Investigating TUDOR domain-containing proteins in the *Drosophila* piRNA pathway

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Abstract
Piwi-interacting RNAs (piRNAs) are a class of evolutionarily conversed, small non-coding RNAs which work to silence expression of transposable elements in gonads through an RNA-induced silencing complex (piRISC). A number of proteins in the piRNA pathways are TUDOR domain-containing (TDRD) proteins which have been implicated in piRNA biogenesis. In this project, we focused on identifying the potential protein interactions of two TDRD proteins involved in piRNA biogenesis – Sister of Yb (SoYb) and Vreteno (Vret).

Introduction
PIWI proteins, first characterized for their role in germline stem cell division[1], associate with small non-coding RNAs to silence expression of transposable elements in the germline through the piRNA pathway[2-5]. While this amplification loop piRNA pathway functions in germline cells, the primary piRNA processing pathway also functions in surrounding somatic gonad tissues without the piRNA amplification loop[6, 7]. Furthermore, germline and somatic piRNAs associate with different proteins during piRNA biogenesis[reviewed in 8]. For example, the Yb body is an organelle found exclusively in somatic cells which has been shown to be involved in primary piRNA processing, but not the piRNA amplification loop[9, 10].

In *Drosophila*, several members of the Tudor superfamily of proteins with TDRD domains have been shown to associate with PIWI proteins or other piRNA biogenesis factors through the symmetrical dimethyl arginines (sDMAs) in PIWI proteins[reviewed in 11]. Further analysis of these TDRD proteins in *Drosophila* and their potential protein interactions may provide insights into piRNA biogenesis in both germline and somatic piRNA biogenesis.

Here, we focused on investigating the protein interactions of two TDRD proteins – Sister of Yb (SoYb) and Vreteno (Vret), both of which have been identified through previous studies as essential proteins in the primary piRNA pathway[12, 13]. Using the biochemical approaches of immunoprecipitation and mass spectrometry analysis, we tried to identify additional proteins which interact with either of these two TDRD proteins.

Materials and Methods

Drosophila Ovarian Somatic Cell (OSC) Culture and Ovaries
To focus on TDRD protein interactions in the primary piRNA processing pathway, we utilized ovarian somatic cell (OSC) culture consisting of only of somatic cells[14]. The OSC line was cultured as previously described: briefly, cells were cultured at 26°C in Cross and Sang's M3

(BF) medium with 0.6 mg mL^{-1} glutathione, 10% FBS, 10 mU mL^{-1} insulin and 10% fly extract from Oregon R flies. OSCs were passaged by first rinsing three times with PBS, detached from the plate by trypsin, and resuspended in supplemented BF medium. For myc-SoYb analysis, OSC lines were transfected (Xfect, Clontech) with either myc-eGFP or myc-SoYb constructs generated by K. Sato. In addition, to probe potential protein interactions in the germline, adult ovaries were dissected from Oregon R flies in ice-old PBS.

Antibodies

The Siomi laboratory generated an anti-Vreteno antibody using a Vreteno-specific peptide sequence in mice. Serum was affinity-purified for laboratory use. K. Sato generated myc-tagged SoYb which could be immunoprecipitated using a commercially available mouse myc antibody (Sigma). Antibody recognition and specificity was confirmed by Western blot analysis.

Immunoprecipitation

Harvested OSC cells or ovaries were lysed in NP 40 buffer (0.5 M Hepes, pH 7.3, 1 M KOAc, 1 M $Mg(OAc)_2$, 1 M DTT, and 10% NP40 alternative (Calbiochem)). The OSC lysates were collected by centrigu. Immunoprecipitation was performed using anti-Vreteno and anti-myc antibodies coupled to magnetic beads (Dynabeads). Immunoprecipitates were washed four times with NP40 buffer at 4°C and eluted from the beads by boiling in 2X SDS sample buffer for 3 min at 95°C. Samples were loaded and run on 8.5% SDS-PAGE gels and visualized by silver staining (SilverQuest™, Invitrogen). A schematic detailing the overall experimental plan is presented in Figure 1.

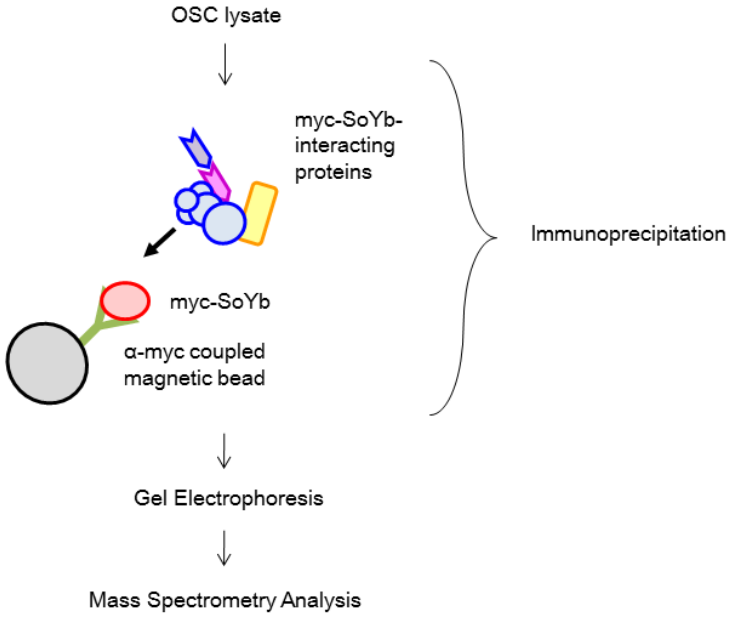

Figure 1 – Experimental Plan Schematic

OSC lysates were incubated with magnetic beads (grey circle) coupled to α-myc antibodies (green Y). In theory, the antibody should pull down myc-SoYb (red circle) and any myc-SoYb interacting proteins (colored shapes) during immunoprecipitation. After elution from the magnetic beads, the elute is run on an SDS-PAGE gel to separate and visualize proteins by size. Bands of interest are excised from the gel and sent for mass spectrometry analysis.

Mass Spectrometry
Bands of interest which observed in multiple silver stained gels from unique immunoprecipitations were excised from the gels and sent to the Institute of Health Biosciences Support Center for Advanced Medical Sciences at the University of Tokushima for mass spectrometry and preliminary analysis.

Results
SoYb potentially interacts with PABP and ribosomal proteins
To identify potential SoYb protein interactions, we transfected OSC cell lines with either a myc-EGFP construct or a myc-SoYb construct. Immunopurification using an antibody against the myc tag allowed us to pull down protein complexes assoaited with either myc-EGFP or myc-SoYb. Consistent with previous literature, we observed that SoYb associates with both Yb and Vret, but does not associate with Maelstrom (Mael), another piRNA biogenesis factor[15] (Figure 2A). Any protein bands which appeared during electrophoresis of myc-SoYb but not myc-EGFP (the control for the myc tag) were excised and sent for mass spectrometry analysis. Preliminary mass spectrometry analysis of these bands confirmed the presence of SoYb and also predicted two additional proteins associated with SoYb – poly(A)-binding protein (PABP) and a ribosomal protein (Figure 2B).

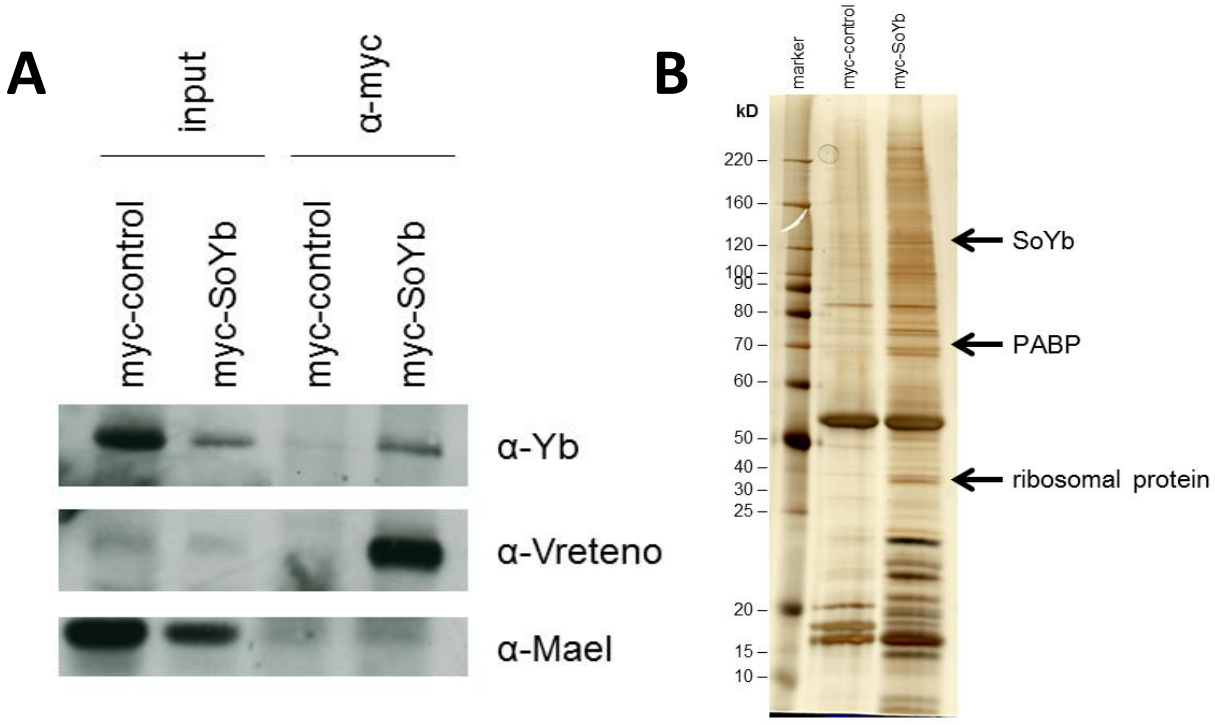

Figure 2 – SoYb Immunopurification
(A) Western blotting confirmed previously reported SoYb interactions with Yb (top) and Vret (middle), but showed no SoYb interaction with Mael (bottom). (B) Silver staining of immunoprecipitated complexes from the myc-EGFP and myc-SoYb transfected cells. Arrows indicate bands sent out for mass spectrometry and predicted protein by preliminary analysis.

Vret may interact with additional proteins which have yet to be identified
Vret complexes from OSC cells and ovaries were immunopurified directly using a Vret-specific antibody. Through immunohistochemistry, we detected Vret in both OSC and ovary cells, noted Vret interaction with Armi in OSC, and observed no Vret interactions with several other known components of the piRNA pathway – Mael, AGO3, Piwi, and Spindle E (SpnE) (Figure 3A). Due to practical difficulties in identifying bands form ovary immunoprecipitation through silver staining, only bands from the OSC immunoprecipitation were sent for mass spectrometry analysis.). We identified three potential Vret protein interactions in the OSC immunoprecipitation through silvering staining (Figure 3B) which were sent for mass spectrometry, but yet to receive preliminary analysis from these samples.

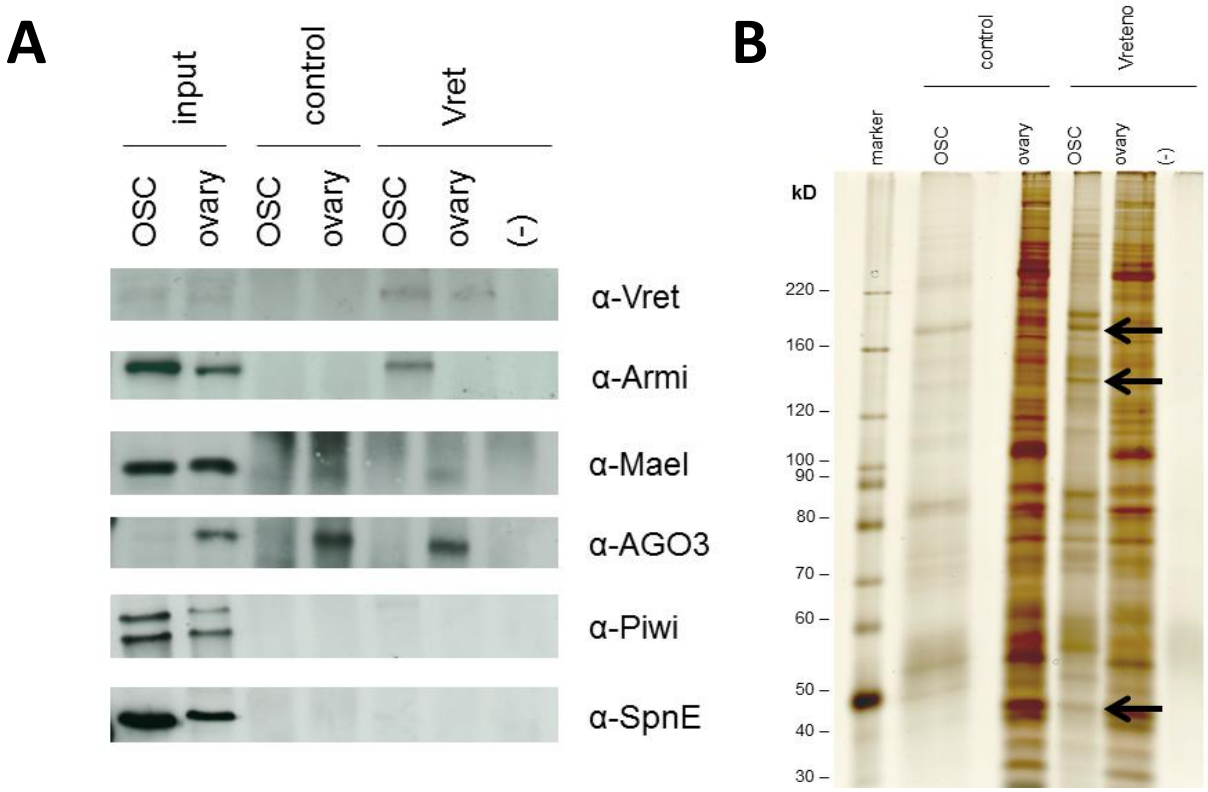

Figure 3 – Vret Immunopurification
(A) Western blotting showed Vret antibody specificity (top), interaction with Armi in OSC, and no specific interactions with Mael, AGO3, Piwi, or SpnE. (B) Silver staining of immunoprecipated complexes from ovaries and OSC. Arrows indicate bands sent from out for mass spectrometry.

Discussion
The preliminary analysis of SoYb interacting proteins predicted PABP and a ribosomal protein as potential protein interactors. As its name suggests, PABP binds to the mRNA poly(A) tail, but To date, PABP has not been shown to participate in piRNA biogenesis, but has recently been implicated to play a role in another RNA silencing pathway, the microRNA (miRNA) pathway, although its role is not currently well understood[16-18]. The analysis for the ribosomal protein was not as definitive, suggesting multiple different possible protein interactions. Further biochemical analysis of these potential SoYb interactors, as well as any identified Vret interactors, is needed to confirm their association and potential roles in the piRNA pathway.

Acknowledgements
We thank Ikuko Sagawa Institute of Health Biosciences Support Center for Advanced Medical Sciences at the University of Tokushima for mass spectrometry and preliminary analysis and the Siomi laboratory for the antibodies. I am grateful to Professor Mikiko Siomi for the opportunity to visit and work in the laboratory, Kaoru Siomi for technical and practical experimental supervision, members of the M. Siomi and H. Siomi laboratories for an intellectually stimulating experience, and the UTRIP office for financial and program support.

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