Short communication

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miR-932 Suppresses the Expression of Germline-Specific *vasa* in Somatic *Drosophila* Testis Hub Cells

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

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Abstract

Germline cells are specified early in embryogenesis and are encapsulated by somatic cells to form the gonads (testis or ovary). This development requires genes with expression restricted to germline cells, such as the DEAD-box RNA helicase Vasa, an evolutionarily conserved protein exclusively expressed in the germline of the testis. However, the mechanisms underlying germline-specific expression remain poorly understood. To identify microRNAs that function in the somatic cells of the testis, we employed the binary Gal4/UAS expression system, which enables the expression of UAS-microRNA sponges in somatic cells driven by somatic Gal4 drivers. The screening identified the miR-932 sponge as a regulator. Testes with hub-specific Gal4 driven expression of the UAS-miR-932 sponge exhibit ectopic Vasa expression in the hub cells. Thus, our findings suggest that miR-932 in the somatic hub cells prevents Vasa expression in these cells.

Keywords: Vasa, Germline, Somatic, *Drosophila*, miR-932, MicroRNA

INTRODUCTION

The soma/germline distinction is essential to the survival of all animal species; in the absence of germline cells, sperm and eggs cannot be produced, leading to termination of the species. In *Drosophila*, primordial germ cells (PGCs) are specified during early embryogenesis in the germ-plasm at the posterior end of the embryo (Lehmann, 2016; Dehghani & Lasko, 2017). Initially, they migrate anteriorly and become encapsulated with somatic cells to form embryonic gonads (Boyle & DiNardo, 1995; Rongo et al., 1997; Okegbe & DiNardo, 2011; Anllo et al., 2019; Anllo & DiNardo, 2022). Later, the PGCs differentiate into germline stem cells (GSCs) while the gonadal somatic cells differentiate into hub and cyst stem cells (CySCs), forming the adult testis (DiNardo et al., 2011; Losick et al., 2011). At the tip of the adult testis is the hub, comprised of ~10 cells, to which are attached intermingled CySCs and GSCs (Yamashita et al., 2003, 2005; Davies & Fuller, 2008). The hub secretes signaling molecules including Unpaired (Upd), Bone morphogenetic protein, and Hedgehog, which stimulate CySCs and GSCs for hub attachment, asymmetric cell division, and stemness maintenance (Kiger et al., 2001; Tulina & Matunis, 2001; Leatherman & Dinardo, 2008, 2010; Amoyel et al., 2013). The CySCs produce cyst cells that encapsulate GSC-derived differentiating germ cells (de Cuevas & Matunis, 2011; Spradling et al., 2011).

Germline-specific genes are exclusively expressed in the PGCs of embryos and the germline cells of adult gonads (Lehmann & Nüsslein-Volhard, 1991; Rongo et al., 1997; Slaidina & Lehmann, 2014;

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Conceptualization: Jang W, Kim C.
Data curation: Lee YC, Kim C.
Methodology: Lee JA, Jang W.
Software: Lee JA, Jang W.
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Investigation: Lee JA, Jang W.
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This article does not require IRB/IACUC approval because there are no human and animal participants.

Trcek & Lehmann, 2019). The mechanisms that restrict expression of germline genes are yet poorly understood. The evolutionarily conserved DEAD-box RNA helicase Vasa (also known as DDX4) is one such germline-specific gene, being expressed in germline cells from the early embryo to the late adult gonad (Lasko & Ashburner, 1990; Rongo et al., 1997; Van Doren et al., 1998; Wang et al., 2015; Jeske et al., 2017). In *Drosophila*, it is involved in germline specification in embryos and in GSC maintenance and differentiation in adults (Lasko, 2013; Dehghani & Lasko, 2017; Durdevic & Ephrussi, 2019; Adashev et al., 2024). Mechanistically, Vasa binds hundreds of mRNAs, is required for the enrichment of several hundred mRNAs at the posterior pole in embryos, and is involved in the translational regulation of selected mRNAs (Lasko, 2013; Kotov et al., 2024). In this communication, we report expression of a miR932 sponge to result in ectopic *vasa* expression in hub cells, revealing a microRNA-based mechanism regulating *vasa* expression in the adult testis.

MATERIALS AND METHODS

1. *Drosophila* stocks and husbandry

Animals were maintained on a standard cornmeal diet (68 g dry yeast, 90 g sugar, 43 g cornmeal, 9 g agar, 4.5 mL propionic acid, 1 g methyl-4-hydroxybenaoate per 1-liter water) at 25℃ and 40% relative humidity under 12-hour light/dark cycle conditions. All flies harboring esg^{ts}, upd^{ts}>*UAS-miR.sponge* were raised at 22℃ to restrict Gal4 unless otherwise noted. Flies were shifted to 29℃ for three days to inhibit $Gal80^{ts}$ and activate Gal4.

The following lines were generous gifts from colleagues in the fly community: esg^{ts} driver refers to *esg-Gal4, UAS-GFP/Cyo; tub-*$Gal80^{ts}$ (Micchelli & Perrimon, 2006), and upd^{ts} driver refers to *upd-Gal4;* $tubP\text{-}Gal80^{ts}$ (Albert et al., 2018). The following lines were obtained from the *Bloomington Drosophila Stock Center: C587-Gal4* (BL67747), *UAS-mCherry.scramble.sponge* (BL61501), *UAS-mCherry.miR-932.sponge* (BL61439), and *UAS-mCherry.miR-let7.sponge* (BL61635).

2. Immunohistochemistry

Testes were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in 1XPBS for 30 minutes at room temperature. Fixed samples were washed twice with 0.3% triton X-100 in 1XPBS (1XPBST) for 15 minutes at room temperature, then blocked with 5% normal goat serum in 1XPBST (blocking solution). Primary antibodies were diluted in blocking solution and incubated overnight at 4℃. Testes were washed twice with 1XPBST for 15 minutes each time and incubated with secondary antibodies for two hours at room temperature. After the incubation, the testes were again washed twice with 1XPBST for 15 minutes, then mounted in Fluoromount-G® (Southern Biotech, Birmingham, AL, USA) on a glass slide. Primary antibodies were rabbit anti-mCherry (PA5-34974, 1:200 Invitrogen, Waltham, MA, USA), rabbit anti-eGFP (CAB4211, 1:500 Invitrogen), rat anti-Vasa (760351, 1:400 Developmental Studies Hybridoma Bank, DSHB, Iowa, IA, USA), and mouse anti-FasIII (7G10, 1:30 DSHB). Secondary antibodies were as follows: Alexa Fluor 488-conjugated goat anti-rabbit (A11008, Invitrogen, diluted 1:800), Alexa Fluor 488-conjugated goat anti-mouse (A11001, Invitrogen, 1:800), Alexa Fluor 555-conjugated donkey anti-mouse (A31570, Invitrogen, 1:800), and Alexa Fluor 555-conjugated goat anti-rabbit (A21429, Invitrogen, 1:800). Images were taken with a Leica Application Suite X confocal microscope system and image analysis was performed using the Leica LAS X software.

RESULTS AND DISCUSSION

Vasa is expressed in germline cells, including GSCs, the goniablast (GB), and GB-derived

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germline cells of the adult testis; conversely, it is not expressed in somatic cells of the adult testis, including hub cells, CySCs, and the cyst (Fig. 1A). To identify microRNAs functioning in the *Drosophila* adult testis, we employed the Gal4/UAS binary expression system (Brand & Perrimon, 1993), which enables expression of UAS-transgenes under a tissue-specific Gal4 driver. This study used the temperature-sensitive somatic Gal4 driver termed esg^{ts}, which harbors *esg-Gal4* (somatic-Gal4) and $tub\text{-}Gal80^{ts}$ (tubulin promoter linked to a temperature-sensitive form of the Gal4 inhibitor Gal80) (Micchelli & Perrimon, 2006). After a temperature shift from 25℃ to 29℃, which renders Gal80 nonfunctional, esg^{ts} can drive expression of UAS-transgenes in somatic cells (Fig. 1B). To knock down microRNAs, we employed UAS-microRNA sponges (Fulga et al., 2015) which consist of the mRNA for mCherry with twenty concatenated copies of a sequence complementary to a microRNA inserted into its 3'UTR. These antisense sequences can sequester microRNAs, allowing expression of the transcripts the microRNAs would otherwise repress.

Flies were reared at 22℃ for three days after eclosion, then shifted to 29℃ for three days. Testes were dissected out and immunostained with a hub-specific antibody (FasIII), germline-specific antibody (Vasa), and mCherry-specific antibody to label cells in which the Gal4 driver was active. Vasa was detected in germline cells of control and experimental testis (Fig. 2A–C). Vasa was not

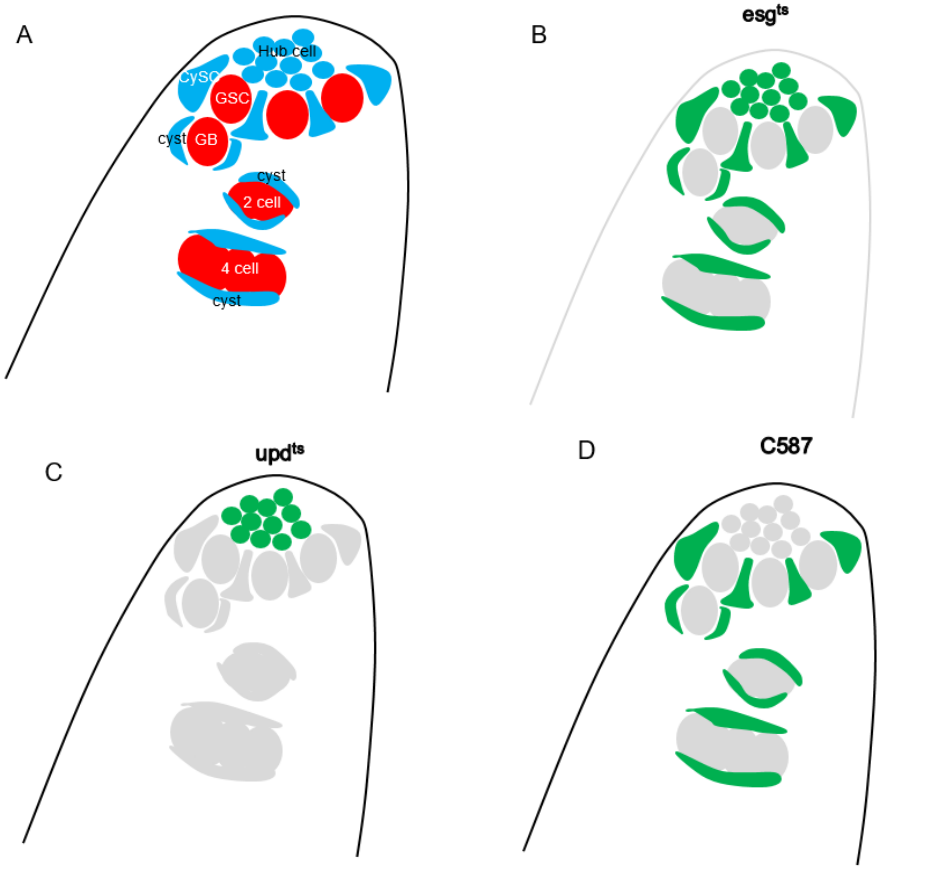

Fig. 1. Cartoons depicting gene expression in the *Drosophila* testis tip. (A) Vasa is exclusively expressed in germline cells (red), and not in somatic cells (blue). (B–D) Gal4 drivers employed in this study. (B) The temperature-sensitive somatic cell driver esg^{ts} comprises *esg-Gal4* and $tub\text{-}Gal80^{ts}$. At 29℃, esg^{ts} drives expression of a UAS-transgene in somatic cells (green). (C) The temperature-sensitive hub cell driver upd^{ts} comprises *upd-Gal4* and $tub\text{-}Gal80^{ts}$. At 29℃, upd^{ts} drives expression of a UAS-transgene in hub cells (green). (D) The CySC- and cyst-cell-specific driver C587-Gal4 drives expression of a UAS-transgene in CySCs and cyst cells (green). CySC, cyst stem cell; GSC, germline stem cell; GB, goniablast.

detected in hub cells of control testis (*esg^{ts}>UAS-mCherry.scramble.sponge* and *esg^{ts}>UAS-mCherry.miR-let7.sponge*) (Fig. 2A and B), but was found to be ectopically expressed in hub cells of the *esg^{ts}>UAS-mCherry.miR-932.sponge* testis (Fig. 2C), indicating that knockdown of miR-932 by the miR-932 sponge induced ectopic expression of *vasa* in hub cells. This suggests that miR-932 acts to silence *vasa* expression in hub cells of the wild-type testis.

Esg^{ts} drives expression of UAS-transgenes in somatic cells, inclusive of hub cells, CySCs, and cyst cells (Fig. 1B). To examine whether the effect of miR932 sponge expression on *vasa* expression in hub cells is intrinsic (cell-autonomous) or extrinsic (non-cell-autonomous) in mechanism, we employed the hub-specific *upd^{ts}* driver (Albert et al., 2018) that harbors *upd-Gal4* (a hub-specific Gal4) and *tub-Gal80^{ts}* (Fig. 1C). At three days post-shifting to 29℃, ectopic *vasa* expression was also observed in the hub cells of *upd^{ts}>UAS-mCherry.miR-932 sponge* flies (Fig. 3), supporting a cell-autonomous (or intrinsic) effect of miR-932 sponges on expression of *vasa* in hub cells. Ectopic *vasa* expression was not observed in control testis (*upd^{ts}>UAS-mCherry.scramble.sponge* and *upd^{ts}>UAS-mCherry.miR-let7.sponge*) (Fig. 3). Expression of the miR-932 sponge under the *C587-Gal4* driver (specific to CySCs and cyst cells) (Fig. 1D) (Le Bras & Van Doren, 2006) did not produce ectopic expression of *vasa* in CySCs or cyst cells (Fig. 4). Thus, the miR-932 sponge, which is likely to knock down miR-932, acts intrinsically to facilitate ectopic expression of *vasa* in hub cells, but not in CySCs or cyst cells.

CONCLUSION

We demonstrated that the specific expression of the miR-932 sponge in hub cells resulted in the ectopic expression of *vasa*. These findings suggest that *vasa* is inhibited by miR-932 in hub cells from wild-type testis. An examination of the *vasa* transcript did not identify any miR-932 complementary sequences, which excludes the possibility of its direct inhibition by miR-932. Thus, miR-932 might target other regulators that control *vasa* expression in hub cells (Fig. 5). MiRNA target prediction software (TargetScanfly 7.2) identified 163 transcripts with miR-932 binding sites, including histone deacetylase 4 (HDAC4) and 65 transcripts of unknown function. The knockdown of these targets in hub cells using UAS-RNAi lines, available in *Drosophila* stock centers from the USA, Japan, and Europe, could lead to the identification of miR-932 targets.

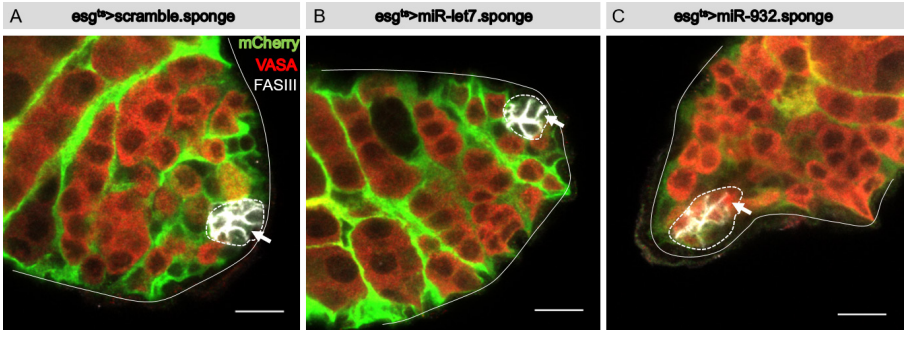

Fig. 2. Confocal images of immunostained cells in testis tips showing expression of microRNA sponges under the *esg^{ts}* driver. mCherry labels cells (green) expressing mCherry.miR.sponges. Vasa labels germline cells (red). FasIII labels hub cells (white). *Esg^{ts}* refers to *esg-Gal4* and *tub-Gal80^{ts}*. *Esg^{ts}>scramble.sponge*, *esg^{ts}>miR-let7.sponge*, and *esg^{ts}>miR-932.sponge* respectively denote *esg^{ts}>UAS-mCherry.scramble.sponge*, *esg^{ts}>UAS-mCherry.miR-let7.sponge*, and *esg^{ts}>UAS-mCherry.miR-932.sponge*, in which mCherry is fused to miR sponges. Testes were analyzed at three days post-temperature shift from 25℃ to 29℃. A representative testis is shown from three independent experiments. More than five animals were observed for each genotype. Vasa was not detectable in the hub cells of control testis (arrow, A,B), but was detected in most hub cells (92%) of *esg^{ts}>miR-932.sponge* testis (arrow, C). Scale bar, 10 μm.

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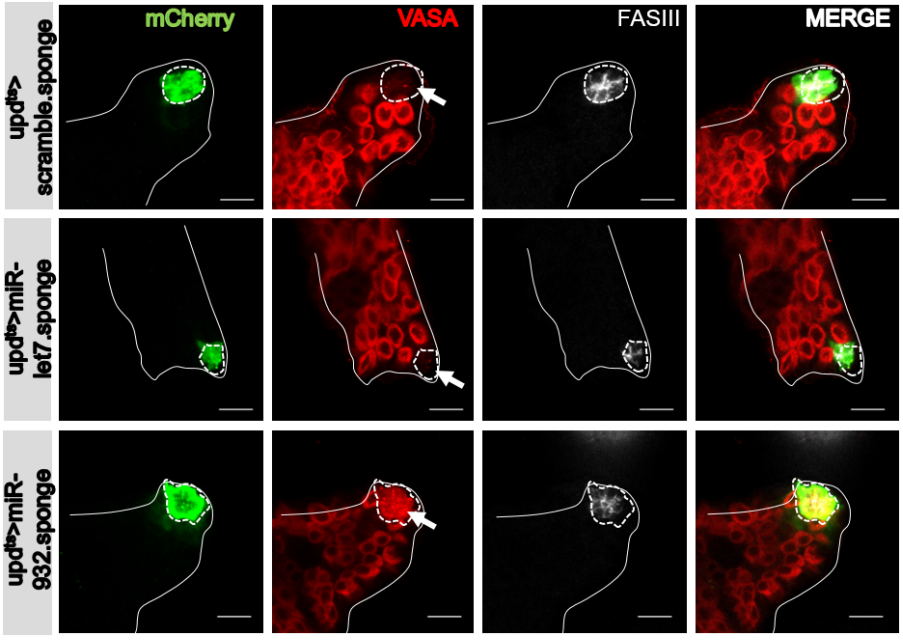

Fig. 3. Confocal images of immunostained cells in testis tips showing expression of microRNA sponges under the *upd^{ts}* driver. mCherry labels cells (green) expressing mCherry.miR.sponges. Vasa labels germline cells (red). FasIII labels hub cells (white). *Upd^{ts}* refers to *upd-Gal4*, *tub-Gal80^{ts}*. *upd^{ts}>scramble.sponge*, *upd^{ts}>miR-let7.sponge*, and *upd^{ts}>miR-932.sponge* respectively denote *upd^{ts}>UAS-mCherry.scramble.sponge*, *upd^{ts}>mCherry.UAS-miR-let7.sponge*, and *upd^{ts}>UAS-mCherry.miR-932.sponge*. Testes were analyzed at three days post temperature shift from 25℃ to 29℃. A representative testis is shown from three independent experiments. More than five animals were observed for each genotype. Vasa was not detectable in the hub cells of control testis (arrow, top and middle rows), but was detected in most (95%) hub cells of *upd^{ts}>miR-932.sponge* testis (arrow, bottom row). Scale bar, 10 μm.

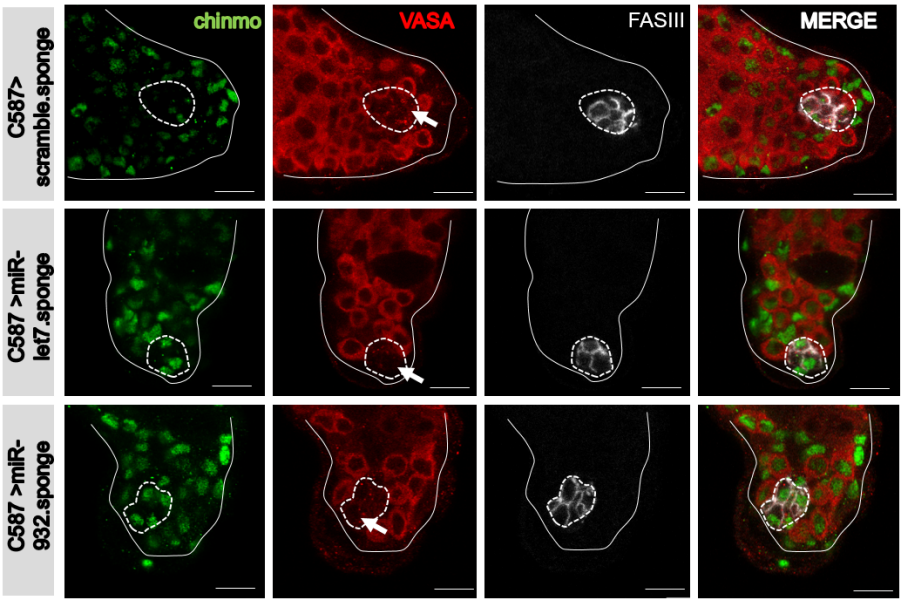

Fig. 4. Confocal images of immunostained cells in testis tips showing expression of microRNA sponges under the *C587* driver. Chinmo labels somatic cells (green). Vasa labels germline cells (red). FasIII labels hub cells (white). *C587>scramble.sponge*, *C587>miR-let7.sponge*, and *C587>miR-932.sponge* respectively denote *C587>UAS-mCherry.scramble.sponge*, *C587>UAS-mCherry.miR-let7.sponge*, and *C587>UAS-mCherry.miR-932.sponge*. Testes were analyzed at three days post temperature shift from 25℃ to 29℃. A representative testis is shown from three independent experiments. More than five animals were observed for each genotype. Vasa was not detectable in the hub cells of control testis (arrow, top and middle rows) or in testis expressing the miR-932 sponge (arrow, bottom row). Scale bar, 10 μm.

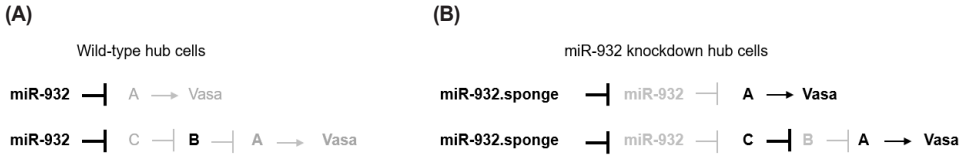

Fig. 5. Models illustrating the putative function of miR-932 in hub cells. (A) miR-932 in hub cells inhibits expression of *vasa* via inhibiting A, a hypothetical positive regulator of Vasa expression. Alternatively, miR-932 in hub cells inhibits C, which inhibits B, a negative regulator of A. (B) miR-932 sponges knock down miR-932 and thereby allow ectopic expression of *vasa*.

Future research identifying hub-cell-specific miR-932 targets with ectopic *vasa* expression is required to elucidate the mechanisms that prevent *vasa* expression in hub cells of the adult testis. It is worth noting that the expression of the miR-932 sponge in other somatic cells (CySCs and cyst cells) did not result in ectopic *vasa* expression. Thus, mechanisms other than miR-932 may exist for inhibiting vasa expression in CySCs and cyst cells.

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