Transcriptional regulatory regions that promote innexin2 expression in somatic support cells in the Drosophila ovary

著者（英）	Taisuke Kimura, Hiroaki Kita, Seiji Hira, Atsushi Kuhara, Takehiro G. Kusakabe, Masanao Michinomae, Satoru Kobayashi, Masanori Mukai
journal or publication title	Memoirs of Konan University. Science and engineering series
volume	60
number	1
page range	1-9
year	2013-01-31
URL	http://doi.org/10.14990/00000186

Transcriptional regulatory regions that promote *innexin2* expression in somatic support cells in the *Drosophila* ovary

Taisuke Kimura[1], Hiroaki Kita[1], Seiji Hira[1, 5], Atsushi Kuhara[1, 3], Takehiro G. Kusakabe[1, 3], Masanao Michinomae[1, 2], Satoru Kobayashi[4], Masanori Mukai[1, 3]*

[1]Graduate School of Natural Science, Konan University, Okamoto, Higashinada, Kobe 658-8501, Japan; [2]Department of Biology, Faculty of Science and Engineering, Konan University; [3]Institute for Integrative Neurobiology, Konan University; [4]Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Higashiyama, Myodaiji, Okazaki 444-8787, Japan; [5]Research Fellow of Japan Society for the Promotion of Science, Tokyo, Japan

(Received August 26, 2013)

*Author for correspondence (e-mail: mmukai01@center.konan-u.ac.jp)
To whom correspondence should be addressed.
Department of Biology, Faculty of Science and Engineering,
Konan University, Okamoto, Higashinada,
Kobe 658-8501, Japan
Telephone number: +81-78-435-2547
Fax number: +81-78-435-2539
E-mail: mmukai01@center.konan-u.ac.jp

Number of text pages: 15
Number of figures: 4
Total number of words: 2,964

Abstract

Germ cells normally differentiate while they are in contact with somatic support cells. The interaction between the germ cells and somatic support cells is essential for the production of functional gamates. Germ cells are closely associated with somatic support cells via gap junctions during oogenesis. We have previously reported that *innexin2* (*inx2*) gene, which encodes an invertebrate gap junction protein, is involved

in the regulation of germ cell development in *Drosophila* ovaries. *inx2* is expressed in escort cells and follicle cells. However, the regulatory mechanism underlying *inx2* expression in these somatic support cells remains elusive. We investigated transcriptional regulatory regions of the *inx2* gene using the Gal4/UAS system. Here, we show that the genomic fragment encompassing the upstream region of *inx2* is required for *inx2* expression in escort cells and follicle cells. Our data indicate that the regulatory elements to promote *inx2* expression in the somatic support cells are located in the upstream region and also in the intron of the *inx2* gene. Our results imply that *inx2* expression in the somatic support cells may be differentially regulated by distinct sets of cis-elements.

Key words: *Drosophila*, oogenesis, gap junction, gene expression, Gal4 driver

Introduction

Germ cells normally differentiate while they are in contact with somatic support cells. The interaction between the germ cells and somatic support cells is essential for the production of functional gamates. In *Drosophila* ovaries, somatic cells at the tip of germarium form the niche, in which germline stem cells (GSCs) are maintained. Upon GSC division, the daughter cell removed from the niche becomes to be wrapped by escort cells at the germarium region 2a (Decotto and Spradling, 2005). Then, the daughter cell initiates differentiation into a cystoblast that undergoes incomplete mitotic divisions to yield a 16-cell cyst; one becomes an oocyte and the other 15 germ cells form nurse cells. Next, the 16-cell cyst is associated with somatic prefollicular cells at the boundary of region 2a and 2b. The cyst moves into region 2b and becomes encapsulated by follicle cells to form an egg chamber (Nystul and Spradling, 2010). An egg chamber budded off from the germarium proceeds to the late stages of oogenesis.

Germ cells are closely associated with somatic support cells via gap junctions during oogenesis (Mahowald, 1972; Bohrmann and Zimmerman, 2008). Gap junctions are involved in intercellular communication between germ cells and somatic support cells. *innexin* genes, which encode invertebrate gap junction proteins, are involved with germline development. The function of *zpg* gene, which is also known as *innexin4*, in germ cells is required for the survival of germ cells in ovaries. It has been proposed that Zpg in germ cells forms gap junctions with other Innexins in escort cells to mediate signals between germ cells and escort cells in order to promote germ cell differentiation (Tazuke et al., 2002). We previously reported that *inx2* is expressed in escort cells and follicle cells, and that *inx2* function in escort cells is required for germ cell survival and promotes cyst formation. *inx2* function in follicle cells promotes egg chamber formation. Moreover, genetic experiments show that *inx2* interacts with *zpg* (Mukai et al., 2011). These results strongly suggest that Inx2-gap junctions are involved in intercellular communication between germ cells and somatic support

cells to promote germ cell differentiation. The regulatory mechanism underlying *inx2* expression in escort cells and follicle cells would provide insight into the important aspects of oogensis. It would be also applicable to experimental tools to study gene function in a particular cell type-specific manner. We investigated transcriptional regulatory regions of the *inx2* gene that control expression in ovarian somatic support cells using the Gal4/UAS system.

Materials and Methods

Fly stocks. The wild-type strain used was Oregon-R (OR). The *y w* fly strain was used for the generation of transformants. *UAS-mCD8-GFP* was obtained from the Bloomington Stock Center. All stocks were maintained at 25°C or room temperature in a standard *Drosophila* medium.

DNA construction. pPTGal plasmid vector was obtained from the Drosophila Genomics Resource Center. To generate *up 2.5kb inx2(WT)-Gal4*, the upstream region of *inx2* was amplified from wild-type genomic DNA by polymerase chain reaction (PCR) using the inx2-up-KF01(NotI) (5' –atttgcggccgcGATGTATAAAAGAGCCACAGG-3') and inx2-5'UTR-KR01(EcoRI) (5' –cggaattcGGTTCCTCACTCGTTGGCCAC-3') primers; this step was followed by digestion with *Not*I and *Eco*RI and subcloning into the multi cloning sites (MCS), *Not*I/*Eco*RI sites of pPTGal (Sharma, et al., 2002). To generate *inx2 up0.9kb-Gal4*, the upstream region of *inx2* was amplified from wild-type genomic DNA by PCR using the inx2-up-KF02 (XbaI) (5'-gctctagaCACTCCAGCAGCCACATGTAC-3') and inx2-up-KR02(EcoRI) (5'-cggaattcGCTTGGCTGCGCTAAACGCCG-3') primers. The fragment was digested with *Xba*I and *Eco*RI, and then subcloned in the *Xba*I/ *Eco*RI sites of pPTGal. To generate *inx2 up0.5kb-Gal4*, the upstream region of *inx2* was amplified from wild-type genomic DNA by PCR using the inx2-up-TF02(XbaI) (5'-gctctagaATCGTGCATGGCCGTAATGAA-3') and inx2-up-KR02(EcoRI) (5'-cggaattcGCTTGGCTGCGCTAAACGCCG-3') primers. The fragment was digested with *Xba*I and *Eco*RI, and then subcloned in the *Xba*I/*Eco*RI sites of pPTGal. To generate *inx2-up 0.9~2.5kb-Gal4*, the upstream region of *inx2* was amplified from wild-type genomic DNA by PCR using the inx2-up-KF01(NotI) (5'-atttgcggccgcGATGTATAAAAGAGCCACAGG-3') and inx2-up-TR01(EcoRI) (5'-cggaattcGTACATGTGGCTGCTGGAGTG-3') primers. The fragment was digested with *Xba*I and *Eco*RI, and then subcloned in the *Xba*I/*Eco*RI sites of pPTGal. To generate *int 1.2kb inx2 (WT)-Gal4 and int 1.2kb inx2 (mut)-Gal4,* 3'-UTR region of *inx2*, which contains the intron, was amplified from wild-type and $inx2^{FA42}$ mutant genomic DNA, respectively, by PCR using the inx2-int-F01 (5'-gctctagaAAGTCTCGTATATACCATCCC-3') and inx2-3'UTR-R03 (5'-GCTAACGTTTCTGGCTGCGGC-3') primers. These

fragments were digested with *Xba*I and *Eco*RI, and then subcloned in the *Xba*I/*Eco*RI sites of pBSK. The construct, which contains wild-type intron, termed int2.7kb inx2(WT)/pBSK and the construct, which contains mutant intron, termed int2.7kb inx2(FA42)/pBSK, were used as templates to obtain intron region by PCR using the T3 (5'-AATTAACCCTCACTAAAGGG-3') and inx2-int-RR1(EcoR1) (5'-gggaattcACATTGGGCAACGCAGCC-3') primers. These fragments were digested with *Xba*I and *Eco*RI, and then subcloned in the *Xba*I/*Eco*RI sites of pPTGal. The constructs were injected in *y w* flies by using a standard procedure (Rubin and Spradling, 1982).

in situ hybridization and immunostaining of ovaries. *in situ* hybridization of ovaries was performed by using DIG-labeled antisense *inx2* RNA probe, as previously reported (Mukai et al., 2011). Immunostaining was carried out as described (Mukai et al., 2011). The following antibodies were used: rabbit anti-Vasa (1:500, Kobayashi), rabbit anti-Inx2 (1:50, M. Hoch) (Bauer et al., 2004) and mouse anti-GFP (1:200, Wako Pure Chemicals). Alexa Fluor 488- and 568-conjugated second antibodies (Molecular Probes) were used at 1:1000. Stained ovaries were observed under confocal microscopy (TCS NT, Leica or FV1200, Olympus).

Results

inx2 has been reported to be expressed in somatic support cells, such as escort cells and prefollicular cells, which are located in germarium region, and also in follicle cells in egg chambers of adult ovaries (Bohrmann and Zimmerman, 2008; Mukai et al., 2011). In order to determine the genomic region required for *inx2* expression in the ovarian somatic cells, we isolated ~2.5kb genomic fragment encompassing the upstream region of *inx2* and inserted in the MCS of pPTGal vector (Sharma, et al., 2002) (Fig. 1). This construct, termed *up 2.5kb inx2(WT)-Gal4*, was then introduced into the fly genome to obtain Gal4 lines. We crossed the Gal4 lines with *UAS-mCD8-GFP* to examine the expression of GFP in the ovaries of F1 adults. GFP signal was detected in the somatic support cells in *up 2.5kb inx2(WT)-Gal4>UAS-mCD8-GFP* ovaries. The distribution of GFP signal was similar to that of endogenous *inx2* mRNA (Fig. 2) and Inx2 protein (data not shown) (Mukai et al., 2011). GFP was detected in escort cells in germarium region 1~2a, in prefollicular cells in region2a/b, and in follicle cells in egg chambers (Fig. 2 and 3). These observations indicate that the ~2.5kb genomic fragment encompassing the upstream region of *inx2* contains regulatory elements.

In order to locate regulatory elements that control *inx2* expression in somatic support cells, we introduced a series of deletions into the *up 2.5kb inx2(WT)-Gal4* construct. These derivatives were introduced into the fly genome to obtain Gal4 lines. The Gal4 lines were mated with *UAS-mCD8-GFP* and investigated GFP expression in the F1 ovaries. We generated three constructs with deletions in the upstream region (Fig. 1).

We obtained two independent Gal4 lines (M15-3 and M15-6) carrying *inx2 up0.9kb-Gal4*. In *inx2 up0.9kb-Gal4 (M15-3) >UAS-mCD8-GFP* ovaries, GFP signal was detected in escort cells and prefollicular cells in germaria (Fig. 2 and 3). But GFP signal was hardly detectable in nascent egg chambers and egg chambers at stage 4-5. In *inx2 up0.9kb-Gal4 (M15-6) >UAS-mCD8-GFP* ovaries, GFP signal was detected in follicle cells in egg chambers but not in escort cells in germaria. These results suggest that the 0.9kb genomic fragment encompassing the upstream region of *inx2* contains regulatory elements that promote *inx2* expression both in escort cells and follicle cells. We found that further deletion to the position –0.5kb results in a dramatic reduction in the GFP expression in escort cells and prefollicular cells. In *inx2 up0.5kb-Gal4>UAS-mCD8-GFP* ovaries, GFP was detectable in only subsets of follicle cells containing stalk cells. These results suggest that the genomic region between -0.9kb and -0.5kb is critical for *inx2* expression in somatic support cells in germarium region. We also examined GFP expression in *inx2 up 0.9-2.5kb-Gal4>UAS-mCD8-GFP* and found that GFP signal was detected in only a subset of follicle cells containing stalk cells, but not in escort cells and prefollicular cells (Fig. 2 and 3). This suggests that regulatory elements, which control *inx2* expression in follicle cells, are located in the upstream region between -2.5kb and -0.9kb.

We have previously reported that the $inx2^{FA42}$ mutation decreases *inx2* expression in escort cells and follicle cells, and have identified a small deletion in the intron of *inx2* gene in the mutant flies (Mukai et al., 2011). Therefore, we speculated that regulatory elements responsible for the expression in somatic support cells may also be located in the intron of the *inx2* gene. To confirm this, we isolated genomic fragments in the intron of *inx2* from wild type and $inx2^{FA42}$ mutant flies, and the fragments were both subcloned into the pPTGal vector. Theses constructs, termed *int 1.2kb inx2 (WT)-Gal4* and *int 1.2kb inx2 (mut)-Gal4,* were then introduced into the fly genome to obtain Gal4 lines (Fig. 4). We investigated GFP expression in *int 1.2kb (WT)-Gal4>UAS-mCD8-GFP* and *int 1.2kb (mut)-Gal4>UAS-mCD8-GFP* ovaries. We found that GFP signal is detectable in escort cells and prefollicular cells in *int 1.2kb (WT)-Gal4>UAS-mCD8-GFP* but not in *int 1.2kb (mut)-Gal4>UAS-mCD8-GFP* ovaries (Fig. 4). This suggests that regulatory elements responsible for the expression in escort cells and prefollicular cells are located in the intron of the *inx2* gene, and that the deletion in the $inx2^{FA42}$ mutant disrupts the regulatory elements.

Discussion

In this paper, we report novel Gal4 lines, which effectively induce GFP expression in somatic support cells in *Drosophila* ovaries. By introducing a series of deletions into the *up 2.5kb inx2(WT)-Gal4* construct, we show that the genomic region between -0.9kb and -0.5kb is critical for *inx2* expression in escort cells. The upstream region between -2.5kb and -0.9kb exhibits enhancer activity in follicle cells. Furthermore,

our data suggest that regulatory elements in escort cells and prefollicular cells are located also in the intron of the *inx2* gene. These results suggest that *inx2* expression in somatic support cells is regulated by multiple regulatory elements lying within both the upstream region and the intron of the *inx2* gene. Our results support an idea that *inx2* expression in the somatic support cells may be differentially regulated by distinct sets of cis-elements.

We have generated two independent Gal4 lines (M15-3 and M15-6) carrying *inx2 up0.9kb-Gal4*. The M15-3 Gal4 line preferentially drives *UAS-GFP* reporter gene in escort cells and prefollicular cells, but the M15-6 Gal4 line preferentially induces GFP expression in follicle cells in egg chambers. It has been previously reported that genes expressed using the Gal4/UAS system show variable expression levels (Skora and Spradling, 2010). The Gal4/UAS variegation may influence GFP expression in these lines. Moreover, flanking sequences can have an effect on cis-element function (Goode et al., 2011). Genomic DNA flanking the *inx2 up0.9kb-Gal4* constructs may affect the function of regulatory elements in the constructs.

Our expression studies show that the upstream region of *inx2* is sufficient to drive GFP expression in ovarian somatic support cells. However, we have previously reported that a deletion in the intron of *inx2* reduces *inx2* expression in somatic support cells and impairs oogenesis (Mukai et al., 2011). Therefore, the regulatory elements in both the upstream region and the intron of *inx2* may cooperatively act to increase *inx2* expression in the somatic support cells in order to promote germ cell development. Because the Gal4 is a potent transcription activator, we could detect enhancer activity of different regulatory elements in the upstream region and the intron of *inx2*, respectively. It is also plausible that GFP may be more stable than endogenous Inx2 protein.

Somatic support cells, escort cells, prefollicular cells and follicle cells in ovaries play distinct roles during oogenesis. Thus, it is important for clarifying the molecular mechanisms that control oogenesis to investigate gene function in cell type. The Gal4 driver lines developed in this study, *inx2 up0.9kb-Gal4 (M15-3)* and *int 1.2kb (WT)-Gal4* are able to induce gene expression preferentially in escort cells and prefollicular cells in the germarium region. These Gal4 driver lines may be beneficial tools to study gene function in oogenesis.

Acknowledgements

We are grateful to the Bloomington Stock Center and the Drosophila Genomics Resource Center for providing fly strains and reagents. We are grateful to Dr. Y. Watanabe for critical reading and comments on the manuscript. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology (C) 21570233 and by Grant-in-Aid for Scientific Research (KAKENHI) on Innovative Areas, 'Mechanisms regulating gamete formation in animals' (#25114002).

References

Bauer R., Lehmann C., Martini J., Eckardt F., and Hoch M. (2004). Gap junction channel protein innexin 2 is essential for epithelial morphogenesis in the *Drosophila* embryo. *Mol. Biol. Cell* **6** 2992-3004.

Bohrmann J., and Zimmerman J. (2008). Gap junctions in the ovary of *Drosophila melanogaster*: localization of innexins 1, 2, 3 and 4 and evidence for intercellular communication via innexin-2 containing channels. *BMC Dev. Biol.* **8** 111-122.

Decotto E., and Spradling A. C. (2005). The *Drosophila* ovarian and testis stem cell niches: similar somatic stem cells and signals. *Dev. Cell*. **4** 501-510.

Goode D. K., Callaway H. A., Cerda G. A., Lewis K. E., and Elgar G. (2011). Minor change, major difference: divergent functions of highly conserved cis-regulatory elements subsequent to whole genome duplication events. *Development* **138** 879-884.

Mahowald A. P. (1972). Ultrastructural observations on oogenesis in *Drosophila*. *J. Morphol.* **137** 29-48.

Mukai M., Kato H., Hira S., Nakamura K., Kita, H. and Kobayashi S. (2011). Innexin2 gap junctions in somatic support cells are required for cyst formation and for egg chamber formation in *Drosophila*. *Mech. Dev.* **128** 510-523.

Nystul T., and Spradling A. C. (2010). Regulation of epithelial stem cell replacement and follicle formation in the *Drosophila* ovary. *Genetics* **184** 503-515.

Rubin G. M., and Spradling A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218** 348-358.

Skora A. D., and Spradling A. C. (2010). Epigenetic stability increases extensively during *Drosophila* follicle stem cell differentiation. *Proc. Natl. Acad. Sci. U S A.* **107** 7389-7394.

Sharma Y., Cheung U., Larsen E. W., and Eberl D. F. (2002). pPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in *Drosophila*. *Genesis* **34** 115-118.

Tazuke S. I., Schulz C., Gilboa L., Fogarty M., Mahowald A. P., Guichet A., Ephrussi A., Wood C. G., Lehmann R., and Fuller M. T. (2002) A germline-specific gap junction protein required for survival of differentiating early germ cells. *Development* **129** 2529-2539.

Figure legends

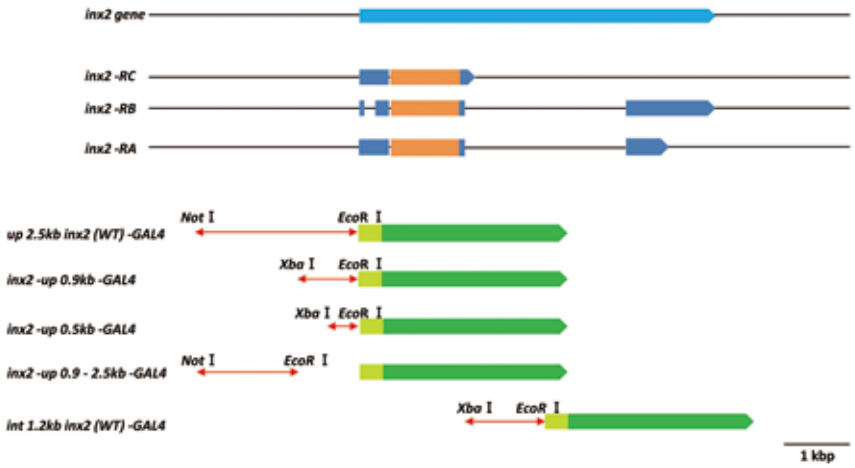

Fig. 1 Schematic representation of the *inx2* locus, *up 2.5kb inx2(WT)-Gal4* construct and its derivatives. Orange boxes indicate the inx2 open reading frame (ORF). The basal promoter (light green) and ORF of Gal4 (green) in the pPTGal vector are shown.

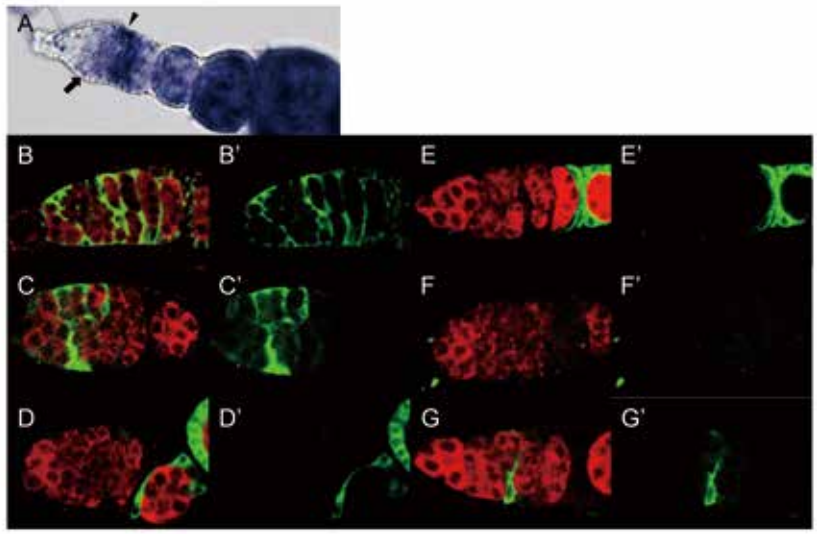

Fig. 2 (A) *inx2* mRNA was expressed in escort cells (arrow), prefollicular cells (arrowhead) and follicle cells in wild-type ovary. (B-G) Germaria of *up 2.5kb inx2(WT)-Gal4 (F25-1-1) >UAS-mCD8-GFP* (B), *inx2 up0.9kb-Gal4 (M15-3) >UAS-mCD8-GFP* (C), *inx2 up0.9kb-Gal4 (M15-6) >UAS-mCD8-GFP* (D), *inx2 up0.5kb-Gal4 (M43-M1) >UAS-mCD8-GFP* (E), *inx2-up 0.9-2.5kb-Gal4 (F7-F1) >UAS-mCD8-GFP* (F) and *int 1.2kb (WT)-Gal4 (M14-2-1) >UAS-mCD8-GFP* (G) were double-stained with anti-Vasa (red) and anti-GFP (green) antibodies. (B'-G') The GFP channel is shown its own.

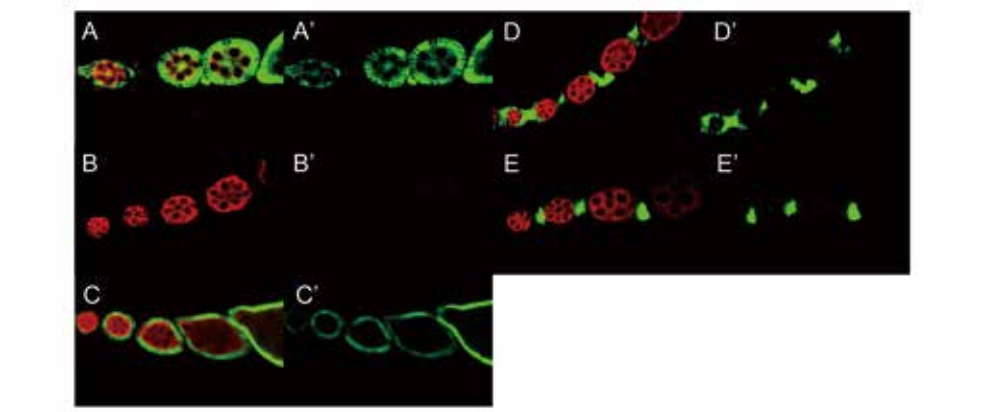

Fig. 3 (A-E) Egg chambers of *up 2.5kb inx2(WT)-Gal4 (F25-1-1) >UAS-mCD8-GFP* (A), *inx2 up0.9kb-Gal4 (M15-3) >UAS-mCD8-GFP* (B), *inx2 up0.9kb-Gal4 (M15-6) >UAS-mCD8-GFP* (C), *inx2 up0.5kb-Gal4 (M43-M1) >UAS-mCD8-GFP* (D), *inx2-up 0.9-2.5kb-Gal4>UAS-mCD8-GFP (F7-F1)* (E) were double-stained with anti-Vasa (red) and anti-GFP (green) antibodies. (A'-E') The GFP channel is shown its own.

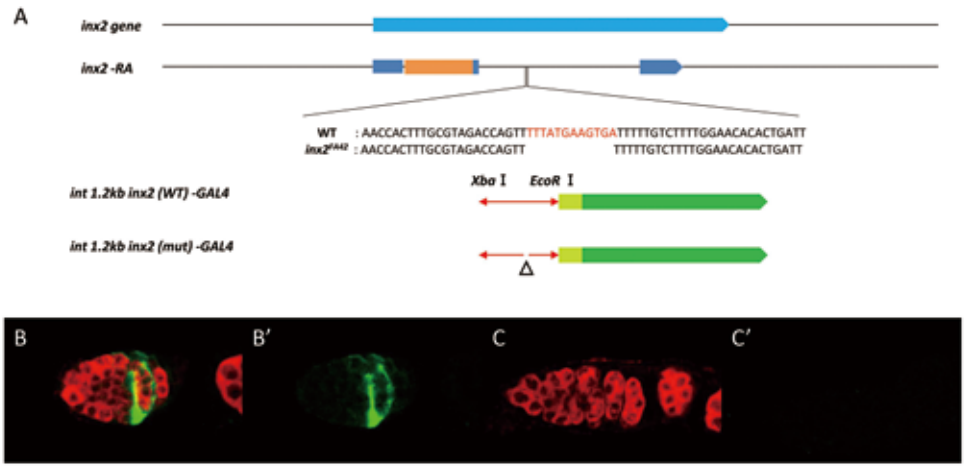

Fig. 4 (A) Schematic representation of the *int 1.2kb inx2 (WT)-Gal4* and *int 1.2kb inx2 (mut)-Gal4* constructs. (B and C) Ovarioles of *int 1.2kb inx2 (WT)-Gal4 (M14-2-1) >UAS-mCD8-GFP* (B) *and int 1.2kb inx2 (mut)-Gal4 (M03-2-1) >UAS-mCD8-GFP* (C) were double-stained with anti-Vasa (red) and anti-GFP (green) antibodies. (B'and C') The GFP channel is shown its own.