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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

The replicative histone chaperone CAF1 is essential for the maintenance of identity and genome integrity in adult stem cells

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ABSTRACT

Chromatin packaging and modifications are important to define the identity of stem cells. How chromatin properties are retained over multiple cycles of stem cell replication, while generating differentiating progeny at the same time, remains a challenging question. The chromatin assembly factor CAF1 is a conserved histone chaperone, which assembles histones H3 and H4 onto newly synthesized DNA during replication and repair. Here, we have investigated the role of CAF1 in the maintenance of germline stem cells (GSCs) in *Drosophila* ovaries. We depleted P180, the large subunit of CAF1, in germ cells and found that it was required in GSCs to maintain their identity. In the absence of P180, GSCs still harbor stem cell properties but concomitantly express markers of differentiation. In addition, P180-depleted germ cells exhibit elevated levels of DNA damage and de-repression of the transposable I element. These DNA damages activate p53- and Chk2-dependent checkpoints pathways, leading to cell death and female sterility. Altogether, our work demonstrates that chromatin dynamics mediated by CAF1 play an important role in both the regulation of stem cell identity and genome integrity.

KEY WORDS: Stem cells, Replication, Germline, Chromatin, *Drosophila*, Transposon

INTRODUCTION

Adult stem cells play essential roles in the maintenance of tissue homeostasis. They allow both the continuous production of differentiated cells for normal tissue integrity and the regeneration of damaged tissue after injury. At the same time, a pool of stem cells with identical nuclear (genetic and epigenetic information) and cytoplasmic properties needs to be maintained. How this is achieved over multiple rounds of DNA replication and cell divisions remains a challenging issue.

S phase is a central event in the transmission of chromatin states across cellular divisions. Indeed, the replication of DNA in S phase triggers a genome-wide alteration of the chromatin structure, enabling the replication machinery to access DNA, and it is followed by *de novo* chromatin assembly onto newly synthesized DNA. Although crucial for maintaining the epigenome of particular cell types, the re-assembly of chromatin onto the daughter DNA strands also provides a unique opportunity for generating different chromatin states on sister chromatids that would potentially influence gene expression patterns and cell fate in the daughter cells (Weintraub, 1974). A major player in the regulation of chromatin dynamics in S phase is chromatin assembly factor 1 (CAF1). CAF1 is a largely conserved histone chaperone that was first isolated for its ability to assemble new histones H3 and H4 onto newly synthesized DNA, during DNA replication and repair (Gaillard et al., 1996; Smith and Stillman, 1989). It has also been shown to participate in the maintenance of heterochromatin and it is specifically required for the duplication of pericentric heterochromatin in mice (Quivy et al., 2004, 2008). By virtue of its central role in replication-coupled chromatin assembly, CAF1 appeared to be a candidate of choice in the search for molecules involved in the inheritance of chromatin states in mitotic cells.

In that respect, the asymmetric division of stem cells is an interesting case study. Using this type of division, stem cells generate two daughter cells with distinct identities, allowing their self-renewal and the production of differentiating daughter cells at the same time. Yet the involvement of chromatin dynamics in this process remains poorly appreciated. Recently, differential inheritance of chromatin states has been proposed to underlie the acquisition of distinct identities upon asymmetric divisions of *Drosophila* male germline stem cells (GSCs) (Tran et al., 2012; Xie et al., 2015). In this context, parental histones remain preferentially in the stem cell, while the differentiating daughter cell inherits mostly newly synthesized histones.

In the present study, we address the role of the replicative histone chaperone CAF1 in the regulation of *Drosophila* female GSC homeostasis. *Drosophila* oogenesis provides an ideal system in which to study stem cell biology *in vivo* (Spradling et al., 2011), as well as the role of chromatin in germline development (Flora et al., 2017; Molla-Herman et al., 2014). Each *Drosophila* ovary is composed of a group of 16-20 individual substructures called ovarioles that correspond to chains of progressively maturing egg chambers. The continuous production of egg chambers originates from a specialized structure at the anterior end of each ovariole: the germarium. The latter is organized into four morphological regions (Huynh and St Johnston, 2004) (Fig. 1A). In region 1, two or three GSCs are in contact with somatic niche cells that are essential for their maintenance and self-renewal. Each GSC divides asymmetrically to self-renew and produce a differentiating daughter cell: the cystoblast (CB). The balance between stem cell self-renewal and differentiation is controlled by both extrinsic and intrinsic cues (Xie, 2013), and a key factor in this process is Bag of marbles (Bam), which is necessary and sufficient to promote the differentiation of germline cyst cells (McKearin and Ohlstein, 1995). Upon expression of Bam, CBs undergo four rounds of synchronous mitoses with incomplete cytokinesis, giving rise to a germline cyst of 16 cells interconnected via stable cytoplasmic bridges named ring canals.

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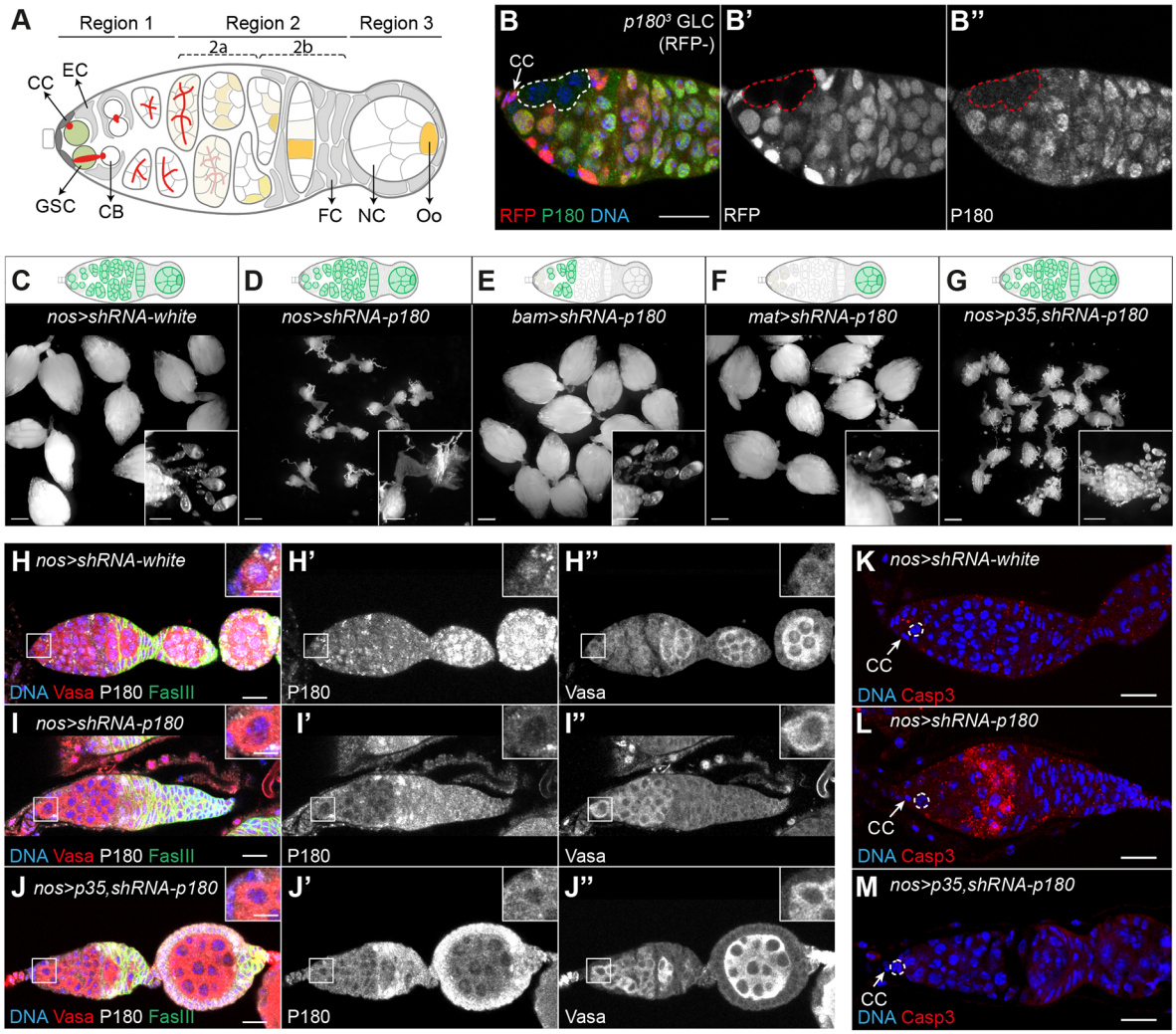

Fig. 1. P180 is required in GSCs for the maintenance of the female germline. (A) Schematic view of the *Drosophila* germarium showing the germline stem cells (GSCs, green) in contact with cap cells (CCs, dark grey). GSCs divide asymmetrically to self-renew and produce a cystoblast (CB) in contact with escort cells (ECs). The CB undergoes differentiation by dividing four times, leading to a cyst of 16 cells, comprising 15 nurse cells (NCs) and one oocyte (Oo, yellow). The cyst is encapsulated by follicular cells (FCs) and buds out of the germarium to form an egg chamber. The different shades of yellow represent the progressive restriction of oocyte-specific factors to the future oocyte. In red are the spectrosome (in GSCs and CBs) and the fusome (in cysts). Regions 1, 2a, 2b and 3 of the germarium are indicated. (B) *p180*^{3} mutant germline clone (GLC, dotted lines), identified by the absence of RFP fluorescence (B′) and the absence of P180 staining (B″) combined with a positive DNA staining (blue). Cap cells (CCs) are indicated. Scale bar: 20 µm. (C-G) Ovaries from flies expressing a control shRNA (C) or a shRNA targeting *p180* (D-G) under the control of Gal4 drivers expressed in different regions of the germarium, represented in green in the schemes at the top of each panel. Insets show higher-magnification views of a dissected ovary. Scale bars: 200 µm; 100 µm in insets. (H-J″) Fragments of control (H-H″), P180-depleted (I-I″) and P35-expressing rescued P180-depleted (J-J″) ovarioles, with DNA (blue), P180 (white and H′,I′,J′), Vasa (red and H″,I″,J″) and Fasciclin III (FasIII, green) staining. Insets show higher-magnification views of framed GSCs. Scale bars: 15 µm; 7 µm in insets. (K-M) Control (K), P180-depleted (L) and P35-expressing rescued P180-depleted (M) germaria, with DNA (blue) and cleaved caspase 3 (Casp3, red) staining. The dotted circles delineate GSCs, attached to CCs. Scale bars: 15 µm.

Later, in region 2a of the germarium, meiosis is initiated and the future oocyte differentiates from one of the two cells of the cyst that are connected to the others via four ring canals. In region 2b, meiosis appears to be restricted to the future oocyte that arrests in prophase I. The cyst flattens to become a one-cell thick disc spanning the whole width of the germarium. Somatic follicular cells then start to migrate and surround the cyst. Finally, in region 3 the follicular cells form a monolayer around the cyst, which rounds up in a sphere with the oocyte at its posterior side. The oocyte genome becomes highly condensed to form the karyosome, while the 15 other cells of the cyst differentiate into polyploid nurse cells. Eventually, the cyst buds out of the germarium: this corresponds to the stage 1 of egg chamber development (Huynh and St Johnston, 2004).

Here, we report that the large subunit of the *Drosophila* CAF1 complex is essential to support oogenesis. Intriguingly, its function is required in GSCs and early germ cells, and seems dispensable in more differentiated germ cells. We show that the large subunit of *Drosophila* CAF1 regulates GSC identity, conceivably by maintaining proper transcriptional repression of the gene encoding the differentiation factor Bam. In addition, this large subunit is required to preserve genome integrity of germ cells. Indeed, its depletion in GSCs and daughter cells induces de-repression of the transposable I element, together with increased DNA damage, as well as replicative stress at ribosomal DNA (rDNA) loci. These genotoxic stresses lead to the activation of p53- and Chk2-dependent checkpoint pathways that eventually trigger apoptosis and oogenesis arrest.

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RESULTS

Drosophila CAF1 large subunit is required in GSCs for the maintenance of the female germline

CAF1 is a heterotrimeric complex, composed of P180, P105 and P55 subunits in *Drosophila* (Kamakaka et al., 1996; Tyler et al., 1996). Loss-of-function mutations in the genes encoding each subunit were shown to induce growth defects and lethality at the larval stage (Anderson et al., 2011; Klapholz et al., 2009; Song et al., 2007; Wen et al., 2012; Yu et al., 2013). This lethality precluded a direct analysis of *Drosophila* CAF1 loss-of-function phenotypes during oogenesis. Thus, we used the Flp/FRT system to induce germline clones (GLCs) homozygous mutant for a null allele to knockout *p180* (*Caf1-180* – FlyBase) in germ cells.

It has previously been reported that GLCs mutant for either *p180* or *p55* (*Caf1-55* – FlyBase) did not yield any eggs, a defect that was rescued by expression of the wild-type proteins, demonstrating that the function of CAF1 is essential during oogenesis (Klapholz et al., 2009; Song et al., 2007; Wen et al., 2012). Here, we found that the vast majority of $p180^3$ mutant GLCs were restricted to the germarium (~90%, n=24), regardless of the time at which the ovaries were dissected following the induction of GLC. Immunostaining for P180 indicated that the amount of P180 was significantly reduced in $p180^3$ GLCs (Fig. 1B). We concluded that P180 is required early during germline development, prior to egg chamber formation.

In order to define more precisely at which stage(s) of germline development the function of P180 was required, we used RNA interference (RNAi) combined with the *UAS/gal4* system (Ni et al., 2011) to specifically deplete the protein in germ cells at different stages of oogenesis. *nanos-gal4* drives the expression of transgenes placed under the control of a *UAS* promoter in all germ cells of the ovaries, including GSCs (Dietzl et al., 2007) (Fig. 1C). Females expressing a shRNA against *p180* under the control of *nanos-gal4* were sterile and exhibited severely atrophied ovaries (Fig. 1D). These rudimentary ovaries were mainly composed of germaria, with germ cells devoid of detectable P180 protein and with no egg chamber attached (Fig. 1I). This is in agreement with the observation that $p180^3$ mutant GLCs generally did not develop outside the germarium (Fig. 1B). The same phenotype was obtained when the depletion was induced with a different shRNA transgene targeting *p180* (not shown). In addition, the global amount of histone H3, as estimated with a *H3:RFP* transgene, was visibly decreased in the nucleus of P180-depleted germ cells (Fig. S1A-B), arguing that RNAi with shRNAs targeting *p180* constitutes an efficient strategy for altering CAF1 function. Moreover, as previously described in *Drosophila* imaginal discs (Yu et al., 2013), depletion of P180 induced a concomitant downregulation of P105, the median subunit of CAF1 (Fig. S1C,D).

We then depleted P180 at later stages of germ cell differentiation. Using one copy of the *bam-gal4* driver, described as being expressed in two- to eight-cell cysts (Chen and McKearin, 2003b, Fig. 1E, scheme), we found no visible phenotype during oogenesis (Fig. 1E). However, we observed a significant knockdown of P180 protein starting at the eight-cell cyst stage only (not shown). Increasing the number of *bam-gal4* transgenes (up to 6 copies) did not allow significant depletion of P180 in earlier germline cysts (Fig. S2A-B). Similarly, knocking down P180 with the *maternal-tubulin-gal4* (*mat-gal4*) driver, which is strongly expressed from stage 1 egg chambers onwards (Staller et al., 2013), produced mature egg chambers with no obvious defects (Fig. 1F and Fig. S2C,D). Therefore, P180 is required in stem cells and early germline cysts but is likely dispensable at later stages of germline development.

Importantly, the rudimentary ovaries induced by the depletion of P180 in germ cells under the control of *nanos-gal4* are not associated with a complete loss of germ cells. Indeed, Vasa-positive germ cells with undetectable P180 protein in their nucleus persisted in the anterior region of the germarium (Fig. 1I). Furthermore, immunodetection of cleaved caspase 3, a marker of cells undergoing apoptosis (Fan and Bergmann, 2010), revealed that a vast majority of germaria depleted of P180 in germ cells accumulated apoptotic cells in region 2a/2b (Fig. 1L), corresponding to the area beyond which germ cells were no longer observed in P180-depleted germaria (Fig. 1I). In contrast, cleaved caspase 3 staining was not detected in the anterior region of the germarium, where GSCs are located in contact with the somatic cap cells, arguing that P180-depleted GSCs themselves do not undergo apoptosis. Moreover, when depletion of P180 was induced in eight-cell cysts onwards under the control of *bam-gal4*, we did not observe cleaved caspase 3 staining in germ cells (Fig. S2E). We then inhibited apoptosis in germ cells depleted of P180 by co-expressing the P35 baculovirus protein (Hay et al., 1994) under the control of the same *nanos-gal4* driver (Fig. 1M). Although these female flies remained sterile, the arrest of oogenesis was significantly rescued, as ovaries contained ovarioles with five or six maturing egg chambers (Fig. 1G,J,M), further suggesting that GSCs are still functional upon depletion of P180. Of note, this rescue was not a consequence of a weaker depletion of P180 by titration of the Gal4 activator, as the expression of *UAS-lamin:GFP* in the same background did not rescue the oogenesis arrest (not shown).

Taken together, these results show that *Drosophila* CAF1 large subunit is required in GSCs and early germ cells. Its loss in these cells induces cell death and an arrest of oogenesis later on in differentiating germ cells.

Loss of *Drosophila* CAF1 large subunit leads to the formation of stem cysts expressing Bam

In order to understand how GSCs were affected by the depletion of P180, we first analyzed the distribution pattern of α-spectrin in mutant germaria. α-Spectrin is a particularly useful marker because it is present in both GSCs and cyst cells with distinct characteristic patterns (Lin et al., 1994). In wild-type germaria, it is enriched in a round structure called the spectrosome in GSCs and CBs (Fig. 1A and Fig. 2A, Sp, arrowhead in GSC) and in a branched structure called the fusome in cyst cells (Fig. 1A and Fig. 2A, Fu, arrow). Cysts with a normal fusome were also observed in P180-depleted germaria (Fig. S3), indicating that the remaining germ cells (Vasa positive, Fig. 1I) are able to enter the first steps of differentiation. However, in some P180-depleted germaria, we observed branched fusomes abnormally originating from cells presumed to be GSCs based on their direct contact with cap cells (Fig. 2B). A small α-spectrin-positive dot resembling a scar of spectrosome (Lopez-Onieva et al., 2008) was frequently observed in the same presumed GSCs (Fig. 2B, Sp, arrowhead). Such abnormal α-spectrin staining patterns were also observed in $p180^3$ mutant GLCs (Fig. S4A). This is reminiscent of the phenotype observed in germaria mutant for factors that are directly involved in the regulation of the last step of cytokinesis: abscission. This process is developmentally regulated and its duration varies according to cell types and organisms (Agromayor and Martin-Serrano, 2013). In *Drosophila* ovaries, abscission takes place in GSCs after completion of S phase of the following cell cycle (de Cuevas et al., 1998), whereas it is completely blocked during the four divisions of CBs, thereby forming a syncytium of germ cells. Recently, it was shown that gain of function of Aurora B kinase, an upstream regulator of abscission,

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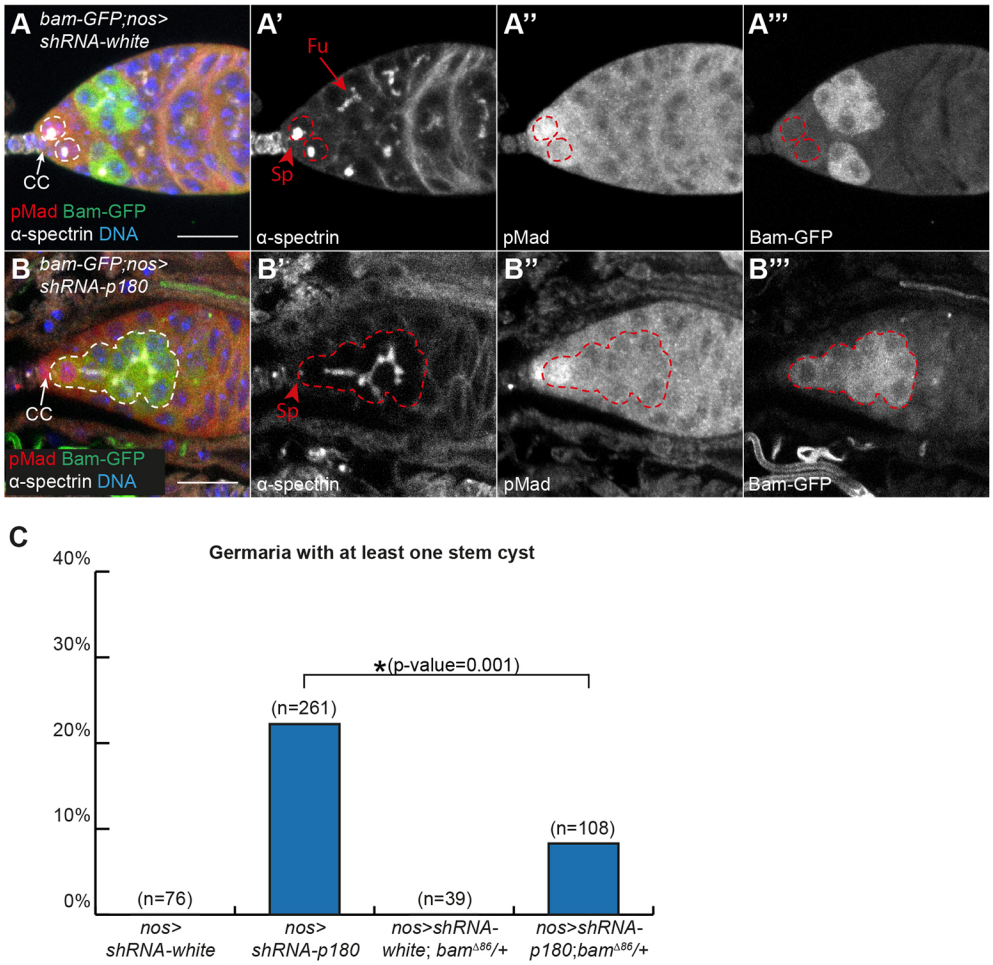

Fig. 2. Bam-expressing stem cysts form upon depletion of P180 in germ cells. (A-B‴) Control (A) and P180-depleted (B) germaria expressing the *bam-GFP* transgene (GFP fluorescence in green and A′″,B′″), with DNA (blue), phosphorylated Mad (pMad, red and A″, B″) and α-spectrin (white and A′,B′) staining. The dotted lines outline either single GSCs (A) or a stem cyst (B) with a pMad positive GSC (B″) aberrantly expressing Bam-GFP (B′″) and connected to eight other Bam-GFP positive germ cells by a branched fusome (B′). Cap cells (CCs), spectrosomes (Sps, red arrowheads) and fusome (Fu, red arrow) are indicated. Images are maximum intensity projections of several confocal sections. Scale bars: 15 µm. (C) Fraction of control and P180-depleted germaria exhibiting at least one stem cyst in otherwise wild-type or $bam^{\Delta 86}$/+ heterozygous flies. Stem cysts were scored on germaria immunostained using α-spectrin antibody: they were identified as a group of at least three cells, linked by a fusome, with the most anterior cell being attached to the niche. *n* indicates the number of germaria analyzed for each genotype and the asterisk indicates a significant difference, as evaluated using a chi-squared test.

and loss of function of Alix or Shrub, two proteins that directly participate in the abscission process, further delay abscission in GSCs and lead to the formation of structures called 'stem cysts' (Eikenes et al., 2015; Mathieu et al., 2013; Matias et al., 2015). These correspond to groups of cells that include a GSC abnormally connected to several daughter cells by a branched fusome. The observation of similar structures upon depletion of P180 in germ cells suggests that abscission might be perturbed in GSCs lacking P180. Alternatively, the presence of branched fusomes in P180-depleted GSCs could result from premature differentiation, as a branched fusome is a typical feature of differentiating germ cells.

Hence, we intended to determine whether depletion of P180 affected germ cells differentiation. As mentioned above, Bam is an essential factor in this process (McKearin and Ohlstein, 1995). The transcription of *bam* is actively repressed in wild-type GSCs and this is achieved mostly through the bone morphogenetic protein (BMP)-like signaling pathway involving the secretion of Decapentaplegic (Dpp) by the cap cells of the niche (Xie and Spradling, 1998). The binding of Dpp to its cognate receptors present at the surface of GSCs leads to the phosphorylation of Mothers against Dpp (Mad), which then associates with Medea (a homolog of Smad4) and translocates to the nucleus where it binds to a silencing element at the *bam* locus and favors the recruitment of transcriptional repressors (Chen and McKearin, 2003a). We observed that P180-depleted germ cells in contact with cap cells were positively stained with an antibody recognizing the phosphorylated form of Mad (pMad, Fig. 2B). Therefore, the first steps of BMP signaling are functional in P180-depleted germ cells. Bam-GFP, a reporter for Bam protein expression (Chen and McKearin, 2003b), was detected in P180-depleted cysts (Fig. S3), which further confirms that Vasa-positive germ cells persisting in P180-depleted germaria (Fig. 1I) are able to initiate differentiation. Surprisingly, we observed that, in some P180-depleted stem cysts, pMad-positive cells concomitantly expressed Bam-GFP (Fig. 2B), which was also expressed in the other cells of the stem cyst connected to the GSC by a branched fusome. We also detected the presence of *bam* transcripts in these stem cysts (Fig. S5). This suggests that P180-depleted GSCs may abnormally express *bam*, although it remains possible that the Bam-GFP and *bam* RNA observed in GSCs that are part of stem cysts are not produced by the GSC itself but inherited by diffusion from more-posterior cells of the stem cyst. This contrasts with what was seen in stem cysts formed as a consequence of a delayed abscission in GSCs, which do not express Bam (Eikenes et al., 2015; Mathieu et al., 2013; Matias et al., 2015). We examined whether the stem cysts that we observed upon depletion of P180 were a consequence of ectopic expression of Bam in GSCs and tested genetic interactions between *bam* and *p180*. We removed one copy of *bam* in germaria depleted of P180 by RNAi and observed that the number of branched fusomes originating from GSCs was significantly reduced (Fig. 2C). Hence, we concluded that the ectopic expression of Bam in P180-depleted GSCs contributes to the formation of stem cysts.

Altogether, our observations indicate that the large subunit of *Drosophila* CAF1 plays a crucial role in maintaining GSC identity. Upon its depletion, presumed GSCs exhibit both stem cell features (phosphorylation of Mad) and differentiating cysts features (Bam expression and incomplete abscission).

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Checkpoint pathways are activated in response to depletion of *Drosophila* CAF1 large subunit in germ cells

The formation of stem cysts does not generally lead to cell death and oogenesis arrest (Eikenes et al., 2015; Mathieu et al., 2013; Matias et al., 2015). Thus, we sought to determine the cause of cell death occurring in differentiating cyst cells lacking P180. One plausible trigger for cell death is the activation of checkpoint pathways in response to genotoxic stresses, in which p53 plays a central role (Vousden and Lane, 2007). To determine whether p53 was activated in response to P180 depletion in germ cells, we took advantage of a transgenic reporter of p53 activity previously characterized in fly ovaries: *p53R-GFP.nls* (Lu et al., 2010; Wylie et al., 2014). As reported, *p53R-GFP.nls* was expressed in the meiotic region of control germaria (Fig. 3A), corresponding to regions 2a/2b where meiotic double strand breaks (DSBs) are observed. By contrast, in P180-depleted germaria this reporter was robustly expressed in GSCs and daughter cells (Fig. 3B). Similar results were obtained in $p180^{3}$ GLC (Fig. S4B). We then tested the functional relevance of p53 activation in this context by simultaneously depleting P180 and p53 with cognate shRNAs co-expressed under the control of *nanos-gal4*. Double knockdown (KD) ovaries developed much further compared with P180 single KD ovaries, with ovarioles containing chains of four or five maturing egg chambers (Fig. 3D,F). However, female flies remained sterile upon co-depletion of P180 and p53. This rescue argues that p53 activation contributes to the oogenesis arrest induced by P180 depletion in germ cells.

In flies, the checkpoint effector kinase Chk2 is the primary regulator of p53 activity in response to DNA damage (Song, 2005). Thus, we tested whether Chk2 participated in the oogenesis arrest induced by P180 depletion, using a null allele of the gene encoding Chk2 in *Drosophila*, named mnk^{p6} (Abdu et al., 2002). A significant rescue of the oogenesis arrest was observed upon depletion of P180 in germ cells of flies carrying one copy of the mnk^{p6} mutation (Fig. 3D,H). The rescue was not enhanced in mnk^{p6} homozygous females (not shown).

As abscission can be blocked or delayed in response to the activation of checkpoint pathways following genotoxic stress in budding yeast and mammalian cells (Mackay and Ullman, 2015; Norden et al., 2006), we assessed whether stem cysts forming in P180-depleted germaria were a consequence of checkpoint activation. However, the occurrence of branched fusomes anchored to the niche was not reduced in P180-depleted germaria upon co-depletion of p53 or in a mnk^{p6}/+ context (Fig. 3I), indicating that the formation of stem cysts upon P180 depletion does not result from the activation of p53 or Chk2. This reinforces our assumption that incomplete abscission in P180-depleted GSCs is caused by ectopic Bam expression and premature entry into the differentiation program.

Replicative stress at rDNA arrays contributes to the oogenesis arrest induced by depletion of *Drosophila* CAF1 large subunit in germ cells

Next, we intended to identify the genotoxic stresses initiating the activation of checkpoints in P180-depleted germ cells. In wild-type fly ovaries, Chk2 and p53 are normally activated in response to programmed meiotic DSBs that arise approximatively in region 2a of the germarium (Lu et al., 2010). Given the role of P180 in the repair of DSBs (Song et al., 2007), one possible cause for the strong checkpoint activation in P180-depleted ovaries could be unrepaired meiotic DSBs. This scenario seemed rather unlikely as we detected p53 activity in P180-depleted germ cells at early stages of development, including in presumed GSCs and daughter cells (Fig. 3B), which is prior to the induction of programmed meiotic DSBs in wild-type ovaries. Nonetheless, to rule out this possibility, we induced the depletion of P180 in germ cells of females mutant for the gene encoding Mei-W68, the Spo-11 homolog that catalyzes programmed meiotic DSBs in flies (McKim and Hayashi-Hagihara, 1998). Despite the absence of meiotic DSBs in this context, oogenesis was arrested at the same stage as when the depletion of P180 was induced in otherwise wild-type germ cells (Fig. S6), confirming that this arrest is not primarily due to a failure to repair meiotic DSBs.

Because CAF1-dependent nucleosome assembly and heterochromatin maintenance are tightly associated with DNA replication (Quivy et al., 2004; Smith and Stillman, 1989), we then considered replication stress as a possible source of checkpoint activation in P180-depleted germ cells. The initial event sensed by checkpoints activated in response to replication defects is usually the formation of tracts of single-stranded DNA (ssDNA). These exposed sites are bound by the replication protein A (RPA) complex that consists of three subunits (RpA70, Rpa2 and Rpa3 in *Drosophila*) and recruits checkpoint proteins to sites of stress (Zou and Elledge, 2003). A fluorescent RpA70:GFP reporter was shown to form foci in the nuclei of fly embryos, which gradually increase in both number and intensity under conditions of induced replication stress (Blythe and Wieschaus, 2015). We monitored the localization of this reporter in P180-depleted germ cells. While in control germaria, RpA70:GFP foci were visible only in the meiotic region of the germarium and later in endocycling cells (not shown), foci were observed in GSCs and early differentiating germ cells in P180-depleted germaria (~30%, Fig. 4A-C). This indicates that tracts of ssDNA abnormally arise in P180-depleted early germ cells. By contrast, we did not observe an increase in the number of Rpa70:GFP foci when P180 was depleted at later stages of germ cell development using *bam-gal4* (Fig. S7).

Surprisingly, among the P180-depleted GSCs and CBs harboring RpA70:GFP foci, most (~94%, n=201) had only one or two foci. This suggests either that ssDNA accumulates at specific loci or that these foci correspond to aggregates of ssDNA tracts originating from different loci, or both. Ribosomal DNA (rDNA) arrays, which are particularly prone to replication stress, are good candidates for being part of these loci for two reasons. First, it was recently reported that ribosome assembly factors are required for *Drosophila* germline maintenance and their depletion induces delayed abscission in GSCs and the formation of stem cysts (Sanchez et al., 2015). Second, mutations in the genes encoding CAF1 subunits in *Arabidopsis thaliana* have been shown to induce a progressive loss of the 45S rDNA (Muchová et al., 2015). Accordingly, RpA70:GFP foci in P180-depleted GSCs preferentially accumulated within the nucleolus (labelled with an anti-fibrillarin antibody), where rDNA arrays localize (53.85%, n=13) (Fig. 4B). Interestingly, the nucleolus often looked fragmented in P180-depleted germ cells (35.7% of germaria show at least one germ cell with a fragmented nucleolus, n=14), whereas this was less frequently the case in control germ cells (10% of germaria, n=10, Fig. 4A″-B″). This phenotype resembles that in heterochromatin mutants, such as *hp1* mutants, in which fragmented nucleoli are associated with extra-chromosomal circular DNA containing rDNA sequences (Peng and Karpen, 2007). We next tested whether altering the number of rDNA copies would functionally interfere with P180 depletion. We used the *C(1)DX* attached X chromosome that lacks the *bobbed* locus, which contains rDNA arrays. Females with a *C(1)DX* attached X chromosome therefore have a reduced number of rDNA copies. Strikingly, in presence of the *C(1)DX* chromosome, the oogenesis arrest induced

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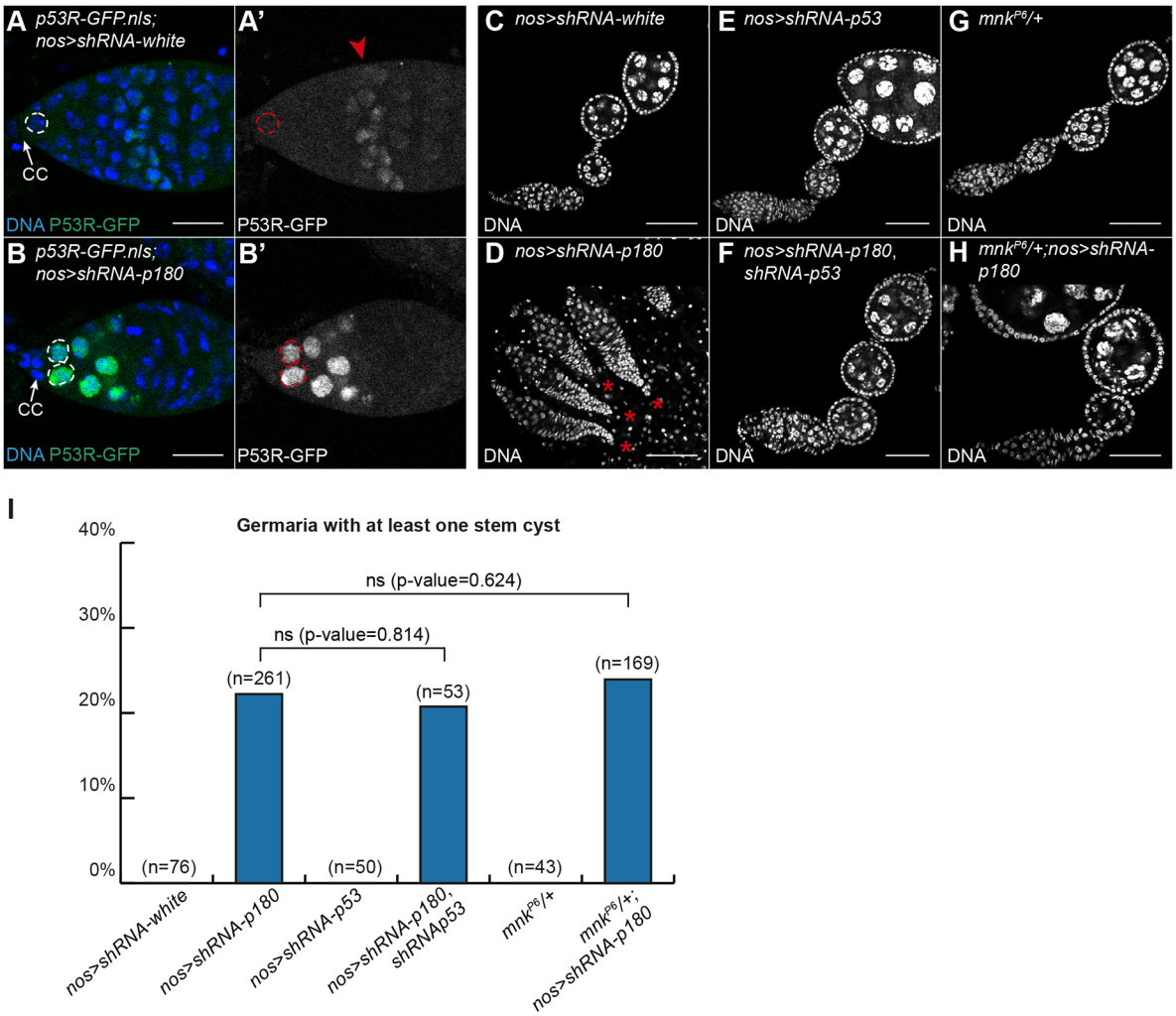

Fig. 3. p53- and Chk2-dependent checkpoint pathways are activated in response to depletion of P180 in germ cells. (A-B′) Control (A) and P180-depleted (B) germaria expressing the *p53R-GFP.nls* transgene (GFP fluorescence in green and A′,B′), with DNA staining (blue). Dotted circles delineate GSCs, attached to cap cells (CCs). The red arrowhead indicates cells with P53R-GFP-associated fluorescence in the meiotic region of the control germarium (A′). Scale bars: 15 µm. (C-H) Control (C,E,G) or P180-depleted (D,F,H) ovarioles from otherwise wild-type (C,D), p53-depleted (E,F) or *mnk*^{P6} heterozygous (G,H) ovaries. Ovarioles are visualized by DNA staining. The red asterisks (D) indicate the absence of egg chambers attached to the germarium in non-rescued P180-depleted ovaries. Scale bars: 50 µm. (I) Fraction of control or P180-depleted germaria exhibiting at least one stem cyst in otherwise wild-type, p53-depleted or *mnk*^{P6}/+ heterozygous ovaries. Stem cysts were scored on germaria immunostained using α-spectrin antibody: they were identified as a group of at least three cells, linked by a fusome, with the most anterior cell being attached to the niche. *n* specifies the number of germaria analyzed for each genotype; ns indicates a non-significant difference, as evaluated using a chi-squared test.

by depletion of P180 in germ cells was significantly rescued (Fig. 4D-G). However, the number of GSCs/CBs containing RpA70:GFP foci was not significantly affected in rescued ovaries (Fig. 4C). Overall, these data argue that the large subunit of *Drosophila* CAF1 is required to prevent replicative stress at given loci, such as the rDNA loci. This precludes, at least in part, the activation of checkpoint pathways that ultimately lead to oogenesis arrest upon depletion of P180 in germ cells.

I elements are de-repressed and DNA damage accumulates in germ cells depleted of *Drosophila* CAF1 large subunit

Another possible source of genotoxic stress in P180-depleted germ cells could be DNA damage arising from de-repression of transposable elements. Indeed, mouse embryos defective for the large subunit of CAF1 do not develop beyond the morula stage and this was shown to be due, at least in part, to de-repression of retrotransposons (Hatanaka et al., 2015). Thus, we used an antibody recognizing the protein product of I elements, a class of retrotransposons present in the fly genome (Bucheton et al., 1992), to evaluate whether I elements were expressed upon loss of P180. We did not detect any significant staining in the germarium of P180-depleted flies (not shown). Nevertheless, we expected that, if abnormally expressed, the I element peptides would progressively accumulate and be more visible at later stages (Seleme et al., 1999). This led us to assess the expression of I-elements in the P180-depleted egg chambers that form in ovaries rescued by the inhibition of cell death or checkpoint pathways. Remarkably, we observed an accumulation of I element peptides forming aggregates in the nuclei of rescued P180-depleted nurse cells but never in control nurse cells (Fig. 5A-D). As p53 has been shown to restrain the expression of transposons (Wylie et al., 2016), there may be a cumulative effect on the expression of I element peptides caused by depleting both p53 and P180 (Fig. 5D), although we did not observe I element peptides when p53 was depleted alone (Fig. 5C).

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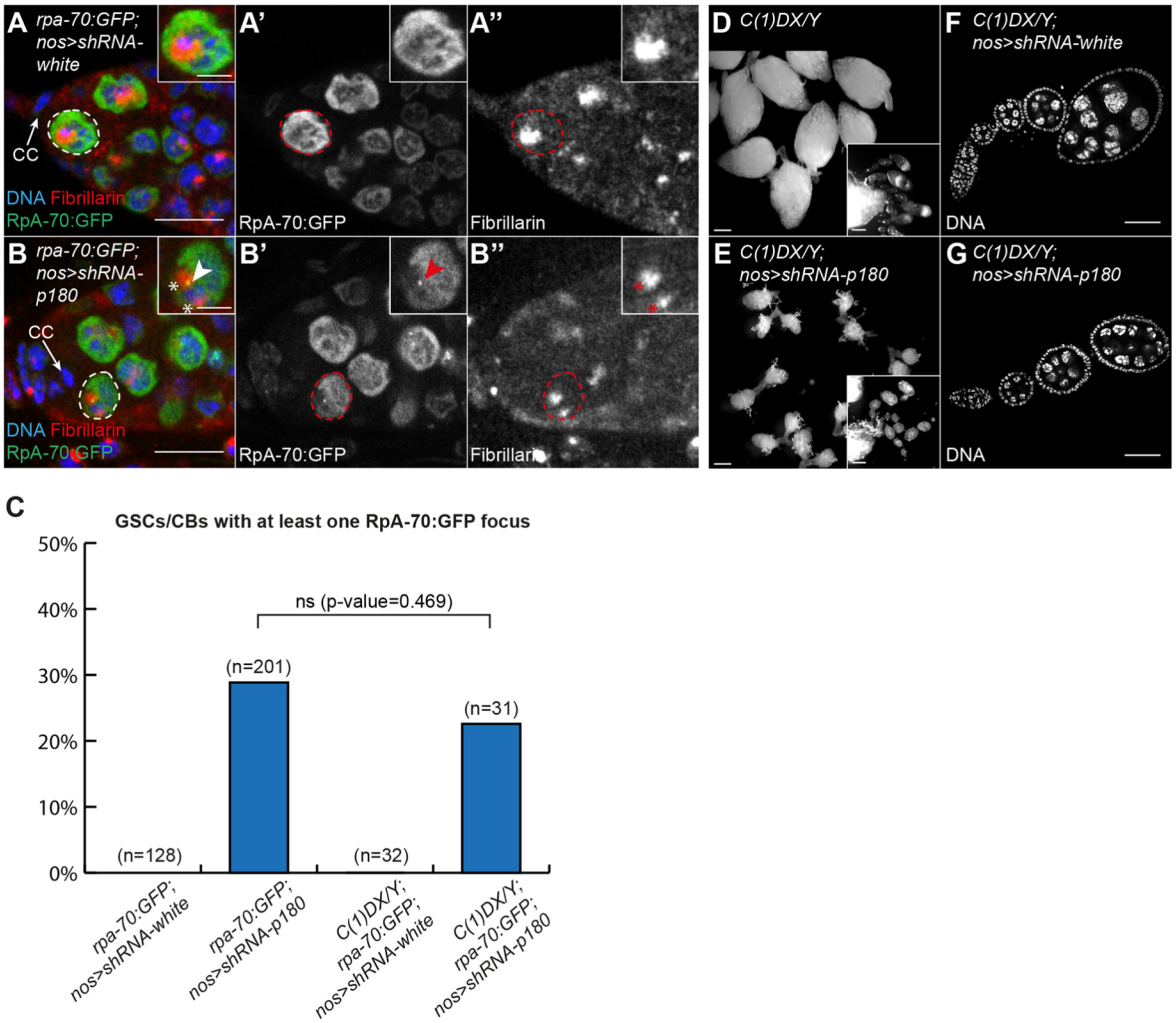

Fig. 4. rDNA arrays are involved in the oogenesis arrest induced by the depletion of P180. (A-B″) Control (A) and P180-depleted (B) germaria expressing the *rpa-70:GFP* transgene (GFP fluorescence in green and A′,B′), with DNA (blue) and fibrillarin (red and A″,B″) staining. Dotted circles mark GSCs in contact with cap cells (CCs). Insets show higher-magnification views of the circled GSCs. The arrowheads (B,B′) indicate a focus of ssDNA and the asterisks (B,B″) indicate fragmentation of the nucleolus. Scale bars: 10 µm; 5 µm in insets. (C) Fraction of control and P180-depleted GSCs and CBs exhibiting at least one focus of RpA-70:GFP in otherwise wild-type or *C(1)DX*-rescued ovaries. *n* specifies the number of GSCs and CBs analyzed for each genotype; ns indicates a non-significant difference, as evaluated using a chi-squared test. (D-G) Control (D,F) and P180-depleted (E,G) whole ovaries (D-E) and ovarioles (F,G) from flies carrying a *C(1)DX* chromosome that lacks rDNA arrays. Insets show higher-magnification views of a dissected ovary. Ovarioles on F and G are visualized by DNA staining. Scale bars: 200 µm in D,E; 50 µm in F,G; 100 µm in insets.

In the germline, transposable elements are primarily repressed by a specialized class of small RNAs: the Piwi-interacting RNAs (piRNAs) (Iwasaki et al., 2015). Interestingly, we observed that the 'nuage', a perinuclear structure containing several piRNA processing factors and whose integrity reflects efficient production of piRNAs (Lim and Kai, 2007), was altered in nurse cells of the rare $p180^{3}$ egg chambers that we observed upon induction of GLC (Fig. S8). This suggests that piRNA production in general may be affected by the loss of P180 in nurse cells.

In addition, the nuclear localization of I element peptides in P180-depleted nurse cells (Fig. 5B,D) suggests that retrotransposition occurs in this context. In line with this hypothesis, rescued P180-depleted nurse cells exhibited a strong γH2Av staining when compared with control nurse cells (Fig. 5E-H), indicating increased DNA damage, which likely contributes to the induction of checkpoint pathways and cell death. These observations indicate that the large subunit of *Drosophila* CAF1 is required to maintain the repression of I element retrotransposons and to prevent DNA damage.

Depletion of histone H3, HP1a and the Eggless H3K9 methyltransferase alters germ cells identity and genome integrity

Finally, we aimed to investigate whether the well-characterized functions of *Drosophila* CAF1 in histone deposition and heterochromatin maintenance were involved in the maintenance of GSC identity and genome integrity. These functions notably rely on interactions with histone H3 and HP1a, respectively (Huang et al., 2010; Roelens et al., 2017; Tyler et al., 1999). Hence, we depleted H3 and HP1a using RNAi in germ cells and compared the resulting phenotypes with those obtained by depleting P180. Transgenes encoding shRNAs targeting these proteins provided efficient depletion in the germline (Fig. S9). Depletion of either H3 or HP1a under the control of *nanos-gal4* induced female sterility. H3-depleted ovaries contained mainly germaria with rare egg chambers attached (Fig. 6A,B), whereas HP1a-depleted ovaries contained ovarioles with approximately five or six egg chambers that increase only a little in size and arrest their development before accumulating yolk (Fig. 6C,D).

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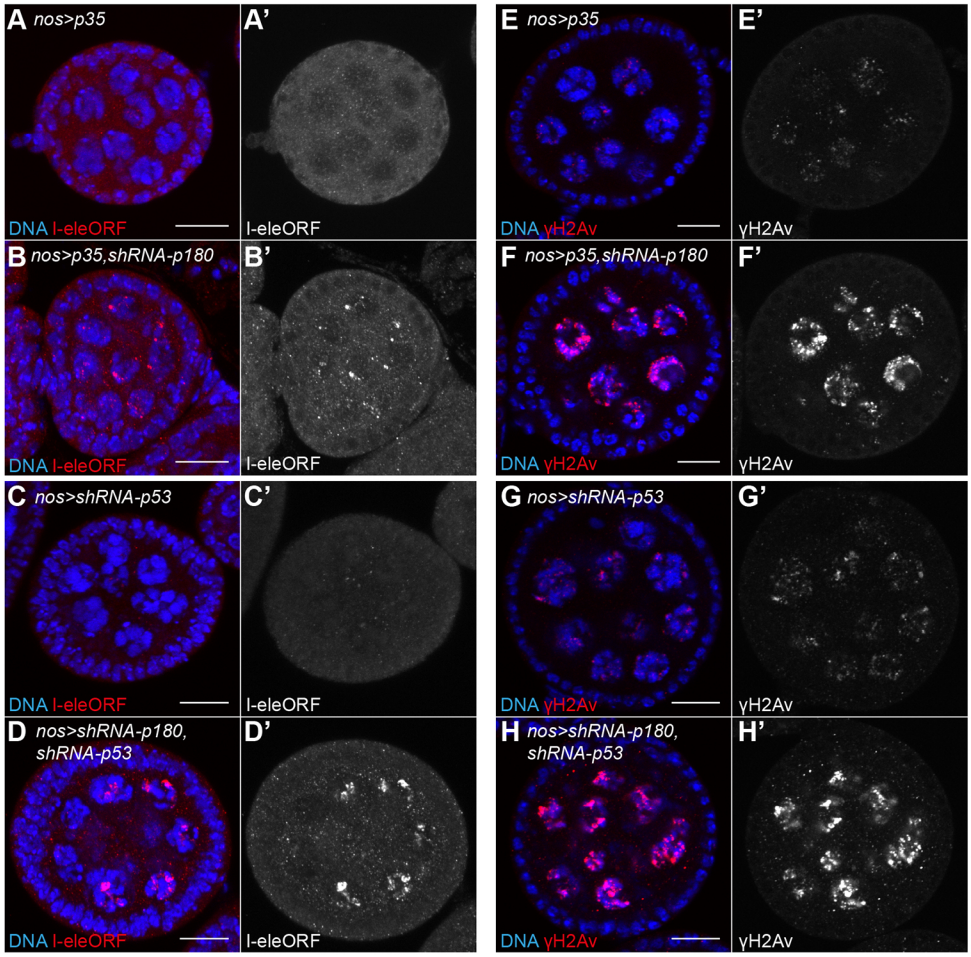

Fig. 5. I-element retrotransposons are expressed and DNA damage accumulates in the nucleus of rescued P180-depleted nurse cells. (A-D′) Control (A,A′,C,C′) and rescued P180-depleted (B,B′,D,D′) egg chambers from otherwise P35-expressing or p53-depleted ovaries, with DNA (blue) and I-element peptide (I-eleORF, red and A′-D′) staining. Images are maximum intensity projections of several confocal images. Scale bars: 15 µm. (E-H′) Control (E,E′,G,G′) and rescued P180-depleted (F,F′,H,H′) egg chambers from otherwise P35-expressing or p53-depleted ovaries, with DNA (blue) and phosphorylated H2Av (γH2Av, red and E′-H′) staining. Scale bars: 15 µm.

Both H3-depleted and HP1a-depleted GSCs harbored branched fusomes and expressed simultaneously pMad and Bam-GFP (Fig. 6E-F), indicating a defect of GSC identity, such as that induced by depletion of P180. CAF1 associates with the H3 lysine 9 methyltransferase SetDB1 during replication of heterochromatin in human cells (Sarraf and Stancheva, 2004). Eggless, the homolog of SetDB1 in flies, is also known to be required for piRNAs production in the germline (Rangan et al., 2011). We therefore checked the phenotype induced by expression of a shRNA against *eggless* under the control of *nanos-gal4* and found that it led to the formation of Bam-GFP positive stem cysts (Fig. 6G).

Despite these strong similarities, genome integrity was affected differently in H3- and HP1a-depleted contexts. In nurse cells depleted of H3, I-element peptides accumulated in nuclei (Fig. 6H), together with γH2Av foci (Fig. 6J), indicative of I element de-repression and increased DNA damage, as observed upon P180 depletion. In contrast, in HP1a-depleted egg chambers, I-element peptides accumulated in a pattern clearly different than observed in P180- and H3-depleted cells: I elements were not enriched in the nuclei of nurse cells but mainly in the cytoplasm of the oocyte (Fig. 6I). This is similar to the pattern described in the ovaries of hybrid flies in which I elements show a high frequency of retrotransposition in mature oocytes (Seleme et al., 1999). Interestingly, the absence of I-element peptides in the nuclei of HP1a-depleted nurse cells coincides with a much lower γH2Av staining when compared with H3-depleted nurse cells (Fig. 6K), suggesting that DNA damage does not accumulate in HP1a-depleted nurse cells.

Similarly to P180 depletion, knockdown of H3, HP1a or Eggless/dSetDB1 in germ cells alters GSC identity and transposon silencing. These results imply that both functions of *Drosophila* CAF1 in nucleosome assembly and heterochromatin maintenance likely contribute to the maintenance of GSC identity and genome integrity.

DISCUSSION

Using RNAi to deplete the large subunit of *Drosophila* CAF1 (P180) at different stages of female germline development, we show that its function is required in the stem cell compartment to ensure proper oogenesis. When the depletion of P180 is induced in all ovarian germ cells, including stem cells, oogenesis is aborted prior to egg chamber formation and this arrest is in part rescued by inhibition of cell death. Remarkably, we observed that, although GSCs persist upon depletion of P180, their identity appears compromised. Indeed, depletion of P180 from GSCs onwards leads to the formation of stem cysts, which are characterized by a GSC anchored to the niche and abnormally linked to other germ cells by a branched fusome. Strikingly, stem cysts forming upon depletion of P180 express Bam, which is in contrast to previously reported stem cysts forming upon perturbation of abscission (Eikenes et al., 2015; Mathieu et al., 2013; Matias et al., 2015). Even though Dpp signaling seems activated in P180-depleted GSCs, as shown by pMad staining, it is not sufficient to repress Bam-GFP and *bam* RNA expression in stem cysts. Although it remains possible that Bam-GFP and *bam* RNA are produced by the

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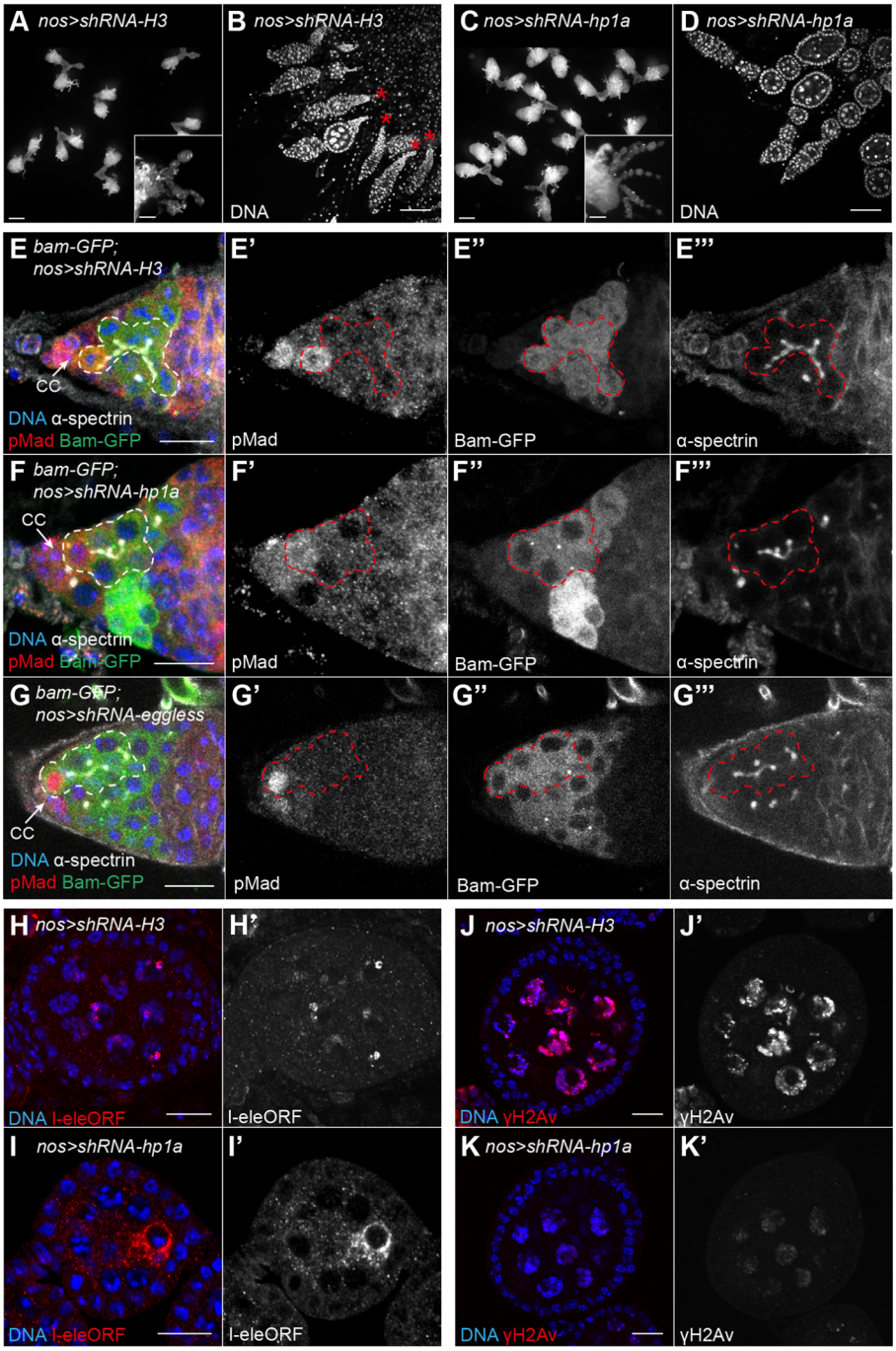

Fig. 6. Depletion of histone H3, HP1a and the Eggless H3K9 methyltransferase alters germ cells identity and genome integrity. (A,B) Histone H3-depleted whole ovaries (A) and ovarioles (B). Ovarioles in B are visualized by DNA staining. The red asterisks (B) indicate the absence of egg chambers attached to the germarium. Inset in A shows a higher-magnification view of a dissected ovary. Scale bars: 200 µm in A; 50 µm in B; 100 µm in inset. (C,D) HP1a-depleted whole ovaries (C) and ovarioles (D). Inset in C shows a higher-magnification view of a dissected ovary. Ovarioles in D are visualized by DNA staining. Scale bars: 200 µm in C; 100 µm in inset; 50 µm in D. (E-G‴) H3-depleted (E-E‴), HP1a-depleted (F-F‴) and Eggless-depleted (G-G‴) germaria expressing the *bam-GFP* transgene (GFP fluorescence in green and E″,G″), with DNA (blue), phosphorylated Mad (pMad, red and E′,G′) and α-spectrin (white and E‴,G‴) staining. Dotted lines delineate stem cysts. Images are maximum intensity projections of several confocal images. CC, cap cells. Scale bars: 15 µm. (H-I′) H3-depleted (H,H′) and HP1a-depleted (I,I′) egg chambers with DNA (blue) and I-element peptides (I-eleORF, red and H′,I′) staining. Scale bars: 15 µm. (J-K′) H3-depleted (J,J′) and HP1a-depleted (K,K′) egg chambers with DNA (blue) and γH2Av (red and J′,K′) staining. Scale bars: 15 µm.

most posterior cells of the stem cysts and diffuse into the anterior GSC, our results strongly suggest that P180-depleted GSCs adopt a mixed fate.

Moreover, expression of P180 in germ cells is essential to preserve genome integrity and its absence triggers p53- and Chk2-dependent checkpoint activation that eventually lead to cell death. This recalls observations in zebrafish, in which a mutation in *caf1b*, the gene encoding the middle subunit of CAF1, leads to apoptosis during the formation of the retina, a phenotype that is rescued upon simultaneous depletion of p53 (Fischer et al., 2007). As a possible source of genotoxic stress causing checkpoint activation, P180-depleted GSCs and their daughter cells exhibit discrete foci of ssDNA, visualized by RpA70:GFP accumulation and evocative of replicative stress. These foci mainly localize within the nucleolus and reduction of rDNA copy number in P180-depleted germ cells results in a partial rescue of the oogenesis arrest, suggesting that replicative stress may occur at rDNA arrays. Yet, RpA70:GFP foci persisted in P180-depleted germ cells rescued by reduction of rDNA copy number. These foci could correspond to remaining rDNA arrays or to aggregates of damage sites (Aymard et al., 2017) containing not only rDNA but possibly other remote loci, potentially explaining why they are not visibly affected by reduction of rDNA copy number. Genotoxic stress in P180-depleted germ cells could also arise from retrotransposon insertions, as I-element retrotransposons are de-repressed in these cells in correlation with increased DNA damage signaled by γH2Av. A possible cause for the de-repression of transposable elements in P180-depleted germ cells could be a defect in piRNA biogenesis, as

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we observed that the 'nuage' was altered in this context. Of note, CAF1 is also involved in the regulation of transposable elements in other organisms, including mammals (Hatanaka et al., 2015; Ishiuchi et al., 2015) and *Paramecium* (Ignarski et al., 2014).

Last, the GSC identity defect reflected by the formation of Bam-expressing stem cysts is recapitulated by depletion of either histone H3, HP1a or Eggless/SetDB1. De-repression of I elements is also induced by depletion of either histone H3 or HP1a. We observed that DNA damage signaled by γH2Av accumulates in P180- and H3-depleted egg chambers, in which I-element peptides localize in the nucleus of nurse cells, but not in egg chambers depleted of HP1a, in which I-element peptides are restricted to the cytoplasm of the oocyte. The correlation between the localization of I-element peptides in the nuclei of nurse cells or in the cytoplasm of oocytes and the respective occurrence or absence of DNA damage suggest that DNA damage may be a consequence of I-element peptide accumulation in the nuclei of nurse cells.

We found that *bam* and I-element loci are abnormally de-repressed upon depletion of the *Drosophila* CAF1 large subunit. It is possible that transcriptional repression dependent on specific chromatin states is particularly sensitive to the loss of CAF1 function. Indeed, at each cycle of DNA replication, nucleosomes are disassembled and in absence of CAF1-dependent deposition of new histones, only the parental histones are expected to be reassembled onto the newly synthesized DNA strands through CAF1-independent histone recycling pathways. Consequently, pre-existing regulatory chromatin states would be progressively diluted at each cycle of replication, thereby alleviating the repression of the underlying loci, such as *bam* and I-element insertions. Dilution of parental histones in the absence of CAF1 would generate a more-accessible chromatin landscape that could also facilitate the binding of transcriptional activators, as previously proposed (Cheloufi et al., 2015). As depletion of H3 or HP1a recapitulated both the de-repression of *bam* and the de-repression of I-element loci, we hypothesize that depletion of P180 in germ cells could lead to a decrease in H3K9me3, a histone modification associated with HP1 binding and transcriptional silencing (Eissenberg and Reuter, 2009), at these loci. Accordingly, we found that depletion of the H3K9 methyltransferase Eggless/SetDB1 in all germ cells also appears to cause a defect in *bam* repression in GSCs. We also observed that the global amount of histone H3 in P180-depleted germ cell nuclei was visibly, but only partially, decreased, suggesting that nucleosome organization might not be globally altered upon loss of *Drosophila* CAF1 function. This is reminiscent of *Arabidopsis thaliana* CAF1 mutants (Ramirez-Parra and Gutierrez, 2007; Schönrock et al., 2006) and murine embryonic stem cells deficient for CAF1 function (Houlard et al., 2006), in which local changes in histone H3 occupancy and histones H3- and H4-associated marks were corresponded to changes in transcriptional activity at specific loci, without global perturbation of nucleosomal organization. Mechanistically, it is not clear how loss of CAF1 function could alter the chromatin structure in a loci-specific manner. One hypothesis is that physical interactions of CAF1 subunits with components of signaling pathways could direct the complex to specific target loci. In *Drosophila*, this was proposed to mediate targeting of CAF1 to the enhancer regions of the target genes in the Notch pathway, in order to maintain a local enrichment in acetylated histone H4 (Yu et al., 2013).

In the context of the fly germline development, our results bring new insights into the mechanism underlying *bam* repression downstream of the BMP signaling pathway at the level of the chromatin in female GSCs. The involvement of HP1a in this process corroborates recent findings in male GSCs, in which RNAi-mediated depletion of HP1 in germ cells leads to premature expression of *bam* in stem cells (Xing and Li, 2015). Interestingly, another epigenetic regulator, histone H1, was also recently shown to be required for the repression of *bam* transcription in female GSCs (Sun et al., 2015), highlighting the importance of the chromatin environment to regulate cell fate in GSCs.

Finally, our findings in the *Drosophila* female germline contribute to reinforce the idea that CAF1 plays an essential role in the maintenance of given cellular identities, as proposed from studies in other organisms (Cheloufi and Hochedlinger, 2017; Cheloufi et al., 2015; Ishiuchi et al., 2015; Kaya et al., 2001; Nakano et al., 2011; Zeng et al., 2013). Our observation that female GSCs depleted of *Drosophila* CAF1 large subunit exhibit a dual identity, simultaneously harboring features of stem cells and differentiating germ cells, particularly recalls the observation that, in plants, stem cells of the root meristem also accumulate markers of differentiated cells in absence of CAF1 (Kaya et al., 2001). Importantly, a common aspect that seems to arise from our study and previous studies in other organisms is that, upon loss of CAF1 function, the identity of less differentiated cells appears to be generally more affected than the one of more differentiated cells. In other words, loss of function of CAF1 seems to have a more pronounced effect on the identity of cells that have a higher degree of plasticity. However, further investigations will be necessary to confirm this trend and to understand the molecular mechanisms that underlie it.

MATERIALS AND METHODS

Fly stocks and genetics

The *Drosophila* alleles or transgenes used in this study were as follows: $p180^{3}$ (Klapholz et al., 2009), mnk^{P6} (a gift from U. Abdu, Ben-Gurion University of the Negev, Beersheba, Israel), $bam^{\Delta 86}$ (BDSC, #5427), *C(1)DX* (BDSC, #23880), $mei\text{-}W68^{1}$ (BDSC, #4932), $mei\text{-}W68^{K05603}$ (BDSC, #10574), *bam:GFP* (a gift from D. McKearin, University of Texas Southwestern Medical Center, Dallas, TX, USA), *p53R:GFP.nls* (a gift from J. Abrams, University of Texas Southwestern Medical Center, Dallas, TX, USA), *rpa-70:GFP* (a gift from E. Wieschaus, Princeton University, NJ, USA), *H3:RFP* (a gift from B. Loppin, Université Claude Bernard, Villeurbanne, France), *FRT19A* (BDSC, #1709), *ubi-mRFP.nls,hsFLP, FRT19A* (BDSC, #31418), *nanos-gal4* (a gift from M. Fuller, Stanford University School of Medicine, CA, USA, and BDSC, #4937), *bam-gal4* (a gift from M. Fuller), *bam-gal4*×3 or ×6 (new insertions generated by standard transposition using the Delta2-3 transposase; two independent insertions on the second chromosome were recovered and recombined), *matα-GAL4-VP16* (*mat-gal4*; BDSC, #7062), *P{TRiP.HMS00480}attP2* (*UAS-shRNA-p180*; BDSC, #32478), *P{TRiP.HM05129}attP2* (*UAS-shRNA-p180*; BDSC, #28918), *P{TRiP.GL01220}attP40* (*UAS-shRNA-p53*; BDSC, #41638), *P{TRiP.HMS00278}attP2* (*UAS-shRNA-hp1a*; BDSC, #33400), *P{TRiP.GL00255}attP2* (*UAS-shRNA-H3*; BDSC, #35344), *P{TRiP.HMS00112}attP2* (*UAS-shRNA-eggless*; BDSC, #34803), *P{TRiP.HMS00045}attP2* (*UAS-shRNA-white*; BDSC, #33644), *UAS-P35* (BDSC, #5072) and *UAS-lamin:GFP* (BDSC, #7376). All fly stocks were raised at 22-25°C on standard food media.

$p180^{3}$ mutant clones in the ovaries were generated using the Flp/FRT recombination technique (Chou and Perrimon, 1992). $p180^{3}$*,FRT19A/FM7i* females were crossed with *ubi-mRFP.nls,hsFLP,FRT19A* males and the resulting third instar larvae were exposed to heat-shock at 37°C for 2 h on 2 consecutive days. Adult females were dissected 4 days after hatching. All crosses to induce RNAi-mediated depletion together with control crosses were performed at 29°C and the resulting adult females were dissected 1-3 days after hatching.

Immunostaining

Adult ovaries were dissected in PBS and fixed in 4% paraformaldehyde for 15 min. Samples were then rinsed and permeabilized in PBT×2 (PBS with 0.2% Triton X-100) for at least 30 min, incubated at 4°C overnight with

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primary antibodies at the proper dilution in PBT×2, washed three times for 15 min in PBT×2, incubated for 2 h with secondary antibodies at the proper dilution in PBT×2 at room temperature and washed three times 15 min in PBT×2. For DNA staining, samples were subsequently stained with DAPI (0.8 µg/ml in PBS) for 10 min. Samples were mounted in Vectashield (Vector laboratories).

The primary antibodies used are as follows: mouse anti-α-spectrin 3A9 (1:500, DSHB), rat anti-Vasa (1:10, DSHB), mouse anti-fasciclin III 7G10 (1:50, DSHB), rabbit anti-cleaved caspase 3 (1:100, Cell Signaling, 9661S), rabbit anti-phospho-Smad5 Ser463/465 (referred to as pMad, 1:100, Epitomic, 2224-1, a kind gift from L. De Koning, Institut Curie, Paris, France), rabbit anti-γH2Av (1:1000, Rockland, 600-401-914), rabbit anti-P180 [1:1000, antibody raised in rabbit against a peptide corresponding to the residues 198-421 of P180 (Roelens et al., 2017)], rabbit anti-fibrillarin (1:500, Abcam), rabbit anti-I-eleORF (1:400, kindly provided by D. Finnegan, Edinburgh University, UK), rabbit anti-H3 (1:200, Abcam, 1791) and rabbit anti-aubergine (1:500, kindly provided by P. Lasko, McGill University, Montreal, Canada). Alexa Fluor 488-, 568- or 633-conjugated secondary antibodies (Life Technologies and Jackson Immunoresearch) were used at a 1:500 dilution.

RNA-FISH

Dual fluorescence detection of proteins and RNA on adult ovaries was performed as previously described for *Drosophila* testes (Toledano et al., 2012). All steps were carried out in RNAse-free conditions, using solutions treated with diethylpyrocarbonate. Adult ovaries from 1- to 3-day-old flies were dissected in PBS, fixed in 4% formaldehyde in PBT (PBS-0.1% Tween-20) for 20 min, washed in PBTH (PBT, 50 µg/ml heparin, 250 µg/ml tRNA) and incubated with primary antibody (mouse anti-α-spectrin 3A9, DSHB, 1:10) in PBTHR (PBTH with 0.2 U/ml RNAse) overnight at 4°C. On the second day, samples were washed in PBTH, incubated with fluorescent secondary antibody (1:100) in PBTHR for 2 h, washed in PBTH and post-fixed for 20 min in 10% formaldehyde in PBT. After being washed in PBTH, samples were then pre-hybridized for 1 h in hybridization buffer (Toledano et al., 2012) supplemented with tRNA (10 mg/ml) before addition of the denatured *bam* RNA-digoxigenin (DIG) probe (1.2 µg). Hybridization was carried out overnight at 65°C. On the third day, samples were washed with decreasing concentrations of hybridization buffer diluted in PBT at 65°C, before being processed for signal detection following instructions provided by the TSA-Cyanine 3 kit manufacturer (Perkin Elmer). Samples were incubated in blocking buffer for 30 min and with sheep anti-DIG-POD in blocking buffer (1:500) overnight at 4°C. Finally, on the fourth day, samples were washed in PBT, incubated with TSA-Cyanine 3 in Amplification Diluent (1:500) provided in the TSA kit for 30 min at room temperature. After washes in PBT, samples were mounted in Vectashield containing DAPI (Vector Laboratories).

The sense and antisense RNA probes directed against *bam* RNA were constructed according to the protocol described in Toledano et al., from a linearized plasmid containing *bam* cDNA sequence flanked by a SP6 and a T7 promoter on 5′ and 3′ respectively. Both probes were labelled using a DIG RNA labeling kit (Roche).

Microscopy and image analysis

Fluorescent images were acquired with a Plan-Apochromat 40×/NA1.4 oil immersion objective on a Zeiss LSM 780 confocal microscope. Image analysis was performed using the ImageJ software.

Stem cyst quantification was carried out on germaria immunostained using α-spectrin antibody: stem cysts were identified as a group of three cells minimum, linked by a fusome, with its most anterior cell being attached to the niche. The percentage of germaria exhibiting at least one stem cyst was calculated. Chi-squared tests were used to compare the percentages observed in the different genotypes. All images shown are representative images of at least three independent experiments.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.C., A.M.-H., J.M., J.-R.H., N.D.; Formal analysis: M.C.; Investigation: M.C., A.M-H., J.M.; Writing - original draft: M.C., J.-R.H., N.D.; Writing - review & editing: M.C., A.M.-H., J.M., J.-R.H., N.D.; Supervision: J.-R.H., N.D.; Funding acquisition: J.-R.H., N.D.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.161190.supplemental

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