Investigating the Binding Mechanism of Germ Plasm Protein Me31B and Tudor

NORTHWEST

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Abstract: *Drosophila* germ plasm proteins Me31B and Tudor are important for germ cell development. The helicase Me31B and the conserved germ plasm protein Tudor have been previously shown to physically interact. To unravel the interaction mechanism, we aim to investigate the Tudor-binding motifs of Me31B. Full length, N-terminal, and C-terminal Me31B expression plasmids were constructed and used to express the protein domains in S2 cell culture. HA-tagged Tudor proteins were purified from fly ovaries and used in an *in vitro* binding assay with the Me31B protein domains above. Preliminary results showed that C-terminal has the highest binding affinity for Tudor, however, some binding was noticed on N-terminal Me31B. To further validate our results, we are conducting the binding assay in reverse: using HA-Tud as "bait" protein and adding purified Me31B domains to the bait. Our study further elucidates the interaction mechanism of germ plasm proteins and how they contribute to germ plasm assembly.

Introduction

The interaction of Me31B with RNAs and other proteins facilitates the proper development of the germline stem cells of *Drosophila melanogaster*. Me31B is found in germ granules and is an ATP-dependent RNA helicase. As a *trans*-acting element Me31B is responsible for the silencing of various mRNAs during transport to the germ plasm. Me31B belongs to the DEAD box helicase family and is a homolog of DDX6, found in humans.

Tudor is an essential protein in the development of *Drosophila* germ cells. Previous studies have indicated Tudor's role as a scaffold element in the Me31B interactome. One function of the interactome is believed to inhibit the premature translation of mRNAs during transport to the germ plasm. In a previous study (DeHaan *et al., 2017*) provided evidence for the physical binding between Me31B and Tudor. In this experiment we examined the Me31B-Tudor binding domain by analyzing the N-terminus and C-terminus for Tudor binding as shown in Figure 1.

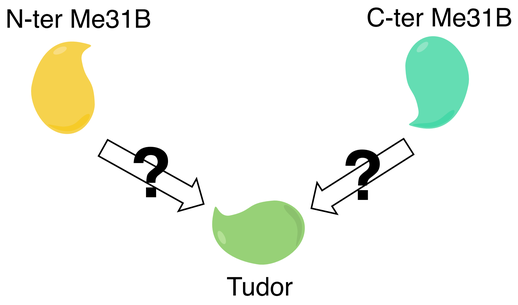

Figure 1: N-ter Me31B or C-ter Me31B containing Tudor binding site

Methods

Mutant Generations

For preliminary analysis, wild-type (WT) and GFP (control) line were used to conduct a binding assay. GFP and WT genes were generated via polymerase chain reaction (PCR) and tagged with c-Myc. After generation, the mutant plasmids were transformed into *Escherichia coli* (*E. coli*) cells. Colonies of *E. coli* that properly expressed the plasmid were grown on antibiotic-treated media and were selected. Collected cells were stored in a 50% glycerol solution at -80°C. *E. coli* previously collected were inoculated into lysogeny broth (LB) + carbenicillin medium. Manufacturer (*ZymoPURE™ II Plasmid Midiprep Kit)* procedure was followed for plasmid extraction. Competent *Drosophila* Schneider 2 (S2) cells were then transfected with the previously collected plasmid following the manufacture procedure (*ThermoFisher Scientific Catalog Number R69007)*. Cells expressing the mutant proteins were stored at -80°C. The amino acid sequence is shown in Figure 2.

MMTEKLNSGHTNLTSKGIINDLQIAGNTSDDMGWKSKLKLPPKDNRFKTTDVTDTRGNEFEEFCLKRELLMGIFEKGWERPSPIQEAAIPIALSGKDVLARAKNGTGKTGAYCIPVLEQIDPTKDYIQALVMVPTRELALQTSQICIELAKHL
DIRVMVTTGGTILKDDILRIYQKVQLIIATPGRILDLMDKKVADMSHCRILVLDEADKLLSLDFQGMLDHVILKLPKDPQILLFSATFPLTVKNFMEKHLREPYEINLMEELTLKGVTQYYAFVQERQKVHCLNTLFSKLQINQSIIFCNSTQRVEL
LAKKITELGYCCYYIHAKMAQAHRNRVFHDFRQGLCRNLVCSDLFTRGIDVQAVNVVINFDFPRMAETYLHRIGRSGRFGHLGIAINLITYEDRFDLHRIEKELGTEIKPIPKVIDPALYVANVGASVGDTCNNSDLNNSANEEGNVSK

Q Motif

Helicase ATP-binding "N-Term"

Helicase "C-Term"

Figure 2: Amino acid sequence of Me31B visualizing N-terminus and C-terminus

Tudor-HA Protein Purification

Tudor-HA protein was isolated from *Drosophila* ovaries. Dissected ovaries (~300μL) were homogenized with 500μL of Standard Lysis Buffer [PBS, 0.1% NP-40, 10% glycerol, and 1X protease inhibitor]) and centrifuged. The protein concentration of the supernatant was calculated via a Bradford assay. The protein concentration was diluted to 1.5 mg/mL with lysis buffer solution. Agarose beads (20μL, *MBL*) were then added to the lysate followed by a 60-minute incubation period at 4°C on a rotary shaker. The manufacture procedure and wash solution were used to isolate the proteins.

Myc-tagged GFP/Me31B WT Protein Purification

Immunoprecipitation (IP) was conducted of each cell sample to purify the proteins GFP and WT. Respective cells (50μL) were homogenized with 500μl of pre-chilled lysis buffer (PBS 1X Protease inhibitor). The lysate was centrifuged, and the protein concentration of the supernatant was conducted via a Bradford assay. The protein concentrations were adjusted to 1.5 mg/mL with lysis buffer. TritonX-100 (20%) in PBS was added to the lysate to bring the concentration of TritonX-100 to 2%. Next, 20μl of Anti-c-Myc beads (*MBL*) were added to the lysate followed by a 90-minute incubation period at 4°C on a rotary shaker. The manufacture procedure and wash solution were then followed.

GFP/WT Me31B - Tudor Binding Assay

Previously purified c-Myc-Me31B WT and c-Myc-GFP were separately equilibrated with 200μL cold IGEPAL solution (PBS, 0.05% IGEPAL CA-630). Then 40μL of Tudor-HA and 0.5% IGEPAL solution was added to 40μL of each c-Myc sample (GFP and WT). The samples were incubated for 60 minutes with gentle agitation every 15 minutes at 4°C. The samples were then centrifuged, and the flow-through was discarded. The samples were then washed 3 times with cold IGEPAL solution (200μL each wash, 0.05%). Proteins were eluded from the beads with 25μL SDS/PAGE sample buffer at 95°C for 5 minutes. Eluted proteins were detected via western blot using anti-c-Myc antibody (Santa Cruz Biotechnology, 1:1000) and HA-Tud was detected via western blot using anti-HA antibody (Roche, 1:2000).

Results of Preliminary Data and Discussion

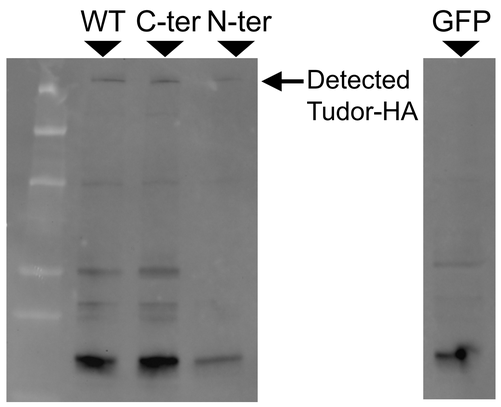

Figure 3: (anti-HA) Early replicate suggests Me31B C-terminus to have a higher binding affinity for Tudor. Tudor (prey) was added to purified *me31B* mutants (bait).

Early replicate shown in Figure 3 indicated Tudor binding to the C-terminus mutant was stronger than the N-terminus. Tudor was not detected to bind with GFP control. Tudor was added to purified Me31B mutants.

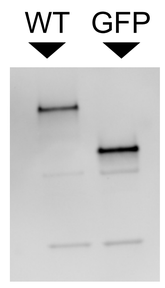

Figure 4: (anti-HA) Replicate showing WT and GFP binding to Tudor. Reverse assay, WT and GFP mutants added to Tudor.

A reverse binding assay, WT and GFP mutants were added to Tudor, was conducted as shown in Figure 4. This assay showed increased binding of Tudor to WT, however, also increased binding to GFP. Furthermore, possible Tudor degradation may have been enhanced with this method.

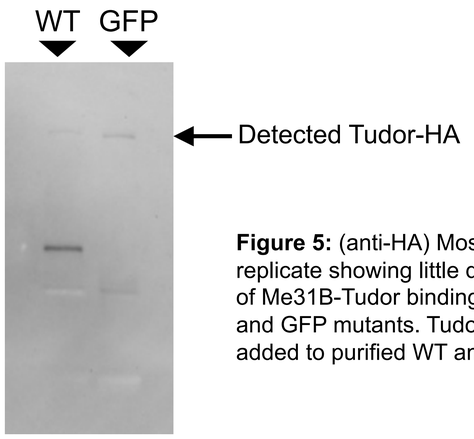

Figure 5: (anti-HA) Most recent replicate showing little difference of Me31B-Tudor binding of WT and GFP mutants. Tudor was added to purified WT and GFP.

Our most recent replicate reverted to adding Tudor to Me31B mutants. Tudor was detected; however, Tudor was binding to the negative control, GFP as shown in Figure 5.

Our future plans include running more replicates with varying testing conditions. We are considering the possibility of including more vigorous washes, using Sf9 cells instead of S2, and isolating Me31B from ovaries with various Tudor domains isolated from S2 cells.

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