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Lin28 Represses Hub Cell microRNAs to Maintain *Drosophila* Testis Niche Function

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Conflict of interests
The authors declare no potential conflict of interest.

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Authors' contributions
Conceptualization: Sreejith P.

Abstract

In the *Drosophila* testis, hub cells form a specialized niche that sustains adjacent stem cells. The RNA-binding protein Lin28 is expressed in hub cells and is essential for their maintenance and niche function; however, the underlying mechanisms remain poorly understood. Lin28 is well-characterized as a suppressor of *let-7* biogenesis, raising the possibility that Lin28 regulates *let-7* and additional microRNAs to preserve hub cell identity. We performed genome-wide microRNA expression profiling and identified a set of microRNAs upregulated in *lin28* mutant testes, including *let-7*, demonstrating that Lin28 represses *let-7* in hub cells to protect *let-7*-targeted hub cell factors. Here, we report genetic analysis of *miR-304*, a microRNA identified from the profiling. *miR-304* contains a seed site complementary to the 3' UTR of the IGF-II mRNA-binding protein (IMP), a critical hub-specific factor. We find that *miR-304* mutant testes exhibit elevated levels of IMP specifically in hub cells, suggesting that *miR-304* targets *imp* mRNAs and reduces *imp* expression in hub cells. Thus, in wild-type hub cells, Lin28 represses *miR-304* to maintain IMP expression. We propose that Lin28 preserves niche function by restraining multiple microRNAs, thereby protecting essential hub cell factors from microRNA-mediated silencing.

Keywords: Lin28, IGF-II mRNA-binding protein (IMP), Testis, Niche, microRNAs, Stem cells, *Drosophila*

INTRODUCTION

The *Drosophila* testis is a powerful model for investigating the establishment, maintenance, and age-related deterioration of the stem cell niche and its regulation of resident stem cells (Matunis et al., 2012; Sinden et al., 2012; Voog et al., 2014; Anllo et al., 2019; Kong et al., 2024). At the apical tip of the testis, a cluster of approximately 10 hub cells forms a specialized structure that provides a niche for two stem cell populations: germline stem cells (GSCs) and cyst stem cells (CySCs) (Fuller & Spradling, 2007; Voog et al., 2014; Greenspan et al., 2015). Hub cells support stem cell maintenance through direct, adhesion-mediated contact and by secreting niche-derived signaling molecules, most notably Unpaired (Upd), which activates Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling to promote stem cell self-renewal (Kiger et al., 2001; Tulina & Matunis, 2001; Yamashita et al., 2005; Leatherman & Dinardo, 2008; Inaba et al., 2010). Physical association with the hub orients the asymmetric divisions of

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both GSCs and CySCs; in each lineage, the daughter cell that remains attached to the hub retains stem cell identity, whereas the displaced daughter initiates differentiation (Yamashita & Fuller, 2008; Inaba et al., 2010; de Cuevas & Matunis, 2011). GSC daughters become gonialblasts (GBs), which undergo four rounds of transit-amplifying mitoses to generate 16-cell spermatogonial cysts that subsequently differentiate into spermatocytes and enter meiosis to produce 64 haploid sperm. Throughout germline development, cyst cells derived from CySCs encapsulate and support the germline cyst, coordinating differentiation and morphogenesis (Fuller & Spradling, 2007; Matunis et al., 2012).

Recent studies have identified the evolutionarily conserved RNA-binding proteins Lin28 and IGF-II mRNA-binding protein (IMP) as key intrinsic regulators of hub cell maintenance and niche function (Toledano et al., 2012; Sreejith et al., 2019; To et al., 2021; Sreejith & Kim, 2023). Lin28 is best known for inhibiting *let-7* microRNA biogenesis by promoting the uridylation and degradation of let-7 precursors, thereby protecting *let-7* target genes from silencing (Heo et al., 2008; Viswanathan et al., 2008; Heo et al., 2009; Yamashita et al., 2019; Shaik Syed Ali et al., 2025). In hub cells, Lin28 levels are high in young males and decline with age (Sreejith et al., 2019), whereas *let-7* expression exhibits the inverse trend, increasing in older hubs (Toledano et al., 2012), suggesting that Lin28 regulates *let-7* in hub cells. To test the hypothesis that Lin28 represses multiple microRNAs, including *let-7*, in hub cells, we employed microRNA expression profiling of wild-type and *lin28* mutant testes. MicroRNA array analysis revealed 20 microRNAs upregulated in *lin28* mutants, including *let-7*, demonstrating that the low levels of *let-7* in young hub cells are due to repression by Lin28. Here, we report genetic analysis of *miR-304*, which was identified through this profiling.

MATERIALS AND METHODS

1. Fly stocks and husbandry

Flies were reared on standard cornmeal/agar medium supplemented with yeast at 25 ℃, 60±5% relative humidity, under a 12 h light/12 h dark cycle. The following stocks were obtained from the Bloomington *Drosophila* Stock Center: w^{1118}, $lin28^{EP915}$ (BDSC #17298), and *miR-304* knockout mutant (BDSC #58918).

2. Total RNA extraction and microRNA array profiling

Testes from 0–1-day-old males (w^{1118} and $lin28^{EP915}$) were dissected in sterile, ice-cold 1× phosphate-buffered saline (PBS) in triplicate. Dissected testes were immediately flash-frozen in liquid nitrogen prior to RNA extraction. Briefly, RNA extraction was performed in two steps. First, testes were lysed in lysis buffer followed by phenol:chloroform extraction. RNA was then further purified using the Ambion microRNA extraction kit. Purified RNA was shipped to Exiqon Services (Exiqon, Vedbæk, Denmark) on dry ice for microRNA profiling. All microRNA array experiments were conducted at Exiqon Services. For each sample, 750 ng total RNA (sample and reference) was labeled with Hy3™ and Hy5™ fluorescent dyes, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon), following the manufacturer's instructions. Hy3™-labeled samples and Hy5™-labeled reference RNA were mixed pairwise and hybridized to the miRCURY LNA™ microRNA Array (*Drosophila melanogaster*) (Exiqon), which contains capture probes targeting microRNAs registered in miRBase v18.0. Hybridization was performed according to the miRCURY LNA™ microRNA Array instruction manual using a Tecan HS4800™ hybridization station (Tecan, Männedorf, Austria). After hybridization, microarray slides were scanned and stored in an ozone-free environment (ozone level<2.0 ppb) to prevent bleaching of fluorescent dyes. Slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA), and image analysis was performed using ImaGene® 9 (miRCURY LNA™ microRNA

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Array Analysis Software, Exiqon). Quantified signals were background-corrected using Normexp with an offset of 10 and normalized using global locally weighted scatterplot smoothing (LOWESS) regression. Upregulated microRNAs in *lin28* mutant testis relative to wild-type testis were visualized in a heatmap, with values representing fold change. Specifically, values of 0.5, 1, and 1.5 correspond to 1.4-, 2-, and 2.8-fold increases, respectively. The microRNA data have been deposited in ArrayExpress (E-MTAB-17143, Hinxton, UK).

3. Immunohistochemistry

Testes from 2~3-day-old males were dissected in 1× PBS. The following primary antibodies were used: mouse monoclonal anti-Fasciclin III [FasIII; 1:50 dilution; Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA, USA) 7G10], rabbit polyclonal anti-IMP (1:500 dilution; a gift from Dr. Paul Lasko), and guinea pig polyclonal anti-Traffic Jam (TJ; 1:2,000 dilution; a gift from Dr. Dorothea Godt). Following primary antibody incubation, samples were washed twice for 15 min in phosphate-buffered saline with tween (PBST) and incubated with secondary antibodies in blocking solution for 2 h at room temperature. Secondary antibodies (all from Invitrogen, unless otherwise noted) included: Alexa Fluor 488-conjugated goat anti-rabbit (A11008, 1:800); Alexa Fluor 488-conjugated goat anti-mouse (A11001, 1:800); Alexa Fluor 555-conjugated donkey anti-mouse (A31570, 1:800); Alexa Fluor 555-conjugated goat anti-rabbit (A21429, 1:800); and Cy3-conjugated goat anti-horseradish peroxidase (HRP) (Jackson ImmunoResearch, West Grove, PA, USA, 1:800). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:1,000) for 30 min. Finally, samples were washed twice in PBST and mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Confocal images were acquired using a Leica Application Suite X (LAS X, Leica Microsystems GmbH, Wetzlar, Germany) confocal microscope. Image processing and analysis were performed using Leica LAS X and ImageJ/Fiji software (National Institutes of Health, Bethesda, MD, USA). To quantify fluorescence intensity in the hub cells, the hub area was outlined using ImageJ, and the mean fluorescence intensity was measured. To account for variations in sample preparation, these values were normalized to the background signal measured in non-expressing regions within the same sample.

4. Bioinformatic prediction of microRNA target sites

Putative microRNA binding sites in the 3' untranslated regions (3' UTRs) of candidate target mRNAs were predicted using TargetScan Fly (version 7.2; http://www.targetscan.org/fly/, Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and miRanda (version 3.3a; http://www.microrna.org, Memorial Sloan Kettering Cancer Center, New York, NY, USA). Predictions were based on seed sequence complementarity (nucleotides 2–7 or 2–8 of the mature microRNA).

5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 11.0.2 software (GraphPad Software, LLC, Boston, MA, USA). Data are presented as the mean±SEM from three independent testes (n=3). Differences between two groups were determined using a two-tailed unpaired Student's *t*-test. Statistical significance is denoted as follows: ns, $p>0.05$; **** $p<0.0001$.

RESULTS

The apical tip of the *Drosophila* testis comprises diverse somatic cells, including hub cells, CySCs, and cyst cells, alongside germline cells, including GSCs and GBs (Fig. 1A) (Matunis et al., 2012). Lin28 is exclusively expressed in hub cells and is absent in other testicular cell types (Fig. 1B) (Sreejith

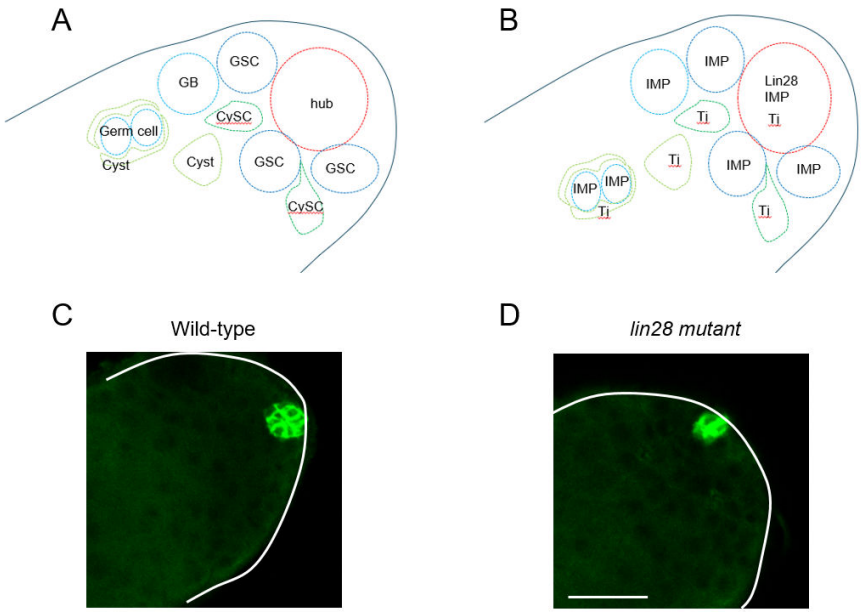

Fig. 1. Schematic of the apical tip of the *Drosophila* testis. (A) Cellular organization at the apical tip. The hub is composed of approximately 10 hub cells and is surrounded by germline stem cells (GSCs) and cyst stem cells (CySCs). GSCs undergo asymmetric division to produce gonialblasts (GBs), which subsequently differentiate into spermatogonia. CySCs produce cyst cells that encapsulate and support the developing germline. (B) Summary of representative genes expressed in each cell type at the apical tip. Lin28 is expressed exclusively in hub cells, whereas IMP and Tj are expressed in hub cells as well as other cell types as indicated. (C, D) Representative confocal images of testis tips stained with anti-FasIII antibody to label hub cells. Note the defective hub architecture and reduced cell numbers in *lin28* mutant testes compared with wild-type controls (n>5). Scale bars: 10 μm. FasIII, anti-Fasciclin III.

et al., 2019). As previously reported, *lin28* mutant testes exhibit hub cell loss, a defective hub structure (Fig. 1C and D) (Sreejith et al., 2019), suggesting that Lin28 plays a crucial role in maintaining hub identity and niche function. Given that the best-characterized biochemical role of Lin28 is to mediate the degradation of the microRNA precursor *pre-let-7*, we hypothesized that the hub defects in *lin28* mutant testes might be caused by the upregulation of *let-7* and other microRNAs. To test this, we performed microRNA array profiling of wild-type and *lin28* mutant testes. The resulting heatmap identified 20 microRNAs that were upregulated in *lin28* mutant testes, including *let-7* (Fig. 2), demonstrating that Lin28 represses *let-7* and multiple other microRNAs in young hub cells.

It was previously shown that *let-7* targets the mRNA of the intrinsic hub factor *imp* (Toledano et al., 2012). The IMP protein protects *upd* mRNAs from degradation by siRNAs in hub cells (Toledano et al., 2012). Upd, a hub-specific self-renewal signaling molecule, is secreted to stimulate the JAK/STAT pathway in adjacent stem cells, which is required for stem cell self-renewal (Kiger et al., 2001; Tulina & Matunis, 2001; Boyle et al., 2007; Issigonis et al., 2009). Thus, our microRNA profiling, which identified *let-7* as upregulated in *lin28* mutant testes, suggests that Lin28 represses *let-7* expression in hub cells to protect *imp* mRNAs from *let-7*-mediated repression.

Among the microRNAs upregulated in *lin28* mutant testes, we selected *miR-304* for further analysis. First, *miR-304* contains a predicted seed site in the 3› UTR of *imp* (Fig. 2) (Toledano et al., 2012). Second, a *miR-304* mutant was available from the *Drosophila* stock center. Third, IMP expression can be monitored using an anti-IMP antibody. These factors enabled us to examine whether *miR-304* targets *imp*. We immunostained wild-type and *miR-304* mutant testes with antibodies to identify specific cell types. In particular, anti-FasIII antibody marks hub cells (Fig. 1C and D).

Immunohistochemical staining revealed that IMP protein levels as visualized by immunofluorescence

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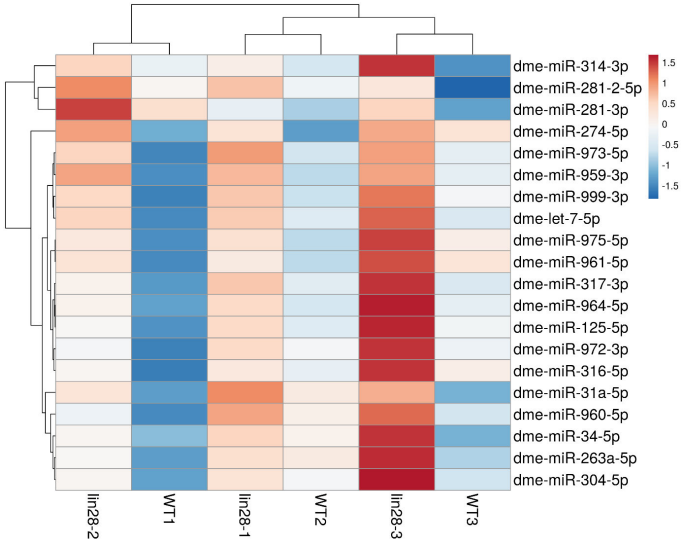

Fig. 2. MicroRNAs upregulated in *lin28* mutant testes. Heatmap showing 20 microRNAs that are significantly elevated in *lin28* mutant testes relative to wild-type controls, including *dme-let-7-5p (let-7)* and *dme-miR-304-5p (miR-304)*. The 3′ UTR of *imp* contains a conserved seed site for *miR-304*.

intensity were noticeably increased specifically in the hub cells of *miR-304* mutant testes compared to wild-type controls (Fig. 3A', B', and C). The IMP signal intensity in germ cells appeared unchanged (Fig. 3A' and B), demonstrating that *miR-304* exerts a hub-restricted effect on *imp* mRNA, which is consistent with the hub-specific expression of Lin28 (Sreejith et al., 2019). As a control, we stained testes with an anti-Traffic jam (Tj) antibody, which labels Tj-expressing cells, including hub cells and various somatic cells (Li et al., 2003; Wingert & DiNardo, 2015). We observed no significant difference in Tj staining intensity in Tj-positive somatic cells between wild-type and *miR-304* mutants (Fig. 3A–C), highlighting the specificity of *miR-304* in restricting IMP, but not Tj, expression in hub cells. Thus, our microRNA profiling, which identified *miR-304* as upregulated in *lin28* mutant testes, suggests that Lin28 represses *miR-304* expression in hub cells to protect *imp* from *miR-304*-mediated repression.

DISCUSSION

Our microRNA array analysis revealed that *lin28* mutant testes contain elevated levels of multiple microRNAs compared to wild-type controls. Given that Lin28 expression in the testis is restricted to hub cells (Sreejith et al., 2019), this upregulation likely reflects the loss of Lin28-mediated repression of these microRNAs within the niche.

Our profiling identified *let-7* as upregulated in *lin28* mutant testes, suggesting that Lin28 represses *let-7* expression in hub cells. These findings are consistent with the known expression dynamics of Lin28 and *let-7*: Lin28 is highly expressed in the young testis and decreases gradually, whereas *let-7* shows an inverse expression pattern (Toledano et al., 2012; Sreejith et al., 2019). Thus, the accumulation of *let-7* in aged testes may, in part, result from the age-dependent decline of Lin28 (Sreejith et al., 2019). Since *let-7* is known to target *imp* mRNA, this regulatory pathway accounts for the decreased IMP levels observed in aged testes (Toledano et al., 2012). Here, we demonstrate that *miR-304*, another microRNA identified as upregulated in *lin28* mutant testes, also targets *imp* mRNA. This conclusion is supported by *miR-304* mutant analysis; however, future research utilizing *UAS-miR-304* transgenic lines and reporter constructs (GFP) reporter constructs containing *miR-304* target sequences will be required to fully establish this relationship.

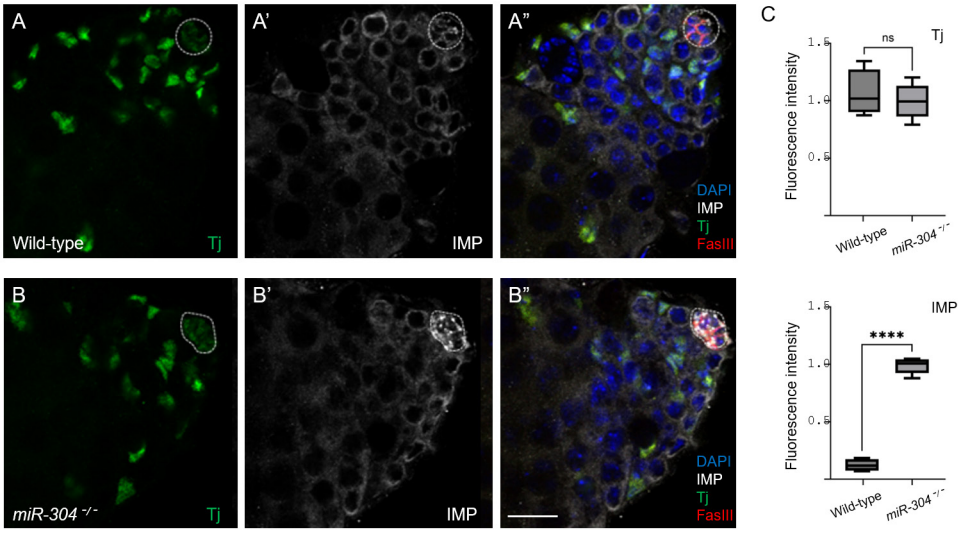

Fig. 3. *miR-304* regulates IMP expression in hub cells. (A–B") Representative confocal images of testes from 2-day-old wild-type and *miR-304* mutant males. (A, B) Traffic jam (Tj; green) marks hub cells (circled) and cyst cells. (A', B') IMP (white) marks hub cells (circled) and germline cells. (A", B") FasIII (red) marks hub cells (circled), and DAPI (blue) marks nuclei. Scale bars: 10 μm. (C) Quantification of Tj and IMP fluorescence intensity in hub cells. Fluorescence levels from wild-type were normalized to the *miR-304* mutant. p-values were determined by two-tailed unpaired Student's t-tests from three independent experiments: ns, $p>0.05$; **** $p<0.0001$. Error bars indicate SEM. IMP, IGF-II mRNA-binding protein; FasIII, anti-Fasciclin III; DAPI, 4',6-diamidino-2-phenylindole.

IMP has recently been identified as a critical intrinsic hub factor required for the stability of *upd* transcripts, which encode a key self-renewal signal expressed in hub cells (Toledano et al., 2012). Thus, our study suggests that Lin28 maintains IMP and Upd expression in hub cells through the coordinated repression of *let-7* and *miR-304*. Additionally, our profiling identified a suite of other microRNAs upregulated in *lin28* mutant testes, whose roles remain unexplored. We propose that Lin28 represses these microRNAs to protect other, as-yet-unidentified essential hub factors (Fig. 4). Future research will focus on characterizing these additional microRNA targets to further elucidate the gene regulatory networks through which Lin28 maintains the stem cell niche.

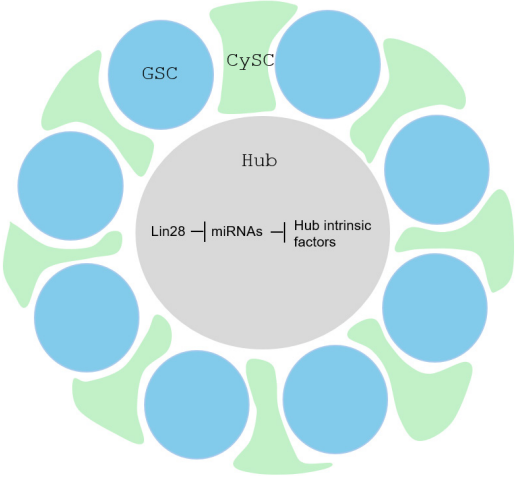

Fig. 4. Proposed model for Lin28-mediated microRNA repression in the maintenance of hub cell function. Lin28 represses a set of microRNAs in hub cells to stabilize essential intrinsic hub factors. GSC, germline stem cell; CySC, cyst stem cell.

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