding suggests that the reduced cell number in ball² mutant lineages is not caused by cell death of pNBs. In addition, we also immunostained larval brains at later stages with antibodies directed against activated Caspase 3, which is a marker for cell death (Xu et al., 2006), but could not detect an increased number of apoptotic cells in ball² mutant cell lineages at 96 h ALH (n=56 lineages). These results indicate that pNBs of ball² mutant have either a reduced rate of proliferation or they stopped proliferation after they have been marked by the MARCM system. To distinguish between these possibilities, we stained larval brains with antibodies directed against the mitotic marker histone H3S10ph at 96 h ALH. We found that ball² mutant pNBs were still dividing at 96 h ALH, but the number of mitotic pNBs was significantly lower than the number of mitotic control pNBs (Fig. 3F). This result indicates that the mutant pNBs did not cease proliferation. Thus, we asked whether the proliferation rate of pNBs was reduced. To address this question, we determined the increase in cell numbers of pNB lineages between 72 h and 96 h ALH, this increase was only about one fourth of that in wild type lineages (Fig. 3E). To further rule out that the reduced number of neurons resulted from an accumulation of GMCs, we determined the numbers of GMCs in wild type and ball² mutant lineages. At 96 h or 72 h ALH, ball² mutant cell lineages contained about half the number of GMCs as compared to wild type control cell lineages (Fig. 3G). This result argues for a reduced rate of pNB proliferation in the absence of ball activity. We also asked whether raising the expression of ball in wild type pNBs causes an increase in the rate of pNB proliferation by over-expressing a UASp-ball transgene in wild type thoracic pNBs (Siegrist and Doe, 2005). To label the pNBs, we used the w0118;Gal4 driver to express the β-Gal marker either together with or, as a control, without the ball transgene. However, the percentage of mitotic ball over-expressing pNBs (17.6%; n=728 pNBs) was not significantly different from mitotic pNBs expressing only β-Gal (19.8%; n=758 pNBs). Together these results establish that BALL, although not essential for cell cycle progression per se, modulates the rate of pNB divisions and has a permissive function to maintain their specific proliferative potential.

ball mutant pNBs fail to maintain neuroblast identity

Most of the ball² mutant MARCM lineages contained morphologically distinct pNBs at 72 h ALH (96%; n=227). Since the brains of homozygous ball² mutant larvae lose

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Fig. 3. ball² mutant thoracic pNBs proliferate at a reduced rate. (A–A’) Control wild type cell lineage labeled by tub-GAL4 dependent β-Galactosidase (β-Gal) expression through the MARCM system. (A) 3D reconstruction from confocal image sections. Also shown are tilted views of the reconstruction (A’) and a single focal plane (A”) of which the position is indicated by a red dashed line in (A). An individual cell lineage is marked by a white dashed outline. (B) Counterstaining for DNA and the neuronal marker Elav (ELAV). Asterisks indicate the position of the pNBs, arrowheads point at GMCs, which lack ELAV expression. (C–C”) ball² mutant cell lineages stained, analyzed and displayed the same way as control wild type lineages (A”). (D) Counterstaining as in (B). (E) Quantification of neuronal cell numbers in wild type control (green) and ball² mutant (red) cell lineages at 96 h and 72 h ALH. The Number of ELAV expressing cells per cell lineage is displayed on the y-axis (neurons). Mean values and the total number of cell lineages analyzed (n) are given above the bars. (F) Quantification of mitotic pNBs in wild type control (green) and ball² mutant (red) cell lineages at 96 h and 72 h ALH, respectively. The fraction of pNBs that were in mitosis, based on H3S10ph positive staining, is indicated on the y-axis. Mean values and the total number of marked pNBs (n) are given above the bars. Scale bar in A–D, 10 μm.
expression of the pNB marker protein MIRA (Fig. 2), we also stained larval brains of MARCM experiments for MIRA, a determinant for pNB identity (Shen et al., 1997). We found that only about half of the ball mutant pNBs expressed MIRA at 72 h ALH (56%; n=112 pNBs; Fig. 4A). Furthermore, the apically localized pNB determinant aPKC (Wodarz et al., 2000) was expressed and properly localized in only about half of the ball mutant pNBs (67%; n=106 pNBs) (Fig. 4B) when compared with the respective control lineages (97%; n=64 pNBs and 100%; n=68 pNBs, respectively) (Fig. 4A,B). These data suggest that about half of the ball mutant pNBs had lost neuroblast identity.

Niche-dependent ball mutant germline stem cells (GSCs) (Herzig et al., 2014) undergo premature differentiation. Thus, we finally asked whether the loss of NB stem cell determinants results also in a premature differentiation of the ball mutant pNB, i.e. both daughter cells develop into GMCs and subsequently into neurons. In wild type, thoracic pNBs undergo self-renewal until about 120 h ALH before they differentiate terminally into neurons (Maurange et al., 2008). This final differentiation step is characterized by a lengthening of the pNB cell cycle, loss of MIRA expression and a reduction of cell size (Maurange et al., 2008). As reported above, the proliferation rate of the ball mutant pNBs was reduced, implying a lengthening of the cell cycle, and MIRA expression was lost from about half of NBs. In addition, we found that the loss of NB determinants that we observed at 72 h ALH became progressively more severe till 96 h ALH. Amongst the ball mutant pNBs that could be identified at 96 h ALH, only few expressed either MIRA (2%; n=44 pNBs) or aPKC (27%; n=141 pNBs), whereas nearly all of the control pNBs expressed MIRA (97%; n=44 pNBs) and aPKC (99%; n=204 pNBs) at the corresponding larval stage (Fig. 4A,B). Moreover, at 96 h ALH a significant number of the ball mutant cell lineages contained no longer a morphologically distinct pNB (26%; n=172 lineages) or a pNB with clearly reduced cell size (16%; n=172 lineages), whereas time matched controls still contained a morphologically distinct pNB (97%; n=250 lineages). Since these observations correspond to the events during the differentiation of wild type pNB, they suggest that ball mutant pNBs differentiated prematurely between 72 and 96 h ALH.

**DISCUSSION**

Our results establish that BALL is essential to maintain the proliferation rate as well as the undifferentiated state of pNBs and therefore interlink these two aspects of stem cell self-renewal. The proliferation rate of ball mutant pNBs was reduced already at 72 h ALH, a time point when approximately half of the pNBs continued to express the stem cell determinants MIRA and aPKC. Therefore, it is plausible that the primary function of BALL is to control the proliferation rate of pNBs as a prerequisite for continuous self-renewal of neuroblasts.

The effects of a reduced proliferation rate were previously studied in epithelial tissue such as wing imaginal discs, which led to the discovery of a phenomenon termed cellular competition (Moreno et al., 2002). It describes that cells with reduced cellular fitness proliferate at a lower rate and are eventually eliminated by apoptosis. We observed this phenomenon after generating ball mutant cells by MARCM in wing imaginal discs, showing that the mutant cells are capable to proliferate and to form cell clones. However, these cell clones fail to compete with wild type cells and subsequently undergo apoptosis (supplementary material Fig. S6). Maintenance of the stem cell character of pNBs is unlikely to
be regulated through a competitive mechanism, since the pNB lineages contain only a single stem cell. Our data suggest that the same process that determines competitiveness of wing disc epithelial cells is a prerequisite to maintain the self-renewal of pNBs.

We have recently shown that BALL is required to sustain self-renewal of niche-controlled stem cells (Herzig et al., 2014). Here, we show that this function of BALL is not restricted to niche-controlled stem cells but is also required in pNBs, which depend on asymmetric distribution of cell fate determinants for self-renewal. Thus, the function of BALL for stem cell self-renewal is not limited by the factors and mechanisms that mediate cell fate decisions in the different stem cell systems. Our study therefore suggests that Drosophila stem cells employ cell intrinsic mechanisms to ensure stem cell self-renewal that are independent of the tissue specific modes of stem cell fate decisions and shared by diverse stem cell populations. The molecular basis of these mechanisms and how BALL is integrated in these processes remains to be established by future studies.

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Competing interests

The authors declare that they have no conflict of interest.

Author contributions

T.Y. and A.H. designed the experiments, T.Y., U.G. and A.H. performed experiments and analyzed data, H.J. and A.H. supervised the study and wrote the manuscript together with U.G.

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References


Supplementary Material
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Fig. S1. BALL protein distribution in embryos. Embryos were stained with antibodies for BALL (green) and against DNA (red). The figures show optical sections from the interior (medial) or close to the epidermal layer (apical) of the embryo. BALL protein was ubiquitous till completion of the early cleavage divisions (A, medial) and subsequent stages (B, apical). During stage 12 of embryogenesis BALL is still present in the epidermis (C, apical) but becomes enriched in the nervous system (D, medial). BALL protein in the epidermal layer is likely persisting from earlier stages as ball mRNA was undetectable in this tissue by this stage (Fig. 1). Clear enrichment of BALL in the nervous system is observed by stage 13 (E, apical; F, medial). Note that BALL protein is highest in the peripheral layer of the nervous system that harbors the eNBs. Stage 17 embryos briefly before hatching into larvae (G,H) showed strong staining only in few cells of the nervous system and in the gonads (G, arrowhead). Scale bar, 100 μm.
Fig. S2. BALL is associated with chromatin.
(A) Whole mount embryo immunostained for BALL and counterstained for DNA. During early embryogenesis, BALL protein is found ubiquitously in epidermal tissue. (B–D) In interphase cells before mitosis 14 (below dotted lines) BALL co-localizes with DNA in the nucleus. During mitosis (between dotted lines) BALL is distributed throughout the cell and re-accumulates in the nucleus during the subsequent interphase of cell cycle 15 (above dotted lines). (E–G) In mitosis BALL co-localizes with chromosomes in the nucleus during prophase (P). During metaphase (M), anaphase (A) and telophase (T) BALL is found cytoplasmic as well as co-localized with DNA. During late telophase and early interphase (I) BALL is still found co-localizing with DNA, although it appears no longer to be enriched on chromatin. (H) BALL-EGFP fusion protein was expressed maternally by using \( P^{(w^{+}mc^{5}-matalpha4-GAL-VP16)} V3 \) maternal driver line. Embryos were collected, dechorionated and live embryos were imaged using a Leica SP2 LSM. Shown are selected images from a movie that show the dynamics of BALL-EGFP subcellular distribution in epidermal cells undergoing mitosis 14 during a 15 min time interval. Arrowheads are used to trace single nuclei. Chromosomal association of BALL-EGFP becomes obvious during prophase and a nucleoplasmic pool of BALL-EGFP appears to distribute into the cytoplasm after nuclear envelope breakdown (blue arrowheads, between 560 s and 650 s). Chromosomal association is visible in metaphase, anaphase and telophase (red arrowheads; 200 s, 300 s and 460 s, respectively). Clearance of BALL-EGFP from the cytoplasm is observed during early interphase (white arrowheads, 200 s to 560 s; starting from telophase at 0 s). Scale bar in A, 50 \( \mu \)m. Scale bar in B–D, 10 \( \mu \)m. Scale bar in E–F, 10 \( \mu \)m.
Fig. S3. BALL expression is strongly reduced in ballmutant eNBs. ballmutant and control sibling embryos were stained for β-Galactosidase (β-Gal, blue), BALL (green) and DNA (red). (A–D) Control embryos (β-Galactosidase positive) show strong BALL staining in the nervous system. (E–H) ballmutant embryos (β-Galactosidase negative) show no accumulation of BALL in the nervous system indicating that this expression domain resembles zygotic transcription of ball. (I–K) High magnification view of the nervous system in a wild type embryo. (L–N) High magnification view of the nervous system in a ballmutant embryo. Scale bar in A–H, 100 μm. Scale bar in I–N, 10 μm.

Fig. S4. BALL is not essential in endoreduplicating tissue. (A–C) Wild type larval salivary gland stained for DNA and BALL. In addition to large endoreduplicating and non-dividing cells the gland contains mitotically dividing cells in the imaginal ring (IR, arrowhead). BALL is expressed in endoreduplicating cells (inset, A) and in IR cells (B,C). (D–F) Endoreduplication of gland nuclei still occurs in ballmutant glands (D) although BALL is no longer detectable (D, inset). The proliferation of IR cells, however, is severely disrupted (E,F), which indicates that BALL is not essential for endoreduplication or S phase progression. (G) Wild type and ballmutant larvae grow to comparable size before pupariation. Since larval growth is largely accomplished by endoreduplication of differentiated tissue this supports the notion that BALL is largely dispensable in this process. (H) ballmutant larvae still form pupae but degenerate subsequently and die. Scale bar in A,D, 200 μm. Scale bar in B,C and E,F, 10 μm.
Fig. S5. BALL is enriched in thoracic pNBs. Wild type brains of larvae 96 h ALH were stained for BALL (green in overlays), DNA (red in overlays) and various marker proteins (blue in overlays). Neuroblasts (pNBs) of thoracic lineages are marked by arrowheads. (A–C) BALL levels are elevated in pNBs but BALL is also present in differentiating neurons that are marked by ELAV. (D–F) BALL is undetectable in glial cells that contact thoracic pNBs and are marked by the REPO protein. (G–I) In thoracic pNB lineages PROS is expressed in neurons that are born early during larval development. We could not detect a differential expression of BALL in PROS positive versus negative cells. (J–L) BALL expression in thoracic neuroblasts was verified by co-localization with the transcription factor Grainyhead (GRH). (M–O) Whole mount brain 96 h ALH stained for BALL (green) and GRH (red). pNBs of the abdominal region of the ventral ganglion (red bracket) only generate small lineages during larval development since they get eliminated by apoptosis. BALL levels in the abdominal region of the ventral ganglion are much lower than in the thoracic region (white bracket) that contains continuously proliferating pNBs. Scale bar in A–L, 5 μm. Scale bar in M–O, 100 μm.

Fig. S6. ball^2 mutant cell clones in wing imaginal discs are eliminated by apoptosis. (A–D) ball^2 mutant MARCM clones were induced at 24 h ALH and wing discs were dissected at 96 h ALH. We observed positively β-Galactosidase marked cells (β-Gal, green in overlay) indicating that ball^2 mutant cells can proliferate. However we found many cells in the clones stained for cleaved Caspase 3 (aCasp3, red in overlay), indicating that ball^2 mutant cells are eliminated by apoptosis. Counterstaining for DNA is blue in overlay. (E–H) Non-mutant control MARCM clones were induced as in (A–D). Cell numbers were significantly higher in the control clones than in (A–D).