miR-9a modulates maintenance and ageing of Drosophila germline stem cells by limiting N-cadherin expression

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Ageing is characterized by a decline in stem cell functionality leading to dampened tissue regeneration. While the expression of microRNAs across multiple species is markedly altered with age, the mechanism by which they govern stem cell-sustained tissue regeneration is unknown. We report that in the Drosophila testis, the conserved miR-9a is expressed in germline stem cells and its levels are significantly elevated during ageing. Transcriptome and functional analyses show that miR-9a directly regulates the expression of the adhesion molecule N-cadherin (N-cad). miR-9a null mutants maintain a higher number of stem cells even in the aged tissue. Remarkably, this rise fails to improve tissue regeneration and results in reduced male fertility. Similarly, overexpression of N-cad also results in elevated stem cell number and decreased regeneration. We propose that miR-9a downregulates N-cad to enable adequate detachment of stem cells toward differentiation, thus providing the necessary directionality toward terminal differentiation and spermatogenesis.
Ageing leads to reduced tissue homeostasis and a decline in the ability to replace damaged cells by new functional ones. Homeostasis and repair of many adult tissues, such as blood, gut, and testis, are supported by small specialized populations of tissue-specific stem cells. In a given tissue, stem cells reside in a local microenvironment (niche) that acts as a control-unit to determine stem cell proliferation rate and protects the overall stem cell pool from depletion.

In the *Drosophila melanogaster* (*Drosophila*) testis, mature sperm cells are generated by germline stem cells (GSCs) that are located at the apical tip of the testis. These cells, together with Cyst stem cells (CySCs) co-habitat in the niche and adhere around a cluster of somatic cells called the hub. The hub is a spherical three-dimensional (3D) structure of approximately 12 cells, the great majority of which (Fig. 1a) are associated on several planes with all the surrounding stem cells. The GSCs are in direct contact with the hub via microtubule-based nanotubes that protrude directly into the hub. The hub expresses signaling and adhesion molecules that maintain the stem cells within the niche. Upon GSC division, one of the two daughter cells remains adherent to the hub for self-renewal, while the other, a displaced progenitor cell, undergoes transit amplification divisions to generate spermatogonia progenitor cells before becoming a terminally differentiated spermatocyte. GSCs have an age-dependent limited half-life of 14 days and are lost mainly via detachment from the niche. In contrast, spermatogonia cells can dedifferentiate back into GSCs during ageing to repopulate the niche (Fig. 1a), thus representing an intermediate cell population that can either differentiate or dedifferentiate according to the needs of the tissue. Therefore, although ageing results in significantly smaller testis with reduced cells of all types, unlike females, aged males (30-days) tend to remain fertile. Nonetheless, since all testicular germ cells originate from a minute number of stem cells (approximately seven) even a small change of miRNA levels can significantly affect testis size and cell numbers.

**Fig. 1** miR-9a increases during ageing and is expressed in GSCs and spermatogonia. **a** Side-view representation of the apical tip of the *Drosophila* testis. GSCs (red) are anchored to the hub (blue) and spermatogonia germ cells (red) can terminally differentiate to spermatocytes (crimson) or dedifferentiate to GSCs (two-directional arrows). GSCs and spermatogonia are encapsulated by cyst cells (green). Dashed-line separates spermatogonia from spermatocytes. **b** Testes from 1-day, 15-days, or 30-days-old males (green, mature sperms; Imp^CB04573^ immunostained for Vasa (red, germ cells) and Fas3 (blue, hub and seminal vesicles). Note an age-related decrease in cell numbers and in overall testis size. Scale bars, 100 µm. Asterisks mark the hub. **c** Heatmap of fold-change of *Drosophila* miRNAs in testes of 1-day, 15-days, or 30-days-old wild-type (*w^{1118}*). Top 11 most age-altered miRNAs out of a total of 100 are shown. DESeq defined significance between 30-days and 1-day for each miRNA (P<0.05; n=2 replicates for each time point). **d** qRT-PCR for mature miR-9a relative to control (25S rRNA) in the testes of 1-day, 15-days, or 30-days-old wild-type (*w^{1118}*). Levels are normalized to 1-day-old adults. Error bars denote s.d. of three biological repeats each in triplicate measurements. Note a 10-fold increase of miR-9a in 30-days. Statistical significance was determined by one-way ANOVA and post hoc analysis was performed with Tukey multi-comparison test. **P ≤ 0.005** between 15 and 1-day and between 30 and 1-day. **e-g** Testes of control GFP sensor (**e, e’**) and miR-9a sensor 1-day (**f, f’**), and 30-days (**g, g’**) stained for Fas3 (blue) to mark the hub (asterisks), Vasa to mark the germ cells (red) and GFP (green). A control sensor is expressed in all cells at the apical tip of the testis including; hub, GSCs, CySCs, cyst cells (arrowhead), spermatogonia and spermatocytes. The miR-9a sensor detect endogenous levels of miR-9a in GSCs and spermatogonia. Dashed-line separates spermatogonia from spermatocytes. Note that miR-9a is not expressed in spermatogonia or the somatic niche (hub and cyst cells). All images in all figures are single sections. Scale bars, 10 µm
change in their overall number or division frequency is expected to impact tissue regeneration.

MicroRNAs (miRNAs) are negative regulators of mRNA targets that prevent their translation into proteins. Recent data reveals that the levels of some miRNAs change with age across organisms, yet little is known about the molecular pathways that are regulated by these changes. Here, we aimed to identify the miRNAs that change in the course of ageing and to find how these changes affect stem cell functionality. We found that miR-9a levels increase in the GSCs during ageing. Furthermore, miR-9a directly downregulates Neural-Cadherin (N-cad) to control the adhesion between GSCs and the hub, thus promoting GSCs detachment from the niche to allow differentiation and functional spermatogenesis.

Results

miR-9a levels increase in testis of aged males. To determine whether the levels of specific miRNAs are age-altered, we analyzed the miRNAome of testes dissected from 1-day (young), 15-days (mid-aged), and 30-days (aged) wild-type flies by NanoString technology (Fig. 1b, c). The resulting miRNAome showed that of a total of approximately 100 miRNAs, the expression level of a small cohort of 11 was elevated by more than two-folds in aged flies (Fig. 1c). The cohort included let-7 that was found to regulate GSCs niche ageing, thus supporting library reliability. Interestingly, one of the top candidates identified was the evolutionary conserved miR-9a. miR-9a was also included in the top four most abundant miRNAs in the testis and in aged flies represents ~1% of the entire miRNAome. A subsequent qRT-PCR analysis revealed that compared to young males, miR-9a levels were increased by five-fold in testis of mid-aged males and by ten-fold in aged males (Fig. 1d).

miR-9a is expressed in GSCs and progenitor germ cells. To identify the cells that express miR-9a in the testis we used a green fluorescent protein (GFP) sensor that utilizes the unique property of miRNAs to silence protein expression. The GFP-miR-9a sensor contains two repeats of the complementary sequence of miR-9a in an artificial 3' untranslated region (3'UTR) following a reporter GFP sequence. Therefore, cells that endogenously express miR-9a create a silencing mechanism that prevents the expression of GFP. We used this method to compare the expression pattern of GFP-control and GFP-miR-9a sensor both driven under a tubulin promoter. GFP of the control sensor is expressed in all the cells of the testis, with a brighter signal in cyst cells as expected from high Tubulin expression in these long thin cells (Fig. 1e). However, in tests of both young and aged flies, the miR-9a sensor revealed that miR-9a is expressed in GSCs and spermatogonia progenitor germ cells. Moreover, miR-9a is not expressed in terminally differentiated spermatocytes, mature sperms, somatic cyst, and, notably, not in hub cells (Fig. 1f, g). Expressing miR-9a sensor in a miR-9a null mutant background of miR-9a[E39] resulted in GFP expression in GSCs and spermatogonia cells, indicating that in the sensor flies GFP is specifically repressed by miR-9a in these cells (Supplementary Fig. 1a, b). In support of these data, miR-9a fluorescence in situ hybridization (FISH) shows that miR-9a is expressed in GSCs and spermatogonia and absent from the hub. Moreover, no miR-9a FISH signal was obtained in testis of miR-9a[E39] null mutants (Supplementary Fig. 1c–e).

Fig. 2 miR-9a mutants increase GSCs maintenance but reduce spermatogenesis. a–h Testes from 1-day (a, c) or 30-days (e, g) old control (w1118) or 1-day (b, d) or 30-days (f, h) old miR-9a[E39] mutants immunostained for Fas3 (blue, hub), Vasa (green, germ cells), and with pH3 (green, c–d). White dots denote GSCs. i–l Rescue of miR-9a[E39] mutants by ectopic expression of DsRed-miR-9a in GSCs and progenitor germ cells (nos-GAL4, UAS-DsRed-miR-9a; miR-9a[E39])/TM6, young (i) and aged (k); Vasa, Fas3, and pH3 (green) DsRed (red) and DAPI (blue). Asterisks mark the hub, arrowhead denote mitotic GSCs and arrow mitotic spermatogonia. Scale bars 10 μm (a–f, i–l) and 100 μm (g, h). Shown are average number of GSCs per testis along with 95% confidence interval (error bars). The total number of testes scored: control (w1118) 1-day (n = 47), 30-days (n = 60); miR-9a[E39] 1-day (n = 71), 30-days (n = 70); Con. rescue 1-day (n = 45), 30-days (n = 24); miR-9a[E39] rescue 1-day (n = 33), 30-days (n = 16); Statistical significance was determined as in Fig. 1d. **P ≤ 0.005, *P ≤ 0.01 between miR-9a[E39] and the other three genotypes (Con. w1118, Con. rescue, and miR-9a [E39] rescue) of the same age. n Fertility assay of aged males (30 days) from control (w1118, n = 36) and miR-9a[E39] mutants (n = 41), miR-9a[E39] rescue (n = 20). Note decreased fertility of aged miR-9a[E39] mutants and rescue in DsRed-miR-9a ectopic expression in GSCs and spermatogonia of the mutant background.
**miR-9a null present higher GSCs number with reduced division.** To define the function of miR-9a in stem and progenitor germ cells, we compared the niche of young and aged miR-9a [E39] mutant males to age-matched controls. Immunofluorescence microscopy with germ and hub cell markers was used to evaluate the number of GSCs that are defined as germ cells that are physically attached to the hub. Our analysis showed that compared to control (w1118), miR-9a[E39] young mutants contain 37% more GSCs in their niche (Fig. 2a, b, m). Furthermore, these differences were maintained also in aged males, where miR-9a[E39] mutants showed 45% more GSCs (Fig. 2e, f, m). Similar to miR-9a[E39], a second miR-9a null allele, miR-9a[j22]14, also maintains a high average number of GSCs in the niche of young and aged males (Supplementary Fig. 1f).

To our surprise, the marked increase in GSCs number in miR-9a[E39] mutants did not result in improvement of the ageing phenotype. Similar to age-matched controls, testes of aged miR-9a[E39] mutants remained small, and presented with an even worse fertility phenotype. Similar to age-matched controls, testes of aged miR-9a[E39] mutants did not result in improvement of the ageing phenotype.
UAS-DsRed-miR-9a null mutants consists of a higher number of GSCs, these cells fail to maintain spermatogenesis in aged males due to enylation and decay\(^{19}\). Thus, the mRNA levels of direct targets in the testis are expected to be elevated in aged mutant males (Fig. 2n).

Validation of N-cad as a miR-9a target. Consistent with the transcriptome analysis, immunofluorescence staining of controls and miR-9a\(\text{E39}\) mutants with anti-N-cad revealed higher levels in adherent junctions of mutants, among hub cells and between hub and GCSs (Fig. 3b). Examination of image Z-sections and a 3D projection revealed that the majority of hub cells generate N-cad boundaries with GSCs in several planes (Supplementary Fig. 3a, b). Therefore, what may appear as an increase in N-cad among hub cells following miR-9a knockdown is in fact an increase that occurs mainly between hub and GSCs. Signal quantification of images taken at the exact same exposure showed a 1.9-fold increase of N-cad in miR-9a\(\text{E39}\) mutants compared to controls (Fig. 3c). In accordance with this observation, reducing N-cad levels in GSCs of the miR-9a\(\text{E39}\) mutant flies resulted in an overall reduction of N-cad expression (Supplementary Fig. 3c, d).

Two miR-9a canonical recognition sites are located within UTR of N-cad (Targetscaen Fly, Table 1 and Fig. 3d). Western analysis in Schneider 2 (S2R+) cells showed that miR-9a causes a 90% reduction in the expression of a GFP reporter containing the N-cad 3′UTR\(^{\text{WT}}\). This effect was abolished by mutating the predicted target sites (gfp-N-cad-3′UTR\(^{\text{Mut}}\)), indicating that seed mutations rendered the reporter resistant to translation inhibition (Fig. 3d–f). Consistent with these observations, flow cytometry (FACS) analysis of the GFP-expressing cells population revealed a marked 83% decrease in the mean fluorescent intensity (MFI) when miR-9a was co-expressed with gfp-N-cad-3′UTR\(^{\text{WT}}\) reporter (Fig. 3g). The transfection efficiency of miR-9a co-expression with gfp-N-cad-3′UTR\(^{\text{WT}}\) or gfp-N-cad-3′UTR\(^{\text{Mut}}\) reporters was measured by qRT-PCR for mature miR-9a, and confirmed similar expression levels (Fig. 3h). Moreover, miR-9a overexpression did not affect cell viability. Quantification of gfp by qRT-PCR revealed a 90% reduction in mRNA levels of a previously characterized target of miR-9a, confirming library reliability (Fig. 3a and Table 1)\(^{14, 17}\). However, antibody staining did not reveal the presence of senseless in the testis. Notably, the list also included N-cad, a typical member of the cadherin family of proteins that forms Ca\(^{2+}\)-dependent homophilic interactions to mediate dynamic cell–cell adherent junctions\(^{36}\). N-cad levels were significantly higher in testis of both young and aged miR-9a\(\text{E39}\) mutants compared to age-matched controls (Table 1). In contrast, the levels of Epithelial-cadherin (E-cad), an N-cad family member that is not a predicted miR-9 target, were unchanged (Table 1). At the apical tip of the testis N-cad is expressed both in the adherent junctions that connect hub cells to each other and in the junctions that connect GSCs to the hub\(^{11, 21}\).

### Table 1: Six potential mRNA targets for miR-9a in the testis

| Gene               | 1-day young         | 30-days aged        | Seeds |
|--------------------|---------------------|---------------------|-------|
|                    | logFC   | Significance (P-value) | Average CPM per gene | logFC   | Significance (P-value) | Average CPM per gene |
|                    | WT     | Mir-9a mutant        |                   | WT     | Mir-9a mutant        |                   |
| N-cad herin        | 1.6    | 1.3E\(^{-26}\)       | 20.7               | 63.4   | 0.9                 | 4.9E\(^{-08}\)    | 27.0               | 49.3   | 2     |
| sticks and stones  | 1.2    | 1.08E\(^{-05}\)      | 3.08               | 7.1    | 1.0                 | 0.0005            | 2.57               | 5.23   | 1     |
| CG10512            | 1.5    | 5.25E\(^{-25}\)      | 23.56              | 67.0   | 1.5                 | 1.5E\(^{-22}\}    | 41.29              | 115.62 | 1     |
| lame duck          | 2.0    | 1.26E\(^{-09}\)      | 1.02               | 4.2    | 1.1                 | 0.0009            | 1.6                | 3.43   | 1     |
| mos18e             | 1.3    | 7.35E\(^{-11}\}      | 9.79               | 23.6   | 1.0                 | 1.6E\(^{-6}\)     | 11.9               | 23.5   | 1     |
| CG34136            | 1.5    | 0.0002               | 1.03               | 3.0    | 2.2                 | 1.6E\(^{-6}\)     | 0.7                | 3.1    | 1     |
| Senseless          | 1.0    | 0.0001               | 3.8                | 7.7    | 1.0                 | 0.001             | 3.5                | 6.4    | 1     |
| E-cad herin        | 0.1    | 0.453                | 54.5               | 59.3   | 0                   | 0.95              | 63.4               | 64.1   | 0     |

Transcriptome filtration based on logFC \(\geq 0.9\), significance cutoff (P-value \(\leq 0.05\)), and minimal reading levels (sum reads per each gene \(\geq 1\)) obtained a group of 231 genes that showed higher expression in miR-9a\(\text{E39}\) mutant vs. control in either young or aged testis. Comparison of this list to the predicted miR-9a targets resulted in the presented six candidates. The negative control, E-cad, is shown at the bottom of the table.

Identification of miR-9a targets in the testes. miRNAs repress mRNA translation, which is often followed by the mRNA deadenylation and decay\(^{19}\). Thus, the mRNA levels of direct miR-9a targets in the testis are expected to be elevated in miR-9a\(\text{E39}\) mutants. To facilitate miR-9a target identification we analyzed the transcriptome of cDNA libraries (Illumina) of four RNA samples (each in at least two biological repeats) prepared from testes of young (1-day), aged (30-days), control (\(w^{1118}\)), and miR-9a\(\text{E39}\) mutants. Reads were aligned to the Drosophila genome and gene expression levels were quantified using Htsseq-count. This provided a list of 11,416 genes that are expressed in the testis (Supplementary Fig. 2). Differential gene analysis using edgeR classic method provided count per million (CPM) values and P-values. After filtration based on log Fold Change (logFC \(\geq 0.9\)), significance cutoff (P-value \(\leq 0.05\)), and minimal CPM per each gene (\(\geq 1\)), we obtained a group of 450 genes that showed higher expression in young \(\text{miR-9a}[\text{E39}]\) mutant vs. control, and 446 genes that increased in old mutants vs. control. Of these, 231 genes showed higher expression in \(\text{miR-9a}[\text{E39}]\) mutant vs. control in both young and aged testis (Fig. 3a). A comparison of this list to the 194 in silico predicted miR-9a targets ( Targetscaen Fly) yielded six potential direct targets, one of which was senseless, compared to 40% control (Fig. 2g, h, n). To determine why the increase in GSCs of miR-9a\(\text{E39}\) mutants does not improve spermatogenesis, we immunostained testes with anti-Thr 3-phosphorylated histoneH3 (pH3) to mark mitotic cells (Fig. 2c, d) and counted pH3H3-positive GSCs. In agreement with previous findings, the division frequency of control GSCs was decreased from ~6% (13/223 GSCs) to ~3% (7/258 GSCs) in aged flies\(^{5}\). However, GSCs of miR-9a\(\text{E39}\) and miR-9a\(\text{fl22}\) mutant alleles completely arrested division in aged flies \((n = 233 \text{ and } n = 178, \text{ respectively})\). Thus, although the niche of miR-9a null mutants consists of a higher number of GSCs, these cells fail to maintain spermatogenesis in aged males due to reduced division frequency.

To determine whether these mutant phenotypes are due to lack of miR-9a in GSCs and spermatogonia cells, we ectopically overexpressed UAS-DsRed-miR-9a\(\text{E39}\)\(^{15}\) in these cells of \(\text{miR-9a}[\text{E39}]\) mutants \((\text{nos-GAL4, UAS-miR-9a-DsRed; miR-9a}[\text{E39}]\)). DsRed fluorescent signal was used to mark the miR-9a-positive cells (Fig. 2i–l). This ectopic expression was sufficient to return the average number of GSCs associated with the hub back to normal numbers both in young and aged adults (Fig. 2m). Moreover, overexpression of miR-9a in \(\text{miR-9a}[\text{E39}]\) mutant regained GSCs division frequency to 3% \((n = 102)\) and rescued fertility of the aged mutant males (Fig. 2n).

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the wild-type reporter only when miR-9a was co-expressed, with no effect on the mutant (Fig. 3i). These data confirm that miR-9a directly inhibits N-cad protein expression and destabilizes its mRNA through canonical sequences within the 3′UTR.

Misregulation of miR-9a in the stem cell niche. Given that miR-9a is expressed in GSCs and not in the hub, we tested the possibility that it regulates the adherent junctions between GSCs and hub cells via N-cad levels. We used UAS-miR-9a-DsRed13 to overexpress miR-9a either in GSCs and spermatogonia cells (nos-GAL4; UAS-miR-9a-DsRed) or in the hub (upd-GAL4; UAS-miR-9a-DsRed), whereas the DsRed fluorescent signal marks the positive-expressing cells. miR-9a overexpression in GSCs and spermatogonia cells that normally express miR-9a resulted in fragmented N-cad expression in the adherent junctions between GSCs and hub cells (Fig. 4a, b). Moreover, ectopic expression of miR-9a-DsRed in hub cells, which do not express miR-9a in wild-type, was partial as only some of the tested expressed DsRed. However, all the samples where DsRed was detected (8/21) showed a dramatic decrease in N-cad expression in adherent junctions among hub cells (Fig. 4c–e). Simultaneous expression of UAS-miR-9a-DsRed in both the hub and GSCs (upd-GAL4; nos-GAL4, UAS-miR-9a-DsRed) also caused a dramatic reduction in N-cad expression (Fig. 4f). However, here too, clear detection of the DsRed signal in both hub and GSCs was only apparent in a few samples (14/39), while the rest of the samples expressed miR-9a-DsRed only in the germline (25/39, Fig. 4g). Together, these data suggest that miR-9a expression in GSCs regulates the dynamic adhesion between these cells and the hub.

N-cad overexpression in germline stem and progenitor cells. To determine whether N-cad overexpression presents a similar phenotype to that of miR-9a mutants, we measured the stem cell number, division frequency, and fertility of N-cad overexpressing flies in stem and spermatogonia germ cells (UAS-N-cad; nos-GAL4) compared to controls (nos-GAL4 outcrossed to w1118). N-cad overexpression in young and aged males resulted in a significantly higher number of stem cells that were attached to the niche (Fig. 5a–e). However, GSC division frequency as measured by pH3-positive cells was markedly reduced (0.7%) in aged N-cad overexpressing males (n = 277). Fertility tests showed that 45% of these flies are sterile compared to 19% of controls (Fig. 5f). Taken together, these findings indicate that the phenotype of miR-9a mutants originates at least partially from N-cad overexpression in stem cells.

N-cadRNAi in GSCs rescues the miR-9a[E39] phenotype. Lastly, to directly address the possibility that the miR-9a[E39] mutant phenotypes of increased GSCs and decreased fertility are due to the presence of elevated N-cad at the hub-GSC junctions, we reduced its expression in GSCs of the mutants (nos-GAL4, UAS-N-cadRNAi; miR-9a[E39], Fig. 5g–i). This restored normal GSC number in both young and aged adults (Fig. 5k), regained normal division rate of GSCs in aged males (n = 178), and importantly rescued the age-related sterility of miR-9a[E39] mutants (Fig. 5l).

Discussion

In summary, our results suggest that miR-9a levels increase significantly in the testis during ageing to promote stem cell differentiation and detachment from their niche. While both young and aged miR-9a mutants hold a much higher number of stem cells in their niche, this does not improve spermatogenesis. On the contrary, GSC division frequency is reduced and spermatogenesis of miR-9a mutants is gradually decreased leading to premature sterility in aged males.

The appearance of high levels of N-cad in the niche of the miR-9a null mutants may be explained by the fact that GSCs are directly connected to the majority of the hub cells. Alternatively, it is possible that N-cad expression in the hub is non-autonomously regulated by N-cad levels or by other miR-9a targets in the GSCs. We present several lines of evidence to support that N-cad is a direct target of miR-9a. Two miR-9a binding consensus sites are located at the 3′UTR of N-cad to mediate translation inhibition and mRNA destabilization in vitro and in vivo. Furthermore, our data suggest that at the apical tip of the testis miR-9a serves to disconnect GSCs from the hub by downregulating the expression of N-cad. GSCs adherent junctions are dynamic and constantly subjected to regulation. We propose...
that the differential expression of miR-9a in GSCs and progenitor germ cells (N-cad OE: nos-GAL4, UAS-N-cad) increases GSCs number but reduces their division frequency compared to control (Con.: nos-GAL4 outcrossed to w1118). G-I N-cadR(s) in GSCs and progenitor germ cells of miR-9a[E39] mutants rescue mutant phenotype (rescue: nos-GAL4, UAS-N-cadR(s); miR-9a[E39]). Con. rescue: nos-GAL4, UAS-N-cadR(s); miR-9a[E39]/TM6). A-D, G-J Representative images of testes immunostained for N-cad (red), Vasa and pH3 (green) and DAPI (blue; scale bars 10 μm). Arrowheads denote mitotic germ cells and arrows denote N-cad ectopic expression (C-D). G-K Shown are average number of GSCs per testis along with 95% confidence interval (error bars). Statistical significance was determined as in Fig. 1d. G The total number of tests scored: Con. 1-day (n = 28) 30-days (n = 41); N-cad OE 1-day (n = 35), 30-days (n = 41). P < 0.005 between N-cad OE and control in 1-day and P < 0.01 in 30-days. K The total number of tests scored: Con. (w1118) 1-day (n = 23), 30-days (n = 35); miR-9a[E39] 1-day (n = 27), 30-days (n = 40). Con. rescue 1-day (n = 27), 30-days (n = 29); miR-9a rescue 1-day (n = 30), 30-days (n = 31). P < 0.005 between N-cadR(s); miR-9a[E39] rescue and miR-9a[E39] in young and aged males. F I Fertility assay of aged males. F Con. (n = 21) and N-cad OE (n = 21). Note decreased fertility of aged N-cad overexpression. I Con. rescue (n = 29), miR-9a[E39] (n = 21), and N-cadR(s); miR-9a[E39] rescue (n = 24). Note that N-cadR(s) rescues miR-9a[E39] sterility in spermatogenesis and promotes detachment toward sperm maturation. The abnormal increase in stem cell maintenance in miR-9a mutant testes and the age-related loss of fertility illustrate the severe consequences of failure to restrain stem cell adherence to their niche.

**Methods**

*Drosophila* stocks, ageing, and fertility. Flies were raised at 25 °C on standard cornmeal molasses agar medium freshly prepared in our lab. Young flies were selected upon hatching and dissected in the first 3 days of their life. Young flies designated for ageing were placed in vials (20 males and 20 females per vial) that were replaced three times a week to prevent second generation from hatching and adult fly loss. Middle-aged flies were dissected at 15 days and aged flies at 30 days. Control and experiments were aged and tested at the same time. Fertility assays of aged males were performed by mating single males of each genotype (n ≥ 21) with three wild-type (w1118) virgin females. Males in vials that did not contain progeny were replaced three times a week to prevent second generation from hatching and adult fly loss. Middle-aged flies were dissected at 15 days and aged flies at 30 days.

Control and experiments were aged and tested at the same time. Fertility assays of aged males were performed by mating single males of each genotype (n ≥ 21) with three wild-type (w1118) virgin females. Males in vials that did not contain progeny were replaced three times a week to prevent second generation from hatching and adult fly loss. Middle-aged flies were dissected at 15 days and aged flies at 30 days.
heated DNase and RNase free water and kept in 100 µL TRIzol nitrogen (RNA extraction, frozen samples were thawed at 37 °C and re-frozen in liquid RNA extraction and blocked with TNB buffer (TSA Fluorescence Kit, PerkinElmer). Following washes in PBTH (PBT, 50 °C), samples were re-

miR-9a FISH. Whole-mount tests were dissected in PBS diethyl pyrocarbonate (DEPC) and placed in Terasaki plates in 10 µl of 99% ethanol was added and the samples and total RNA was extracted using a Miniprep kit (ZYMO Research), according to manufacturer instructions. RNA was kept in RNase-DNase free water in 4 °C until future use. RNA quality was measured by a bioanalyzer and samples were used for miRNAome, transcriptome analysis and statistics.

miRNA analysis and statistics. Total purified RNA samples (0.5 µg at 100 ng/µl) of young, mid-aged, and aged w+118 were used to determine the identity and levels of miRNAs with nanoString Technologies (nCounter fly miRNA expression kit). Raw data were normalized using the nCounter software and differential expression was analyzed using DESeq.

Generation of DNA constructs. The nucleotide numbers of the following sequences refer to their numbers in the 3’UTRs as shown by FlyBase. N-cad 3’UTR was amplified by PCR from genomic DNA with forward primer: N-cad’3’UTR_Xho1: CGCGTCAAGGCTGGTGGAGCGACAGTGATGAG, reverse primer: N-cad_3’UTR_Uln3: CCAACGCTGGCTCCTAACTAAGACATGGTAGTGCCTCT. PCR fragment was ligated into pGEM T Easy vector. To generate pGEM-N-cad 3’UTR-Mut nucleotides 180–182 (5’-CCAAGT = TGGA; Mut1) and 148–150 (5’-CCAGT = TGG; Mut2) were mutated by site-directed mutagenesis (Strategene) with primers: n-cad_3’UTR_Mut1_For: CACACACACAAAGAAAGAGCGCACTGGGAAGGACTCATACGCTG and n-cad_3’UTR_Mut1_Rev: CAGCATGAGTTGCTTCCGTCGTTTCTTTGTTGTGTG, and n-cad_3’UTR_Mut2_For: GATTTGTATGATGTAGGAGAGCCTGG, and n-cad_3’UTR_Mut2_Rev: GGGCTCCTAACCATAACCCGATGTGCTCTCTCCTCTATCATCAACAATG. pAc-EGFP was a gift from D. Ideses (The Juven-Gershon Lab) in which eGFP ORF was ligated via EcoRI and NotI into pAc5.1. N-cad 3’UTR WT or Mut was digested from pGEM with XhoI and Hinc3 and ligated into pAc5-EGFP to generate pAc gfp-N-cad’3’UTR transfected according to the manufacturer’s recommendations (LipoJet*). 48 h transformation was collected for flow cytometry, RNA/protein extraction.

Flow cytometry. Cells were washed twice and resuspended in 200 µl PBS. The samples were analyzed using BD FACSComp II flow cytometer with DACS4dwa software (BD Biosciences). Gates were set to exclude necrotic cells and cellular debris, and the fluorescence intensity of events within the gated regions was quantified. Data were collected from 10,000 events for each sample.

Western blotting. Cells were washed twice in PBS and total cell proteins were lysed with 1× Laemmli buffer. Proteins were separated on 12.5% SDS-PAGE gels, followed by western blotting and detection using a CCD camera and Image-J software. The uncropped western blot can be found in Supplementary Fig. 4.

qRT-PCR analysis. The sequence of qRT-PCR primers will be deposited with DnaSel (Promega) and reverse- transcribed with random hexamer mixture and the High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific), according to the manufacturer instructions. Quantitative real-time PCR was performed with a StepOnePlus™ Real-time PCR System using TaqMan® Gene Expression Assay (Applied Biosystems). Relative gfp (assay ID: Mm03337667_m1) levels were compared to Ribosomal Protein L32 (RPL-32) (assay ID: Dd01215192_g1). For miRNA analysis 10 ng RNA was used to prepare cDNA with TaqMan miRNA-specific reverse-transcription primers (Applied Biosystems) for mir-9a (Mir-9 assay ID: 005853) and ribosomal RNA 25S (V00206). Real-time PCR results were analyzed using StepOne software (Applied Biosystems) and significance was determined using Student’s t-test. An average of three experiments (each performed in triplicate measurements) is shown (mean ± s.d.). P-values were generated after a two-tailed Student’s t-test was used to compare ΔCy at between time points or genotypes across three independent biological replicates.

Statistics. For quantification of GSC numbers and for densitometric analysis of pixel intensity, the mean ± 95% confidence interval and the number (n) of testes examined are shown. P-values were generated after a two-tailed Student’s t-test was carried out by one-way ANOVA and post hoc analysis was performed with Tukey multi-comparison test if samples are normally distributed and have equal variances.*P ≤ 0.005 and **P ≤ 0.01.

Data availability. The transcriptome and miRNAome data have been deposited in Dryad Digital Repository (Epstein et al. 2017): DOI: http://dx.doi.org/10.5061/dryad.3cn0.
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Author contributions
Y.E., N.P., M.V., M.Z.F., R.B., L.P.K., and H.T. designed and carried out the experiments and interpreted the results. H.T. wrote the manuscript. N.P. and M.V. corrected the manuscript.

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