

Lin28 and Imp are Required for Stability of Bowl Transcripts in Hub Cells of the *Drosophila* Testis

Van To, Hyun Ju Kim, Wijeong Jang, Perinthottathil Sreejith, and [†]Changsoo Kim

School of Biological Sciences and Technology, Chonnam National University, Gwangju 61186, Korea



Received: October 15, 2021
Revised: November 28, 2021
Accepted: December 15, 2021

[†]Corresponding author

Changsoo Kim
School of Biological Sciences and
Technology, Chonnam National University,
Gwangju 61186, Korea.
Tel: +82-62-530-5201
E-mail: changgk2001@hanmail.net

Copyright © 2021 The Korean Society of
Developmental Biology.
This is an Open Access article distributed
under the terms of the Creative Commons
Attribution Non-Commercial License
(<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted
non-commercial use, distribution, and
reproduction in any medium, provided
the original work is properly cited.

ORCID

Van To
<https://orcid.org/0000-0002-3296-4344>
Hyun Ju Kim
<https://orcid.org/0000-0003-4819-7431>
Wijeong Jang
<https://orcid.org/0000-0002-3056-4566>
Perinthottathil Sreejith
<https://orcid.org/0000-0001-5445-3265>
Changsoo Kim
<https://orcid.org/0000-0002-2852-9649>

Conflict of interests

The authors declare no potential conflict of interest.

Acknowledgements

This work was supported by grants from the National Research Foundation of Korea (NRF-2021R1A2C1010334 to CK, NRF-2020R111A1A01074292 to WJ, NRF-2017R1A6A3A11032327 to WJ). We thank the Bloomington *Drosophila* Stock Center (BDSC) and Vienna *Drosophila* RNAi Center (VDRC) for flies, and the *Drosophila* Genetic Resource Center (DGRC) for Bowl cDNA. We are grateful to F. Michon for sharing Lin28^{ds30}, T. Guy for Upd-Gal4, and

Abstract

Hub cells comprise a niche for germline stem cells and cyst stem cells in the *Drosophila* testis. Hub cells arise from common somatic gonadal precursors in embryos, but the mechanism of their specification is still poorly understood. Here we find that RNA binding proteins Lin28 and Imp mediate transcript stability of Bowl, a known hub specification factor; Bowl transcripts were reduced in the testis of Lin28 and Imp mutants, and also when RNA-mediated interference against Lin28 or Imp was expressed in hub cells. In tissue culture Luciferase assays involving the Bowl 3'UTR, stability of Luc reporter transcripts depended on the Bowl 3'UTR and required Lin28 and Imp. Our findings suggest that proper Bowl function during hub cell specification requires Lin28 and Imp in the testis hub cells.

Keywords: Lin28, Imp, Niche, Stem cells, Testis, Bowl, *Drosophila*

INTRODUCTION

Adult stem cells are present in most tissues and provide differentiated cells when needed throughout an animal's entire life (Kretzschmar & Clevers, 2017). This ability is realized through a stem cell's unique capability for self-renewing cell division, in which it divides to simultaneously generate a daughter stem cell and a daughter cell that initiates differentiation (Funk et al., 2020). This unique property of stem cells is provided by the niche, a local environment that houses stem cells: a daughter cell within the niche's area of influence maintains stemness and does not undergo differentiation, while one outside the niche loses stemness and starts differentiation (Spradling et al., 2008; Losick et al., 2011). Accordingly, stem cells are attached to niche cells and influenced by signaling molecules from them.

The *Drosophila* testis offers an incisive genetic animal model for dissection of molecular mechanisms underlying niche specification, aging, and niche-stem cell interaction (Herrera & Bach, 2019). An assembly of ~12 cells known as hub cells is located at the terminal tip of the testis and provides the niche for both germline stem cells (GSCs) and cyst stem cells (CySCs) (Yamashita et al., 2005). Hub cells are directly attached to GSCs and CySCs via adherens junctions, and express and secrete signaling molecules that stimulate GSCs and CySCs to promote stemness, cell division, and their attachment to hub cells (Kiger et al., 2001; Tulina & Matunis, 2001; Shivdasani & Ingham, 2003; Kawase et al., 2004; Leatherman & DiNardo, 2010; Michel et al., 2011).

Within the *Drosophila* testis, the RNA binding protein Lin28 is exclusively expressed in hub cells (Sreejith et al., 2019). Lin28 not only acts as a repressor of Let-7 biogenesis but also controls the

N Sokol for Lin28^{Δ1}.

Authors' contributions

Conceptualization: Kim C.
Data curation: To V, Kim HJ.
Methodology: Jang W, Sreejith P.
Writing-original draft: To V, Kim HJ
Writing-review & editing: Kim C.

Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

stability and translation of target mRNAs including Unpaired (Upd), an extrinsic stem cell self-renewal factor that is expressed and secreted from hub cells (Kiger et al., 2001; Tulina & Matunis, 2001; Sreejith et al., 2019). A second RNA binding protein from a conserved protein family, IGF-II messenger RNA binding protein (Imp), is also required for stabilizing Upd transcripts (Toledano et al., 2012). However, what other factors Lin28 and Imp may act to stabilize in hub cells is yet largely unknown.

Hub cells and CySCs are both specified during embryogenesis from somatic gonadal precursors (SGPs) (D Le Bras & Van Doren, 2006; Kitadate & Kobayashi, 2010; iNardo et al., 2011; Okegbe & DiNardo, 2011). For hub cells, specification, aggregation, and assembly are known to require Notch signaling and the transcription factor Bowl, while CySC specification requires Lines (DiNardo et al., 2011; Okegbe & DiNardo, 2011). Notably, the embryonic gonads of Bowl mutants contain fewer hub cells, while increased Bowl activity transforms CySCs to the hub cell fate (DiNardo et al., 2011); these findings indicate that Bowl is a hub cell specification factor. Similarly, Lin28 mutant embryonic gonads contain fewer hub cells (Sreejith et al., 2019), suggesting that Lin28 could function in hub cell specification together with Bowl. Here we find that in hub cells, Bowl transcripts require Lin28 and Imp for stability.

RESULTS AND DISCUSSION

Lin28 was previously shown to be required for stabilizing transcripts of Upd, an important hub cell factor (Sreejith et al., 2019). This observation prompted us to identify other targets that are stabilized by Lin28 in hub cells of the *Drosophila* testis. To identify new target transcripts, we carried out reverse transcription polymerase chain reaction (RT-PCR) and compared transcript levels in Lin28 mutant and wild-type testis. This screening identified Bowl transcripts as reduced in the Lin28 mutant (Fig. 1). Lin28 is exclusively expressed in hub cells, suggesting that Bowl reduction is due to Lin28 mutation specifically in hub cells. To confirm this notion with an independent method, we used the bipartite GAL4-UAS system (Brand et al., 1994) in combination with RNA-mediated interference (RNAi) to reduce Lin28 function exclusively in hub cells. Transgenic flies bearing Upd-Gal4 (hub cell-specific Gal4) and UAS-Lin28 RNAi were generated (Upd-Gal4>UAS-Lin28 RNAi), in which Lin28 RNAi was specifically expressed in hub cells. RT-PCR of testis samples from two independent Lin28 RNAi lines confirmed the reduction of Bowl transcripts (Fig. 2). We likewise examined the relation of Bowl and Imp; however, Imp null mutation was lethal, so we instead relied on Imp knockdown in hub cells of the adult testis. Testis from three independent lines of Upd-Gal4>UAS-Imp RNAi flies expressing Imp RNAi in hub cells likewise showed reduction of Bowl transcripts (Fig. 2). Taken together, these data suggest that maintenance of Bowl transcripts in hub cells requires both Lin28 and Imp.

To further investigate the molecular mechanisms underlying promotion of Bowl transcript stability by Lin28 and Imp, we carried out cell culture experiments using Luciferase (Luc) constructs bearing the Bowl 3'UTR (Luc-Bowl 3'UTR), employing RT-PCR to examine whether those hybrid transcripts were stabilized by Lin28 and Imp, which stabilizes target mRNAs through 3'UTR (Toledano et al., 2012; Lee et al., 2019; Sreejith et al., 2019). When either Lin28 or Imp was co-transfected with the construct, Luc-Bowl 3'UTR transcript levels were increased (Fig. 3A). Meanwhile, levels of control Luc transcripts lacking the Bowl 3'UTR were not affected by the presence of either Lin28 or Imp (Fig. 3B). These results suggest that Lin28 and Imp stabilize Luc-Bowl 3'UTR transcripts through the Bowl 3'UTR.

Fig. 4 illustrates our proposed model of the roles of Lin28 and Imp in the testis. We and others

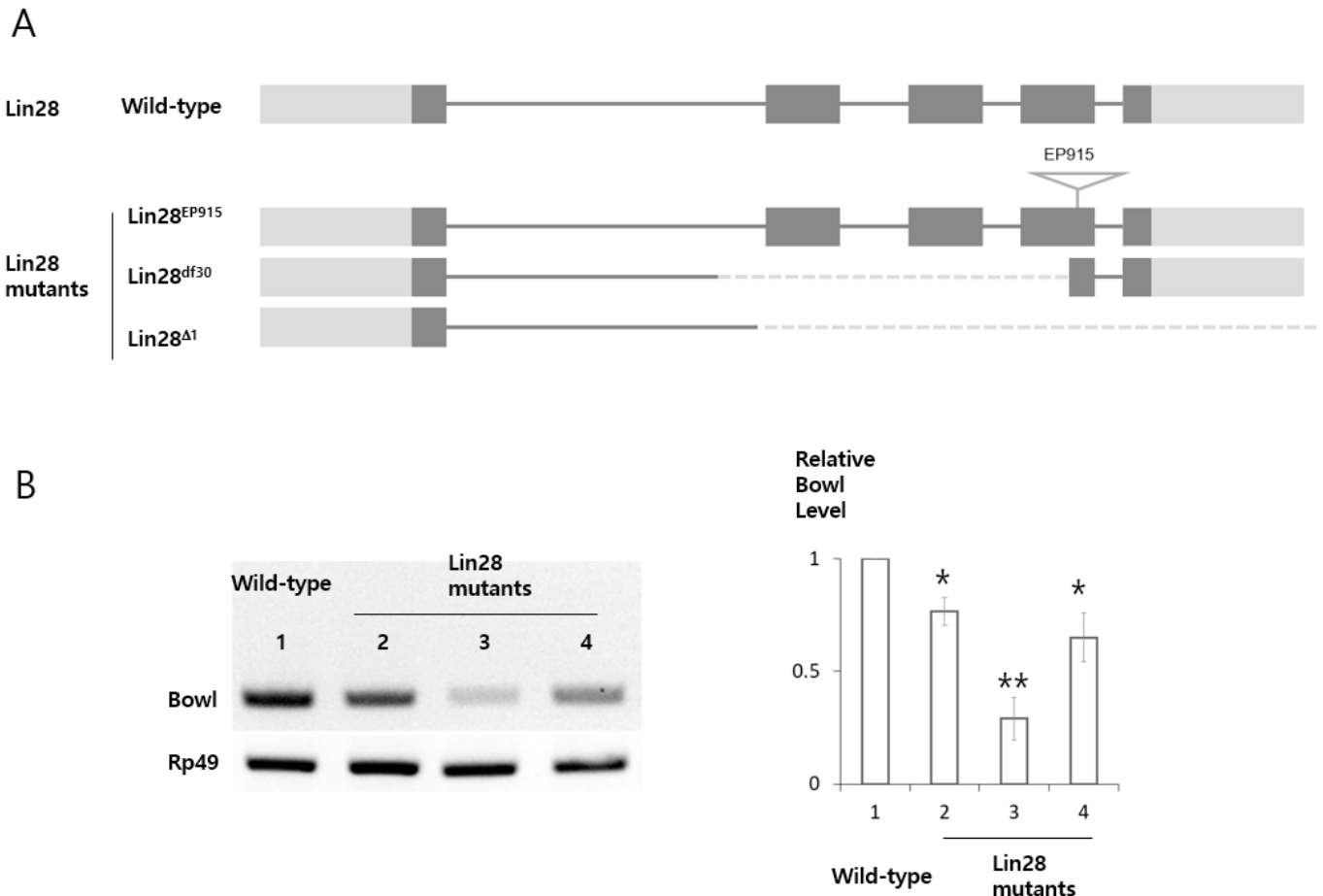


Fig. 1. Reduced Bowl transcripts in Lin28 mutant testis. (A) Lin28 mutants with a P-element insertion in the 4th exon (Lin28^{EP915}) and with deletions in the middle of an exon as indicated (Lin28^{df30} and Lin28^{Δ1}). (B) (Left) Agarose gel image of RT-PCR products from testis with Lin28 mutations. Rp49 used as a loading control. 1. Wild type, 2. Lin28^{EP915}, 3. Lin28^{df30}, 4. Lin28^{Δ1}. (Right) Quantification of RT-PCR bands by Image J analysis, with Bowl band intensity normalized by control Rp49 band intensity. 1. Wild type, 2. Lin28^{EP915}, 3. Lin28^{df30}, 4. Lin28^{Δ1}. *p*-values by student's *t*-test in Sigma-Aldrich Plot. * *p*<0.05, ** *p*<0.01. RT-PCR, reverse transcription polymerase chain reaction.

previously showed that in adult testis hub cells, Lin28 and Imp are required to sustain hub cell maintenance and function and act through stabilizing mRNAs encoding Upd, a critical hub cell factor that stimulates stemness and cell division of GSCs and CySCs (Fig. 4A) (Toledano et al., 2012; Sreejith et al., 2019). In this communication, we identify a new hub cell-specific mRNA stabilized by Lin 28 and Imp, the Bowl transcript (Fig. 4B). Bowl was previously shown to be a hub cell specification factor during early development of the embryonic gonad (DiNardo et al., 2011). Our findings suggest that Lin28 and Imp are present in hub cells and act to protect both Bowl transcripts in embryos and Upd transcripts in adults. An interesting question remains regarding the mechanistic particulars of how Lin28 and Imp stabilize Upd and Bowl mRNAs. Future studies are required to address this question.

MATERIALS AND METHODS

1. *Drosophila* strains

Flies were grown in standard fly food. W¹¹¹⁸, Lin28^{EP915}, and Imp^{MI05901} were from the

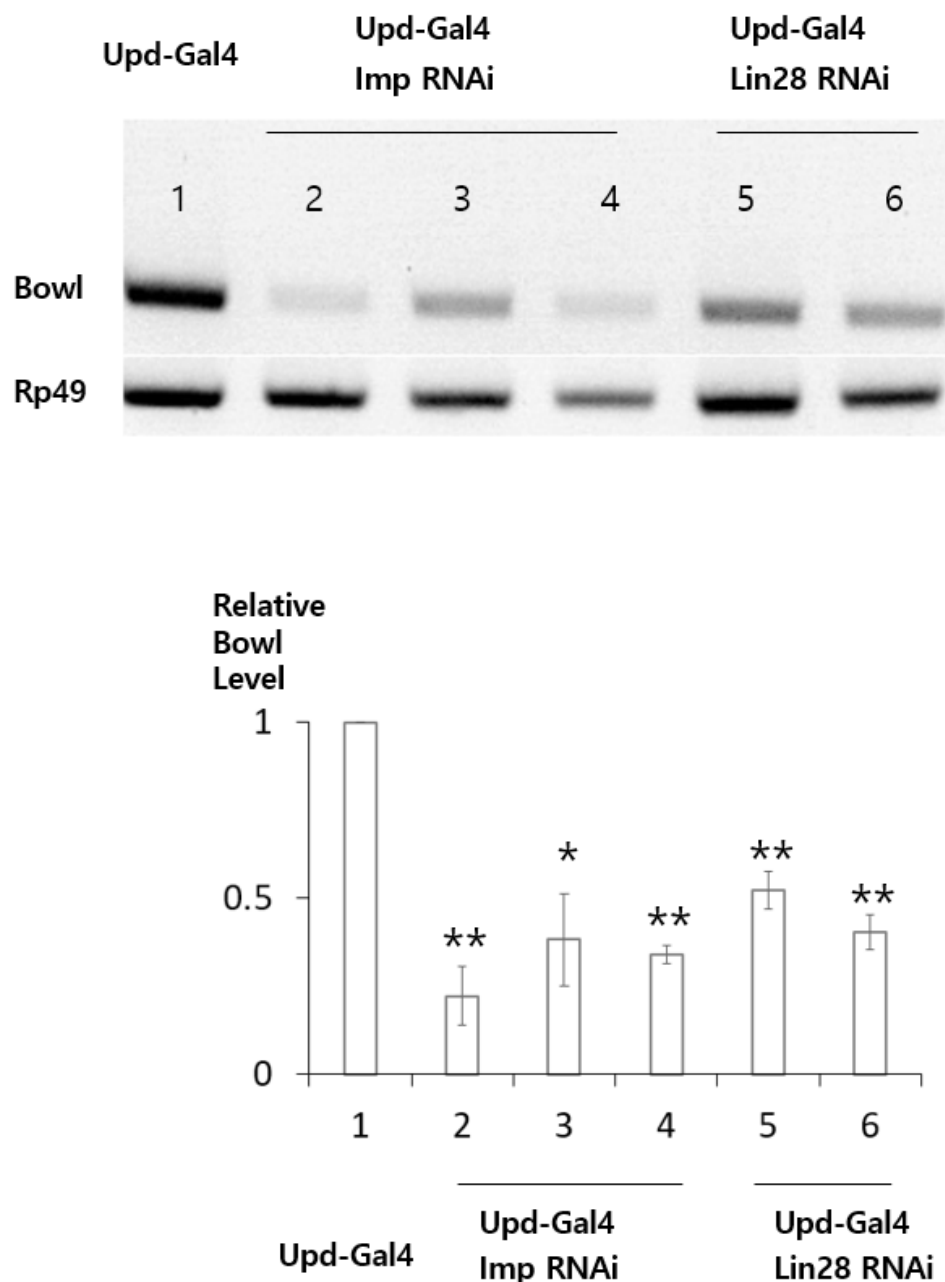


Fig. 2. Reduced Bowl transcripts in the testis with Lin28 and Imp RNAi expressed in the hub cells. (Up) Agarose gel image of RT-PCR products from testis with RNAi expressed in hub cells. Rp49 used as a loading control. 1. Upd-Gal4, 2–4. Upd-Gal4>UAS-Imp RNAi (2032, 20322, 34977), 5–6. Upd-Gal4>UAS-Lin28 RNAi (50679, 29564). (Down) Quantification of RT-PCR bands by Image J analysis, with Bowl band intensity normalized by control Rp49 band intensity. 1. Upd-Gal4, 2–4. Upd-Gal4>UAS-Imp RNAi (2032, 20322, 34977), 5–6. Upd-Gal4>UAS-Lin28 RNAi (50679, 29564). *p*-values by student's *t*-test in Sigma-Aldrich Plot. * *p*<0.05, ** *p*<0.01. RT-PCR, reverse transcription polymerase chain reaction; RNAi, RNA-mediated interference; Upd, unpaired.

Bloomington *Drosophila* Stock Center (BDSC). Lin28^{dl30} was from F. Michon and Lin28^{dl1} from N. Sokol. UAS-Lin28 RNAi (50679, 29564) and UAS-Imp RNAi (34977) were from the BDSC and UAS-Imp RNAi (20321, 20322) from the Vienna *Drosophila* RNAi Center (VDRC).

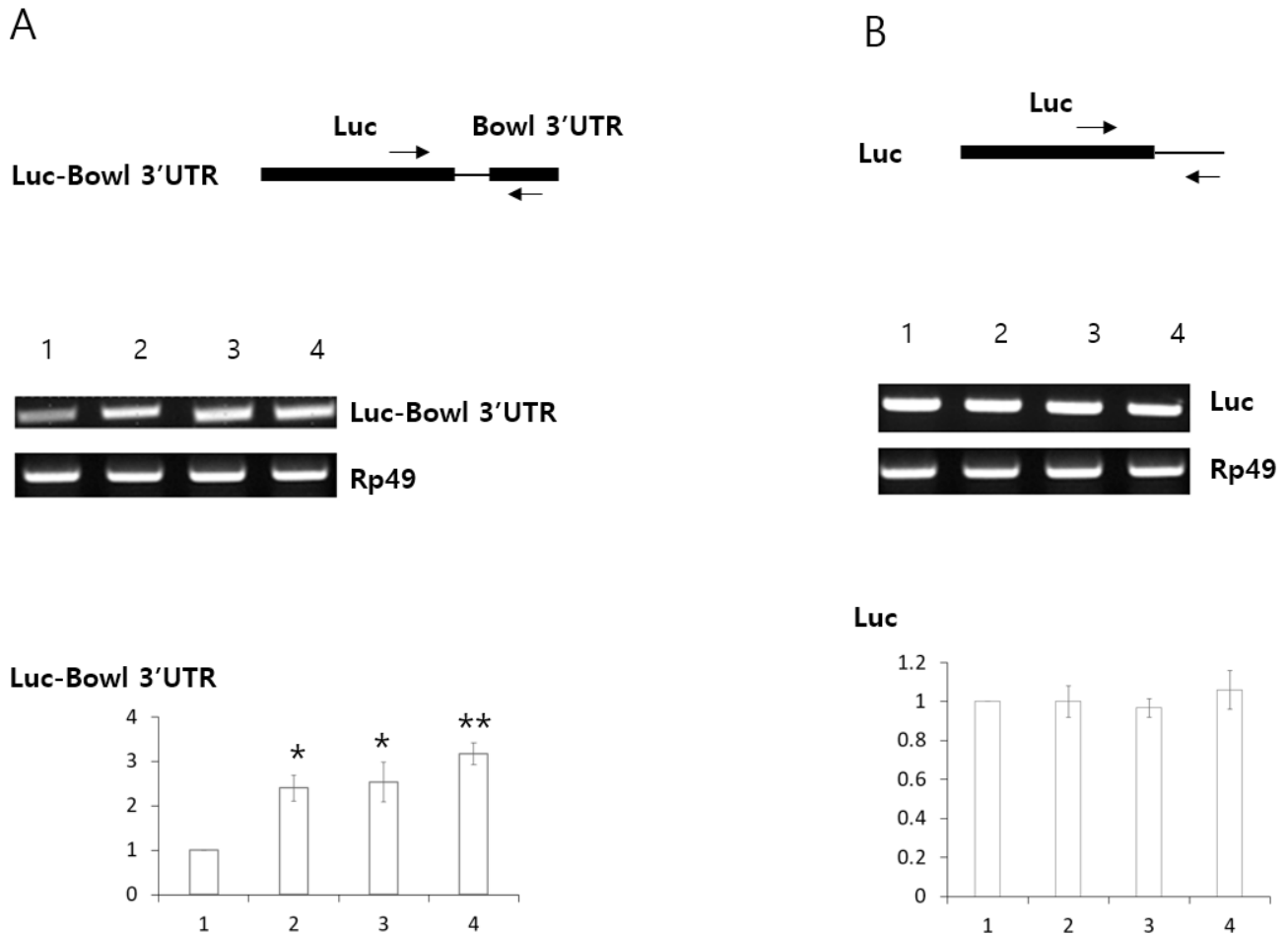


Fig. 3. Bowl 3'UTR mediates stability of Bowl transcripts. Agarose gel image of RT-PCR products from S2 cells co-transfected with the Luc-Bowl 3'UTR hybrid construct (A) and parental Luc construct (B) in combination with parental vector (1), Lin28 expression vector (2), Imp expression vector (3) and Lin28+Imp (4). Rp49 used as a loading control. Primers used for RT-PCR indicated as arrows. (Bottom) Quantification of RT-PCR bands by Image J analysis, with Bowl band intensity normalized by control Rp49 band intensity. p -values by student's t -test in Sigma-Aldrich Plot. * $p < 0.05$, ** $p < 0.01$. RT-PCR, reverse transcription polymerase chain reaction.

2. Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted using standard TRIzol (Invitrogen) procedure. Complementary DNA synthesis was performed with oligodT primers using one microgram RNA. PCR was performed with the following primers: Rp49: Forward 5'-CACCAGGAACCTTCTTGAATCCGG-3', Reverse 5'-AGATCGTGAAGAAGCGCACC-3'; Bowl: Forward 5'-CTGCTCATCCACGAGAGG-3', Reverse 5'-TGTGGACAGCCAAGGTTTC-3'; Luc-Bowl 3'UTR: Forward 5'-CCCTCGAGCAATCCATTAATGGAG-3', Reverse 5'-GCTCTAGACATTATTAATGCATACTTTATTTGA-3'. RT-PCR were carried out three replicates in Fig. 1, 2, and 3.

3. Cloning of Luc-Bowl 3'UTR

pAc5.1A-Luc (S2 cell expression vector) was created using the Luc gene from the pGL3-Basic vector (Promega, Madison, WI, USA), which was ligated into the EcoRI-NotI sites of pAc5.1/V5-HisA (Invitrogen). The Bowl 3'UTR (853 bp) from Bowl cDNA (RE05342, *Drosophila* Genetic Resource Centre) was ligated into the XhoI-XbaI sites of the pAc5.1A-Luc vector.

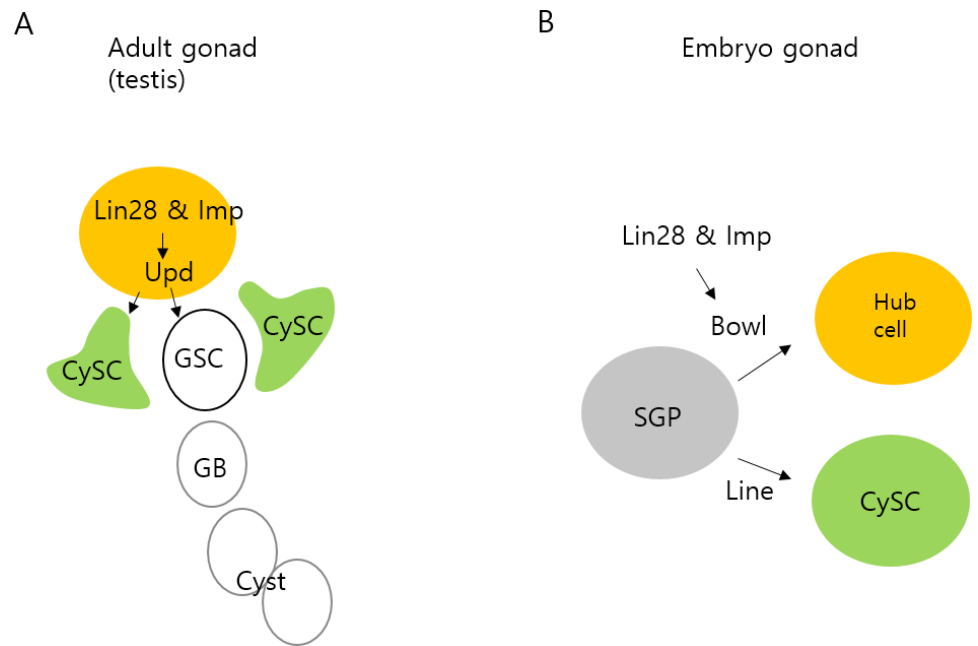


Fig. 4. Models depicting roles of Lin28 and Imp in the *Drosophila* testis. (A) Roles of Lin28 and Imp in hub cells of the adult testis. Upd transcripts are stabilized by Lin28 and Imp. GSC, germline stem cell; CySC, cyst stem cell; GB, goniablast. (B) Roles of Lin28 and Imp in hub cell specification in the embryonic gonads. Bowl transcripts are stabilized by Lin28 and Imp. SGP, somatic gonadal precursor. Upd, unpaired.

4. S2 cell culture and transfection

Schneider 2 cells were grown and maintained in Shields and Sang M3 insect Media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% insect media supplement (Sigma-Aldrich) with appropriate antibiotics at 25 °C. Cells were subcultured every five days at a 1:5 ratio when confluency reached around 100%. The DDAB method of transfection was employed with some modifications for 24-well plates (Han, 1996). Cells were collected and assayed three days after transfection.

REFERENCES

- Brand AH, Manoukian AS, Perrimon N (1994) Ectopic expression in *Drosophila*. *Methods Cell Biol* 44:635-654.
- DiNardo S, Okegbe T, Wingert L, Freilich S, Terry N (2011) Lines and bowl affect the specification of cyst stem cells and niche cells in the *Drosophila* testis. *Development* 138:1687-1696.
- Funk MC, Zhou J, Boutros M (2020) Ageing, metabolism and the intestine. *EMBO Rep* 21:e50047.
- Han K (1996) An efficient DDAB-mediated transfection of *Drosophila* S2 cells. *Nucleic Acids Res* 24:4362-4363.
- Herrera SC, Bach EA (2019) JAK/STAT signaling in stem cells and regeneration: From *Drosophila* to vertebrates. *Development* 146:dev167643.
- Kawase E, Wong MD, Ding BC, Xie T (2004) Gbb/Bmp signaling is essential for maintaining germline stem cells and for repressing *bam* transcription in the *Drosophila* testis. *Development*

- 131:1365-1375.
- Kiger AA, Jones DL, Schulz C, Rogers MB, Fuller MT (2001) Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science* 294:2542-2545.
- Kitadate Y, Kobayashi S (2010) Notch and Egfr signaling act antagonistically to regulate germline stem cell niche formation in *Drosophila* male embryonic gonads. *Proc Natl Acad Sci USA* 107:14241-14246.
- Kretzschmar K, Clevers H (2017) Wnt/ β -catenin signaling in adult mammalian epithelial stem cells. *Dev Biol* 428:273-282.
- Le Bras S, Van Doren M (2006) Development of the male germline stem cell niche in *Drosophila*. *Dev Biol* 294, 92-103.
- Leatherman JL, DiNardo S (2010) Germline self-renewal requires cyst stem cells and *stat* regulates niche adhesion in *Drosophila* testes. *Nat Cell Biol* 12:806-811.
- Lee M, Nguyen TMT, Kim K (2019) In-depth study of *lin-28* suggests selectively conserved let-7 independent mechanism in *Drosophila*. *Gene* 687:64-72.
- Losick VP, Morris LX, Fox DT, Spradling A (2011) *Drosophila* stem cell niches: A decade of discovery suggests a unified view of stem cell regulation. *Dev Cell* 21:159-171.
- Michel M, Raabe I, Kupinski AP, Pérez-Palencia R, Bökel C (2011) Local BMP receptor activation at adherens junctions in the *Drosophila* germline stem cell niche. *Nat Commun* 2:415.
- Okegbe TC, DiNardo S (2011) The endoderm specifies the mesodermal niche for the germline in *Drosophila* via Delta-Notch signaling. *Development* 138:1259-1267.
- Shivdasani AA, Ingham PW (2003) Regulation of stem cell maintenance and transit amplifying cell proliferation by *tgf- β* signaling in *Drosophila* spermatogenesis. *Curr Biol* 13:2065-2072.
- Spradling AC, Nystul T, Lighthouse D, Morris L, Fox D, Cox R, Tootle T, Frederick R, Skora A (2008) Stem cells and their niches: Integrated units that maintain *Drosophila* tissues. *Cold Spring Harb Symp Quant Biol* 73:49-57.
- Sreejith P, Jang W, To V, Hun Jo Y, Biteau B, Kim C (2019) Lin28 is a critical factor in the function and aging of *Drosophila* testis stem cell niche. *Aging* 11:855-873.
- Toledano H, D'Alterio C, Czech B, Levine E, Jones DL (2012) The *let 7*-Imp axis regulates ageing of the *Drosophila* testis stem-cell niche. *Nature* 485:605-610.
- Tulina N, Matunis E (2001) Control of stem cell self-renewal in *Drosophila* spermatogenesis by JAK-STAT signaling. *Science* 294:2546-2549.
- Yamashita YM, Fuller MT, Jones DL (2005) Signaling in stem cell niches: Lessons from the *Drosophila* germline. *J Cell Sci* 118:665-672.