Stem Cell Reports
ISSCR
Article
OPEN ACCESS

Ras promotes germline stem cell division in *Drosophila* ovaries

Qi Zhang,[1] Yanfang Wang,[1] Zhenan Bu,[1] Yang Zhang,[1] Qian Zhang,[1] Le Li,[1] Lizhong Yan,[1] Yuejia Wang,[1] and Shaowei Zhao[1,2,*]
[1]Department of Genetics and Cell Biology, College of Life Sciences, Nankai University, Tianjin 300071, People's Republic of China
[2]Lead contact
*Correspondence: swzhao@nankai.edu.cn
https://doi.org/10.1016/j.stemcr.2024.06.005

SUMMARY

The *Ras* family genes are proto-oncogenes that are highly conserved from *Drosophila* to humans. In *Drosophila*, Ras^{V12} is a constitutively activated form of the Ras oncoprotein, and its function in cell-cycle progression is context dependent. However, how it influences the cell cycle of female germline stem cells (GSCs) still remains unknown. Using both wild-type GSCs and *bam* mutant GSC-like cells as model systems, here we determined that Ras^{V12} overexpression promotes GSC division, not growth, opposite to that in somatic wing disc cells. Ras performs this function through activating the mitogen-activated protein kinase (MAPK) signaling. This signaling is activated specifically in the M phase of mitotic germ cells, including both wild-type GSCs and *bam* mutant GSC-like cells. Furthermore, Ras^{V12} overexpression triggers polyploid nurse cells to die through inducing mitotic stress. Given the similarities between *Drosophila* and mammalian GSCs, we propose that the Ras/MAPK signaling also promotes mammalian GSC division.

INTRODUCTION

The *Ras* family genes encode small GTPases that regulate many cellular processes, including cell-cycle progression, cell metabolism, cell migration, and cell survival (Gimple and Wang, 2019). Remarkably, The Cancer Genome Atlas (TCGA) project reveals that the Ras pathway is the most frequently altered oncogenic pathway in cancer (Sanchez-Vega et al., 2018). Ras mutations alone contribute to 20%–30% of all human cancers (Gimple and Wang, 2019). Among three *Ras* genes in the *Drosophila* genome, *Ras oncogene at 85D* (*Ras85D*, hereafter *Ras*) has the highest homology to human *Ras* (Neuman-Silberberg et al., 1984). Ras^{V12} is a constitutively activated form of the fly Ras oncoprotein (Fortini et al., 1992). Its overexpression promotes the growth, not the division, of imaginal wing disc cells (Prober and Edgar, 2000). In intestinal stem cells, its overexpression may promote both their growth and division through elevating the cell-cycle regulators Cyclin E and String/Cdc25 (Jin et al., 2015). In primordial germ cells, coactivation of Ras and signal transducer and activator of transcription (STAT) by a gain-of-function mutation of a receptor tyrosine kinase gene, *torso*, is required for their proliferation and invasive migration (Li et al., 2003). Therefore, it seems that the function of the Ras signaling in cell-cycle progression is context dependent. However, it still remains unknown how Ras^{V12} overexpression influences the cell cycle of germline stem cells (GSCs).

GSCs are well defined in fly ovaries. Each ovary has 16–20 individual ovarioles, and the anterior structure of each ovariole is called germarium. The tip region of each germarium holds a stem cell niche, which is composed of cap cells, escort cells, and terminal filament cells. Each stem cell niche contains two or three GSCs. Typically, GSCs perform asymmetric divisions individually to generate a GSC daughter to self-renew and a cystoblast daughter committed to differentiate. During the differentiation process, each cystoblast first takes exactly 4-round transit-amplification divisions with incomplete cytokinesis to form a 16-cystocyte germline cyst. Then, each germline cyst is enclosed by epithelial follicle cells to make up an egg chamber. Among the 16 germ cells in each egg chamber, one differentiates into the oocyte while the rest 15 become nurse cells (Figure 1A). Of note, nurse cells are endocycling polyploid cells, which only have G and S phases. They perform robust cellular growth to accumulate abundant proteins and RNAs. In late oogenesis, they sacrifice these contents to the oocyte and then disappear. Finally, the oocyte occupies the whole egg chamber and develops into a mature egg (de Cuevas et al., 1997).

Mechanistically, GSC stemness is maintained by both extrinsic and intrinsic factors. The major extrinsic factors secreted by niche somatic cells are the bone morphogenetic protein (BMP) ligands. They activate the downstream BMP signaling components as the key intrinsic factors in GSCs to repress the transcription of a key differentiation gene, *bag of marbles* (*bam*), to maintain the stemness of GSCs (Chen and McKearin, 2003a; Song et al., 2004; Xie and Spradling, 1998). Different from GSCs, cystoblasts are displaced from stem cell niches. Consequently, they are driven to differentiate by key differentiation factors, Bam and benign gonial cell neoplasm (Bgcn) (Lavoie et al., 1999; Li et al., 2009; Ohlstein et al., 2000). Of note, *bam* or *bgcn* mutant GSCs are unable to differentiate, and they perform uncontrolled proliferation to form GSC-like

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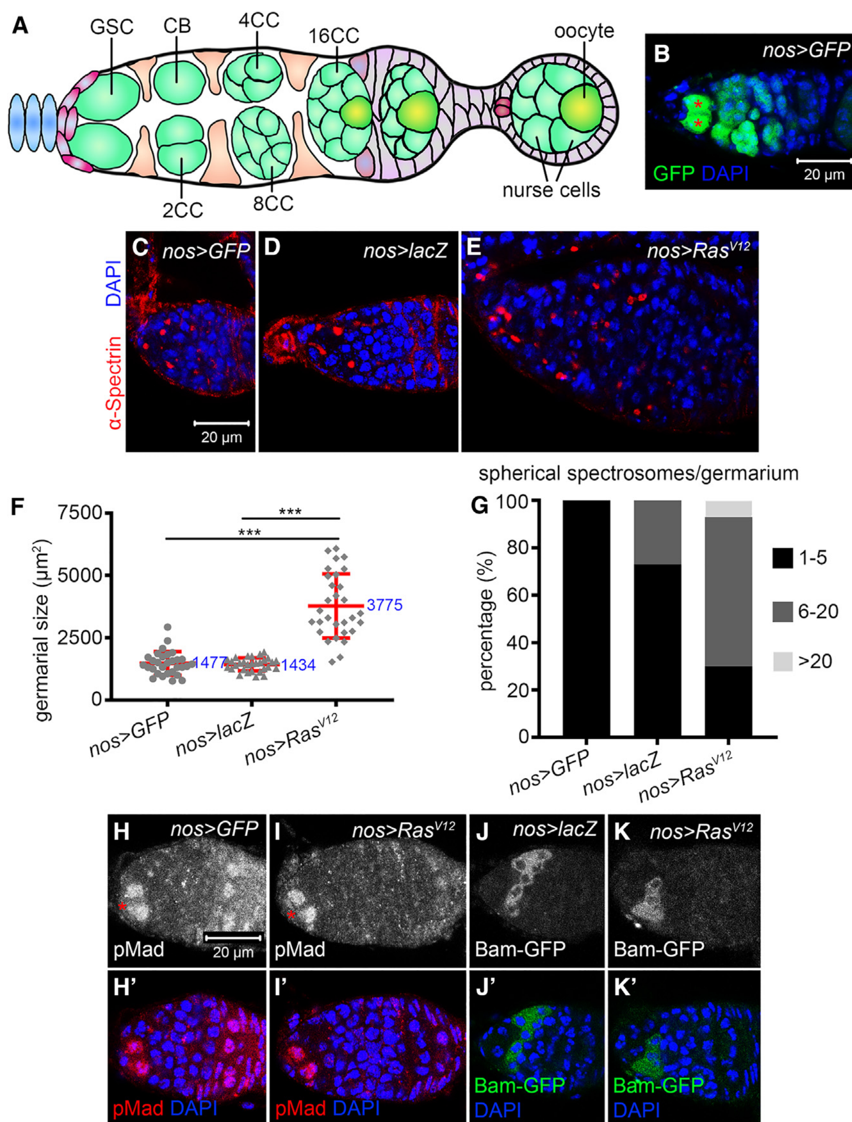

Figure 1. Ras^{V12} overexpression induces GSC over-proliferation

(A) Schematic diagram of a germarium and an early-stage egg chamber. GSC, germline stem cell; CB, cystoblast; 2, 4, 8, 16CC, 2-, 4-, 8-, 16-cell cystocytes. The oval red cells to the left of GSCs are cap cells, and the oval blue cells to the left of cap cells are terminal filament cells. The triangular yellow cells are escort cells and inner sheath cells. The dark and light pink cells in the posterior part of the germarium are follicle stem cells and follicle cells, respectively. The two red cells in the anterior part of the egg chamber are polar cells.

(B) The activation pattern of *nos-GAL4-VP16*. The two red asterisks denote germline stem cells.

(C–E) Representative germarial images. α-Spectrin labels spectrosomes in GSCs/cystoblasts (spherical shape) and fusomes in cystocytes (branched shape). All images are of the same magnification.

(F) Quantification of germarial sizes. For each genotype, 30 germaria were quantified, and, for each germarium, its biggest 2D size scanned by confocal microscope in one focal plane was used to reflect its 3D size. Data represent mean ± SD, and, for each group of data, the mean number is labeled right. Statistical significance was determined by t test. ***: $p < 0.001$.

(G) Quantification of spherical spectrosomes per germarium. For each genotype, 30 germaria were quantified.

(H–K′) Representative germarial images. The red asterisks in (H and I) denote cap cells, and the pMad^{+} germ cells to the right of them are GSCs. All images are of the same magnification. These experiments were performed at 29°C, and 7-day-old flies were used. See all genotypes in Table S1 and the raw quantification data in Table S2. See also Figures S1 and S2.

tumors (Lavoie et al., 1999; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990). These GSC-like tumor cells are also an invaluable model system to study GSC cell-cycle progression.

In this study, using both wild-type GSCs and *bam* mutant GSC-like cells as model systems, we determined that Ras^{V12} overexpression promotes GSC division, not growth, opposite to that in somatic wing disc cells. Ras performs this function through activating the mitogen-activated protein kinase (MAPK) signaling. This signaling is activated specifically in the M phase of both wild-type GSCs and *bam* mutant GSC-like cells. Also, we found that Ras^{V12} overexpression triggers polyploid nurse cells to die through inducing mitotic stress.

RESULTS

Ras^{V12} overexpression drives GSCs to over-proliferate

We first set out to examine how GSCs respond to Ras^{V12} overexpression. Such overexpression was induced by the *nos-GAL4-VP16/UAS* system (Figure 1B) (Brand and Perrimon, 1993; Rørth, 1998). Given that *UASp*, not *UASt*, fly strains work efficiently in fly female germ line (Brand and Perrimon, 1993; Rørth, 1998), we generated *UASp-Ras^{V12}* transgenic flies. To enhance the gene-overexpression efficiency, we performed the experiments at 29°C. Remarkably, compared with either *nos>GFP* or *nos>lacZ* controls, *nos>Ras^{V12}* germaria underwent drastic overgrowth (Figures 1C–1F). To determine whether these overgrowing

germaria contain more GSC-like cells, we did anti-α-Spectrin immunofluorescent staining, which labels a germ line-specific organelle called spectrosome in GSCs/cystoblasts and fusome in cystocytes. Spectrosome exhibits a spherical shape while fusome is branched (Lin et al., 1994). Of note, more spherical spectrosomes were observed in *nos>Ras^{V12}* germaria than those in the controls (Figures 1C–1E and 1G). To determine if this phenotype is caused by the ectopic activation of the BMP signaling, we did anti-phosphorylated mothers against dpp (pMad) immunofluorescent staining, which specifically labels GSCs in wild-type fly ovaries (Kai and Spradling, 2003). Strikingly, pMad signals were still restricted to the GSCs within stem cell niches in *nos>Ras^{V12}* germaria (Figures 1H–1I′), excluding the possibility that Ras^{V12} overexpression activates BMP signaling in germ line. In addition, *nos>Ras^{V12}* ovaries also contained differentiating germ cells that express Bam (Figures 1J–1K′) and even egg chambers (Figure S1), indicating that Ras^{V12} overexpression does not block GSC differentiation. Taken together, these results suggest that Ras^{V12} overexpression drives GSCs to over-proliferate.

Next, we asked whether Ras^{V12} overexpression influences the 4-round transit-amplification of cystocytes. A cystocyte-specific driver, *bam-GAL4-VP16* (Chen and McKearin, 2003b), was used (Figures S2A and S2B), and these overexpression experiments were also performed at 29°C. Surprisingly, *bam>Ras^{V12}* ovaries did not contain any >16-germ-cell egg chambers (Figures S2C and S2D), implying that their cystocytes do not divide >4 rounds. Furthermore, *bam>Ras^{V12}* female flies were fertile (>20 flies examined), indicative of a normal oogenesis process. Therefore, it seems that Ras^{V12} overexpression specifically influences GSC proliferation.

Ras antagonizes the role of Bam in promoting GSC differentiation

Opposite to that of Ras^{V12}, Bam overexpression promotes GSC differentiation prematurely, resulting in germ cell loss (Ohlstein and McKearin, 1997). Therefore, we were curious about whether overexpressing both of them has an antagonistic effect. Notably, *nos>bam+Ras^{V12}* ovaries contained a few egg chambers, none of which was observed in *nos>bam* ovaries alone (Figures 2A–2C).

bam heterozygous-mutant (*bam$^{+/-}$*) ovaries did not exhibit an obvious germline overgrowth phenotype. However, such an overgrowth phenotype was observed in almost all germaria and most egg chambers in *nos>Ras^{V12}; bam$^{+/-}$* ovaries, and it was also much stronger than that of *nos>Ras^{V12}* ovaries alone (Figures 2D–2H). Collectively, these studies imply that Ras^{V12} overexpression is able to partially antagonize the role of Bam in promoting GSC differentiation.

Ras promotes GSC division, not growth

Next, we focused on determining how Ras^{V12} overexpression influences GSC cell cycle. Given that *nos-GAL4-VP16* was strongly activated in GSCs (Figure 1B), we used *nos>GFP* to outline the GSC areas. 30 GSCs with either *nos>GFP+Ras^{V12}* or *nos>GFP+lacZ* (control) genotype were quantified. For each GSC, its biggest 2D size in one confocal focal plane was used to reflect its 3D size. Of note, *nos>GFP+Ras^{V12}* GSCs were comparable to the *nos>GFP+lacZ* controls in cell size (Figures 3A–3C). Then, we used anti-phosphohistone H3 (pH3) immunofluorescent staining to reflect the mitotic index of GSCs (Wei et al., 1999). Remarkably, the mitotic index of *nos>GFP+Ras^{V12}* GSCs was 2.6 times that of the *nos>GFP+lacZ* controls (Figures 3D–3F). Taken together, these studies suggest that Ras promotes GSC division, not growth, opposite to that in somatic wing disc cells (Prober and Edgar, 2000).

To address whether Ras is required for wild-type GSC division, we tried using its null allele, *Ras$^{\Delta C40B}$* (Hou et al., 1995), to generate mutant germline clones. Strikingly, no such clones were recovered (Figure S3, >20 ovaries examined), implying that Ras is required for germline viability.

Ras promotes the division, not the growth, of *bam* mutant GSC-like cells

As what we have introduced earlier, *bam* or *bgcn* mutant germ cells are locked in a GSC-like state and undergo uncontrolled proliferation to form germline tumors (Lavoie et al., 1999; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990). Therefore, we were curious about how their cell cycle is influenced by Ras^{V12} overexpression. Remarkably, Ras^{V12} overexpression markedly enlarged either of these two mutant ovaries (Figures 4A–4C and S4). Between these two mutant germ cells, *bam* mutant ones have been reported to be highly resistant to cell death, even with severe cell-cycle defects (Zhang et al., 2023; Zhao et al., 2018). Therefore, we chose to use them to address our aforementioned question.

For the quantification assays, we used a Vasa-EGFP reporter to outline the germline areas and anti-pH3 staining to reflect the mitotic index of *bam$^{-/-}$* GSC-like cells. 30 germaria with either *nos>Ras^{V12}; bam$^{-/-}$* or *nos>GFP; bam$^{-/-}$* (control) genotype were scanned by confocal microscope in one focal plane. For germ cells in each germarium, their averaged 2D size was used to reflect their 3D size. Of note, *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells were significantly smaller than the *nos>GFP; bam$^{-/-}$* controls (Figures 4D–4F). By contrast, the mitotic index of the former cells was about twice that of the latter control ones (Figures 4D, 4E, and 4G). These results support that Ras also promotes the division, not the growth, of *bam* mutant GSC-like cells, similar to that of wild-type GSCs.

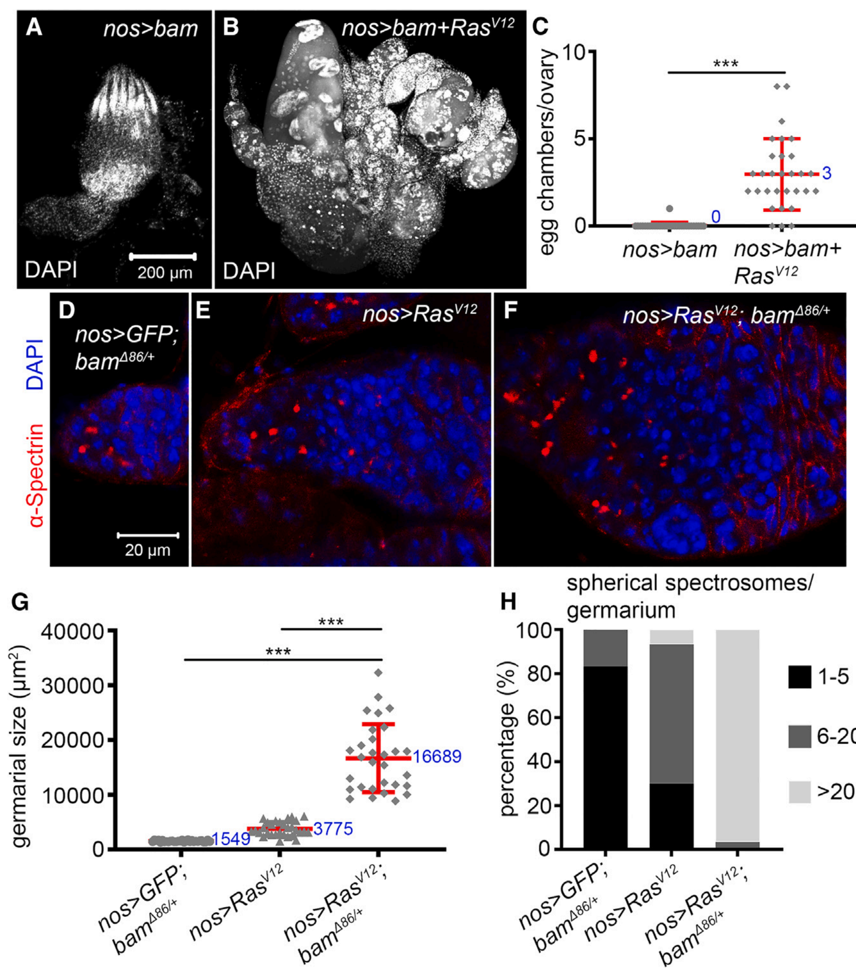

Figure 2. Ras^{V12} overexpression partially antagonizes the role of Bam in promoting GSC differentiation

(A and B) Representative ovarian images. Both images are of the same magnification.

(C) Quantification of egg chambers per ovary. 30 ovaries were quantified for either genotype. Data represent mean ± SD, and, for either group of data, the mean number is labeled right. Statistical significance was determined by t test. ***: $p < 0.001$.

(D–F) Representative germarial images. All images are of the same magnification.

(G) Quantification of germarial sizes. For each genotype, 30 germaria were quantified. Data represent mean ± SD, and, for each group of data, the mean number is labeled right. Statistical significance was determined by t test. ***: $p < 0.001$.

(H) Quantification of spherical spectrosomes per germarium. For each genotype, 30 germaria were quantified. These experiments were performed at 29°C, and 7-day-old flies were used. See all genotypes in Table S1 and the raw quantification data in Table S2.

Ras promotes GSC division through activating the MAPK signaling

The Ras small GTPases could function to activate the downstream MAPK, phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), or Rac/Rho signaling pathways (Gimple and Wang, 2019). Strikingly, knocking down either *downstream of raf1* (*Dsor1*, encoding *Drosophila* mitogen-activated protein kinase kinase) or *rolled* (*rl*, encoding *Drosophila* MAPK) drastically suppressed the GSC over-proliferation phenotype induced by Ras^{V12} overexpression (Figures 5A–5F). By contrast, knocking down any of *PI3K21B*, *PI3K59F*, *PI3K68D*, and *Rac1*, the essential genes controlling PI3K/AKT and Rac/Rho pathways, did not exhibit obvious suppression effects (data not shown). These results support that Ras promotes GSC division through activating the MAPK signaling.

MAPK signaling is activated specifically in the M phase of mitotic germ cells

Then, we sought to determine in which cell-cycle phase(s) the MAPK signaling is activated in both wild-type GSCs and *bam* mutant GSC-like cells with no Ras^{V12} overexpression. It should be noted that very few germ cells are GSCs in wild-type ovaries while all germ cells are GSC-like cells in *bam* mutant ovaries (Lin et al., 1994; McKearin and Ohlstein, 1995). Given this, we chose to examine *bam* mutant GSC-like cells at first. Typically, anti-phosphorylated extracellular signal-regulated kinase (pERK) immunofluorescent staining is used to reflect the activation of the MAPK signaling (Gabay et al., 1997). Of note, percentage of pERK^{+} germ cells in *nos>Ras^{V12}; bam$^{-/-}$* germaria was about 13 times that in the *nos>GFP; bam$^{-/-}$* controls (Figure S5), indicative of MAPK signaling overactivation. To distinguish different cell-cycle phases, we performed the Fly-FUCCI experiments, which can label G1-phase cells green, S-phase cells red, and G2/M-phase cells yellow (Zielke et al., 2014). Previous studies have found that most of *Drosophila* female GSCs are in the G2 phase (Hinnant et al., 2017). Here we obtained similar results in *bam* mutant germ cells (Figure 6A and data not shown, yellow/total = 1045/1225 = 85.31%), further confirming that they are GSC-like cells. Strikingly, only 2.20% (27/1225)

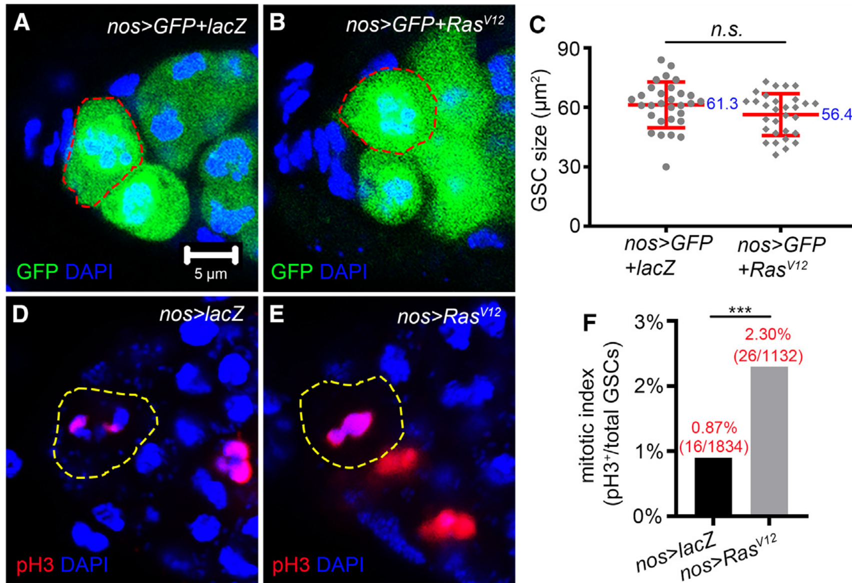

Figure 3. Ras promotes GSC division, not growth

(A and B) Representative GSC images. The red dashed lines outline the GSCs that are biggest in this focal plane. (A, B, D, and E) The images are of the same magnification.

(C) Quantification of GSC sizes. For either genotype, 30 GSCs were quantified. Data represent mean ± SD, and, for either group of data, the mean number is labeled right. Statistical significance was determined by t test. *n.s.*: $p > 0.05$.

(D and E) Representative pH3$^+$ GSCs. These pH3$^+$ GSCs are outlined by yellow dashed lines.

(F) Quantification of GSC mitotic index. The mitotic index was calculated by "pH3$^+$/total GSCs ×100%." Only GSCs within stem cell niches were quantified, and the quantified cell numbers are labeled above. Statistical significance was determined by chi-squared test. ***: $p < 0.001$. These experiments were performed at 29°C, and 7-day-old flies were used. See all genotypes in Table S1 and the raw quantification data in Table S2. See also Figure S3.

of these GSC-like cells were pERK$^+$ (Figure 6A′ and data not shown), and, among them, 25 were labeled yellow and 2 were labeled red. These pERK$^+$ GSC-like cells usually contained condensed chromatids with broken-down nuclear envelopes (Figures 6B and 6B′), both of which are the features of M-phase cells.

To provide more direct evidence, we stained anti-pERK and anti-pH3 (M-phase marker) antibodies together in *bam* mutant GSC-like cells. Remarkably, all pH3$^+$ mitotic germ cells (n > 100) that are in prophase, metaphase, anaphase, or telophase were also pERK$^+$ (Figures 6C–6F″), confirming that the MAPK signaling is activated specifically in the M phase of *bam* mutant GSC-like cells.

Then, we asked whether it is also the case in wild-type GSCs. w^{1118} flies were used as the wild type. Similar to that of *bam* mutant GSC-like cells, all pH3$^+$ germ cells (n > 100), including GSCs, cystoblasts, and cystocytes, were also pERK$^+$ (Figures 6G–6I″). Taken together, these studies indicate that the MAPK signaling is activated specifically in the M phase of mitotic germ cells, including both wild-type GSCs and *bam* mutant GSC-like cells.

Ras^{V12} overexpression triggers polyploid nurse cells to die through inducing mitotic stress

Different from diploid GSCs, nurse cells are polyploid, and they undergo G/S endocycling, losing the division ability (Edgar et al., 2014). Of note, none of wild-type nurse cells was positive for pERK staining (>20 w^{1118} ovaries examined), further confirming that the MAPK signaling is not activated in germline G1 and S phases. Given this, we were very curious about how nurse cell endocycle is influenced by Ras^{V12} overexpression. *nos-GAL4-VP16* was activated not only in early-stage germ cells (Figure 1B) but also in late-stage ones in egg chambers, including nurse cells (Figures 7A and 7B) (Rørth, 1998). Therefore, it could also help us to address how nurse cells respond to Ras^{V12} overexpression. It should be noted that *nos>Ras^{V12}* female flies were sterile (>20 flies examined) at either 25°C or 29°C. At 29°C, many of their germaria and egg chambers were filled with over-proliferating diploid germ cells (Figure S1B); at 25°C, their germaria were also overgrowing but many of their egg chambers were filled with dying nurse cells (Figures 7C–7F and 7H). Thus, it seems that Ras^{V12} overexpression is able to elicit cell-death-causing stress to polyploid nurse cells. *cyclin A* (*cycA*) and *cyclin-dependent kinase 1* (*cdk1*) are two key genes promoting the cell-cycle transition from G2 to M phase (Edgar and Lehner, 1996). Remarkably, either *nos>Ras^{V12}; cycA$^{+/-}$* or *nos>Ras^{V12}; cdk1$^{+/-}$* ovaries contained much fewer degrading egg chambers than those of the *nos>Ras^{V12}* controls (Figures 7E–7H), supporting that this cell-death-causing stress is mitotic stress.

Then, we sought to address how these dying nurse cells undergo cell death. In the fly genome, *Death regulator Nedd2-like caspase* (*Dronc*) and *Death related ced-3/Nedd2-like caspase* (*Dredd*) encode two caspases essential for cell apoptosis (Chen et al., 1998; Dorstyn et al., 1999). Knocking down either of them significantly suppressed the nurse-cell-death phenotype (Figures 7I–7K), indicating

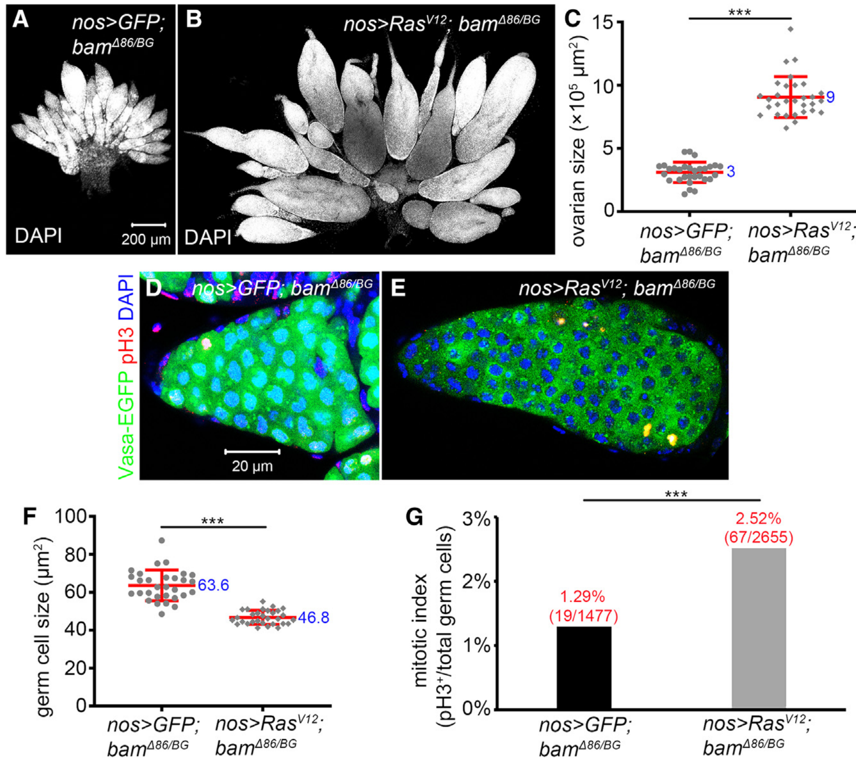

Figure 4. Ras^{V12} overexpression promotes the division of *bam* mutant GSC-like cells

(A and B) Representative ovarian images. *bam$^{\Delta86}$* is a null, and *bam^{BG}* is a strong loss-of-function allele of the *bam* gene. Both images are of the same magnification.

(C) Quantification of ovarian sizes. For either genotype, 30 ovaries were quantified, and, for each ovary, its biggest 2D size under microscope was used to reflect its 3D size. Data represent mean ± SD, and, for either group of data, the mean number is labeled right. Statistical significance was determined by t test. ***: $p < 0.001$.

(D and E) Representative germarial images. Vasa-EGFP can outline the germline areas, and pH3 is a mitotic marker. Both images are of the same magnification.

(F) Quantification of germ cell sizes. For either genotype, 30 germaria were scanned by confocal microscope, and, for each germarium, its averaged germ cell size was calculated by "germline area/germ cell numbers." Data represent mean ± SD, and for the either group of data, the mean number is labeled right. Statistical significance was determined by t test. ***: $p < 0.001$.

(G) Quantification of the mitotic index. Statistical significance was determined by chi-squared test. ***: $p < 0.001$. These experiments were performed at 29°C, and 7-day-old flies were used. See all genotypes in Table S1 and the raw quantification data in Table S2. See also Figure S4.

that such cell death is at least partially apoptosis dependent.

DISCUSSION

In this study, we provided evidence that oncogenic Ras^{V12} promotes GSC division, not growth, through activating the MAPK signaling. This signaling is activated specifically in the M phase of mitotic germ cells, including GSCs. Its overactivation partially antagonizes the role of Bam in promoting GSC differentiation. Also, we found that oncogenic Ras^{V12} triggers polyploid nurse cells to die through inducing mitotic stress.

Although all *bam* mutant germ cells are GSC-like cells (McKearin and Ohlstein, 1995), the ones that are out of stem cell niches are pMad negative (Kai and Spradling, 2003). So were the out-of-niche GSC-like cells in *nos>Ras^{V12}* ovaries, indicating that BMPs are not expanded in both conditions. It should be noted that *bam* mutant GSC-like cells are unable to undergo further differentiation (McKearin and Ohlstein, 1995). By contrast, *nos>Ras^{V12}* germ cells were able to, implying that Ras^{V12} overexpression does not block GSC differentiation, different from that of *bam* mutant. Opposite to the phenotype of *bam* mutant, Bam overexpression promotes GSC differentiation prematurely, leading to a germ-cell-loss phenotype (Ohlstein and McKearin, 1997). Our studies demonstrate that this phenotype was partially antagonized by Ras^{V12} overexpression. It is possible that Ras^{V12} overexpression could slow the GSC differentiation induced by Bam overexpression.

Interestingly, the *nos>Ras^{V12}* GSCs staying in stem cell niches were comparable to the controls in cell size while *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells were smaller than the controls. For *nos>Ras^{V12}* GSCs, it is probably because they reside in stem cell niches, which are rich in nutrients. By contrast, most of *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells do not reside in niches. Because only 2.52% of them were in the M phase, it can rule out the possibility that many of them have just undergone cytokinesis and do not have enough time to grow bigger. To our observation, these cells were generally smaller than the controls. Thus, it seems that they divide fast at the expense of cell size.

A critical question is how Ras^{V12} overexpression dramatically increases the numbers of *bam* mutant GSC-like cells. Of note, 2.52% of *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells were

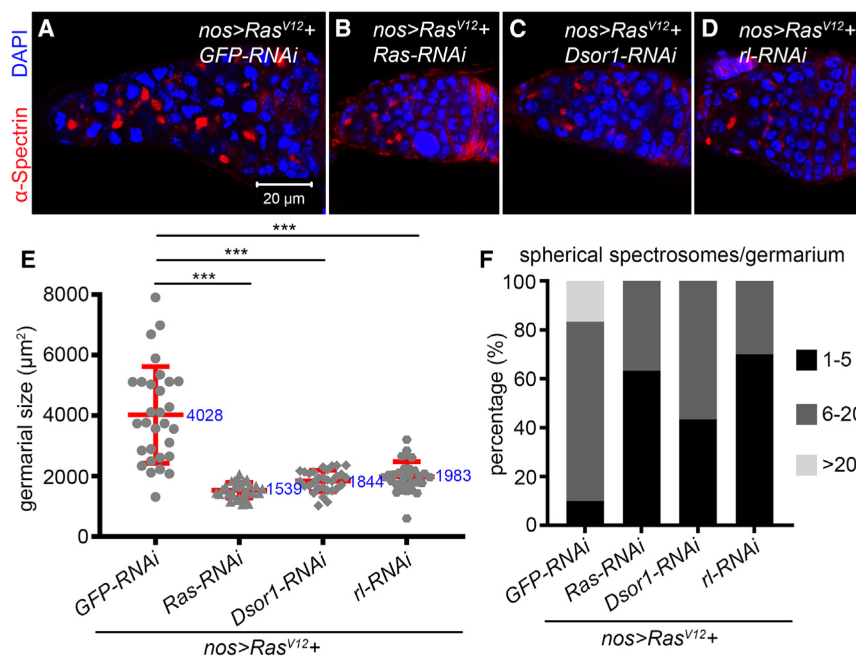

Figure 5. Ras^{V12} overexpression induces GSC over-proliferation through activating the MAPK signaling

(A–D) Representative germarial images. All images are of the same magnification.

(E) Quantification of germarial sizes. For each genotype, 30 germaria were quantified. Data represent mean ± SD, and, for each group of data, the mean number is labeled right. Statistical significance was determined by t test. ***: $p < 0.001$.

(F) Quantification of spherical spectrosomes per germarium. For each genotype, 30 germaria were quantified. These experiments were performed at 29°C, and 7-day-old flies were used. See all genotypes in Table S1 and the raw quantification data in Table S2.

pH3 positive, nearly twice that of the *nos>GFP; bam$^{-/-}$* controls (1.29%), indicating that the former cells divide much faster than the latter ones. This would lead to an exponential increase in cell number. Furthermore, 12.91% of *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells were pERK positive, 13 times that of the controls (0.97%), suggesting that the MAPK signaling is overactivated upon Ras^{V12} overexpression. Given that only 2.52% of *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells were in the M phase ($pH3^+$), the rest 10.39% (12.91%–2.52%) of $pERK^+$ *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells were not in the M phase. It is known that the MAPK signaling could promote both the mitotic entry and mitotic progression of some mammalian cells (Shapiro et al., 1998; Zecevic et al., 1998). Therefore, such non-M-phase MAPK may also promote the mitotic entry of *nos>Ras^{V12}; bam$^{-/-}$* GSC-like cells.

Similar to GSCs, cystocytes also activate the MAPK signaling specifically in their M phase. However, their transit-amplification rounds were not increased upon Ras^{V12} overexpression, unlike that of GSCs. One possibility is that *bam-GAL4-VP16* is too weak. *nos-GAL4-VP16* may be a stronger driver. Unfortunately, *nos>Ras^{V12}* led to severe nurse cell death and egg chamber degradation, which hinders the analyses of cystocyte over-proliferation. Alternatively, the transit-amplification feature itself would make cystocytes resistant to Ras^{V12} overexpression. Previous studies have found that it takes cystocytes 6 h to divide once while it takes GSCs as long as 24 h (Mathieu et al., 2022; Morris and Spradling, 2011). Therefore, it is also possible that fast-dividing cystocytes become tolerant to the division-promoting Ras^{V12} stimulus because of a ceiling effect. Consistently, it has been found that murine $KRas^{G12D}$, similar to fly Ras^{V12}, is unable to drive the transit-amplifying cells of hair follicles, but able to drive their stem cells, to form squamous skin tumors (Lapouge et al., 2011; White et al., 2011). Further studies are still required to distinguish these two possibilities.

It is known that Ras hyperactivation is able to induce the senescence and death of diploid cells (Overmeyer and Maltese, 2011; Zhu et al., 2020). Different from diploid cells, polyploid cells are post-mitotic; that is, they have lost the division ability (Edgar et al., 2014). Notably, our studies demonstrate that fly polyploid nurse cells are sensitive to Ras hyperactivation. Given that polyploid cells are widespread across the animal kingdom (Edgar et al., 2014), it would be interesting to determine whether other types of polyploid cells are also sensitive to Ras hyperactivation. Furthermore, we found that the Ras^{V12}-induced cell death of nurse cells is partially apoptosis dependent. Given that both apoptotic and non-apoptotic pathways are known to regulate nurse cell death (Peterson et al., 2015), it would also be interesting to determine whether non-apoptotic pathways are involved.

Previous studies have demonstrated that the Ras/MAPK signaling promotes the proliferation of intestinal stem cells (Jiang and Edgar, 2009), Malpighian tubule stem cells (Zeng et al., 2010), and primordial germ cells (Li et al., 2003) in flies; embryonic stem cells (Heo et al., 2006), embryonic neural precursor cells (Yanagisawa et al., 2005), intestinal stem cells (Gierut et al., 2015), neural stem cells (Bender et al., 2015), and spermatogonial stem cells (He et al., 2008) in mice; and hematopoietic stem cells in humans (Fatrai

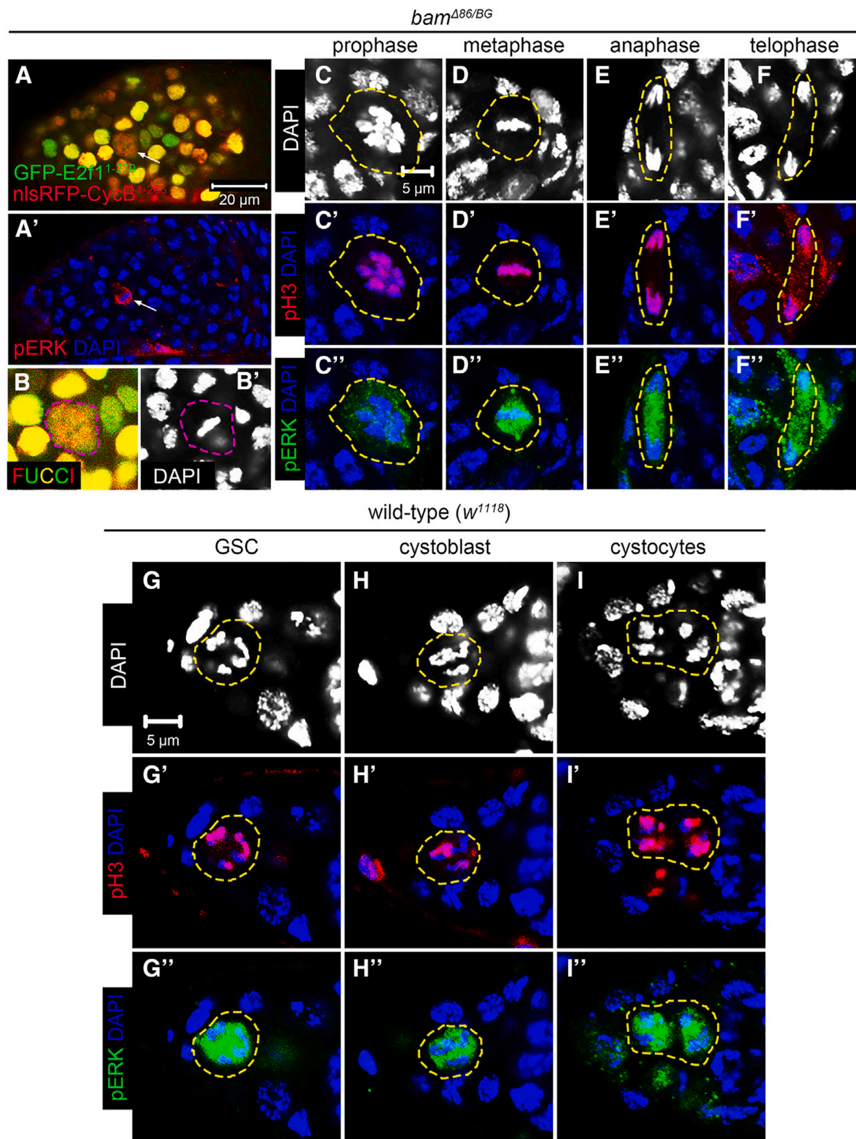

Figure 6. MAPK signaling is activated specifically in the M phase of mitotic germ cells

(A–B′) Representative images of *bam* mutant germ cells with *nos>Fly-FUCCI*. The arrows in (A and A′) indicate a pERK$^+$ germ cell that is enlarged in (B and B′). The magenta dashed lines in (B and B′) outline this pERK$^+$ germ cell. These experiments were performed at 25°C, and 7-day-old flies were used.

(C–F″) Representative images of mitotic *bam* mutant germ cells. The prophase, metaphase, anaphase, and telophase were judged by the morphology of condensed chromatids. The yellow dashed lines outline these mitotic germ cells. All images are of the same magnification.

(G–I″) Representative images of wild-type GSCs, cystoblasts, and cystocytes. w^{1118} flies were used as the wild type. The yellow dashed lines outline these mitotic germ cells. All images are of the same magnification. These experiments were performed at 29°C, and 7-day-old flies were used. See all genotypes in Table S1. See also Figure S5.

et al., 2011). These studies support a universal role of the Ras/MAPK signaling in stem cell proliferation. As to its detailed role in cell-cycle progression, it is known that this signaling promotes both the growth and division of fly intestinal stem cells (Jin et al., 2015). However, in this study, we found that it only promotes the division, not the growth, of fly female GSCs, indicative of a context-dependent regulation manner. It should be noted that Ras small GTPases could be stimulated by diverse extracellular and intracellular signals and could also activate various downstream pathways (Gimple and Wang, 2019). Therefore, to make such context-dependent regulation mechanisms clear, systematic studies should be performed across different types of stem cells. Finally, given that fly and mammalian GSCs share many similarities (Spradling et al., 2011), we propose that the Ras/MAPK signaling also promotes GSC division in mammals, including mice and humans.

EXPERIMENTAL PROCEDURES

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Shaowei Zhao (swzhao@nankai.edu.cn).

Materials availability

Plasmids and fly strains are available upon request.

Data and code availability

All raw data for quantification are included in Table S2. For more information, please contact the corresponding author.

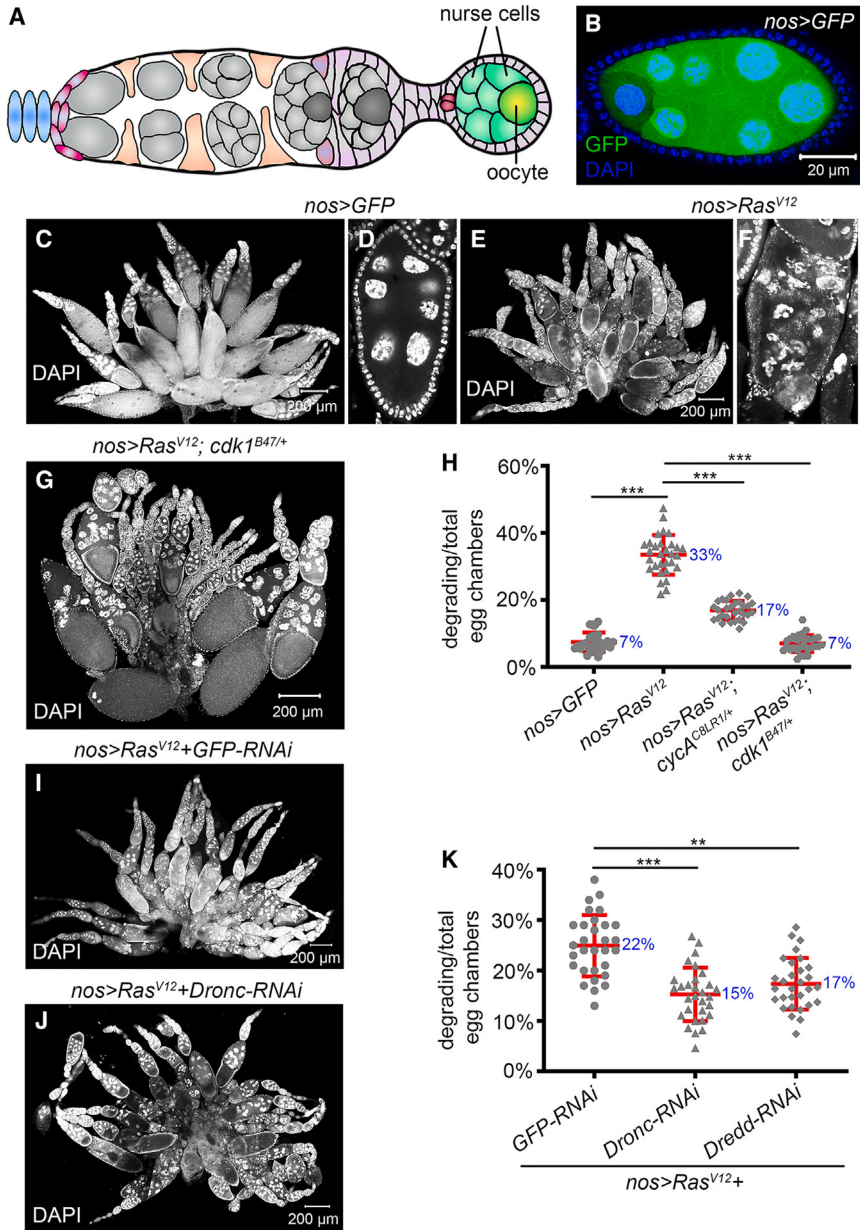

Figure 7. Ras^{V12} overexpression triggers nurse cells to die

(A) A schematic diagram, and nurse cells and the oocyte are highlighted in green.

(B) *nos-GAL4-VP14* is activated in nurse cells and oocytes.

(C, E, G, I, and J) Representative ovarian images.

(D) Representative image of a wild-type egg chamber with the *nos>GFP* genotype.

(F) Representative image of a degrading egg chamber with the *nos>Ras^{V12}* genotype.

(H and K) Quantification of degrading/total egg chambers. Both *cycA^{C8LR1}* and *cdk1^{B47}* are null alleles. 30 ovaries were quantified for each genotype. Data represent mean ± SD, and, for each group of data, the mean number is labeled right. Statistical significance was determined by t test. **: $p < 0.01$; ***: $p < 0.001$. These experiments were performed at 25°C, and 7-day-old flies were used. See all genotypes in Table S1 and the raw quantification data in Table S2.

Fly strains and husbandry

Flies were raised at either 25°C or 29°C as noted in the figure legends. The *Ras$^{\Delta C40B}$*, *bamP-bam-GFP*, and *bam-GAL4-VP16* fly strains were gifts from Celeste Berg, Judith Leatherman, and Erika Matunis, respectively. The fly strain of *nos-GAL4-VP16* on the second chromosome was a gift from Ruth Lehmann. The *bam$^{\Delta 86}$* and *UASp-GFP* fly strains were gifts from Michael Buszczak. These fly strains were gifts from Zhaohui Wang: *bam^{BG}*, *w^{1118}*, *UASp-bam*, and *vasa-EGFP*. The following fly strains were ordered from Bloomington *Drosophila* Stock Center: *bgcn^{1}* (#6054), *bgcn^{MI06696}* (#40815), *cycA^{C8LR1}* (#6627), *cdk1^{B47}* (#6643), *FRT82B* (#86313), *FRT82B EGFP* (#32655), *nos-GAL4-VP16* (#4937), *UASp-GFP-E2f1$^{1-230}$ UASp-nlsRFP-CycB$^{1\text{-}266}$* (#55110), and *UAS-GFP-RNAi* (#44412, #44415). These fly strains were ordered from TsingHua Fly Center (Beijing, China): *UASp-Ras-RNAi* (TH201501177.S), *UASp-Dsor1-RNAi* (THU0677), *UASp-rl-RNAi* (THU3530), *UASp-PI3K21B-RNAi* (TH201501182.S), *UASp-PI3K59F-RNAi-1* (THU3552), *UASp-PI3K59F-RNAi-2* (TH201500700.S), *UASp-PI3K68D-RNAi* (THU1527), *UASp-Rac1-RNAi-1* (THU1493), *UASp-Rac1-RNAi-2* (TH201501180.S), *UASp-Dronc-RNAi* (THU1155), and *UASp-Dredd-RNAi* (THU0601). The fly strain of *UASp-FLP* on the second chromosome was generated by our laboratory previously (Zhang et al., 2023). The *UASp-Ras^{V12}* and *UASz-lacZ* fly strains were generated in this study.

Transgenic flies

UASp-Ras^{V12}

The coding sequence of *Ras^{V12}* was cloned into the *pUASp* vector (from Neal Silverman) (Rørth, 1998), and the plasmids jointly with the *P* element transposase-expressing (helper) plasmids were microinjected into the *w^{1118}* fly embryos. One transgenic fly strain on the 2nd and one on the 3rd chromosomes were used in this study.

UASz-lacZ

The coding sequence of the *lacZ* gene was cloned into the *pUASz1.0* vector (*Drosophila* Genomics Resource Center, DGRC #1431) (DeLuca and Spradling, 2018). The plasmids were microinjected into the *attP40* fly strain to generate site-specific transgenic flies on chromosome 2L.

Immunofluorescent staining

Ovaries were dissected in PBS, fixed in 4% paraformaldehyde (PBS-diluted) for 3 h, washed by PBST (PBS +0.3% Triton X-100) for 1 h at room temperature (RT), incubated with primary antibodies (PBST-diluted) overnight at 4°C, washed by PBST for 1 h at RT, incubated with secondary antibodies and 0.1 μg/mL DAPI (PBST-diluted) overnight at 4°C, washed by PBST for 1 h at RT, and then mounted in autoclaved 70% glycerol. The primary antibodies were used at the concentrations as following: rabbit anti-pERK (Cell Signaling Technology, #4370) at 1:200, mouse anti-pH3 (PTM Biolabs Inc, PTM-757) at 1:200, rabbit anti-pH3 (EMD Millipore Corporation, #06–570) at 1:2,000, rabbit anti-pMad (Ed Laufer) at 1:1,000, and mouse anti-α-Spectrin (Developmental Studies Hybridoma Bank, 3A9) at 1:100. The Alexa Fluor-conjugated secondary antibodies were used at 1:2,000.

Image collection and data processing

The florescent images were scanned by a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, BaWü, GER) and processed by Adobe Photoshop (2022) (San Jose, CA, USA) or ImageJ (NIH, Bethesda, MD, USA). The quantification data were processed by Microsoft Excel (Microsoft Corporation, WA, USA), GraphPad Prism (GraphPad Software, MA, USA), or Zeiss ZEN 2.3 lite (Carl Zeiss AG, BaWü, GER).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2024.06.005.

ACKNOWLEDGMENTS

We thank Eric H. Baehrecke and Quan Chen for critical comments on the manuscript and Celeste Berg, Michael Buszczak, Ed Laufer, Judith Leatherman, Ruth Lehmann, Erika Matunis, Neal Silverman, Zhaohui Wang, DGRC, Bloomington and TsingHua *Drosophila* Stock Centers for antibodies, plasmids, and fly strains. This study was funded by the National Natural Science Foundation of China (NSFC) grants 32070871 and 32270841 to S.Z. and the Tianjin Research Innovation Project for Postgraduate Students 2021YJSB098 to Qi Zhang.

AUTHOR CONTRIBUTIONS

S.Z. conceived and supervised this study. Qi Zhang, Yanfang Wang, Z.B., Y.Z., Qian Zhang, L.L., L.Y., Yuejia Wang, and S.Z. performed the experiments. S.Z. wrote the manuscript, and all authors commented on it.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: February 7, 2024
Revised: June 17, 2024
Accepted: June 18, 2024
Published: July 18, 2024

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