Stem Cell Reports, Volume *1*

Supplemental Information

The CCR4 Deadenylase Acts with Nanos and Pumilio in the Fine-Tuning of Mei-P26 Expression to Promote Germline Stem Cell Self-Renewal

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Inventory of Supplemental information

Supplemental information contains Supplemental Experimental procedures, as well as six supplemental figures and one supplemental table, as follows.

Supplemental Figure 1 related to Figure 1

Supplemental Figure 2 related to Figure 2

Supplemental Figure 3 related to Figure 3

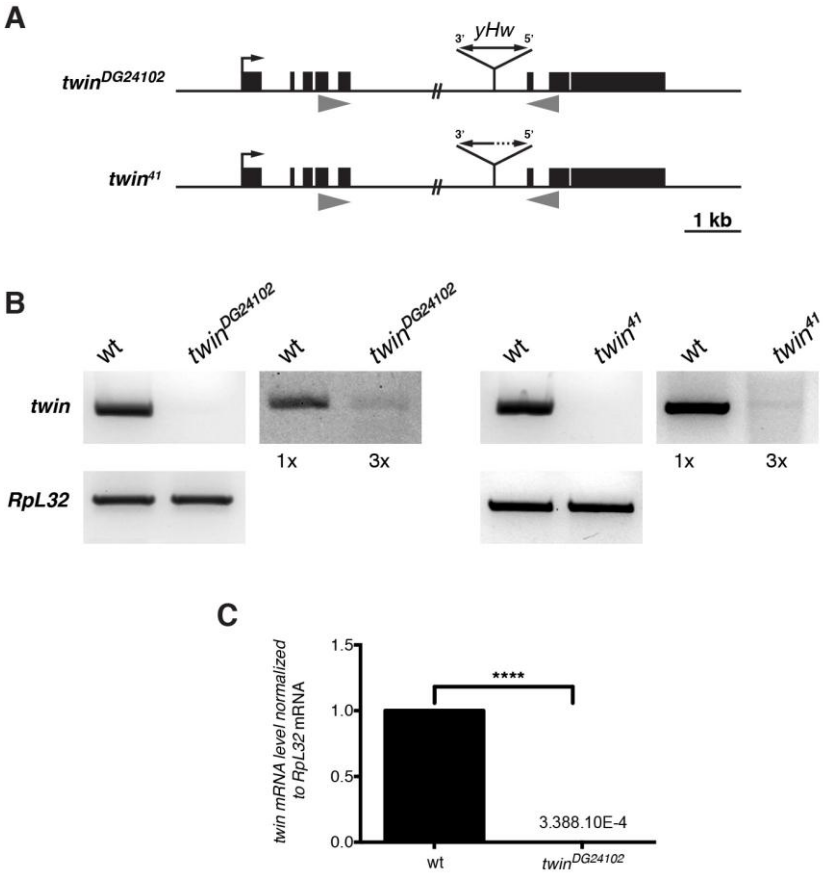
Supplemental Figure 4 related to Figure 4

Supplemental Figure 5 related to Figure 6

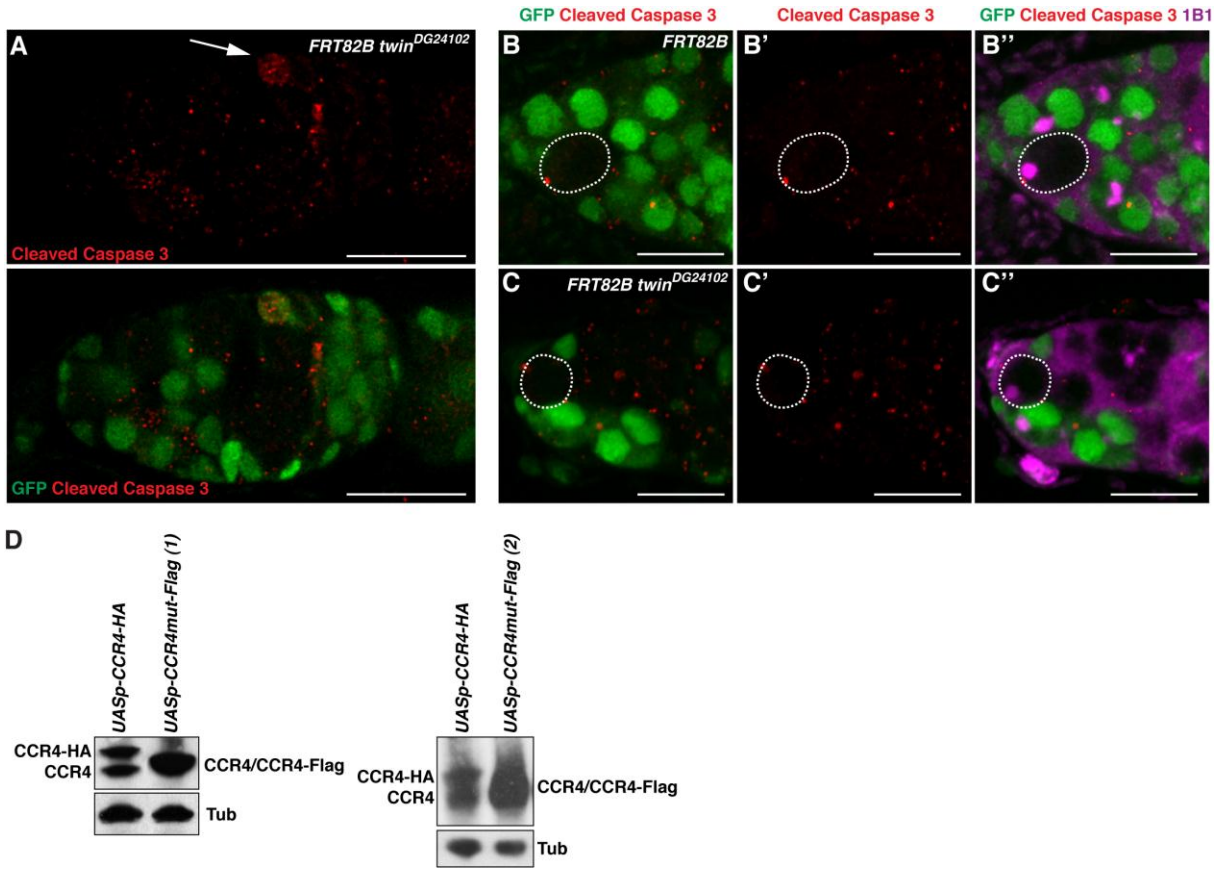
Supplemental Figure 6 related to Figure 5

Supplemental Table 1: sequences of primers used in the study

Supplemental information



Supplemental Figure 1: Characterization of the *twin*^{DG24102} and *twin*^{41} mutant alleles (related to Figure 1)
(A) Schematic representation of the *twin*^{DG24102} and *twin*^{41} alleles showing the *P-Hobo P{wHy}* transposable element (*yHw*) inserted into the *twin* gene. In *twin*^{41}, a part of *P{wHy}* overlapping *Hobo* and *white*$^{+}$ is deleted.
(B) RT-PCR of wild-type, *twin*^{DG24102} and *twin*^{41} ovaries showing that *twin* mRNAs are in very low amounts in *twin* mutants. 3x indicates that the amounts of cDNA were three fold those in 1x. *RpL32* was used as a control mRNA.
(C) Quantification of *twin* mRNAs in wild-type and *twin*^{DG24102} ovaries by RT-qPCR. Quantifications were done in triplicate from two independent RNA extracts. *RpL32* was used as a control mRNA. The amounts of *twin* mRNAs are strongly reduced in *twin*^{DG241002} ovaries. **** *p*-value < 0.0001 using the t-Test.



Genotype	1 day	3 days	7 days
UAS-RNAi-NOT1/nos-Gal4	0% (n=109)	0% (n=103)	nd
nos-Gal4	nd	100% (n=85)	99.2% (n=117)

Supplemental Figure 2: Lack of apoptosis in *twin* mutant GSCs and role of CCR4 as part of the CCR4-NOT complex in GSC self-renewal (related to Figure 2)

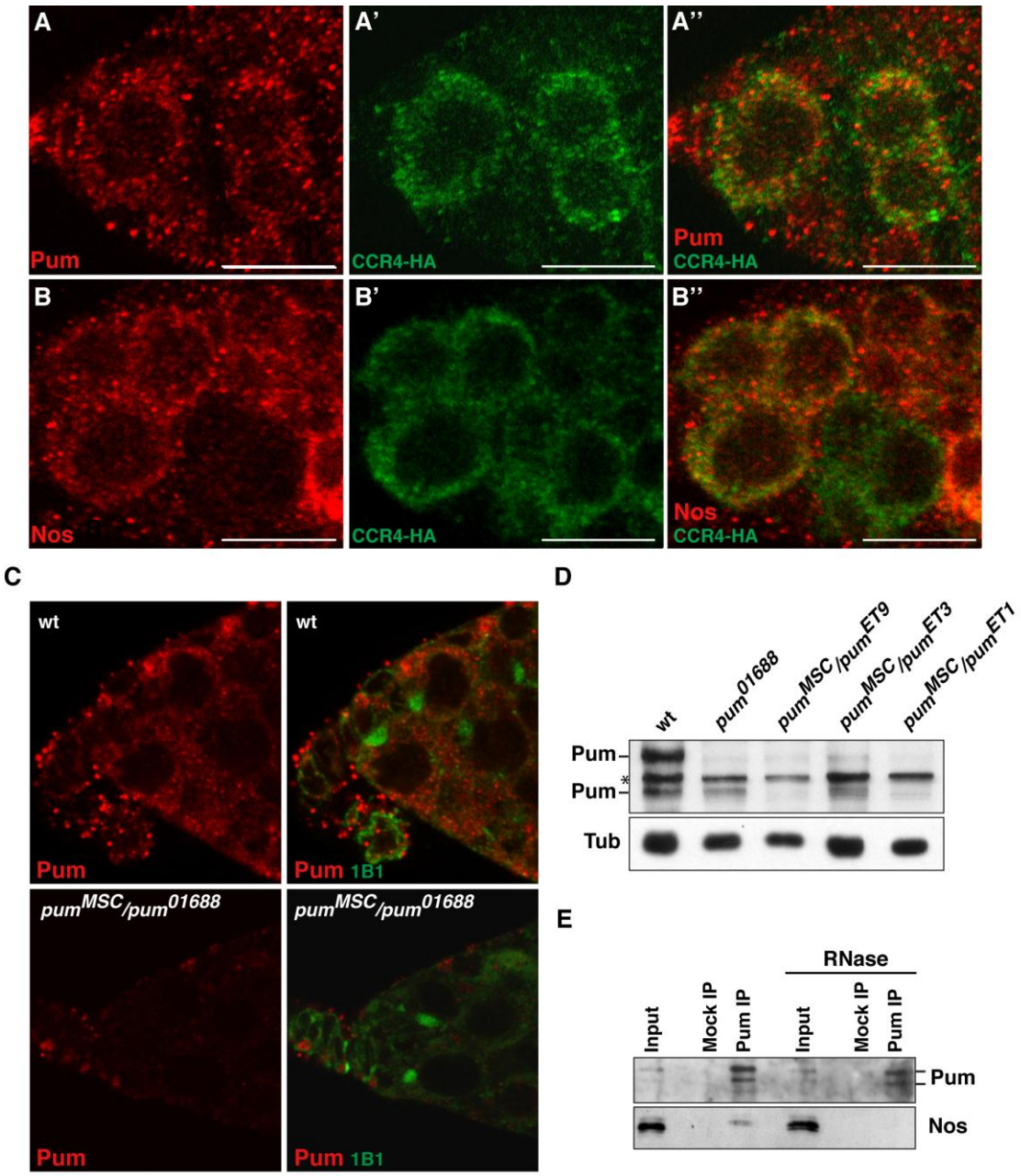
(A) Positive control of activated-Caspase 3 staining in a follicle cell undergoing apoptosis, indicated with a white arrow. Anti-cleaved Caspase 3 staining alone is in the top panel. The merge with GFP is in the bottom panel, showing that the apoptotic follicle cell is not mutant (*twin*^{DG24102}/+). Scale bar: 20 µm.

(B-C'') Mosaic germaria labeled with anti-GFP (green), anti-cleaved Caspase 3 (red) and 1B1 (purple) showing that neither wild-type (B-B'') nor *twin*^{DG24102} (C-C'') clonal GSCs undergo apoptosis. Clonal GSCs, marked by the lack of GFP, are outlined by white dotted lines. They are not stained with anti-cleaved Caspase 3. The merge is shown in (B'') and (C''). Scale bars: 10 µm.

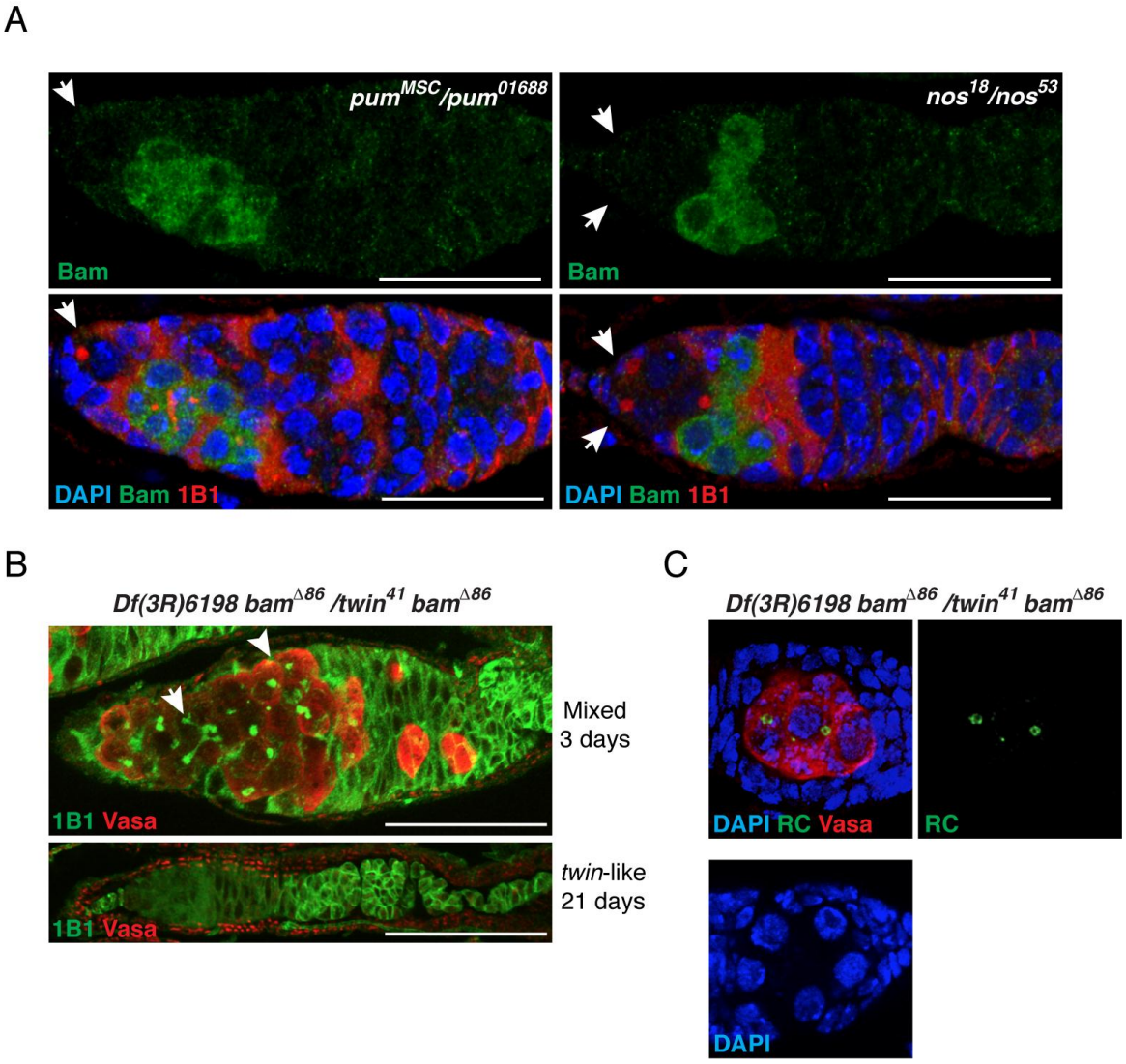
(D) Western blots of ovaries expressing the CCR4 transgenes under the control of *nos-Gal4*. *UASp-CCR4mut-Flag(1)* and *(2)* are two independent genomic insertions of the same transgene. The relative protein levels of CCR4-HA and CCR4-mut-Flag indicate that *CCR4mut-Flag(2)* is highly expressed, consistent with its better rescue activity than that of *CCR4mut-Flag(1)* (Figure 2F). Western blots were revealed with anti-CCR4. α-Tubulin (Tub) was used as a loading control.

(E) RNAi-NOT1 mutant phenotype of lack of GSCs and germ cells. Empty germarium labeled with 1B1 (green) and anti-Vasa antibody (red). Scale bar: 20 µm. Quantification of germaria containing at least one GSC in *UAS-RNAi-NOT1/nos-Gal4* females and in control *nos-Gal4* females. n represents the number of germaria scored. nd: not determined. Note that the decrease in NOT1 levels in RNAi-NOT1 ovaries could not be tested due to the lack of germ cells.

(F) Co-immunoprecipitations with CCR4-HA in GSC-like cells. Ovarian extracts from *bam*^{Δ86} and *bam*^{Δ86}, *nos-Gal4/bam*^{Δ86}, *UASp-CCR4-HA* (*bam*^{Δ86}, *CCR4-HA*) flies were immunoprecipitated with anti-HA, either in the absence, or the presence (RNase) of RNase A. Western blots were revealed with anti-HA, anti-NOT1 and anti-CAF1 antibodies. Input corresponds to the extract before immunoprecipitation.



Supplemental Figure 3: Colocalization of CCR4-HA with Pum and Nos in the GSCs (related to Figure 3)
(A-A") *twin*^{DG24102}, *nos-Gal4/twin*^{DG24102}, *UASp-CCR4-HA* germaria labeled with anti-Pum (red) and anti-HA (green). The merge is shown in A".
(B-B") *twin*^{DG24102}, *nos-Gal4/twin*^{DG24102}, *UASp-CCR4-HA* germaria labeled with anti-Nos (red) and anti-HA (green). The merge is shown in B". Scale bars in (A-B"): 10 μm.
In cells that co-express CCR4-HA and either Pum or Nos, although some colocalization can occur in a few foci, it is mostly seen diffusely in the cytoplasm. Pum and Nos foci are often found in close proximity to, or surrounded by CCR4-HA foci.
(C-D) Validation of the rabbit anti-Pum antibody produced in this study. (C) Wild type and *pum*^{MSC}/*pum*^{01688} mutant germaria labeled with anti-Pum (red) and 1B1 (green) showing that the anti-Pum staining is decreased in *pum* mutant. (D) Western blots of wild type and different *pum* mutant ovaries from 1 day-old females showing the decrease or disapearance of both Pum proteins in the mutants. * indicates a non-specific band recognized by the anti-Pum antibody. Western blots were revealed with anti-Pum. α-Tubulin (Tub) was used as a loading control.
(E) Co-immunoprecipitations (IP) with Pum in GSC-like cells showing that proteins are not degraded in the extract containing RNase A (Input). This indicates that the lack of Nos in Pum IP in the presence of RNase is due to a lack of co-IP. Ovarian extracts from *bam*^{Δ86} flies were immunoprecipitated with anti-Pum (Pum IP), or mock immunoprecipitated (with pre-immune serum, Mock IP), either in the absence, or the presence (RNase) of RNase A. Western blots were revealed with anti-Pum and anti-Nos antibodies.



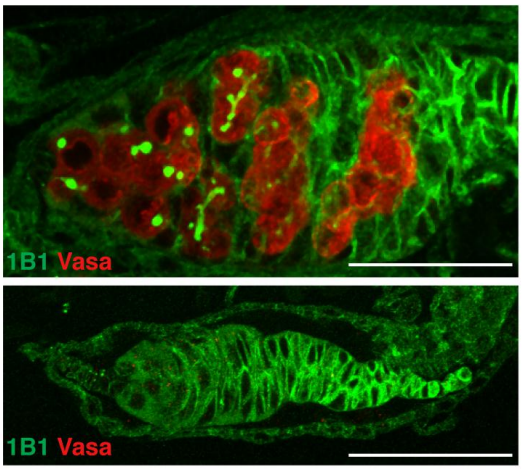
Supplemental Figure 4: Bam is not ectopically expressed in *pum* and *nos* mutant GSCs and *twin* is epistatic to *bam* (related to Figure 4)

(A) *pum*^{MSC}/*pum*^{01688} (left panels) and *nos*^{18}/*nos*^{53} (right panels) germaria labeled with anti-Bam (green), 1B1 (red) and DAPI (blue). Top panels show Bam staining alone, bottom panels show the merge. White arrows indicate GSCs. Bam protein levels are not upregulated in GSCs in *pum* and *nos* mutant germaria. Scale bars: 20 μm in (A, B).

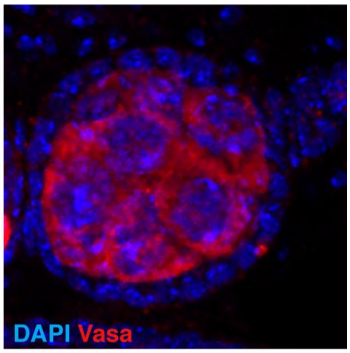
(B) Examples of "Mixed" and "*twin*-like" phenotypes in *twin bam* double mutant germaria quantified in Figure 4E; the "*bam*-like" phenotype is as in Figure 4C. Germaria labeled with 1B1 (green) and anti-Vasa (red). The white arrow indicates a differentiating cyst with a fusome, the white arrow-head indicates GSC-like cells.

(C) *twin bam* double mutant cysts labeled with DAPI (blue), anti-Vasa (red) and anti-Hts-RC (green) which revealed ring canals (RC), showing the presence of ring canals (top panels) and polyploid nuclei (bottom panel), as differentiation markers.

A *UASp-mei-P26/+; nos-Gal4/+* at 21°C



B *UASp-mei-P26/+; nos-Gal4/+*



C

UASp-mei-P26/+; nos-Gal4/+	1 day	3 days	7 days
21°C	72.6% (n=383)	64.5% (n=485)	53.9% (n=284)

UASp-mei-P26/+; nos-Gal4/+	1 day	4 days	7 days
25°C	80% (n=90)	25.5% (n=145)	8.8% (n=194)

Supplemental Figure 5: Mei-P26 overexpression in germ cells leads to GSC loss (related to Figure 6)
(A) *UASp-mei-P26/+; nos-Gal4/+* germaria labeled with 1B1 (green) and anti-Vasa (red) showing the presence of GSC and differentiating cysts with fusome (top), and a lack of germ cells (bottom). Scale bars: 20 μm.
(B) *UASp-mei-P26/+; nos-Gal4/+* cyst labeled with DAPI (blue) and anti-Vasa (red) showing the presence of polyploid nuclei as differentiation marker.
(C) Quantification of germaria containing at least one GSC in *UASp-mei-P26/+; nos-Gal4/+* females showing the loss of GSCs with time and that this loss is increased with higher levels of Mei-P26 at 25°C (Gal4 activity increases with temperature). n represents the number of germaria scored.

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1     TTGCAAATCAAAGCGCGCAACAATCGTCCATCAGTCGATATGAACAAATATAACTACAAATACTCGTCGGGCAAGATATA  80
81    CGGATGTCGACGGCAGCAGCAATCAACAAACACCAGCAATAGCAACACCATCTGCAACAGCAACAGCAACAGCAACAGCA  160
161   ACAGCAACAGTAATAGCAATAGCAATAGCAACCTCAACATAAATATCTTGGCAAGGAGTCAACACACCGTACACTGCAAT  240
241   AACAACAGTCAACTGGACAAGATAAGCAGGATAAGTAGCATGAGCAACATTTGCCTCATCGACAGCAACAGCAACAGCAA  320
321   CATCAATCAACAACCATTGACTACAACAGCGTCAATCATCTAATAGAACCGAGAAGCAAGAATTAGAACAAACTAAGACA  400
401   TTGACACCATCAACAGATGCAGATAGCAGATGCAACTATACCAAATGGGCATATATGTATGAATCAAAATGTATATGTAT  480
481   ATATATGAGTGGGGAATGCTAATAGACTAAATACGAATGATTAACTACGAATACGTGAAAATGAAAAGGTCGAGCTCCTA  560
561   TTTTTTTTTCCCCGTTTATCTGTATAGTCCCCTATCAACGTTCGAATTCTTAAAGCTGATCAAATCTGCGGCTGCACAGA  640   #1
641   CTCGCTCTATATATATGGAGTACAGCTGGAGGCAAATGCAATGCGTTGAATGCGACTTGAAACACATTTTGTTTAATGCG  720
721   AAACACGAGAAACGTTGCCGCTGCAACCGTATGAAACACATAAACAAACCAATAACAGATGATAAACAAAATGCAACAAA  800
                                          ∇position 844
801   TACTATTTTTGATCTCTAATTTAAAAACAATTAGCGCTACTACTAAAAAAAAACAAAAACAAACATCAACAAATGTAACA  880
881   AGGAAAAACAAAACAAAATATAAATATAACAACAAAAACGGTGGAAAAACAAGAAGAATCGACATGAAAACTTCGAATAT  960
961   TTTCAAAACGTTTATAAAAACAAAAAACTCTTTTTAAATTGTTTTTGATTATATTCCTTTTTAATTGAATTTTTGTTCTT  1040  #2
1041  GTTAAAATTGTCATAACACACACACACACACACGCCACACTCACACAAACACGACTACTTTACATGTTACTTATTATC  1120
1121  TATATAGACATATATATTGTTATATCGACCGATTAGCTTTTTTGTGCATACCCTTCCAGAATCTTTTTCTAAAAAAACGA  1200
          ∇poly(A) site 1
1201  AAACAAGAAAAAAAAAAAAAAAACGAAACACGCATATATATATCTACACCTAGTATACAACATCTATGTATATCTAGCAA  1280
1281  AATGTGCAACTTTTGTGCACAGGTTTTTTGCTAACAAATAAGGAAGTAAACAATTTGTTGAGGGCGTCATCAGAGGTCGC  1360
1361  GTACAGGCAATAATTATAGAAGAAGATGAAATCTAAGTAGTTTATTTCGATTTCAAACTGAAACCAAAACACTAAGATAC  1440
1441  AATTTCAGAAAATAACAAAAACTGTTTAGGTTTTTCCTTACGCTGCTTATATAAGGTGATATCTATTTATATATAAATCT  1520
1521  ATATGAGTATATGAAAACAAAACAAAACAAACTATATATATCTTAAACATATATGTGAAATCCTATGACTATATGACTTT  1600
1601  GCCATATAATATACAAAGCAGGACGAGAGAAGAGAGTAGGGAGTTAGGCATATAGGGATAGCAAGGGACGGAGGATGACG  1680
1681  GAACGAATGGAGGCAACACACAACACAACACACCACACGACAGATATGATATCCAGAGACCCAGAGATCTAGAGATTCAT  1760
1761  AGATCCAGAGAATACATTCGATATGTATCAACTAAGTGCGTTCATCACGATAGAGATCATCCGACATAAACTACAGACAA  1840
1841  GAACTCAACATACAAATTAATCTGTAAGCTAAAAAAAAAAACAAACCAAACAAAACGAATGCAGATCGCATTAGGCCAACA  1920
1921  ACTATAATAACTAATACATCAGATTTGATTGAACCTACAGAGATGACATACGCGTATCTGTTAAGTTCTTATATACATTG  2000
2001  TTAAAGAAGCAAAACACACAAAAAGAAAAAAACAAAAACAAACGATATTCCTTTTGCTTATAAACAAACAAAACAAAAAT  2080
2081  GCAATACACACGTGCGCATTTAAGCGACAGATGTGCACACAAACAAACACACACACACGTACACCACAAAACAAAGCA  2160
2161  AAAACAACACCCAAAAACACTCACGATTTATATAGACGATATATAGACAACCATTTAAGATGATATATATATATATATAT  2240  #3
2241  ATAAATAAATAAATATATATTTATACATATGTATATATATATATATATATATATGTTCGGATACATGCAACATTTCTT  2320
2321  TCCCGTTGTAATACAATATAAGGCTGGTTTACTCCCAAGACATTATTATTTATCTATATACAAAATAACATTTTTTAATT  2400
2401  GTGTGAATTTTATCATGTGAGAGGCGTTAGGTAGAACAAAAAATGGCGATTAATTTAAACGAAGTTGGGCGAGAAAGCAA  2480
2481  AGCGAAGCAAGCAACTTATTTTTTATAGGCTGTAACAACCACATTTATTATATACATATACATCTATGTGTACATCTACA  2560
2561  TTAATATGTGACACACACACACACACACACACACGCCCATACACCCATACTCATCCACACACATAAGGCATAACAACGAA  2640  #4
2641  ACGAATCCTTTGTATTGTGAACATTATGATATCCTGTTATTTGTTAGTTGTGTTCGAAACTATGCGATTCCTTTTTTAAA  2720
2721  TGTTTTAAACAGCAAAAATCTTATGTTAATCCCATCATTGGCATTTGTGTACCACAATGCGGCGCGGAGCTAAAAACTGT  2800
2801  TTTAAATCTTGTCATTTTTCTAATTTTAATTGCTAGTTTTAGTCTAGTTTAAACAACACGAGAGCATTAAAGAGCGTGTT  2880
2881  TGGTTTATGTTAAATGTTGAGTTGAGTTGAGTACTAAACAAATGGACTGTGGTCGATCGCGAAGGCCGCACCGTTTGCAT  2960
2961  TAACCATCATTTACTATTGTCATTATTATTAATTATTATTAATTATTATTATTATTATTGTTTAGTTTTTACGTTGT  3040
3041  TACATTGTGAGGCTACACACAATTCTGTTTACATCATTATGATGTAGTGTAATCTATATGTAATAACAACAAACTGCGAA  3120
3121  AGCCAATGTGAAACGCTTCTGTTTATGTTATACCACTGAAATTATGATCATTTTACTATATACATTATATTTAAAACACT  3200
3201  ATGTACCCCAAAGTTGTTGATGGTAGACGAGACGAGGATCTTACAAATGCAAAATGATACTAAGCAAGAGCGAAAGAGAG  3280
3281  AGAAAAAGAGCGAGAGAGAGATAGATAGATAGAGCGAGAGAGAGAGAGAGGGTAAAAAATGCAATTTTTTATGAAACAAT  3360
3361  TTGGCTGTTGAAATATATAATTTTTAATTTAATTACGTTTTCGCATTTCGTTTATTACAACTTAGGCCCCAAAGCAAATG  3440
3441  TTATAGATTATGAATTGAATTTACAGCGAAATCGCCAGGATTTCGGTTGAGCACCTTTCCTTAAACCACCTAAACTTTCC  3520
3521  TTTCCATTTTCGCATAAATACTTTAAAATCTTGTCGCGCTGCATTAACTACGGAAGTATTAAGATGAAACATTTTGCTAA  3600
3601  AGACAATGGAAACACTCAAATGCAAAGTATACCAAAAGAATTGTTTTTAATATCCTAATCGTATTTGTATGTGTATATGT  3680
3681  ATGTTTACCTTTACCTTTTTACTTGATTGCGTTTAAATTCGATTTAAACTTCTTGTTTCCCAATTGATTGTCTTTTTGTC  3760
3761  TTTGTCTTAGTCAGCAGAATTTTCTGAATTTCTGTGACCACCTAATTAGGAAAACCAAACTCGAAAACTTTTATAGAAAT  3840
3841  ATACGAAACAACAAATAACAAATGAAAAACAAAAATCCAATAAAAAAAATACCATTTTACATTTGTTTATAGTTTTTATG  3920  #5
3921  GAAAGCCCTCAAAATGAAATTGTAATTCCACACGCTTTTATTTTACAAACTTAAACGATCACAACATAAAGAGGTTACAT  4000
4001  TTACTATATTAATTTTGTGTTTTATTTTTATTTCCTCTCTCTTTGTTGAAATCACAAAATGGAAAAAGTCAATTGTTGCT  4080
4081  TTAACGAAAATTATGAATTATGAATTATAATTTAAGTTTTAGGTTGGATAAGCAAATGATATTAAGTGAAATTGTATATG  4160
4161  ATTTTTAAAACAAACAAACAAACAAACAACTTTTCCACACGAAACACACAAAACGAAGAGGAAAACAACAAATTTATTTC  4240  #6
                                                    ∇ poly(A) site 2
4241  CTATGGCATACAAATTTTTAAAATGAAAAAAAAAACCAAATAAACATAAATGTGCTTAAGTTTAAATCAAATTTACAATT  4320
4321  TAGTTGAGATTACGCAACTTAACGA                                                        4345
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Supplemental Figure 6: Sequence of *mei-P26* 3'UTR (related to Figure 5)
Annotated sequence of *mei-P26* 3'UTR, starting after the stop codon. Potential Pum binding motifs are indicated in red, including the non-canonical motif in fragment #2. The NRE is indicated in green. The Vasa binding site is highlighted in blue. Position 844 which potentially corresponds to a poly(A) site, as well as poly(A) site 1 and poly(A) site 2 that were identified by mRNA circularization are indicated with a purple triangle and a purple nucleotide. Fragments #1 to #6 that were used in RNA pull-down assays are underlined (#1, #3, #5) or highlighted in grey (#2, #4, #6).

Supplemental Table 1: List of primers used

RT-PCR from the RNA-IP

	Forward primer	Reverse primer
mei-P26	CGAGCCCTCCGACATCGCTATACG	TCAGTGAACATAGAGGGTGTTCAG
sop	CACCCCAATAAAGTTGATAGACCT	ATCTCGAACTCTTTGATGGGAAGC

RT-qPCR from the RNA-IP

	Forward primer	Reverse primer
mei-P26	ACAATCGTCCATCAGTCGATATG GACACCATCAACAGATGCAGATAG	ACTGTTGCTGTTGCTGTTGC GCAGCCGCAGATTTGATCAGCTTT
sop	CACCCCAATAAAGTTGATAGACCT	ACCACCACGAGAGCCAAAT

RNA circularization

	Forward primer	Reverse primer
mei-P26 RT-PCR	TATTGCAGTGATCCTCCTCC	
mei-P26 first PCR-poly(A) site 1	CGACATGAAAACTTCGAA	TATTGCAGTGATCCTCCTCC
mei-P26 nested PCR-poly(A) site 1	CGACATGAAAACTTCGAA	CTTGTTGCACAAGGACGAAAT
mei-P26 first PCR-poly(A) site 2	CTTGTTGCACAAGGACGAAAT	CCTCTCTCTTTGTTGAAATCACAAAATGG
mei-P26 nested PCR-poly(A) site 2	GAAGAACACGGCGATGCAAAT TGG	GGAAAAAGTCAATTGTTGCTTTAACG

PAT assays

	Forward Primer
mei-P26 position 844/poly(A) site 1	GCGGCTGCACAGACTCGCTCTATA
mei-P26 poly(A) site 2	CCTCTCTCTTTGTTGAAATCACAAAATGG
sop	GCTACATATGCCGCCATCG

Primers used to quantify *mei-P26* transcripts by RT-qPCR

	Forward primer	Reverse primer
Primers 1	ACAATCGTCCATCAGTCGATATG	ACTGTTGCTGTTGCTGTTGC
Primers 2	GACACCATCAACAGATGCAGATAG	GCAGCCGCAGATTTGATCAGCTTT
Primers 3	CTGAATTTCTGTGACCACCTAATTAGG	CCATTTTGTGATTTCAACAAAGAGAGAGG

RNA pull down assays

	Forward primer	Reverse primer
oskar	AATCAATTTGGCTCCACCTG	GCATTCGCTTCGGATAAACT
hunchback	GTTCCCCATCACCATCACCTTG	GAACTGAGTGTTATGCGCATATACG
mei-P26 #1	GACACCATCAACAGATGCAGATAG	TCAAAAATAGTATTTGTTGCATTTTG
mei-P26 #2	ATGCAACAAATACTATTTTTGATCTC	GCGAGCTCCGCGGCCGCGTTTTTTTTTTT TVN
mei-P26 #3	CACACGTGCGCATTTAAGCGACAG	CGCCATTTTTTGTTCTACCTAACGCC
mei-P26 #4	GGCGTTAGGTAGAACAAAAAATGGCG	CTAGCAATTAAAATTAGAAAAATGACAAG
mei-P26 #5	CTGAATTTCTGTGACCACCTAATTAGG	GCGAGCTCCGCGGCCGCGTTTTTTTTTTT TVN
mei-P26 #6	TAATACGACTCACTATAGGGCGATCAC AACATAAAGAGGTTAC	GTCGTTAAGTTGCGTAATCTCAAC

Primers used to quantify *twin* transcripts by RT-PCR and RT-qPCR

	Forward primer	Reverse primer
RT-PCR	GGAGCTGTTGCTGAACAACAAC	CTGGTCGCGTTTTGTTGGGCT
RT-qPCR	AAGCTGTTCCACCTCGTCAT	GCGGTGGATTCACGGTAA

Supplemental Experimental Procedures

***Drosophila* stocks and genetics**

The w^{1118} stock was used as a control. Mutant and transgenic stocks used were $twin^{DG24102}$ (Bloomington Stock Center), $twin^{8115}$ (Zaessinger et al., 2006), *Df(3R)Exel6198* (Bloomington Stock Center), pum^{MSC} (Barker et al., 1992), pum^{01688} (Lin and Spradling, 1997), nos^{18} and nos^{53} (Wang et al., 1994), $bam^{\Delta 86}$ (McKearin and Ohlstein, 1995), *nos-Gal4* (Rorth, 1998), *FRT82B* $twin^{DG24102}$, *UASp-ccr4-HA* (Semotok et al., 2005), *UASp-mei-P26* (Neumuller et al., 2008), TRIP.HMS00526 (RNAi-NOT1) (Bloomington Stock Center), *FRT82B*, *FRT82B Ubi-GFP* and *hs-FLP; TM3, Sb/Dr* (Bloomington Stock Center), $mei\text{-}P26^{fs1}$ and $mei\text{-}P26^{mfs1}$ (Page et al., 2000). The $twin^{41}$ allele was generated by mobilization of the *Hobo* element of the *P{wHy}* insertion in $twin^{DG24102}$ as described previously (Huet et al., 2002). New *twin* alleles were selected on the basis of loss of the $white^{+}$ marker and analyzed by PCR. The $twin^{41}$ allele harbors a deletion of the 5' region of the *P-Hobo*-element and shows a similar phenotype to $twin^{DG24102}$. Once collected, females of interest were maintained on standard culture medium supplemented with dry yeast until dissection, at 25°C unless specified otherwise. For the clonal analysis, 2 to 3 day-old females were heat-shocked twice daily at 37°C for 1 h with 8 h of recovery, for three consecutive days. Females were dissected 3, 7, 14 or 21 days after the last heat shock (clone induction) and ovaries were fixed and immunostained as previously described (Song and Xie, 2002).

Immunostaining

For immunostaining, the following antibodies were used, mouse anti-Hts (1B1) and anti-Hts-RC (1/10, Developmental Studies Hybridoma Bank), rabbit anti-Vasa (1/1000, Santa Cruz Biotechnology), rabbit anti-cleaved caspase-3 (1/500, Cell Signaling Technology), rabbit anti-CCR4 (1/300, (Temme et al., 2004)), rabbit anti-Nos (1/1000, a gift from A. Nakamura), rabbit anti-Pum (1/1000), rabbit anti-Mei-P26 (1/1000, (Liu et al., 2009)), rabbit anti-Bam (1/2000, a gift from D. Chen), rabbit anti-GFP (1/1000, Invitrogen), mouse anti-HA (1/2000, ascites produced from 12CA5, Developmental Studies Hybridoma Bank). DNA was revealed with 0.5 µg/ml DAPI. Data were collected with a Zeiss LSM510 confocal microscope. Fluorescence quantification was performed using ImageJ software. For each wild-type or mutant germarium, the fluorescence was quantified both in GSCs and in the most posterior cyst where Mei-P26 expression peaks. Two different cytoplasmic regions of the same size were quantified within a single GSC, while a single cytoplasmic region of the same size was quantified per cell in cysts, for two independent cells of the same cyst. The ratio was calculated by dividing the mean fluorescence in GSCs by that in cysts. Quantification in mosaic germaria was performed using mean fluorescence in whole heterozygous or mutant GSCs, in two confocal sections.

Immunoprecipitations and western blots

For protein co-immunoprecipitations, the ovaries from 100 females were used per point. Ovaries from $bam^{\Delta 86}$ or $bam^{\Delta 86}$ females overexpressing CCR4-HA were dissected and homogenized as described previously (Zaessinger et al., 2006). 500 µl of cleared supernatant were mixed with 50 µl of Dynabeads Protein A (Invitrogen), covalently linked with dimethyl pimelimidate dihydrochloride (Sigma) to 5 µl of mouse monoclonal anti-HA antibody (acite produced from 12CA5, Developmental Studies Hybridoma Bank), or to 4 µl of rabbit anti-Pum antibody (Mock IP with 4 µl of rabbit pre-immune serum). Co-immunoprecipitations were performed in the presence of either RNasin (0.25 $U.\mu l^{-1}$, Promega) or RNase A (0.1 $\mu g.\mu l^{-1}$, Sigma). Western blots were performed as reported (Benoit et al., 1999) with antibodies used at the following dilutions: rabbit anti-CCR4 (1/1000), rabbit anti-CAF1 (1/1000, (Temme et al., 2004)), mouse anti-NOT1 (1/250, (Temme et al., 2010)), mouse anti-HA (1/2000), rabbit anti-Nos (1/2000), rabbit or guinea pig anti-Pum (1/2000). For RNA co-immunoprecipitations, the ovaries from 500 $bam^{\Delta 86}$ females were used per point. Ovaries were homogenized as described previously (Zaessinger et al., 2006) in 300 µl of DxB supplemented with RNasin. The supernatant was pre-cleared with 40 µl of Dynabeads Protein A and then incubated with either 4 µl of rabbit anti-Pum antibody (Mock IP with 4 µl of rabbit pre-immune serum) or 5 µl of mouse monoclonal anti-HA antibody (ascites produced from 12CA5), for 3 hours on a wheel at 4°C. 50 µl of Dynabeads Protein A were then added to each sample and incubated for 45 minutes on a wheel at 4°C. After six washes with DxB, a phenol-chloroform extraction was performed to extract RNA. DNA was removed with Turbo DNA-*free* (Ambion). The reverse transcription was performed on the total amounts of RNA, which were then used for PCR.

RNA pull-down assays

The different fragments used in pull-down assays were PCR amplified from ovarian cDNAs and either used directly for transcription (using a T7 promotor in the primer) or cloned into pCRII-TOPO (Invitrogen). The primers used for PCR amplification are listed in Supplemental Table 1. UTP-biotinylated RNA baits were synthesized using Sp6 or T7 Megascript kits (Ambion) and 1.5 µg of each RNA bait was linked to streptavidin magnetic beads (Roche). An equal amount of HA-PumC, synthesized by *in vitro* translation in reticulocyte lysate (TNT coupled reticulocyte lysate system, Promega), was incubated in binding buffer (10 mM HEPES-KOH at pH 7.9, 3 mM MgCl2, 40 mM KCl, 5 mM EDTA, 5% glycerol, 2 mM DTT, 0.5% NP40, 3 mg/ml Heparin, and 0.5 mg/ml tRNA) during 1h30 at 4°C with the different baits. After two washes in binding buffer, and two washes in binding buffer containing 1M NaCl, the bound proteins were analyzed by western blots using mouse anti-HA.

References

Barker, D.D., Wang, C., Moore, J., Dickinson, L.K., and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the Drosophila abdominal determinant Nanos. Genes & development *6*, 2312-2326.

Benoit, B., Nemeth, A., Aulner, N., Kühn, U., Simonelig, M., Wahle, E., and Bourbon, H.M. (1999). The *Drosophila* poly(A)-binding protein II is ubiquitous throughout *Drosophila* development and has the same function in mRNA polyadenylation as its bovine homolog *in vitro*. Nucleic Acids Res *27*, 3771-3778.
Huet, F., Lu, J.T., Myrick, K.V., Baugh, L.R., Crosby, M.A., and Gelbart, W.M. (2002). A deletion-generator compound element allows deletion saturation analysis for genomewide phenotypic annotation. Proceedings of the National Academy of Sciences of the United States of America *99*, 9948-9953.
Lin, H., and Spradling, A.C. (1997). A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. Development *124*, 2463-2476.
Liu, N., Han, H., and Lasko, P. (2009). Vasa promotes Drosophila germline stem cell differentiation by activating mei-P26 translation by directly interacting with a (U)-rich motif in its 3' UTR. Genes Dev *23*, 2742-2752.
McKearin, D., and Ohlstein, B. (1995). A role for the Drosophila bag-of-marbles protein in the differentiation of cystoblasts from germline stem cells. Development *121*, 2937-2947.
Neumuller, R.A., Betschinger, J., Fischer, A., Bushati, N., Poernbacher, I., Mechtler, K., Cohen, S.M., and Knoblich, J.A. (2008). Mei-P26 regulates microRNAs and cell growth in the Drosophila ovarian stem cell lineage. Nature *454*, 241-245.
Page, S.L., McKim, K.S., Deneen, B., Van Hook, T.L., and Hawley, R.S. (2000). Genetic studies of mei-P26 reveal a link between the processes that control germ cell proliferation in both sexes and those that control meiotic exchange in Drosophila. Genetics *155*, 1757-1772.
Rorth, P. (1998). Gal4 in the *Drosophila* female germline. Mech Dev *78*, 113-118.
Semotok, J.L., Cooperstock, R.L., Pinder, B.D., Vari, H.K., Lipshitz, H.D., and Smibert, C.A. (2005). Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early Drosophila embryo. Curr Biol *15*, 284-294.
Song, X., and Xie, T. (2002). DE-cadherin-mediated cell adhesion is essential for maintaining somatic stem cells in the Drosophila ovary. Proceedings of the National Academy of Sciences of the United States of America *99*, 14813-14818.
Temme, C., Zaessinger, S., Meyer, S., Simonelig, M., and Wahle, E. (2004). A complex containing the CCR4 and CAF1 proteins is involved in mRNA deadenylation in Drosophila. EMBO J *23*, 2862-2871.
Temme, C., Zhang, L., Kremmer, E., Ihling, C., Chartier, A., Sinz, A., Simonelig, M., and Wahle, E. (2010). Subunits of the Drosophila CCR4-NOT complex and their roles in mRNA deadenylation. Rna *16*, 1356-1370.
Wang, C., Dickinson, L.K., and Lehmann, R. (1994). Genetics of nanos localization in Drosophila. Dev Dyn *199*, 103-115.
Zaessinger, S., Busseau, I., and Simonelig, M. (2006). Oskar allows nanos mRNA translation in Drosophila embryos by preventing its deadenylation by Smaug/CCR4. Development *133*, 4573-4583.