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SHORT COMMUNICATION

Histone variant His2Av is implicated in maintaining Piwi protein abundance in the *Drosophila melanogaster* female germline

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ABSTRACT The PIWI-interacting RNA (piRNA) pathway protects the germline from the deleterious effects of uncontrolled transposon expression in animals. Although the piRNA pathway and its components, such as Piwi, have been extensively studied, their regulation is not fully understood. We revealed a reduced mCherry-Piwi signal in *Drosophila* nurse cell nuclei and decreased mCherry-Piwi protein levels in His2Av depleted ovaries. In contrast, we did not detect any significant changes in *piwi* mRNA levels. Here, we report that His2Av is implicated in maintaining Piwi protein abundance in the *Drosophila* female germline, however its precise role remains to be elucidated.
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Introduction

Harmful transposon expression in the animal germline, including *Drosophila*, is controlled by a multi-component defense system, the piRNA pathway (Ozata et al. 2019). Piwi is one of the key components of this pathway. Piwi is indispensable for transcriptional silencing of transposons and for licensing dual-strand piRNA clusters by promoting the deposition of the repressive histone mark H3K9 trimethylation (H3K9me3) (Sienski et al. 2012; Le Thomas et al. 2013; Rozhkov et al. 2013; Akkouche et al. 2017; Ninova et al. 2020). His2Av is the only H2A histone variant in *Drosophila melanogaster* and has multiple roles. It participates in chromatin formation and organization, transcriptional regulation in a context-dependent manner, and DNA damage signaling (Zhang and Pugh 2011; Weber et al. 2014; Giaimo et al. 2019; Scacchetti and Becker 2021). His2Av is incorporated into the genome by the DOM-B complex and is essential for proper embryogenesis in *Drosophila* (Scacchetti et al. 2020; Ibarra-Morales et al. 2021). Its connection to the piRNA pathway has been shown by several RNAi screens (Czech et al. 2013; Handler et al. 2013). While the components of the piRNA pathway are well characterized, only a handful of factors have been identified that regulate the piRNA pathway in *Drosophila* and mice (Bolcun-Filas et al. 2011; Li et al. 2013; Alizada et al. 2025a, 2025b). Given the roles of His2Av and Piwi in chromatin state definition, and the reported link between His2Av and piRNA-mediated transposon silencing, the question arises whether a molecular connection exists between these two factors. Our findings suggest that His2Av is involved in maintaining Piwi abundance in the *Drosophila* female germline, although the underlying mechanism remains unclear.

Material and Methods

Fly stocks

Flies were maintained at 25 °C. For ovary dissection females were aged to 3 days and put on yeast for 1 day. *Drosophila* stocks: *His2Av^{810}* null mutant (BDSC #9264), *shWhite* (white GLKD; BDSC #33623), *shHis2Av* (His2Av GLKD; BDSC #34844) and *nos-Gal4-VP16* (nos-Gal4, BDSC #4937) were ordered from Bloomington Drosophila Stock Center. *hsFLP12; neoFRT82B, Ubi-GFP* and *neoFRT82B* were gifts from David Glover. *mCherry-Piwi* (controlled by its endogenous promoter) was a gift from Gregory Hannon.

RNA extraction and RT-qPCR

Total RNA was extracted from 20 pairs of ovaries using TRIzol Reagent (Invitrogen). Then the RNA (1µg) was treated with DNase I (Invitrogen) and first-strand cDNA was synthesized with SuperScript III Reverse Transcriptase (Invitrogen). qPCR was performed with a Mastercycler RealPlex2 EpGgradientS qPCR machine (Eppendorf), using

Table 1. List of primers used for RT-qPCR

Name	Sequence (5'-3')	Reference
qHis2Av-F	GCGGTAAAGCAGGCAAGGATT	This study
qHis2Av-R	ACGCGTCCATGTGACGTAGT	This study
qpiwi-F	ACTTCCCGAGGTAGTGGTGA	This study
qpiwi-R	TCGGGTCACCTGATGAAGAT	This study
qGapdh1-F	ATTAACGGATTTGGCCGCATC	This study
qGapdh1-R	CCCTTGAAACGACCGTGAGTC	This study
qRP49-F	CCGCTTCAAGGGACAGTATCT	Godneeva et al. 2023
qRP49-R	ATCTCGCCGCAGTAAACGC	Godneeva et al. 2023

MyTaq HS Polymerase Mix (Thomas Scientific) and SYBR Green I stain (Invitrogen). All steps were done according to manufacturer's instructions. Expression of target genes was normalized to the geometric mean of *rp49* and *Gapdh1* reference gene expression. Transcript levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three biological replicates were made (n=3). Primers used for the RT-qPCRs are listed in Table 1.

RNA in situ Hybridization Chain Reaction (HCR)

Ovaries were dissected, and for *piwi* (unique identifier: 5486/G065) RNA *in situ* hybridization chain reaction, probes, amplifiers, and buffers were purchased from Molecular Instruments (molecularinstruments.org). RNA *in situ* HCR v3.0 (Choi et al. 2018) was performed according to the manufacturer's instructions for generic samples in solution, then ovaries were imaged by confocal microscopy.

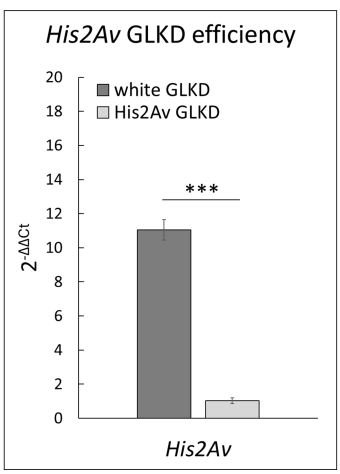

Figure 1. *His2Av* germline knockdown efficiency. Bar plot shows RT-qPCR analyses of *His2Av* mRNA level in white and His2Av GLKD ovaries. Error bars represent standard error, ***p<0.001 (Student's *t*-test; n=3 biological replicates).

Clonal analysis and germline knockdown

The FLP/FRT system was used to generate *His2Av*^{810} null mutant mosaic flies (Theodosiou and Xu 1998; Weasner et al. 2017). *mCherry-Piwi*; *neoFRT82B*, *His2Av*^{810} flies were crossed to *hsFLP12;; neoFRT82B*, *Ubi-GFP* flies and L3 larvae were heat shocked at 37 °C for 60 min for three consecutive days to generate *His2Av*^{810} mutant clones. Ovaries from adult females were dissected and imaged by confocal microscopy (n=15 pairs of ovaries). Germline knockdown (GLKD) was achieved by using nos-Gal4 driver to express shRNAs targeting *white* and *His2Av* genes. Dissected ovaries were imaged by confocal microscopy (n=10 pairs of ovaries). *His2Av* germline knockdown efficiency was verified by RT-qPCR (Fig. 1) and repeated three times (n=3 biological replicates).

Confocal microscopy and image processing

Ovaries were dissected in ice-cold phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde in PBST (PBS, 0.1% Tween-20) for 20 min at room temperature (RT). Ovaries then were washed three times in PBST at RT. DAPI (1:1000, Invitrogen) was added during the second wash. Samples were mounted in VECTASHIELD Antifade Mounting Medium (Vector Laboratories Inc). Images were acquired with Leica Stellaris 8 FALCON confocal microscope (Leica Microsystems) using x63 oil immersion objective and then processed and analyzed with ImageJ (Schneider et al. 2012). Images were acquired under identical microscope settings. Quantification of mCherry-Piwi nuclear fluorescence was performed with ImageJ. For germline knockdown experiments, fluorescence intensities were measured in 40 nurse cell nuclei per genotype (N=40) in stage 5–6 egg chambers from white GLKD and His2Av GLKD ovaries. At least 5 pairs of ovaries were analyzed per genotype. In case of clonal analysis, fluorescence was measured in 32 nurse cell nuclei (N=32) from stage 6–9 egg chambers for both *His2Av*^{810} mutant and control (non-mutant) conditions. Ten pairs of ovaries were analyzed (n=10). To ensure accurate comparison, the same number of nurse cell nuclei were measured

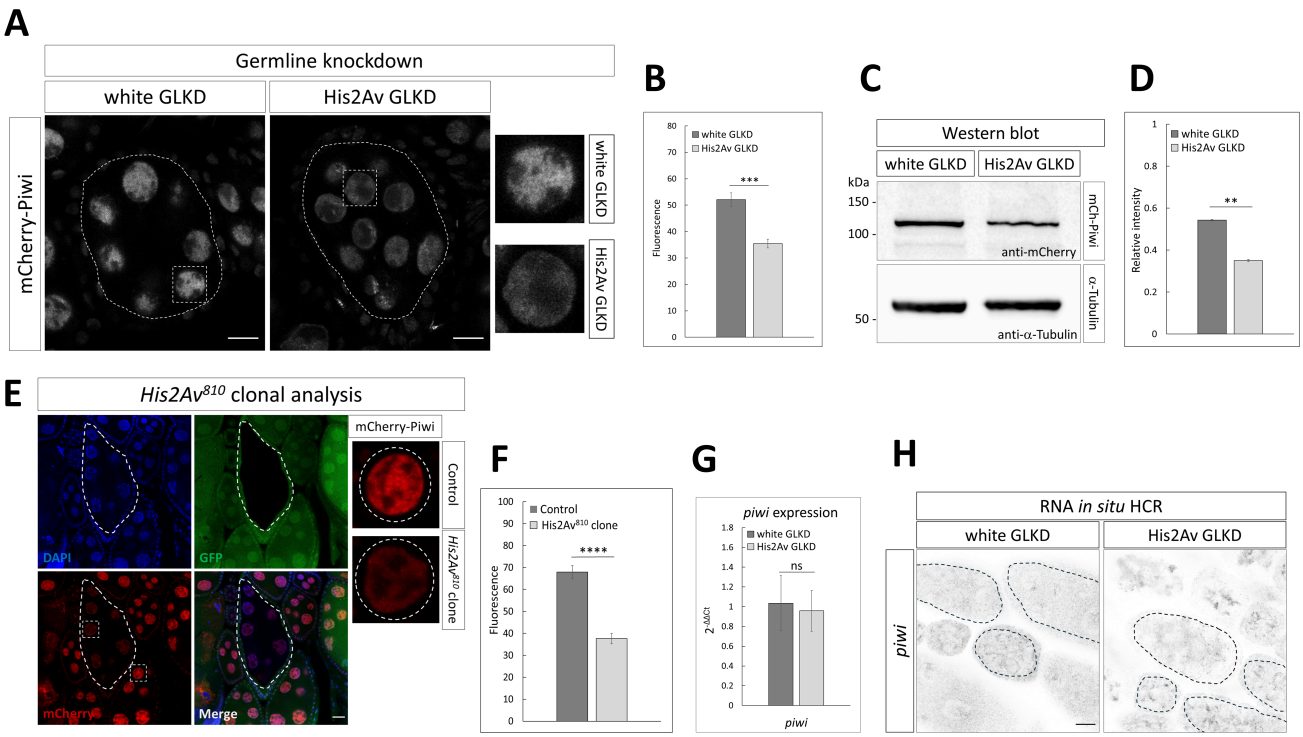

Figure 2. His2Av modulates Piwi protein abundance in female germline. (A) Confocal images show decreased mCherry-Piwi signal intensity in nurse cell nuclei in *His2Av* germline knockdown ovaries. Representative images from white GLKD and His2Av GLKD ovaries are shown. Dashed lines outline egg chambers (stage 5–6) and dashed-line squares indicate the magnified views of nurse cell nuclei. Scale bar = 10μm; n=10 pairs of ovaries. (B) Quantitative evaluation of mCherry-Piwi nuclear fluorescence in white GLKD and His2Av GLKD ovaries. Fluorescence intensity was measured in nurse cell nuclei from stage 5–6 egg chambers (N=40 nuclei/genotype). Error bars represent standard error (***p<0.001, Student's *t*-test). (C) Western blot detection shows reduced mCherry-Piwi (mCh-Piwi) protein levels in His2Av GLKD ovaries compared to white GLKD. α-Tubulin was used as a loading control (n=2 biological replicates). (D) Densitometric quantification of mCherry-Piwi Western blot band intensities from white and His2Av GLKD ovaries. mCherry-Piwi band intensities were normalized to α-Tubulin levels (n=2 biological replicates). Standard errors are shown, **p<0.01 (Student's *t*-test). (E) Consistently, clonal analysis indicates reduced mCherry-Piwi intensity in the nuclei of *His2Av*^{810} mutant nurse cells (*His2Av*^{810} clone). Mutant egg chamber/cells are marked by the absence of GFP signal. Dashed lines outline the mutant egg chamber; dashed-line squares mark the zoomed-in nurse cell nuclei from control and *His2Av*^{810} mutant egg chambers; dashed-line circles surround the zoomed-in nuclei. Representative confocal images of control and *His2Av*^{810} mutant egg chambers (stage 7–8) from mosaic ovaries are shown (scale bar = 20μm; n=15 pairs of ovaries). (F) Quantitative evaluation of mCherry-Piwi fluorescence in nurse cell nuclei from control and *His2Av*^{810} mutant (*His2Av*^{810} clone) egg chambers (stage 6–9). Error bars represent standard error; ****p<0.0001 (Student's *t*-test; N=32 nuclei/condition). (G) RT-qPCR analysis revealed no significant difference in *piwi* mRNA level in His2Av GLKD ovaries compared to white GLKD. Error bars represent standard error (Student's *t*-test, ns – not significant; n=3 biological replicates). (H) *piwi* RNA *in situ* HCR assay shows comparable signal intensity and spatial expression pattern in white and His2Av GLKD ovaries. Dashed lines outline egg chambers (stage 5–7; scale bar = 20μm; n=7 pairs of ovaries).

across identical egg chamber stages. Egg chambers were selected based on standard morphological criteria (egg chamber size, shape and the ratio of oocyte to nurse cells) for developmental stages.

SDS-PAGE and Western blot

Total protein was isolated from 20 pairs of ovaries with RIPA buffer (Thermo Scientific). Then 5 μg total protein was size-separated on 8% SDS-PAGE and transferred onto Immobilon PVDF membrane (Millipore Sigma). After blocking, the membranes were incubated with rat anti-mCherry (1:2000, Invitrogen, 16D7) and guinea pig anti-α-Tubulin (1:5000, ABCD Antibodies, AA345) primary antibodies for 1.5 h. After washing, the membranes were incubated for 1 h with anti-rat and anti-guinea pig (1:5000, Jackson ImmunoResearch Laboratories) horseradish peroxidase-conjugated secondary antibodies. The membranes were subsequently overlaid with Clarity Western ECL Substrate (Bio-Rad) and chemiluminescence was detected with a ChemiDoc MP Imaging System (Bio-Rad). Two biological replicates were made (n=2). Densitometric analysis of Western blot band intensities were carried out with ImageJ (Schneider et al. 2012). Two biological replicates were quantified (n=2).

Results

His2Av depletion reduces Piwi protein abundance in the female germline

Based on the previously reported decline in female fertility and uncontrolled transposon expression (Czech et al.

2013; Handler et al. 2013), a potentially compromised piRNA pathway is implicated in the His2Av depleted flies. To explore the possible connection through other factors, we examined whether His2Av depletion alters the localization and abundance of the key piRNA pathway component Piwi in the *Drosophila* female germline. First, we assessed the efficiency of *His2Av* germline knockdown driven by nos-Gal4. Our RT-qPCR analysis revealed an almost 90% reduction of *His2Av* mRNA level in *His2Av* germline knockdown (hereafter His2Av GLKD) ovaries compared to the mRNA level measured in the control *white* germline knockdown (hereafter white GLKD) ovaries (Fig. 1). We also observed an underdeveloped ovary phenotype in His2Av GLKD flies, with oogenesis arresting primarily at stages 5–6 of egg chamber development. Next, we carefully analyzed mCherry-Piwi nuclear fluorescence in nurse cells of stage 5–6 egg chambers. Confocal microscopy of His2Av GLKD ovaries revealed a reduced mCherry-Piwi signal intensity in nurse cell nuclei (Fig. 2A, B). To ensure that this reduced intensity was independent of the underdeveloped ovary phenotype, we examined the effects of the $His2Av^{810}$ null allele on mCherry-Piwi localization, using clonal analysis to overcome the late larval to early pupal lethality associated with the homozygous mutant (van Daal and Elgin 1992; Kotova et al. 2011; Grigorian et al. 2017). Consistently, confocal analysis of $His2Av^{810}$ homozygous clones in ovarian germline cells revealed a similar decrease in nuclear mCherry-Piwi levels (Fig. 2E, F). Western blot analysis of the His2Av GLKD ovaries showed a decrease in mCherry-Piwi protein levels (Fig. 2C, D). This result suggests that the reduced mCherry-Piwi signal reflects possible protein-level changes, not solely altered mCherry-Piwi distribution. To determine whether this reduction in protein level originates from reduced gene expression, we analyzed potential changes in *piwi* transcription using RT-qPCR and RNA *in situ* HCR assays. Interestingly, no significant changes in *piwi* mRNA levels were observed in His2Av GLKD ovaries (Fig. 2G, H). These results suggest that His2Av might affect Piwi abundance at the protein level, possibly through post-transcriptional regulation or altered protein stability. However, a more detailed analysis of the endogenous Piwi protein is needed to strengthen these findings.

Discussion

The piRNA pathway is essential for preserving genome stability in animal gonads by silencing transposable elements (Ozata et al. 2019). Mutations in piRNA pathway machinery cause genome instability in the germline, disrupt gametogenesis, lead to unrestrained transposon expression, and result in impaired fertility or sterility (Volpe et al. 2001; Carmell et al. 2007; Chen et al. 2007; Klattenhoff et al. 2007; Pane et al. 2007; Rozhkov et al. 2013). Indeed, previous studies showed uncontrolled transposon expression and reduced female fertility in His2Av depleted flies (Czech et al. 2013; Handler et al. 2013), pointing to a potentially compromised piRNA pathway. Our findings suggest that His2Av is involved in maintaining the protein levels of the key piRNA pathway component Piwi in the *Drosophila* female germline (Fig. 2), further pointing to a possible connection to the small RNA-mediated defense mechanism. Previous studies reported that His2Av interacts and forms a complex with HP1 during kinetochore-driven microtubule formation in *Drosophila* (Verni and Cenci 2015) and physically interacts with Suv(var)2-10, the E3 component in *Drosophila* SUMOylation pathway (Tang et al. 2021). However, whether His2Av regulates Piwi directly or via other factors remains to be determined. Our knowledge regarding the regulation of the piRNA pathway is relatively limited. For example, the importance of the transcription factor A-MYB in piRNA production in the mouse male germline was described by Li et al. (2013), whereas only recently Ovo and Traffic jam were shown to transcriptionally regulate the piRNA pathway in ovarian germline and somatic cells, respectively (Alizada et al. 2025a, 2025b). Overall, our results suggest a possible connection between His2Av and the piRNA pathway through the modulation of its core component. However, the precise mechanism by which His2Av affects Piwi abundance remains to be determined. Future work may investigate whether His2Av regulates Piwi post-transcriptionally by altering its translation, stability or protein turnover, or if it acts through indirect, chromatin-dependent processes.

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