RESEARCH ARTICLE

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

Toma Yakulov¹,*, Ufuk Günesdogan²*, Herbert Jäckle and Alf Herzig³,⁴

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 the stem cell niche or asymmetric distribution of cell fate determinants during cell division. 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 inherited 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 Gs1, 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 (Gorjánacz 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 processes is based on self-renewing stem cells. Stem cells are

Max-Planck-Institut für Biophysikalische Chemie, Abteilung Molekulare Entwicklungsbiologie, Am Fassberg 11, 37077 Göttingen, Germany.
¹Present address: Renal Division, University Hospital Freiburg, Hugstetter Strasse 55, 79106 Freiburg, Germany.
²Present address: Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge CB2 1QQ, UK.
³Present address: Max-Planck-Institut für Infektionsbiologie, Department Cellular Microbiology, Charitéplatz 1, 10117 Berlin, Germany.
⁴These authors contributed equally.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

Received 29 April 2014; Accepted 11 August 2014

881

Downloaded from http://bio.biologists.org/ by guest on December 29, 2017
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 downregulated 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 ball& chromosome was generated by imprecise excision of the P(EP)bal& element integration after recombination to the recessive markers. The deletion associated with the ball& allele removes the ball initiation codon and parts of the kinase domain coding sequence. The chromosome P(neoFRT)72B and P(neoFRT)82B e were constructed by mitotic recombination. The transgene P[w+` UASp-T-Avic/AEP=pbalE]2.1 contains the complete ball coding sequence for GALA dependent expression of a BALL-EGFP fusion protein with the P[wor:GALA4] neuroblast driver line (gift from J. Knoblich). Strains to identify ball mutant animals were w; ru ca e ball&/TM3, Ser1, P[35UZ]2 (embryos) and w; ru ca e ball&/TM3, Ser1, P[ActGFPlJM22 (larvae). For MARCM (Lee and Luo, 2001) we used y1 w1118 P(70FLP)3/Fw1 P[UAS-lacZ.p]; P[tub-Gal4]/+; P(neoFRT)82B P[tub-Gal80/L3/P(neoFRT)82B e ball& (ball mutant clones), y1 w1118 P(70FLP)3/Fw1 P[UAS-lacZ.p]; P[tub-Gal4]/+; P(neoFRT)82B P[tub-Gal80/L3/P(neoFRT)82B e ball& (control wild type clones) and y1 w1118 P(70FLP)3/Fw1 P[UAS-lacZ.p]; P[tub-Gal4]/+; P(pbalE]2.1; P(neoFRT)82B P[tub-Gal80/L3/P(neoFRT)82B e ball& (rescued ball 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

Embryos were fixed with 7.4% paraformaldehyde for 20 min and hybridized at 57°C using standard protocols. Digoxygenin (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.

In situ hybridization

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 marking 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 chromat in 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 null 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 aPKC (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 μm$^3$, SD=391.6 μm$^3$, n=30) and ball$^2$ mutant cell lineages (862 μm$^3$, SD=254.2 μm$^3$, n=18) at 96 h ALH in the MARCM experiments. Anti-ELAV antibody staining revealed that the ball$^2$ 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 ball$^2$ mutant neurons was rescued by re-expression of BALL through a UASp-ball-EGFP transgene in ball$^2$ mutant lineages (40.2 neurons; SD=6.9, n=12 lineages), indicating that the ball$^2$ mutant phenotype is indeed caused by the lack of BALL. It is important to note that 96% of the ball$^2$ 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 ball$^2$ 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 ball$^2$ mutant cell lineages at 96 h ALH (n=56 lineages). These results indicate that pNBs of ball$^2$ 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 ball$^2$ 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 ball$^2$ mutant lineages. At 96 h or 72 h ALH, ball$^2$ 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 worniu-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 ball$^2$ mutant MARCM lineages contained morphologically distinct pNBs at 72 h ALH (96%; n=227). Since the brains of homozygous ball$^2$ mutant larvae lose...
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.

Acknowledgements
We thank Sabine Häder for technical assistance, Jürgen Knoblich for sharing reagents and the Max-Planck-Society for support.

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.

Funding
This work was supported by the Max-Planck-Society and U.G. was supported by a fellowship of the Boehringer Ingelheim Fonds.

References