Published in final edited form as:

Nature. 2008 July 10; 454(7201): 241–245. doi:10.1038/nature07014.

Mei-P26 regulates microRNAs and cell growth in the *Drosophila* ovarian stem cell lineage

Ralph A. Neumüller¹, Joerg Betschinger^{1,†}, Anja Fischer¹, Natascha Bushati², Ingrid Poernbacher¹, Karl Mechtler^{1,3}, Stephen M. Cohen^{2,†}, and Juergen A. Knoblich¹

¹Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Dr Bohr Gasse 3, 1030 Vienna, Austria

²European Molecular Biology Laboratory (EMBL), Meyerhofstraße 1, 69117 Heidelberg, Germany

³Research Institute of Molecular Pathology (IMP), Dr Bohr-Gasse 7, 1030 Vienna, Austria

Abstract

Drosophila neuroblasts¹ and ovarian stem cells^{2,3} are well characterized models for stem cell biology. In both cell types, one daughter cell self-renews continuously while the other undergoes a limited number of divisions, stops to proliferate mitotically and differentiates. Whereas neuroblasts segregate the Trim-NHL (tripartite motif and Ncl-1, HT2A and Lin-41 domain)-containing protein Brain tumour (Brat) into one of the two daughter cells^{4–6}, ovarian stem cells are regulated by an extracellular signal from the surrounding stem cell niche. After division, one daughter cell loses niche contact. It undergoes 4 transit-amplifying divisions to form a cyst of 16 interconnected cells that reduce their rate of growth and stop to proliferate mitotically. Here we show that the Trim-NHL protein Mei-P26 (refs ^{7, 8}) restricts growth and proliferation in the ovarian stem cell lineage. Mei-P26 expression is low in stem cells but is strongly induced in 16-cell cysts. In *mei-P26* mutants, transit-amplifying cells are larger and proliferate indefinitely leading to the formation of an ovarian tumour. Like *brat*, *mei-P26* regulates nucleolar size and can induce differentiation in *Drosophila* neuroblasts, suggesting that these genes act through the same pathway. We identify Argonaute-1, a component of the RISC complex, as a common binding partner of Brat and Mei-P26, and show that Mei-P26 acts by inhibiting the microRNA pathway. Mei-P26 and Brat have a similar domain composition that is also found in other tumour suppressors and might be a defining property of a new family of microRNA regulators that act specifically in stem cell lineages.

When *Drosophila* germline stem cells divide (Fig. 1a), 1 daughter cell (the cystoblast) loses niche contact and undergoes 4 transit-amplifying divisions to create a cyst of 16 cystocytes,

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[†]Present addresses: Wellcome Trust Centre for Stem Cell Research (CSCR), University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK (J.B.); Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, 117604, Singapore (S.M.C.).

Author Contributions J.A.K. and R.A.N. designed the study. R.A.N. performed the oogenesis experiments. J.B. and K.M. contributed biochemical data (Fig. 3h and Supplementary Fig. 4a, b). A.F. contributed the S2 luciferase assay (Supplementary Fig. 7c, d). I.P. assisted in the experiment in Fig. 3f, g and performed experiments in the larval brain (Supplementary Fig. 3). S.C. and N.B. designed the microRNA quantitative PCR experiment, which was performed by N.B. (Fig. 4a and Supplementary Fig. 7a, b). J.A.K. wrote the paper.

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which remain connected by the fusome. In each cyst, 1 cell becomes the oocyte, whereas the other cells undergo endoreplication to form 15 so-called nurse cells.

Because *brat*¹⁹² mutant germline clones do not show any obvious defects in oogenesis (data not shown), we analysed the ovarian phenotype of *mei-P26*. Like Brat, Mei-P26 is a Trim-NHL protein⁹ and carries an NHL domain, a coiled-coil region and several B-boxes. Weak *mei-P26* mutants have defects in meiosis⁷ whereas stronger alleles cause tumorous overproliferation⁸. *mei-P26* mutant ovaries were stained using the monoclonal antibody mAb1B1 (ref. ¹⁰), which labels the fusome and the spectrosome—a cytoplasmic organelle only present in stem cells and cystoblasts¹¹ (Fig. 1b–f). Wild-type ovaries contain two stem cells near the tip of the germarium whereas cystoblasts and cysts are separated from the stem cell niche (Fig. 1b). In *mei-P26* mutant ovaries, germaria are filled with individual spectrosome-containing cells (19%) and cysts containing varying numbers of cells (<4 in 39%, >4 in 42%; $n > 400$ cells) connected by a fusome (Fig. 1c, d and Supplementary Fig. 1a, a'). Nurse cells and oocytes are not formed, and fusomes or spectrosomes are maintained at later stages of oogenesis (Fig. 1e, f). In wild-type ovaries, the S-phase marker Cyclin E oscillates with the cell cycle in stem cells and mitotically active cysts, is downregulated as cystocytes exit mitotic proliferation and is re-expressed as nurse cells enter endoreplication¹² (Fig. 1g). In *mei-P26* mutant ovaries, however, Cyclin E is highly expressed at all stages of oogenesis (Fig. 1h). Upregulation of Cyclin E occurs even in small *mei-P26* clones (Supplementary Fig. 1b, c), and is therefore not an indirect consequence of tumour formation. Phospho-histone-H3-positive mitotic germline cells are restricted to the anterior tip of the germarium in wild-type ovaries (Fig. 1i) but are detected throughout the ovarioles in *mei-P26* mutants (Fig. 1j and Supplementary Fig. 1a, a'). Whereas all cells in a wild-type cyst divide synchronously, *mei-P26* mutant cysts frequently contain both mitotic and interphase cells (data not shown). Thus, *mei-P26* is required for proliferation control and differentiation in the female germline stem cell lineage.

To test stem cell niche signalling, we used a green fluorescent protein (GFP) fusion to the *bag of marbles* (*bam*) promoter¹³, which is suppressed by Decapentaplegic (Dpp) from the niche in stem cells but not in cystocytes (Fig. 1k and Supplementary Fig. 1d). In *mei-P26* mutants (Fig. 1l and Supplementary Fig. 1e), *bam* transcription is repressed in niche-contacting cells but is upregulated in cystocytes. Because staining for the niche marker Armadillo (Fig. 1m, n) reveals no structural abnormalities and because ovarian tumours are also observed when *mei-P26* is removed exclusively from the female germ line (Supplementary Fig. 1f, g), *mei-P26* is not required for sending or receiving the niche signal.

mei-P26 mutant tumour cells have branched fusomes and express Bam. To confirm further that these cells do not have stem cell identity, we analysed the expression of oo18 RNA-binding protein (Orb). Orb expression starts in all cystocytes between the 8- and 16-cell stage¹⁴, but is restricted to the oocyte during later stages (Fig. 1o). In *mei-P26* mutants, Orb expression is initiated normally (Fig. 1p) and is detected throughout the tumour. However, Orb is never restricted to a single cell, suggesting that the oocyte is not specified. Thus, *mei-P26* mutants develop a 'cystocytoma' in which tumour cells express markers for the cystocyte fate. Single-spectrosome-containing cells in the tumour might arise from occasional disintegration of fusome-connected cysts, a process that has been described before¹⁵.

Brat can inhibit cell growth and ribosome biogenesis¹⁶. *mei-P26* might also regulate cell growth because overexpression from the *eyeless* promoter reduces eye size (Supplementary Fig. 2a, b), even when cell death is inhibited by co-expressing *p35* (data not shown). To test whether cell growth is differentially regulated in the ovarian stem cell lineage, we quantified the volume of stem cells and cysts after three-dimensional reconstruction (see Methods).

Shortly after stem cell division (elongated spectrosome morphology), stem cells are $314 \pm 13 \mu\text{m}^3$ (mean $\pm$ s.e.m.; $n = 4$) whereas cystoblasts are $330 \pm 30 \mu\text{m}^3$ ($n = 4$). Stem cells grow to a maximum of $600 \mu\text{m}^3$ (average $437 \pm 21 \mu\text{m}^3$, $n = 19$) and double their volume between each mitotic division (about once per day). To measure cyst volumes, mitotic clones were marked by the absence of GFP (Fig. 2a). Three- and four-day-old 16-cell cysts are $1,215 \pm 61 \mu\text{m}^3$ ($n = 7$) and $1,163 \pm 48 \mu\text{m}^3$ ($n = 7$), respectively. Thus, cell growth slows down as cells exit mitotic proliferation. Consistent with this, cystocytes become progressively smaller during transit-amplifying divisions (Fig. 2g). *diminutive* (dMyc), the *Drosophila* Myc homologue, an important regulator of cell growth, is highly expressed in stem cells and cystoblasts but downregulated in 16-cell cysts (Fig. 2e). Nucleoli (stained by anti-Fibrillarin)—the sites of ribosomal RNA transcription—are large in stem cells but much smaller in 16-cell cysts (Fig. 2c and Supplementary Fig. 2c). Because ribosome number is thought to control cellular growth in *Drosophila*^{17,18}, a reduction in ribosome biogenesis might be responsible for the reduced cell growth at the end of mitotic proliferation.

In *mei-P26* mutants, cellular and nucleolar size are increased and dMyc is highly expressed throughout the tumour (Fig. 2d, f, g and Supplementary Fig. 2c): the volume of 16-cell cysts is increased to $3,956 \pm 424 \mu\text{m}^3$ ($n = 4$; Fig. 2b) and cell diameters no longer decrease as cells are displaced from the stem cell niche (Fig. 2g). Thus, *mei-P26* is responsible for the differential regulation of cell growth in the *Drosophila* ovarian stem cell lineage. Like *brat*, it might achieve its function by regulating ribosome biogenesis and controlling the expression of dMyc.

To analyse Mei-P26 expression, we generated a specific antibody (Supplementary Fig. 2d–f). Mei-P26 mRNA and protein levels are low in stem cells but are upregulated in cysts as they decrease growth and exit mitotic proliferation (Fig. 2h, j and Supplementary Fig. 2e, g, h). This regulation is functionally important because *mei-P26* overexpression in ovaries results in stem cell loss and complete depletion of the female germ line (Fig. 3a, b). In *bam* mutant ovaries, Mei-P26 levels remain low suggesting that *mei-P26* is regulated in a Bam-dependent manner (Fig. 2i). *bam* overexpression induces premature differentiation of stem cells in a wild-type (Fig. 3d) but not in a *mei-P26* mutant (Fig. 3e) background, demonstrating that Mei-P26 is essential for Bam to induce cystocyte differentiation. Consistently, germline stem cells do not differentiate when *pumilio* is removed in a *mei-P26* mutant background (Supplementary Fig. 2k, l). To test whether Mei-P26 is the only target of Bam, we overexpressed *mei-P26* in a *bam* mutant background (Fig. 3f, g and Supplementary Fig. 2i, j). *mei-P26* overexpression reduces the size of *bam* mutant cells (from $10.1 \pm 0.17 \mu\text{m}$ (*bam*, $n = 27$) to $7.0 \pm 0.19 \mu\text{m}$ (*bam*, *nanos gal4* $\gg$ *UASP-me-P26*, $n = 27$)) and the enlarged nucleolus in *bam* mutants (Supplementary Fig. 2c) but does not rescue the differentiation defects (Supplementary Fig. 2i, j) observed in these mutants. Thus, *mei-P26* is upregulated by Bam activity in cystocytes and inhibits cell growth and mitotic proliferation.

In the brain, *mei-P26* is weakly expressed in neuroblasts, is absent from ganglion mother cells but is highly expressed in neurons (Supplementary Fig. 3a–d). Although *mei-P26* mutants have no obvious defects in neurogenesis, *mei-P26* overexpression can induce premature neuronal differentiation (Supplementary Fig. 3e, f), suggesting that *mei-P26* and *brat* might have some common targets. Using mass spectrometry, we identified the RNase Argonaute-1 (AGO1) in anti-Brat immunoprecipitates (Supplementary Fig. 4a). Brat and AGO1 can be co-immunoprecipitated from *Drosophila* embryos (Fig. 3h), and co-transfection experiments in S2 cells show that AGO1 also binds Mei-P26 and the Trim-NHL protein Dappled (Supplementary Fig. 4b). This interaction is functionally significant because Mei-P26 lacking the NHL domain no longer binds AGO1 (Fig. 3i) and fails to block self renewal when overexpressed in ovarian stem cells (Fig. 3c).

AGO1 is a core component of the RISC complex¹⁹ and is important for microRNA-mediated translational repression and RNA degradation. MicroRNAs are essential for self renewal in *Drosophila* ovarian stem cells because mutations in *AGO1* (Supplementary Fig. 5), *dicer-1* (ref. ²⁰) or its binding partner *loquacious*²¹ result in premature stem cell differentiation. To test whether Mei-P26 regulates microRNAs, we measured microRNA levels by quantitative reverse transcription polymerase chain reaction (RT-PCR). In *mei-P26* mutants, most microRNAs are significantly upregulated (Supplementary Fig. 7a, b), whereas overexpression of *mei-P26* in *bam* mutants broadly reduces microRNA levels (Fig. 4a and Supplementary Fig. 7a). Importantly, the *mei-P26* mutant phenotype can be partially rescued by removing one copy of *Loquacious* and thereby reducing microRNA levels (Fig. 4b, c and Supplementary Fig. 6a–f), indicating that *mei-P26* acts by inhibiting the microRNA pathway in cystocytes.

One of the best characterized *Drosophila* microRNAs is *bantam*, a regulator of proliferation and apoptosis²². We used a sensor that expresses GFP from the *tubulin* promotor and carries two *bantam* binding sites in the 3' untranslated region²². In wild-type ovaries, this sensor is repressed by *bantam* activity in stem cells but is derepressed in 16-cell cystocytes in which high levels of Mei-P26 are present (Fig. 4e). In *mei-P26* mutants, the sensor is off in all germline cells (Fig. 4g). In contrast, a control sensor lacking microRNA-binding sites shows high expression in all wild-type and mutant cells (Fig. 4d, f). Although *mei-P26* regulates many microRNAs, *bantam* seems to be an important target: even animals heterozygous for a *bantam* null mutation have a reduced number of stem cells (1.15 ± 0.12 per germarium ($n = 32$ germaria) compared to 2.09 ± 0.05 ($n = 32$ germaria) in 14-day-old *bantam* heterozygous and wild-type flies, respectively), suggesting a defect in self renewal (Supplementary Fig. 7e, f). To test whether *mei-P26* regulates *bantam* directly, we expressed a luciferase construct carrying a *bantam*-binding site in its 3' untranslated region in S2 cells. In control cells, the *bantam* sensor is repressed, but a construct lacking the binding site is not affected (Supplementary Fig. 7d). On cotransfection of *mei-P26* (Supplementary Fig. 7c), luciferase expression is significantly derepressed, but no derepression is seen with cotransfection of *mei-P26* lacking the NHL domain; this indicates that Mei-P26 can repress *bantam* activity even in S2 cells.

Our data suggest that *brat* and *mei-P26* might act in a similar manner to control proliferation in stem cell lineages. In both mutants, cells that normally stop self renewal increase ribosome biogenesis, grow abnormally large and fail to exit the cell cycle leading to the formation of a tumour. The general upregulation of microRNAs in *mei-P26* mutants leaves several possibilities for how these proteins might regulate the microRNA pathway. The presence of a RING finger in Mei-P26 suggests a role in protein degradation. The high amounts of AGO1 detected in Mei-P26 immunoprecipitates make it unlikely that AGO1 itself is degraded by Mei-P26. However, another member of the RISC complex might be a degradation target of Mei-P26. Equally likely, Mei-P26 could prevent the incorporation or increase the turnover of microRNAs in the RISC complex.

Many human tumours contain cancer stem cells that drive tumour growth and metastasis^{23, 24}. Although the similarities between *Drosophila* tumours and human cancer are limited, *brat* mutant brains and *mei-P26* mutant ovaries (as well as the other mutant conditions causing stem cell tumours^{25–27}) provide an invertebrate model for stem-cell-derived tumour formation. In *mei-P26* mutants, tumours originate from cystocytes, the transit-amplifying pool of the ovarian stem cell lineage. In *mei-P26* mutants, these cells regain the ability to self-renew: after *bam* overexpression—which leads to premature differentiation of stem cells—the germ line is depleted in a wild-type but not in a *mei-P26* mutant background. Thus, *mei-P26* tumours arise from growth defects in the transit-

amplifying compartment of the ovarian stem cell lineage—a mechanism that could occur in human tumours as well.

Our data establish Trim–NHL proteins as regulators of stem cell proliferation. Vertebrate members of this family exist and are downregulated in human cancer cell lines²⁸ suggesting that their tumour-suppressor function might be conserved in vertebrates as well.

METHODS SUMMARY

For immunofluorescence, ovaries were dissected in PBS, fixed for 20 min in *n*-heptane/PBS (1:1) with 5% PFA, washed three times in PBS containing 0.1% Triton X-100, and stained using standard procedures. Cell volumes were determined after three-dimensional reconstruction from stacks of 0.4-μm sections using IMARIS software. A Rho1–GFP trap (ZCL1957) was used to outline stem cells. To outline individual cysts, mitotic clones, generated by heat-induced expression of FLP recombinase (*hs-FLP*) and marked by the absence of GFP, were stained with rhodamine-phalloidine. Protein lysates were prepared from 20 ovaries dissected in PBS and extracted with Laemmli buffer, and were run on 10% SDS-polyacrylamide gels. Overexpression constructs were created by amplifying *mei-P26* full length, *mei-P26*^{ANHL}, *AGO1*, *dappled* and *brat* coding sequences by PCR from total complementary DNA or expressed sequence tags (ESTs) with appropriate primers containing bacterial attachment (attB) sites to mediate intermolecular recombination for Gateway cloning (Invitrogen) or appropriate restriction sites. MicroRNAs were quantified using the TaqMan microRNA assay and a quantitative PCR machine (Applied Biosystems) with primer sets obtained from Applied Biosystems on 10 ng total RNA extracted from ovaries with TRIzol Reagent (Invitrogen). To measure *bantam* activity, S2 cells were transfected with a plasmid that expresses the yeast transcription factor Gal4 under the control of the actin promoter (*actin-Gal4*), a control construct expressing *Renilla* luciferase, a sensor carrying two copies of a sequence complementary to the *bantam* miRNA downstream of firefly luciferase (FLuc) and the respective pUASp-Mei-P26 constructs. All transfections were performed in triplicate and dual luciferase assays were performed 24 h after transfection following the manufacturer's instructions (Promega).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank V. Siegel, K. Mochizuki, G. B. Cebolla and S. Weitzer for comments on the manuscript, J. Stolte for assistance with quantitative PCRs, C. Richter and the other members of the Knoblich laboratory for discussion, B. Dickson, E. Izaurralde, P. Lasko, L. Luo, S. Hawley, D. McKearin, H. Richardson, F. Schnorrer, J. Skeath, D. Stein, L. Wong, the Bloomington *Drosophila* Stock Center, the Developmental Studies Hybridoma Bank (DSHB) and the *Drosophila* Genomics Resource Center (DGRC) for reagents, and M. Insko and M. Fuller for communicating results before publication. Work in the Knoblich laboratory is supported by the Austrian Academy of Sciences, the Wiener Wissenschafts-, Forschungs- und Technologiefonds (WWTF), the Austrian Science Fund (FWF) and the EU network ONCASYM; K.M. is supported by the Austrian Proteomics Platform (APP) of the Austrian Genome Program (GENAU).

Appendix

METHODS

Cytology and immunofluorescence

Immunofluorescence experiments in larval brains were carried out as described previously⁶. Ovaries were dissected in PBS and fixed for 20 min in a 1:1 mixture of *n*-heptane and PBS

containing 5% PFA. After three washes in PBS containing 0.1% Triton X-100, they were treated as described for brains⁶. For *in situ* hybridizations, DIG-labelled antisense probes were synthesized from the EST GH01646. For clonal analysis, Flp expression was induced by incubating flies at 37 °C for 1 h. *hs-bam* (Fig. 3d, e) was induced by three consecutive 1 h incubations at 37 °C within one day during development. Overexpression of *mei-P26* in the larval brain was carried out with a temperature-sensitive Gal80 (a yeast protein that suppresses Gal4) to circumvent embryonic lethality, and expression was induced for 60 h at 29 °C. The following antibodies were used: mouse anti-1B1 (7H9, Developmental Studies Hybridoma Bank (DSHB), 1:1), rabbit anti-AGO1 (Abcam, 1:100), mouse anti-Armadillo (N2 7A1, DSHB, 1:100), rabbit anti-Brat (1:100), mouse anti-CycE (from H. Richardson, 1:10), guinea pig anti-Deadpan (gift from J. Skeath, unpublished, 1:1,000), mouse anti-Fibrillarin (Abcam, 1:10), mouse anti-GFP (Roche, 1:100), rabbit anti-GFP (Abcam, 1:100), mouse anti-c-Myc (Santa Cruz Biotechnology, 1:100), rabbit anti-d-Myc (from D. Stein 1:5,000), rabbit anti-phosphorylated histone H3 (Upstate Biotechnology, 1:1,000), mouse anti-Prospero (DSHB, 1:10), mouse anti-Orb (4H8, DSHB, 1:10), mouse anti- α -Tubulin (Sigma), rabbit anti-Vasa (gift from P. Lasko, 1:10,000) and goat anti-Vasa (Santa Cruz, 1:200). Rhodamine-conjugated phalloidine (Alexa) was used 1:1,000 and rabbit anti-Mei-P26 (1:300) was raised against the peptide NLKTVLSDDASNSSVLED corresponding to amino acids 23–40. Samples were mounted in Vectashield containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and imaged on a Zeiss LSM 500 confocal microscope equipped with a blue diode laser to visualize DAPI. Images were processed in Adobe Photoshop and Adobe Illustrator.

Constructs

The *mei-P26* full-length, *mei-P26*^{ANHL} (amino acids 1–933), *AGO1*, *dappled* and *brat* coding sequences were PCR-amplified from total complementary DNA or ESTs with appropriate primers containing attB recombination sites (or with primers containing appropriate restriction sites), and were sequenced and recombined into pDONR221 (Invitrogen) and thereafter recombined into either tagged or untagged pUASp or pUAST destination vectors (DGRC). For fly transformation, constructs were co-injected with $\Delta 2-3$ transposase into *Drosophila w¹¹¹⁸* embryos.

To generate a *bantam* luciferase reporter, two copies of a sequence complementary to the *bantam* microRNA were cloned downstream of the FLuc reporter plasmid (*bantam* sensor). A plasmid expressing *Renilla* luciferase (RLuc) (gift from E. Izaurralde) served as a transfection control.

Biochemistry and S2 cell experiments

For ovary lysates, 20 ovaries from the respective genotypes were dissected in PBS. Proteins were extracted with Laemmli buffer and run on a 10% SDS-page. S2 cell transfections, immunoprecipitations, silverstaining and mass spectrometry were performed essentially as described⁶.

For microRNA assays, S2 cells were simultaneously transfected with Actin-*Gal4*, the FLuc microRNA reporter construct, the RLuc transfection control construct and pUASp mei-P26, pUASp mei-P26^{ANHL} or the empty pUASp vector as a control. Dual luciferase assays were performed 24 h after transfection following the manufacturer's instructions (Promega). For each experiment, transfections were performed in triplicates. Three independent experiments showed comparable results, one of which is shown in Supplementary Fig. 7.

Fly strains

We used the following *Drosophila* strains: *AGO1^{k08121}* (gift from T. Uemura), *bantam^{Δ1}*, *mei-P26^{fs1}* (ref. 8), *mei-P26^{mfsl}* (ref. 8), *bam^{Δ86}* (Bloomington), *loqs^{f00791}* (Bloomington), *loqs^{KO}* (gift from D. McKearin), *nanos-Gal4::VP16* (gift from F. Schnorrer), *hs-Bam* (gift from D. McKearin), *brat¹⁹²*, *FRT18A Ubi-GFP.nls*, *hs-Flp/CyO* (Bloomington), *yw hsFLP*, *FRT G13 2xGFP.nls*, *UASP-mei-P26* (gift from S. Hawley, unpublished), *BamP-GFP* (gift from D. McKearin), the *Gal4* lines *OK107* (gift from L. Luo) and *1407*, *tub-Gal80^{ts}* (Bloomington), Rho1–GFP trap (ZCL1957, Fly Trap), and *pum⁰¹⁶⁸⁸* (Bloomington), *pum^{ET3}*. All fly strains were raised on standard food without wet or dry yeast.

microRNA quantitative PCRs

Total RNA from ovaries was extracted from the four respective genotypes (wild type, *mei-P26^{mfsl/fs1}*, *bam^{Δ86}* and *bam^{Δ86} nanos-Gal4::VP16 >> pUASp-mei-P26*) using the TRIzol Reagent (Invitrogen). Primer sets designed to amplify mature microRNAs (and sno RNA227 as a control reaction) were obtained from Applied Biosystems. Products were amplified from 10 ng total RNA samples from the respective genotypes with the TaqMan microRNA assay using a quantitative PCR machine (Applied Biosystems). Wild-type microRNA levels are set to zero in Supplementary Fig. 7a.

Quantification

Wild-type and *mei-P26^{mfsl}* flies carrying *BamP-GFP* were stained with mAb1B1 to identify cell types and rhodamine-phalloidine to outline cells. Cell diameters were determined from image stacks of 0.4-μm sections using the measuring tool of LSM software (Zeiss). Cell types were defined as follows: Stem cells: GFP-negative, niche-contacting single-spectrosome-containing cells. Cystoblasts: GFP-positive single-spectrosome-containing cells. Cystocytes: 4, 8 or 16 GFP-positive cells connected by fusomes. To determine nucleolar:cell size ratio, anti-Fibrillarin and rhodamine-phalloidine were used in stainings. For ellipsoid cells, the mean cell diameter was determined (from the longest and shortest cell diameter) and used in the statistics.

For volume reconstruction of germline stem cells, Rho1–GFP trap (ZCL1957) flies were stained with mouse anti-1B1, GFP and DAPI, and volumes were determined from image stacks of 0.4-μm sections using the IMARIS software. For reconstruction of cyst volume, GFP-negative clones, stained with mouse anti-1B1, rhodamine-conjugated phalloidine (which perfectly colocalizes with the Rho1–GFP trap localization pattern within the resolution of our microscope), GFP and DAPI and were analysed in a similar manner.

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Figure 1. Differentiation and cell cycle defects in *mei-P26* mutant ovaries

a, Overview of *Drosophila* oogenesis showing cap cells (dark green), escort cells (light green), stem cells (orange), cystoblasts and cystocytes (yellow), follicle cells (blue) and the oocyte (dark grey). The spectrosome and fusome are red. **b–f**, Wild type (WT) (**b**, **e**) and *mei-P26* (**c**, **d**, **f**) ovarioles stained with mAb1B1 (1B1, green) and for DNA (red). *fs1* and *ms1* represent two different *mei-P26* alleles. **g**, **h**, Cyclin E (red; green, Vasa; blue, DNA) is downregulated in WT (**g**, arrowhead) but not in the *mei-P26^{fs1}* mutant (**h**, arrowhead) cystocytes. **i**, **j**, Phospho-H3 positive (green, pH3; red, DNA) mitotic cells (arrowheads) are restricted to the tip of the ovarioles in WT (**i**) but are found at all stages in *mei-P26^{fs1}* (**j**) ovarioles. **k**, **l**, GFP under the control of the *bam* promoter (*bamP-GFP*) (green; red, mAb1B1; blue, DNA) is not expressed in WT (**k**) and *mei-P26^{fs1}* mutant (**l**) niche contacting germline cells (arrowheads). **m**, **n**, Anti-Armadillo (green; red, DNA) shows integrity of WT (**m**) and *mei-P26^{fs1}* (**n**) mutant cap cells. **o**, **p**, Orb expression is initiated in WT (**o**) and *mei-P26^{fs1}* mutant (**p**) cystocytes but restricted to the oocyte only in WT (**o**, arrow) and not in *mei-P26^{fs1/ms1}* mutant (**p**, arrowhead) egg chambers.

Figure 2. Mei-P26 regulates cell and nucleolar size

a, b, Three-dimensional reconstruction of WT 16-cell cysts (filled arrowheads; **a**), a WT 8-cell cyst (open arrowhead; **a**) and a *mei-P26^{fs1/mfs1}* mutant cyst containing 14 cells (**b**). **c, d**, Nucleoli (green, anti-Fibrillarin) in *mei-P26^{fs1}* mutant ovaries (**d**) are larger than those in the WT ovaries (**c**). **e, f**, High levels of dMyc (red; green, mAb1B1) are detected in germline stem cells and early cysts (filled arrowhead). In postmitotic cysts, dMyc levels are decreased (open arrowhead) and levels increase again as nurse cells undergo endoreplication (**e**). In *mei-P26^{fs1/mfs1}* mutants, high levels of dMyc are detected throughout the tumour (arrowheads in **f**). **g**, Diameter of the indicated cell types in WT ($n > 24$ cells) and *mei-P26^{fs1/mfs1}* mutant ($n = 11$ cells for Mei-P26 ant., $n > 43$ for all others) ovaries. Abbreviations: Mei-P26 ss, single-spectrosome-containing cells; Mei-P26 3–7 cc (or Mei-P26 >7 cc), cystocytes in *mei-P26^{fs1}* mutant cysts containing either 3–7 (or >7) cells; Mei-P26 ant., anterior niche contacting cells. Error bars, s.e.m. (green bars, WT; red bars, *mei-P26* mutant). **h, i**, Mei-P26 expression peaks in early 16-cell cysts in WT and is not detected at later stages of oogenesis (**h**). In *bam^{Δ86}* mutant ovaries, expression is not upregulated (**i**, compare to WT in **h**). Arrowheads point at equivalent stages. Note that Mei-P26 staining appears more intense in later stages owing to sample thickness and out-of-focus fluorescence (**i**). **j**, Germarium close up: Mei-P26 (red; green, mAb1b1; blue, DNA) expression is low in stem cells (arrowhead), weakly upregulated in 8-cell cysts (open arrowhead) and peaks in 16-cell cystocytes (arrow) (see Supplementary Fig. 2e, f, h).

Figure 3. *Bam* requires the AGO1-binding protein Mei-P26 to induce proper cystocyte differentiation

a, b, c, *mei-P26* (**b**) but not *mei-P26*^{ΔNHL} (**c**) overexpression (OE) (from *nanos-Gal4::VP16*) depletes the germ line (green, Vasa; red, DNA). **d, e,** Transient (heat-shock-induced) *bam* overexpression induces stem cell differentiation and depletes the germ line (red, Vasa; green, mAb1B1; blue, DNA) in a WT (**d**) but not *mei-P26*^{ΔNHL} mutant (**e**) background. **f, g,** *mei-P26* overexpression (from *nanos-Gal4::VP16*) in *bam*^{Δ86} mutants (**g**) reduces cell and nucleolar size (see statistics in Supplementary Fig. 2c) but does not induce stem cell differentiation (Supplementary Fig. 2i, j). **h, i,** The NHL domain proteins Brat, Mei-P26 and Dappled interact with AGO1. Reciprocal immunoprecipitations (IP) of Brat and AGO1 from *Drosophila* embryo extracts (**h**). GFP-tagged Mei-P26 but not GFP-tagged Mei-P26^{ΔNHL} (**i**) coimmunoprecipitates Myc-tagged AGO1 in S2 cells (see Supplementary Fig. 4b).

Figure 4. Me-iP26 regulates miRNAs

a, Quantitative PCR experiment comparing the level of mature microRNAs in *bam⁴⁸⁶* and *bam⁴⁸⁶, nanos-Gal4::VP16>>pUASp-me-iP26*. The *y* axis shows log₂ of the expression ratio between the two genotypes. **b, c**, Loss of one copy of *loquacious* partially rescues the *mei-P26^{fs1}* mutant phenotype (Supplementary Fig. 6). **d–g**, Downregulation of the bantam sensor reveals bantam microRNA expression in stem cells but not cystocytes (**e**), whereas the control sensor is uniformly expressed throughout the germ line (**d**). The *bantam* sensor (**g**) but not the control sensor (**f**) is silenced in the germ line of *mei-P26^{fs1/mfs1}* mutants (arrowheads indicate stem cells).