UC San Diego
UC San Diego Electronic Theses and Dissertations

Title
Analysis of the processing body subunits Winnebago and Trailer Hitch in Drosophila melanogaster

Permalink
https://escholarship.org/uc/item/42k8n0fd

Author
Jiang, Philip Yuan-Zheng

Publication Date
2010

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Analysis of the Processing Body Subunits Winnebago and Trailer Hitch in *Drosophila melanogaster*.

A Thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Philip Yuan-Zheng Jiang

Committee in charge:

Professor James Wilhelm, Chair
Professor Douglas Forbes
Professor Maho Niwa

2010

The Thesis of Philip Yuan-Zheng Jiang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2010

DEDICATION

This Thesis is dedicated to my family for providing me with support and motivation to persevere and achieve my goals. I would also like to show my appreciation to the Wilhelm lab for allowing me to participate in their research and providing me with the opportunity to explore the unknown.

TABLE OF CONTENTS

Signature Page iii

Dedication iv

Table of Contents v

List of Tables and Figures vi

List of Abbreviations vii

Abstract viii

Chapter One

I. Introduction 1

II. Material and Methods 10

III. Results 14

IV. Discussion/Future Experiments 18

Chapter Two

I. Introduction 29

II. Materials and Methods 32

III. Results 33

IV. Discussion/Future Experiments 35

References 39

LIST OF TABLES AND FIGURES

Table 1. RNP component orthologs 7

Figure 1. MTHFS and Winn homology.....8

Figure 2. Hypothesized cyclo-ligase activity.....9

Figure 3. IP of Winn using DXB150 with Chloride.....19

Figure 4. IP of Winn using DXB150 with Acetate.....19

Figure 5. Metal Exchange Chromatography Purification 21

Figure 6. Ion exchange chromatography purification 22

Figure 7. Theorized Assay Results.....23

Figure 8. Characterization ATP/MgCl2 with Bovine Winn 24

Figure 9. Optimizing ATP concentration for activity assay 25

Figure 10. Enzymology of Bovine Winn with 7-methyl GDP as substrate 26

Figure 11. Enzymology of Bovine Winn with NAD as substrate 27

Figure 12. Mutant Bovine Winn assay without use of any substrates 28

Table 2. *orc5* and *tral* genetic interaction.....34

Table 3. Broad genetic screen with *tral* allele.....35

LIST OF ABBREVIATIONS

Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Bruno recognition element	BRE
Bruno	*Bru*
Deoxyribonucleic acid	DNA
Drosophila Extract Buffer	DXB
Eukaryotic initiation factor 4E/F/G	eIF4E/F/G
IGF-II mRNA binding protein	Imp
Maternal expression 31B	Me31B
Messenger Ribonucleic acid	mRNA
Methenyltetrahydrofolate Synthetase	MTHFS
Origin recognition complex	ORC
Ribonucleic acid	RNA
Ribosomal Ribonucleic acid	rRNA
Smaug recognition element	SRE
Sodium chloride	NaCl
trailer hitch	*tral*
Transfer Ribonucleic acid	tRNA
Winnebago	Winn
Ypsilon schachtel	Yps

ABSTRACT OF THE THESIS

Analysis of the Processing Body Subunits Winnebago and Trailer Hitch in *Drosophila melanogaster*

by

Philip Yuan-Zheng Jiang

Master of Science in Biology

University of California, San Diego, 2010

Professor James Wilhelm, Chair

To discover new ribonucleoprotein (RNP) components, Trailer Hitch (Tral), a RNP factor involved in endoplasmic reticulum (ER) exit site function, was immunoprecipitated and Winnebago (Winn) was identified as a novel Tral-associated protein. Analysis of the Winn sequence revealed a RNA binding motif and conservation with the human cyclo-ligase, methenyltetrahydrofolate synthetase (MTHFS). These observations led us to propose that Winn utilizes ATP to catalyze ring closure on RNA 5' caps in a manner similar to that of MTHFS. Experiments included recombinant expression of Bovine Winn protein, purification, and assay for ATPase activity. Despite a multistep protein purification strategy, our preparations were contaminated with low levels of ATPase that interfered with the assays.

In parallel with identifying Winn, we sought to identify mRNA targets of Tral. From microarray analysis of mRNAs that co-immunoprecipitated with Tral, *orc5* mRNA was identified. Orc5 protein is a component of the Origin Recognition Complex involved in DNA replication and membrane trafficking. Orc5 function in membrane trafficking suggested that it might be a functionally relevant target for the ER defects observed in *tral* mutants. We examined *orc5* mutants for the ability to genetically interact with *tral* but these experiments did not reveal an interaction; therefore, *orc5* mRNA is not a target of the Tral complex. We expanded our genetic screen using the *Drosophila* deletion collection to identify regions on Chromosome 3 that could dominantly enhance eggshell defects seen in *tral* mutants. Sixty genomic regions were tested for genetic interaction with *tral*; however, none of the regions tested interacted.

Chapter One

Association of Winnebago and RNP Complex Components is not ATP Sensitive

I. Introduction

RNA is unique amongst biological macromolecules in that it is used for catalysis, information transfer, and as a structural scaffold. Messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) are the primary categories of RNA. mRNA is responsible for transfer of genomic information to generate proteins (amino acids). Ribosomal subunits, 60S and 40S in eukaryotes, contain large and small rRNAs, respectively, that catalyze formation of peptide bonds. The smaller 40S subunit binds and reads the coding sequence of mRNA, starting from a start codon and ending with a stop codon, while the 60S subunit binds to tRNA and facilitates amino acid transfer. Subsequently, tRNA helps facilitate structuring of the peptide chain through use of aminacyl tRNA synthetase and an anticodon. These will base pair to mRNA and feed it through the ribosome to convert codons into amino acids. Of these molecules, mRNA is responsible for specifying amino acids as a blueprint for translation.

A particular aspect of RNA critical to the regulation of gene expression is RNA metabolism. An inherently unstable molecule, mRNA undergoes many post-transcriptional modifications and contains particular sequences that regulate its stability, translation efficiency, specifying codon structure, and localization. Modifications include end terminus additions at both the 5’ and 3’ ends. A modified guanine nucleotide termed the 5’ cap is added on the 5’ end and a long sequence of adenine nucleotides called the poly(A) tail is added to the 3’ end. These additions serve as translational initiation signals as well as protect the mRNA from exonucleases for the 3’ and 5’, respectively. mRNA regulatory sequences are primarily found in the 5’ and 3’ untranslated regions (UTRs) that reside before the start codon and after the stop codon, respectively. These

regulatory regions are not translated into protein, but determine the susceptibility of a particular message for degradation or translation initiation through the interaction of cis-acting and trans-acting components. The cis-acting mRNA sequence, contained in the 3' UTRs, recruits a trans-acting RNA binding protein that further recruits an effector protein. This association of mRNA and proteins forms a ribonucleoprotein (RNP) complex for regulation purposes. For example, Smaug, a trans-acting protein, binds to the Smaug recognition element (SRE), the cis-acting sequence, and recruits the effector CCR4-NOT deadenylation complex the RNP that mediates mRNA degradation (Zaessinger et al. 2006). This particular association exemplifies how mRNA is regulated for translation by shortening poly(A) tails and is destabilized by association with 5' cap binding proteins so that exonucleases can act on the transcript.

RNPs are formed within large supramolecular complexes in the cytoplasm and include enzymes responsible for mRNA stability, localization control, and/or translation regulation. These complexes are termed the processing body (P body). P bodies have been identified to be sites of mRNA turnover and storage (Sheth et al. 2003). In addition, P bodies are complexes involved in the mRNA decay machinery devoid of ribosomes wherein concentrations of RNA decapping enzymes, exonucleases, and miRNA pathway factors occur (Teixera et al. 2005). P body components may shed light to the processes of RNA metabolism.

The subunits of the P body are highly conserved (Table 1). IGF-II mRNA binding protein (Imp) and Ypsilon schachtel protein (Yps) are P body subunits involved in mRNA localization in *Drosophila* . Imp mediates cytoplasmic localization of mRNAs during embryogenesis where they anchor to microfilaments for directing localization (Nielson et al. 2002). Yps is part of a cytoplasmic RNA protein complex that regulates RNA localization in *Drosophila* ovaries (Mansfield et al. 2001).

Cup, a nucleocytoplasmic shuttling protein, is a translational repressor that binds to eIF4E, a subunit of the translation initiator eIF4F. Normally, the translation initiation begins when eIF4E binds to 5' cap and recruits the scaffolding molecule, eIF4G, to the mRNA. The binding of Cup to eIF4E prevents the recruitment of eIF4G and assembly of the remainder of the eIF4F complex, blocking translation initiation (Nelson et al. 2004). The human ortholog of Cup, 4ET, is also a nucleocytoplasmic shuttling protein that binds to eIF4E (Zappavigna et al. 2004). 4ET colocalizes with mRNA decapping factors in the P body. 4ET represses translation to initiate RNP rearrangement and induces the transition from translation to decay.

Me31B and Tral (Trailer Hitch) are two of the most highly conserved subunits of the P body. Me31B is a maternally expressed DEAD-box helicase and mediates translational repression (Nakamura et al. 2001). *me31B* is an essential gene and germ line clones of *me31b* mutants disrupt oogenesis. Two mRNA targets of Me31B-mediated translational repression have been identified: *oskar* and *bicaudal D*. However, it remains unclear whether Me31B acts directly to translationally repress these messages or if it requires additional factors. Consistent with the second model, Cup and Me31B form a complex during oogenesis that is sensitive to the presence of RNase illustrating that the interaction is indirect (Nakamura et al. 2004). The yeast homolog of *me31b*, DHH1, is also localized to P-bodies in yeast. However, Dhh1p acts as an activator of the decapping process through physical interaction with Dcp-1, a necessary component for formation of deadenylation units to remove 5' caps (Fischer et al. 2002). The fact that yeast has no Cup ortholog may explain the functional differences between *DHH1* and Me31B in these two organisms.

The other highly conserved P body component of interest is encoded by the *Drosophila* gene *tral*. Trailer Hitch protein is a component of a RNP complex. It

localizes to the endoplasmic reticulum in the egg chamber nurse cells suggesting a role in endoplasmic reticulum function (Wilhelm et al. 2005). Consistent with this hypothesis mutations in *tral* disrupt ER exit site distribution and function causing defects in the trafficking of both Grk, a ligand for signaling dorsoventral patterning, and the vitellogenin receptor Yolkless (Yl). In particular, Grk and Yl accumulate on the ER at ER exit sites where ER to golgi transport vesicles form. Loss of *tral* function causes both these proteins to be retained in the ER. This suggests that *tral* plays a role in ER exit site function. Surprisingly, Tral copurifies with a number of RNP components involved with regulation of translation and localization including Me31B , Cup, and Yps. This suggests an unexpected link between cytoplasmic mRNA processing and the secretory pathway. The identification of additional Tral associated proteins and the mRNA targets of *tral* would be predicted to help further our understating of the role of *tral* in regulating the secretory pathway.

Role of Winnebago/growl in RNA metabolism

To identify Tral associated proteins and possibly novel P body components, Tral was immunoprecipitated from *Drosophila* . The immunoprecipitate was then analyzed by mass spectrometry to identify Tral associated proteins. This approach identified ~100 candidate proteins that specifically interact with Tral and possible suggest association within a complex. One of the most interesting of these candidates was the putative ATPase, Winnebago/Growl (Wilhelm, unpublished results).

The Winnebago/Growl protein localizes within the oocyte and accumulates in P body cytoplasmic structures where post-transcriptional regulatory factors are found (Snee et al. 2009). Winn's function has not been fully characterized but tissue specific RNAi experiments revealed that it is required for muscle development. We have isolated

a hypomorphic allele of *winn* in *Drosophila* that produced sterility in males and that are incapable of flight. One potential connection between the testes and the flight muscles have high levels of mitochondrial function. Analysis of the Winn sequence revealed interesting homology with the human methenyltetrahydrofolatesynthetase (MTHFS) (Figure 1) and conservation of the RNA binding motif RNP-1.

Human MTHFS is a cyclo-ligase enzyme involved in purine biosynthesis where it catalyzes the formation of carbon-nitrogen bonds in a cyclic fashion in the following reaction (Jolivet et al. 1996):

ATP + 5-formyltetrahydrofolate → ADP + Pi + 5, 10-methenyltetrahydrofolate

The process begins with a nucleophilic attack by ATP on 5-formyltetrahydrofolate resulting in the transfer of phosphate from ATP to 5-formyltetrahydrofolate. An intermediate is formed where nitrogen and carbon form a bond which completes a ring and releases the phosphate group resulting in the 5, 10-methenyltetrahydrofolate product (Figure 2A). This MTHFS reaction is unidirectional and requires Mg2+ along with ATP.

The combination of muscle and testes phenotypes in *winn* mutants and the potential role of Winn as a cyclo-ligase suggested that it might play a role in mitigating effects of oxidation on RNA. Typically, chemical damage to RNA is not considered to be biologically important since mRNA is believed to be highly unstable; however, in germ line cells, it has been observed that mRNA can have a half-life of over two weeks (Park et al. 2007). The existence of long-lived mRNA populations raises the question of how these mRNAs are protected from chemical damage, particularly in tissues that have high levels of mitochondria activity such as the muscles and the testes.

Because RNA is highly susceptible to chemical damage, we hypothesized that Winn acts similarly to MTHFS and that it acts to repair oxidatively opened RNA bases.

The proposed reaction would involve Winn utilizing ATP to form carbon-nitrogen bonds to close ring structures in the mRNA that had been opened by oxidative damage (Figure 2B). Testing this hypothesis would require two approaches to characterize its function in RNA regulation. First, one should test whether Winn is a subunit of a RNP complex in the P body and if association is ATP dependent by immunoprecipitation and immunoblots. Second, one could attempt to develop an activity assay for Winn to determine its ATPase activity and determine if its basal ATPase can be inhibited by two possible reaction products: 7-methyl GDP and NAD.

We have focused on 7-methyl GDP and NAD since both possess ring structures similar to those generated by MTHFS. 7-methyl GDP is a normally structured 5' cap of mRNA, while NAD is a ring structure similar to that found in 7-methyl GDP. Because mutants affect tissues with high levels of mitochondria functions, we favored the hypothesis that it plays a role in protecting RNA from oxidative damage. However, an alternative hypothesis is that Winn has a mitochondria role in NAD recycling since NAD is structurally similar to substrates of a known cyclo-ligase that contributes to mitochondria function. Our enzymatic studies were focused on distinguishing between these two possibilities.

Table 1: RNP component orthologs. Table represents intriguing RNP components that have orthologs that exist in at least two organisms and their general functions.

Drosophila	C. Elegans	Yeast	Human	Functions
Me31B	CGH-1	DHH1	RCK	Translation repression
YPS	CEY-2-4	-	YB1	Localization
Imp	-	-	-	Localization
Cup	Spn2	-	4ET	Translation repression
Tral	Car-1	Scd6	Rap55	Localization

```
 Score =  176 bits (445),  Expect = 3e-48, Method: Compositional matrix adjust.
 Identities = 117/394 (29%), Positives = 188/394 (47%), Gaps = 76/394 (19%)

Query  33   TKRSLRVQTWKKIQEGKVGIGFNNIFNRIPSFVGADKAAALFANEEEFKKAQHIKVNIDR  92
            +K+ +R Q W  ++   +      + +RIP+F GA +AA  F   + FK A+ IKVN D
Sbjct  9    SKQDIREQIWDYMESQNLADFPRPVHHRIPNFKGASRAAEHFPRLQAFKMARTIKVNPDA  68

Query  93   VLHEFKELALLADKSVYLPSTRESSALCLKVDVLADATEEQKKEALRVQDIQKFRSEIGL  152
                 +   L + K++ +P+ R  + L  K+     AT++  ++    Q ++ + + +GL
Sbjct  69   PQKNARFFVLESKKTLLVPTPRLRTGLFNKITPPPGATKDILRKCATSQGVRNYSTPVGL  128

Query  153  DSGLKLDIVVIGSVVVSPEGYRIGRGNCFADLDIGLLIELGAITPKTIIVTIVHDMQVVD  212
            DS + +D+VV+GSV VS +G+RIG+G G+ADL+  +++ +GA++  T +VTIVHD QVVD
Sbjct  129  DSKVLVDLVVVGSVAVSEKGWRIGKGEGYADLEYAMMVSMGAVSQGTPVVTIVHDCQVVD  188

Query  213  SLPPNLFQKYDTPVDIIATPTEIIRVPKRLSFPNGVYWELLSERRLKILPVLQQLKEREE  272
             +P  L + +D  VD I TPT +I        P G+ W  +S   L  +P+L+ L+ +EE
Sbjct  189  -IPEALLEDHDLTVDYILTPTRVITTGCERPKPAGIAWSKISCEMLGKMPILRSLRHQEE  247

Query  273  KAGKSISLKEEDTDVEQHQNNRRRRGPVRRRIQRGNPGRTTSQTDNEQPQGETAQKRTPR  332
            +AGK ++L++                GP      R  PG T                R+PR
Sbjct  248  QAGKDVTLRD---------------GP------RSPPGAT----------------RSPR  270

Query  333  RKGRFVNRRRRTTKSEGDQSGVEAGAKTDDRKLPEAGTDERRSKKNKNSDFCIKLTNLSR  392
                                       D   PE G+    S         +++ NL R
Sbjct  271  ----------------------------DLAPPELGSVPLSS---------VQIGNLPR  292

Query  393  DIRVKDLKSELRKRECTPMSISWKGHFGKCFLHF  426
            D RV +LK  L      P  ++W+G     FLH+
Sbjct  293  DARVSELKRALSALGVAPSRLTWQGPQHGAFLHY  326


 Score = 15.8 bits (29),  Expect = 4.4, Method: Compositional matrix adjust.
 Identities = 5/19 (26%), Positives = 9/19 (47%), Gaps = 0/19 (0%)

Query  41   TWKKIQEGKVGIGFNNIFN  59
            TW  ++ G    GF   ++
Sbjct  443  TWSLLETGSSYSGFGASWS  461
```

Figure 1: MTHFS and Winn homology. This figure presents a protein analysis between MTHFS (Query) and Winn (Sbjct) which presents the conserved regions of interest. Circled region displays the conserved RNA binding motif (RNP-1).

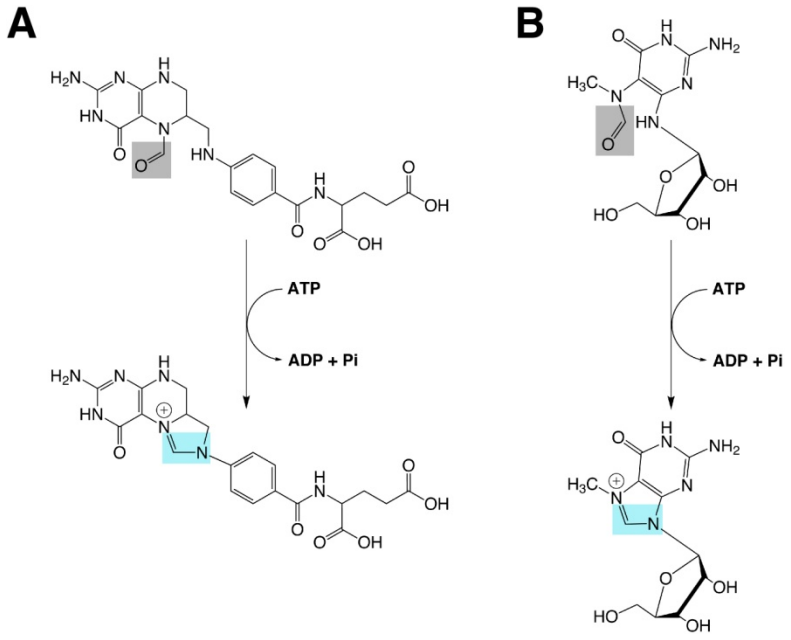


Figure 2. Hypothesized cyclo-ligase activity. A) Depiction of MTHFS catalyzing the ring closure of 5-formyltetrahydrofolate to form 5, 10-methenyltetrahydrofolate. B) Depiction of our hypothesis of how Winnebago mimics the ring closure abilities of MTHFS to regenerate a 5' cap.

II. Materials and Methods

Extract preparation

Drosophila embryo extracts were prepared by collecting 0 to 15 hour embryos as previously described in Gunawardane et al. (2001) but with the following modifications. Dechorionated embryos were resuspended 1:2 (weight/volume) with *Drosophila* extract buffer (DXB: 25mM Hepes [pH6.8], 150mM KCl, 1mM $MgCl_2$, 1 mM DTT, 125 mM sucrose) containing 10ug/ml aprotinin, leupeptin, pepstatin, and 1 mM phenylmethanesulfonyl fluoride. The extracts were homogenized with a sonicator and centrifuged at 10,000 x g for 15 min at 4C in an ultracentrifuge. The supernatant was stored at -80C.

Immunoprecipitation

125 µl of protein A agarose beads (GIBCO BRL) was washed six times with PBS (137 mM NaCl, 2.6 mM KCl, 10 mM $NaHPO_4$, 1.7 mM KH_2PO_4) + 0.1% Triton X-100 (PBST)). 50 µg of anti-Winn antibody was added to the beads in a final volume of 300 µl and mixed for 30 min at room temperature. The beads were washed once with PBST, followed by two washes with 0.2 M sodium borate, pH 9.0. Dimethyl pimelidate was then added to a final concentration of 20 mM and the sample mixed for 1 h at room temperature. The beads were washed three times with 0.2 M ethanolamine, pH 8.0, and mixed for 2 h at room temperature. The beads were pre-eluted with three 1-ml washes of 100 mM glycine, pH 2.5, followed by three washes with DXB.

Fresh 10 µg/ml leupeptin, pepstatin, and 1 mM PMSF were added to 1 ml of frozen *Drosophila* extract (~10 mg/ml) and centrifuged at 10,000 x g for 5 min in a microcentrifuge. 850 µl of supernatant was immunoprecipitated with 50 µl of antibody-coated beads for 1 h at 4℃ with gentle shaking. The beads were washed four times

with DXB150 containing 10 µg/ml leupeptin, pepstatin, and 1 mM PMSF and then eluted with two 150-µl washes and one 200-µl wash of 100 mM glycine, pH 2.5. For experiments with nucleotide in the buffer, 1mM of ATP and ADP were added. The elutions were neutralized with 200 µl of 0.5 M Hepes, pH 7.6, and any residual beads removed by centrifugation at 10,000 x g for 5 min in a microcentrifuge. The supernatant was precipitated with 1/10 volume TCA in the presence of 20 µg aprotinin as a carrier. The sample was resuspended in sample buffer for further analysis by SDS-PAGE.

Immunoblots

Immunoblot analysis involved electrophoresis of samples on SDS–polyacrylamide gels and then transfer to nitrocellulose by semidry transfer. The membrane was blocked in TBS (20 mM Tris-Cl, pH 7.5, 200 mM NaCl), 0.1% Tween 20, 10% nonfat dry milk, and then incubated with 1:2,000 dilution of anti-Me31B, Winn, Cup, or Yps antibodies in TBS, 0.1% Tween 20, 5% BSA. Protein was detected by chemiluminescence using HRP-conjugated donkey anti–rabbit IgG (Amersham Pharmacia Biotech) or anti-rat IgG diluted 1:2,500 in TBS containing 0.1% Tween 20 and 5% BSA. Film exposure ranged from 5 s to 1 min.

Protein expression

Bovine Winn cDNA was obtained from OpenBio.com and the open reading frame was sub-cloned into the vector pET151 and transformed into BL21 competent cells. Colonies were inoculated overnight at 37°C and diluted to 0.05 OD (read at 600nm) in 2L of Luria broth. This was further grown until 0.2 OD and subsequently transferred to 19°C until 0.5-0.7 OD (read at 600nm) was reached. 500µM IPTG was added per liter to induce expression overnight. Cultures were then transferred into large centrifuge tubes

and spun at 4000 rpm for 15 min at 4°C. Pellets were flash frozen with liquid nitrogen and stored at -80°C.

Pellets were resuspended in lysis buffer (50mM Tris-Cl, pH 8.0, 250mM NaCl, 1:1000 BME and protease inhibitor cocktail) and lysed by use of a microfluidizer. Lysates were spun at 15,000 x g at 4°C for 30 min. Supernatant was used for Metal Exchange Chromatography (MEC) which began by adding it to Ni-NTA agarose beads and incubating at 4°C for 30 min. Beads were washed (25mM Tris-Cl, pH 8.2, 150mM NaCl, 1:1000 (40µL) BME) once and eluted (35mM Tris-Cl, pH 8.2, 150mM NaCl, 250mM Imidazole-Cl pH 8.0, 1:1000 (2.5µL) BME) in six 2.5mL fractions. Bradford assays were performed to determine the protein concentration of fractions. SDS-Page stained with was used to ascertain that the protein was of the correct size and to determine the most concentrated elution fractions.

The highly concentrated elution fractions were pooled, diluted 1:5 in dilution buffer (25mM Hepes, pH 7.2, 1:1000 BME), and added to Sepharose beads for Ion Exchange Chromatography (IEC). Beads were washed (25mM Hepes, pH 7.2, 50mM NaCl, 1:1000 BME) and eluted with (25mM Hepes, pH 7.2, that contained increasing concentrations of NaCl beginning with 100mM and ending with 1:1000 dilution of 1M BME. SDS-Page was stained with Coomassie Blue to determine the highest concentration elution fractions.

Enzymology

A master Mix of buffer (1M Hepes, pH 7.2, 1mM $MgCl_2$, phosphate-free water) with or without ATP and substrate at concentrations 3.3, 10, 33, 100, 330µM, and 1mM. This was prepared fresh and placed on ice. The most concentrated elution fractions from Ion exchange chromatography was added to a final concentration of ~3ug. The

reaction was incubated at 37°C. An aliquot of sample (40µl) was extracted at every 10 min interval, starting at 0 min, and added to 40µl of phosphate-free water on ice. Activity was measured by using a PiColorLock Gold detection kit (activity description in Results section), where detection agent and accelerator were added to each time interval sample. These were transferred to 96 well plates and read for absorbance at 595nm with a 96 well plate spectrophotometer. Absorbances were recorded and plotted for comparison.

III. Results

Because Winn protein was identified through mass spectrometry to have association with Tral, we aimed to clarify whether Winn is also part of a RNP. In order to determine if there were any known RNP subunits that were associated with Winn and if that association was ATP dependent, we immunoprecipitated Winn in the presence or absence of nucleotide and immunoblotted for known P body components. Immunoprecipitation was performed on 0-15 hour old *Drosophila* embryo extracts using anti-Winn IgG or control IgG antibody-coupled Protein A beads with *Drosophila* extract buffer in the presence of 1mM ATP, ADP, or no exogenous nucleotide. 0-15 hour embryos have proteins of interest present as opposed to older embryos which begin to decrease expression for particular proteins. Washes were performed using the same nucleotide conditions as the extract. IgG served as a negative control for non-specific binding.

Winn is known to bind specifically to Me31B, Yps, and Imp in the absence of nucleotide. Our data confirmed that Winn is biochemically associated with these known P body components (Figure 3). We next compared immunoprecipitations performed with and without ATP to determine whether the presence of nucleotide would alter the composition of the Winn-associated complex. We found that there was no ATP dependence in the ability of Me31B, YPS or Imp to co-immunoprecipitate with Winn (data not shown).

Because chloride-based buffers can inhibit a number of ATPases (Schultz et al.), we next sought to confirm our results by using an extract buffer where chloride was substituted with acetate salt. Figure 4 shows immunoprecipitation with anti-Winn antibody in the presence of acetate salt containing buffer. Me31B and YPS co-immunoprecipitated with Winn, giving blots identical to our chloride-based blots. These

experiments confirmed our previous findings that Winn co-immunoprecipitates with Me31B, Yps, and Imp in an ATP-independent manner. Furthermore, these results show that Winn is a novel P body component which is associated with a number of highly conserved subunits of the P body.

ATPase activity assay

The first step towards testing whether Winn has any role in RNA repair was to determine whether Winn was an ATPase. Winn proteins from *Danio rerio*, *Xenopus*, and *Bos taurus* (Bovine) were expressed in bacteria in order to identify a recombinant source of Winn that was stable, soluble, and that could be expressed at high levels. Bovine Winn proved to be the most soluble and stable protein when expressed and purified, so it was used as the sole enzyme for the remainder of the experiments.

Purification processes took advantage of both the His-tag sequence from the pET151 plasmid vector on the Winn protein. Consecutive metal exchange chromatography (Figure 5) and Ion exchange chromatography (Figure 6) steps were used to purify His-tagged Bovine Winn protein. SDS-Page was used to confirm purification and determine the most concentrated elution fractions for use in testing Bovine Winn for ATPase activity. In order to assay the ability of Winn to hydrolyze ATP, we developed an assay to detect the release of free phosphate from ATP hydrolysis using the PiColorLock Gold detection agent, as described in Materials and Methods. This particular detection agent was manufactured for assays sensitive for ATPases, Phosphatases, and Nucleotidases. The agent operates by binding a dye complex to any free phosphates present in the solution. Activity was measured with a spectrophotometer set to 595nm in wavelength. Absorbance was recorded and was plotted versus time in a line graph format to determine reaction rates. This agent was

optimal for our test conditions because it directly tests for our hypothesis by binding to free phosphates that would be generated if ATP was hydrolyzed for catalysis of cyclo-ligase. The predicted results are illustrated in Figure 7.

I performed all ATPase assays using Bovine Winn as the enzyme and assay buffer comprised of Na-Hepes, pH 7.2, $MgCl_2$, ATP, and phosphate-free water. To optimize the buffer, I varied the concentrations of $MgCl_2$ and ATP (Figures 8 and 9) to determine the basal ATPase activity present within the Bovine Winn for added nucleotides. These initial procedures revealed that Winn had ATPase activity within the testing parameters.

Over the course of several experiments to optimize basal activity, I noticed that the slope of 1mM ATP reaction varied by almost 50% even though the same prep of enzyme was used. This sudden drop in activity suggested that the protein was rapidly degrading. During the protein purification process I observed that protein from the MEC eluates precipitated out of solution roughly 24 hours after purification. To determine whether it was variability in the purification or sudden degradation of protein affecting the experiment, I repeated the purification procedure. After completing the IEC protocol, the elutates were left on ice at 4C for 4 days to replicate the age at which the sample was previously used in our ATPase assays. These experiments revealed that the Bovine enzyme prepared with Metal Exchange and Ion Exchange Chromatography retained full activity for roughly only 4 days and, moreover, that the enzyme activity varied widely from preparation to preparation.

If Winn acts to close opened forms of the 5’ Cap, we would predict that addition of the closed ring substrates 7-methyl GDP (Figure 10) and NAD (Figure 11) would inhibit the basal ATPase activity of the enzyme. When the ATPase assay was performed in the presence of 7-methyl GDP, however, I observed no change in ATPase

activity. The difference of initial origins of each reaction can be attributed to the phosphates in 7-methyl GDP since higher concentration of substrate lead to a higher origin point. From this, we concluded that the observed ATPase activity is not inhibited by 7-methyl GDP. Given this result, we explored the possibility that Winn might play a role in NAD metabolism since NAD possesses a closed ring structure similar to that of 7-methyl GDP. In order to explore this possibility we tested the ability of NAD to inhibit the Winn ATPase. Figure 11 shows that the addition of NAD does not alter the rate of ATP hydrolysis found in our previous characterization of Winn (Figures 8 and 9; 10µM ATP reactions). These results argued that either our ATPase activity was a contaminant or that neither 7-methyl GDP nor NAD is the substrate for Winn.

To rule out the possibility that our Winn preparations contained a contaminating ATPase, I mutated the predicted ATP binding motif (GWRIGK) of Winn by mutating R to a K. This mutation should inhibit any Winn ATPase and eliminate the basal ATPase activity in our sample. This assay (Figure 12) showed that mutant Winn protein showed no significant difference in ATPase activity compared to the wild type Winn protein (Figure 9).This result indicates that there appears to be a low level of ATPase contamination of our purified protein and that it likely is interfering with the sensitivity of our assay. Thus, we need to develop a better purification scheme in order to provide a definitive test of the hypothesis that either 7-methyl GDP or NAD is a substrate for Winn.

IV. Discussion/Future Experiments

Our immunoprecipitation experiments demonstrated that Winn is biochemically associated with the P body and that that association is not ATP sensitive. These results suggest that Winn has a role in the stability, translation, and/or localization of RNA and that its ATPase activity does not play a role in the regulation of assembly of the complex. Future experiments directed at identifying which P body subunits bind directly to Winn would help greatly in understanding the role of Winn in P body function.

The use of Metal Exchange Chromatography and Ion Exchange Chromatography to purify Bovine Winn protein has allowed us to generate large amounts of a highly pure and stable form of Winn enzyme. However, two technical difficulties have limited our ability to study the enzymatic activity of Winn. First, the precipitation of Winn out solution four days after MEC purification presented us with the problematic issue of stability. Either the chromatography protocol or the elution buffer components appear to cause the protein to become unstable. A systematic study of different buffer conditions to identify a storage buffer that can be used to stabilize Winn would greatly advance our efforts.

The second major technical hurdle that will need to be overcome in future work is to refine our purification procedure so that all contaminating ATPases are removed from the preparation. The ability of either 7-methyl GDP and NAD to inhibit the basal ATPase of Winn was our first major test of the hypothesis that Winn may act as an enzyme in RNA repair catalyzing closing of rings on RNA biochemical structures to facilitate the long-term storage of RNA. Thus, the failure to obtain Winn free of contaminating ATPases has hampered our studies and ability to test hypothesis. Refining the purification scheme with additional chromatography steps will help address this problem and allow us to test our hypothesis.

Another concern associated with our enzymology assays is the sensitivity of the PiColorLock Gold detection agent. It is an ideal agent because of its sensitivity for our primary interests: ATPase, Phosphatase, and Nucleotidase. However, it may not be sensitive enough for our purposes. The threshold of the agent can be interpreted as concentration of phosphates due to hydrolysis or as ATPase rate. Since Winn enzymology activity is hypothesized and barely characterized, ATPase activity may be occurring at rate undetectable by the agent. The opposite is also possible. Addition of enzyme to the reaction may induce an immediate reaction, which could also explain why we have only witnessed insignificant rates of ATPase.

Many variables exist to complicate the experiment, but one solution stands to solve many at once i.e., crystallography. With little to no previous documentation of Winn function, obtaining a crystal structure will provide information regarding what conditions stabilize it and what factors are needed to do so. This should immediately assist us in working toward decreasing the observed precipitation after MEC and may prolong enzymatic activity past four days. A crystal structure should also help pinpoint the true catalytic ability of Winn and point us towards a potential substrate candidate. In the immediate future, further Winn protein purification needs to be explored, as well as development of further open ring candidate substrates, for us to directly test the RNA repair hypothesis.

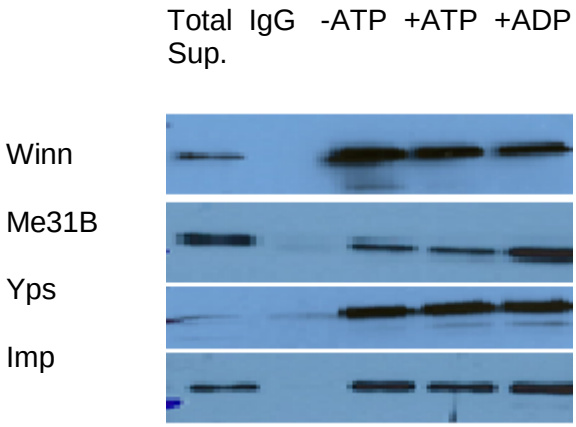


Figure 3: IP of Winn using DXB150 with Chloride. Immunoprecipitation of Winn using DXB150 with chloride salt and 1mM of nucleotides. Winn blot is the positive control while IgG lane is the negative control. Specific co-immunoprecipitation is shown in this immunoblot with RNP subunits Me31B, Yps, and Imp. However, after numerous reproductions, we concluded that blots with nucleotides proved to have no sensitivities to nucleotide addition. Figure represents clearest experiment performed but results were factored from an average of all reproductions.

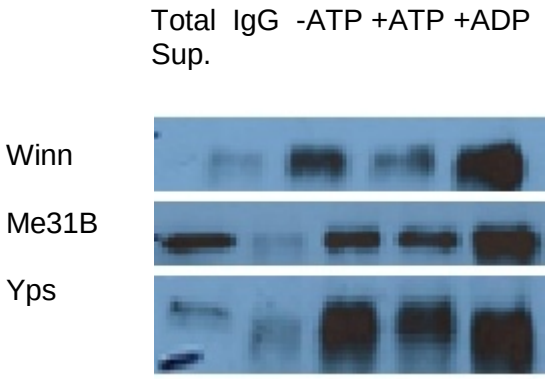


Figure 4: IP of Winn using DXB150 with Acetate. Immunoprecipitation of Winn using DXB150 with acetate salt and 1mM nucleotides. To test ATPase inhibition by chloride, acetate was substituted for chloride. Even though acetate increased the amount of non-specific binding, we can still conclude that the results mirrored figure 1 in which subunits Me31B and Yps co-immunoprecipitated with Winn.

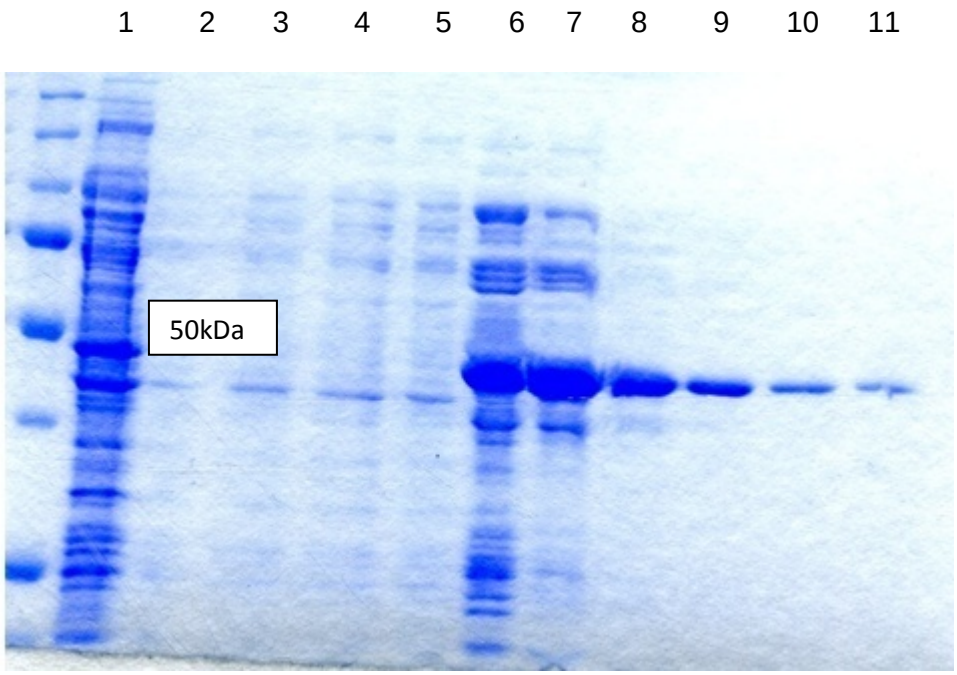


Figure 5: Metal Exchange Chromatography Purification. This 12% polyacrylamide gel depicts the results from metal chromatography. The lane contents are as follows:

1. Pre-Ni-NTA bead sample
2. Post Ni-NTA bead sample
3. Wash
4. Wash
5. Wash
6. Elution 1
7. Elution 2
8. Elution 3
9. Elution 4
10. Elution 5
11. Elution 6

This purification took advantage of the His-tag derived from the pET151 vector. Elution 1, 2 and 3 were combined for further purification through Ion exchange chromatography.

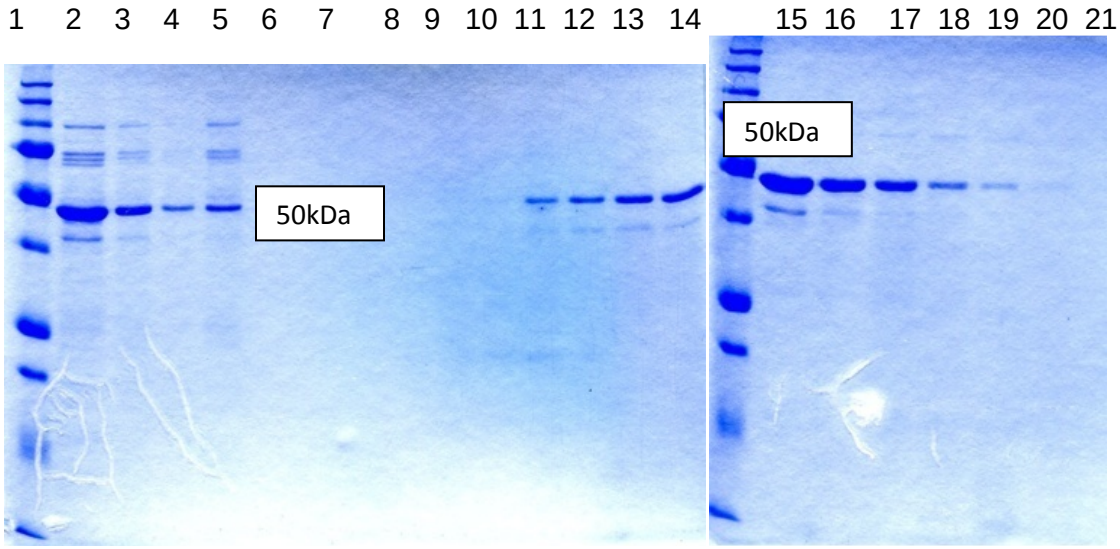


Figure 6. Ion exchange chromatography purification. IEC purified MEC eluates even further by taking advantage of Bovine Winn's ionic characteristics. The lane contents are as follows:

1. Pre-Pooling
2. 1:5 Dilution
3. Sepharose flow through
4. Sepharose flow through
5. Wash
6. Elution A1 (100mM NaCl)
7. Elution A2 (100mM NaCl)
8. Elution B1 (200mM NaCl)
9. Elution B2 (200mM NaCl)
10. Elution C1 (300mM NaCl)
11. Elution C2 (300mM NaCl)
12. Elution D1 (400mM NaCl)
13. Elution D2 (400mM NaCl)
14. Elution E1 (500mM NaCl)
15. Elution E2 (500mM NaCl)
16. Elution F1 (600mM NaCl)
17. Elution F2 (600mM NaCl)
18. Elution G1 (750mM NaCl)
19. Elution G2 (750mM NaCl)
20. Elution H1 (1M NaCl)
21. Elution H2 (1M NaCl)

IEC proved to be quite powerful for purifying the protein. There is substantial decrease in contaminant bands as seen in Figure 4 with MEC. The fractional dilution executed revealed that Bovine Winn elutes at ~500mM NaCl.

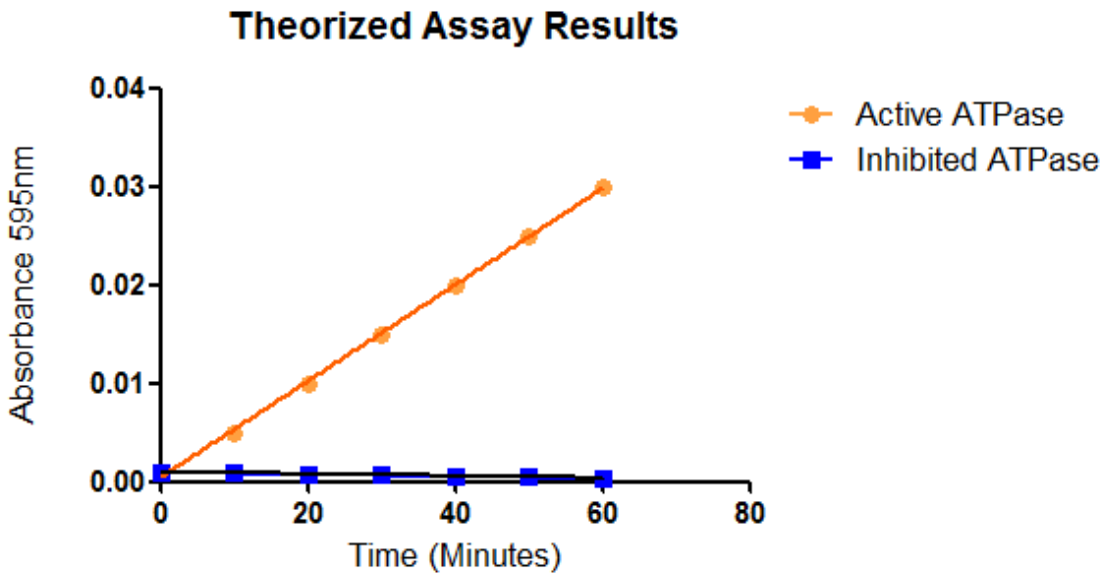
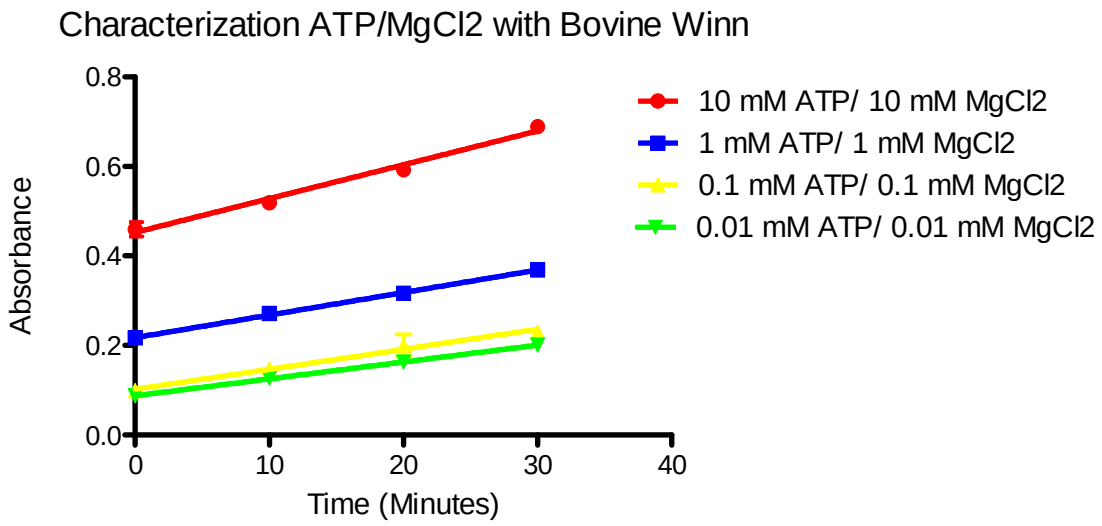


Figure 7: Theorized Assay Results. Expected enzymatic results in which Active ATPase has an obvious slope which represent ATPase activity. Inhibited ATPase should provide a graph with a small or 0 value slope which represents little or no activity.

A

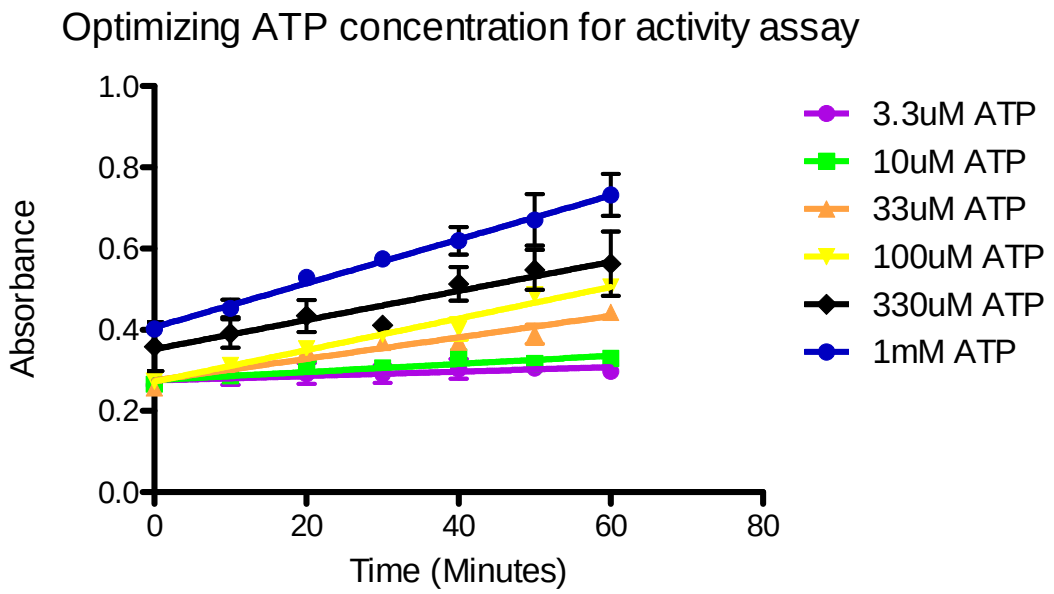


B

10 mM ATP/ 10 mM MgCl2	**1 mM ATP/ 1 mM MgCl2**
0.0075 ± 0.0006	0.0050 ± 0.0002
0.1 mM ATP/ 0.1 mM MgCl2	**0.01 mM ATP/ 0.01 mM MgCl2**
0.0044 ± 0.0005	0.0037 ± 0.0001

Figure 8: Characterization ATP/MgCl2 with Bovine Winn. A) To optimize buffer conditions, four different concentrations of ATP and $MgCl_2$ (10mM, 1mM, 0.1mM, and 0.01mM) were used along with purified Bovine Winn and compared. Graph illustrates that 1mM of ATP/MgCl2 had optimal level of activity and background. B) Slope values of the given testing perimeters show the increase of activity which results with the increase of added nucleotide and MgCl2.

A.

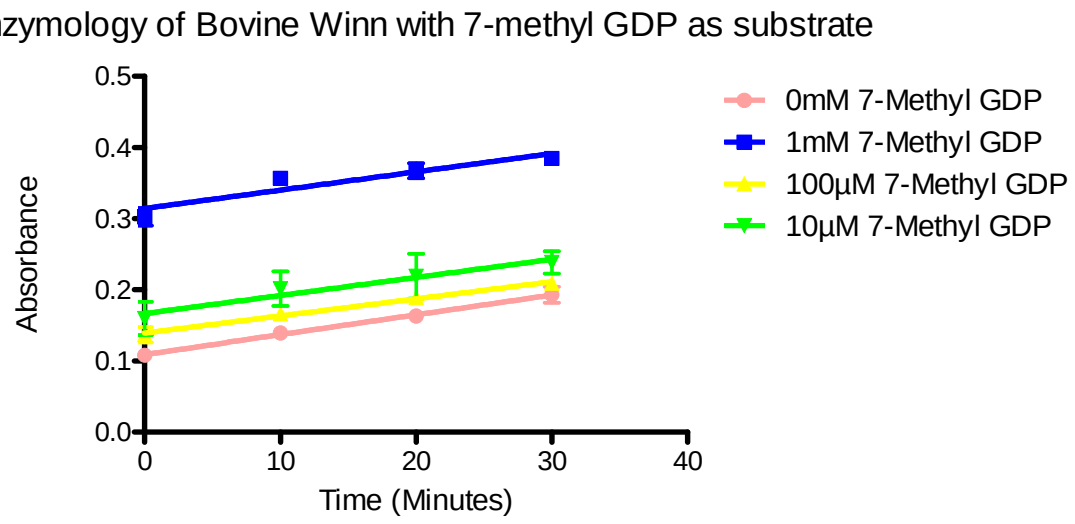


B.

3.3µM ATP	10µM ATP	33µM ATP
0.0005 ± 0.0003	0.0009 ± 0.0002	0.0026 ± 0.0002
100µM ATP	330µM ATP	1mM ATP
0.0038 ± 0.0002	0.0035 ± 0.0007	0.0054 ± 0.0005

Figure 9: Optimizing ATP concentration for activity assay. This data displays our attempt to determine how to minimalize intrinsic ATPase of Bovine Winn. This was performed with 1mM $MgCl_2$ and varying ATP concentrations from 0 minutes to 60 minutes in presence of Bovine Winn. Concentrations above 33µM proved to significantly increase background. B) Slope values displayed the basal ATPase activity inherent to the testing buffers and enzyme without presence of substrate.

A.

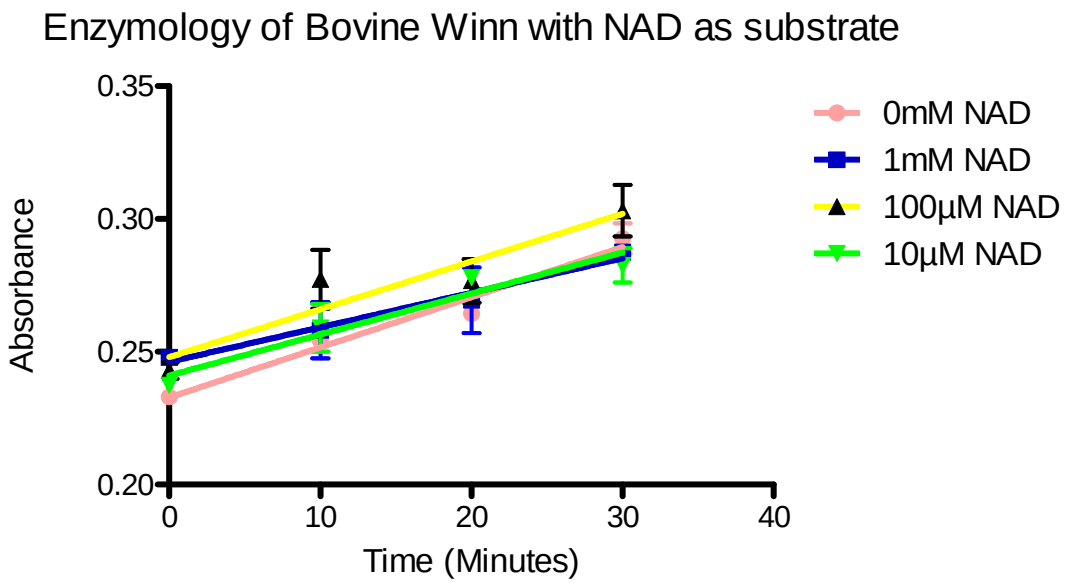


B.

0mM 7-Methyl GDP	1mM 7-Methyl GDP
0.0027 ± 0.0002	0.0025 ± 0.0004
100µM 7-Methyl GDP	10µM 7-Methyl GDP
0.0024 ± 0.0003	0.0025 ± 0.0009

Figure 10: Enzymology of Bovine Winn with 7-methyl GDP as substrate. A) A substrate assay performed with 7-methyl GDP as substrate and buffer containing 10µM ATP, 1mM $MgCl_2$, and Bovine Winn was done in order to determine if any ATPase activity results. Numerical values and graphical data revealed neither a significant difference in ATPase between the different substrate concentrations nor any inhibition activity. B) Slope values of the substrate activity with Bovine enzyme display similarity amongst all testing conditions.

A.

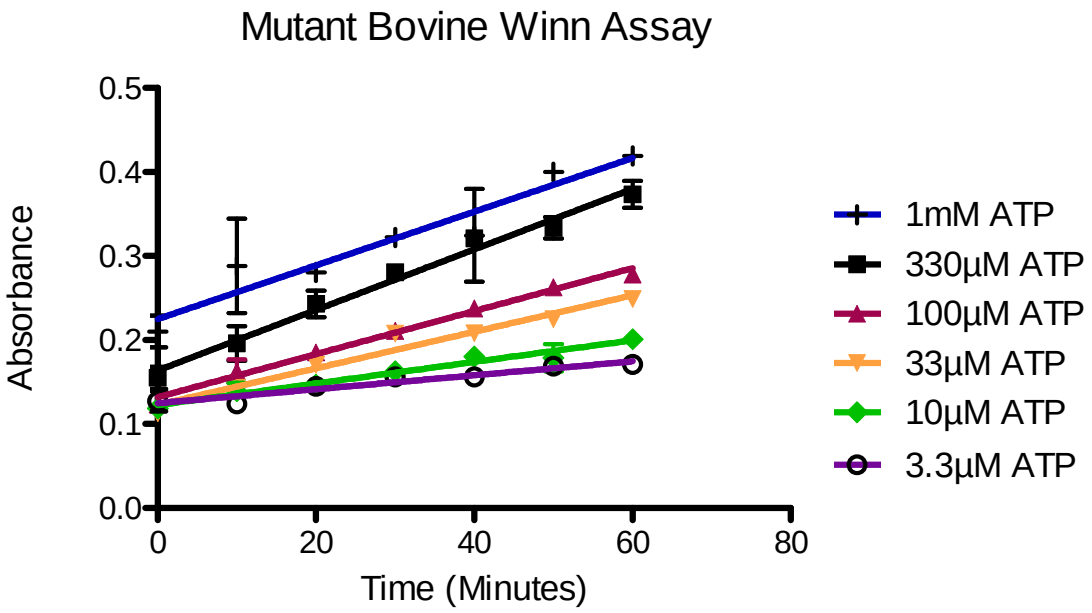


B.

0mM NAD	1mM NAD
0.001888 ± 0.0001786	0.0013 ± 0.0003123
100µM NAD	10µM NAD
0.001799 ± 0.0004085	0.001543 ± 0.0002597

Figure 11: Enzymology of Bovine Winn with NAD as substrate. A) Substrate assay with NAD as the substrate and buffer including 10µM ATP, 1mM $MgCl_2$, and Bovine Winn. This test showed no significant difference in ATPase between the different substrate concentrations or any inhibition. In fact, the rates mirrored previously noted 10µM ATP reactions. B) Slopes values of the substrate activity with Bovine Winn.

A.



B.

1mM ATP	330 µM ATP	100µM ATP
0.0021 ± 0.0002	0.0022 ± 0.0002	0.0019 ± 0.0001
33 µM ATP	10 µM ATP	3.3µM ATP
0.0013 ± 0.0001	0.0003 ± 0.0001	0.0002 ± 0.0001

Figure 12: Mutant Bovine Winn assay without use of any substrates. A) The GWRIGK region of full length Bovine Winn protein was mutated (R into K) to theoretically eliminate all ATPase activity. However, the apparent ATPase activity of mutant Bovine Winn resembles that from previous non-mutated enzyme assays. Reaction parameters include 10µM ATP, 1mM $MgCl_2$, and mutant Bovine Winn. B) Slope values of the mutant protein which displays strong similarity to non-mutant enzyme assays.

Chapter Two

Identification of candidate *tral* targets

I. Introduction

Trailer Hitch, a RNP complex component that localize to the ER exit sites in the nurse cells and oocyte of the *Drosophila* egg chamber. Disruption of *tral* has shown defects in trafficking of both Grk, a ligand for signaling dorsoventral patterning, and Yolkless (Yl), the vitellogenin receptor. Grk and Yl normally localize to the dorsal-anterior region and the plasma membrane of the oocyte, respectively. However, both Grk and Yl accumulate in the ER in *tral* mutant egg chambers (Wilhelm et al. 2005).

Interestingly, two subunits of the E2F transcription factor have been found to phenocopy a *trailer hitch* mutant: e2f2 and Dp. The E2F/Dp complex is known to regulate cell cycle progression via transcriptional repression of critical DNA replication factors (Cayirlioglu et al. 2003). This results in a block in DNA replication and repression of the cell cycle. Surprisingly, E2f2 and Dp mutants also disrupt Grk signaling. This *grk* defect is similar to that observed in *tral* mutants with Grk protein accumulating in large puncta in the ER. *tral* mutants are also phenocopied by mutations in *rhino* (*rhi*). Rhino is a chromodomain transcription factor whose orthologs in other systems are known to form complexes with E2f/Dp (Myster et al. 2000). Together these results suggest that Tral and E2f/Dp may have overlapping targets.

Our lab used an immunoprecipitation microarray strategy to identify mRNAs that were biochemically associated with Tral. This screen identified ~300 candidate Tral targets. Among the most promising candidates were subunits of the Origin Recognition Complex (ORC). In particular, Orc5 was previously identified as a target of E2f2/Dp in *Drosophila* (Cayirlioglu et al. 2005)

ORC is a multiprotein complex directly involved in replication initiation and silencing; however, the complex has been found to have additional roles outside of DNA replication. For instance, it acts to coordinate processes of mitosis and cytokinesis to ensure proper distribution of the replicated genome to daughter cells. It has also been located at high expression concentrations in the nervous system especially at neuronal somatodendritic compartments and membrane fractions. Experiments performed on mouse brains revealed that ORC core subunits, *Orc*2-5, act together as a complex to regulate dendrite branching and form post-mitotic neurons (Huang at el. 2005). Orc2 and Orc6 also have an association with the mitotic spindle midzone, cleavage furrow, and centrosomes. Both subunits are needed for coordination of major cytoskeletal reorganization processes to achieve proper division of the cytoplasm and accurate transmission of daughter cell genome (Prasanth et al. 2002, 2004; Chesnokov et al. 2003). Furthermore, o*rc*3 has been shown to localize at the *Drosophila* neuromuscular junction and *orc*3 mutants show impaired development and function of the neuromuscular junction. ORC's known functions in mitosis and cytoskeletal arrangements thus sparked our interest because the processes disrupted are similar to those observed in *tral* phenotypes.

The *Caenorhabditis elegans* (*C. elegans*) ortholog of Tral, Car-1 also has similar phenotypes to those of *tral*. Mutations in *car-1* disrupt cytokinesis and membrane trafficking in *C. elegans* embryos (Squirrell et al. 2006). Normally, the cleavage furrow accumulates localized membrane factors; however, mutation of *car-1* results in cytokinesis failure where the cleavage furrow ingresses and regresses, the spindle midzone fails to form, and the ER is disorganized. Car-1 protein localizes to P body-like structures, in *C. elegans* suggesting that a link can exist between RNA membrane trafficking and cytokinesis (Squirrell et al., 2006). The ER morphology defects observed

in *car*-1 mutants resemble those observed in *tral* mutants in *Drosophila* . This suggests that the regulation of membrane trafficking is conserved in which *tral* has roles in both ER and membrane trafficking.

Given the membrane functions of o*rc5* and the fact that E2f/Dp regulates transcription and *orc5* has ER phenotypes similar to those of *tral*, we hypothesized that *orc5* is a target of *tral*. Using eggshell ventralization as an assay, I tested multiple alleles of *tral* for genetic interactions with *orc5*. Eggshell ventralization is an excellent assay for *tral* function since decrease of *tral* results in decrease of *grk.* This proportional relationship causes eggs to ventralize which is highly sensitive to changes in *grk* function and easily scored. If *orc5* is a target of *tral*, we predict a *tral* and *orc5* double mutant to increase egg ventralization.

II. Materials and Methods

The following set of crosses were performed in order to generate the w; *tral*^{KG8052}/MKRS; *orc5*^{CG7833}/cyo stock:

1) $\frac{tral}{\mathrm{TM3,Sb}}$ (virgin) X w; $\frac{\mathrm{Xa}}{\mathrm{Cyo}}$; $\mathrm{TM6b, Tb, Hu}$ (male)

2) w; $\frac{orc5}{\mathrm{Cyo}}$ (virgin) X w; $\frac{\mathrm{Sco}}{\mathrm{Cyo}}$; $\frac{\mathrm{MKRS}}{\mathrm{TM6b,Tb,Hu}}$ (male)

Generation two is comprised of virgins with the phenotypes Tb, Hu, Cyo from cross #1 and males with Sco and Sb from cross #2 :

w; $\frac{tral}{\mathrm{TM6b}}$; $\frac{+}{\mathrm{Cyo}}$ (virgin) X w; $\frac{orc5}{\mathrm{Sco}}$; $\frac{+}{\mathrm{MKRS}}$

Lastly, males from generation two with the CyO were mated back with a tra1 allele.

$\frac{tral}{\mathrm{TM3,Sb}}$ (virgin) X w; $\frac{tral}{\mathrm{MKRS}}$; $\frac{orc5}{\mathrm{CyO}}$

Females with non-CyO and non-Sb (w; *tral*^{KG8052}/ *tral*^{KG8052}; +/*orc5*^{CG7833}) were used to test for gene enhancement or suppression. This progeny was crossed with control yellow white (YW) males and the eggs were collected on grape agar plates. Eggs were examined under a microscope for dorsal appendage defects.

III. Results

If *orc5* is a mRNA target of *tral*, one would predict that *orc5* mutations would enhance the dorsal-ventral phenotype observed in *tral* mutants. Phenotypes would be consistent with decreased Grk ligand and show ER exit site To test this prediction, I generated strains that were heterozygous for both *tral* and *orc5.* Eggs were collected and grouped based on observed appendage phenotypes showing 0, 1, or 2 appendages. Eggs laid by the double heterozygote females displayed a high level of egg shell ventralization (29.8% had 0 or 1 appendages) (Table 2), suggesting that o*rc5* and *tral* interact genetically. In order to rule out possible interactions between the mutants and the balancer chromosomes, we also tested the genetic interaction in the absence of balancers. This led to the observation of significant drops in the level of egg shell ventralization, but not to 0%. A relatively high percentage of 9.7% indicates that most of the genetic interaction is between the mutants and the balancers. Interestingly, we found that *orc5* alone produced 5% ventralization. Therefore, *orc5* has a dominant effect on egg shell ventralization and this can be enhanced by the presence of mutations in *tral*. This weak genetic interaction suggests that *orc5* may be a target of *tral*; however, further studies with multiple alleles of *orc5* and *tral* will be necessary in order to test this hypothesis.

Based on the results shown in Table 2, I attempted to broaden the scope of the experiment and conduct a large genetic screen to search for other genes that may enhance the *tral* phenotype. The deficiency collection covering chromosome three was ordered from Bloomington *Drosophila* Stock Center. This collection contains a large series of *Drosophila* stocks each with a deficiency on the third chromosome at a certain locus. Sixty stocks were crossed with the *tral* allele e03082, which is a strong *tral* allele,

to obtain the genotype of Df#/e03082 (Table 3). Unfortunately, none of the deficiencies I tested provided any dominant enhancement of the *tral* ventralization phenotype.

IV. Discussion/Future Experiments

In the first portion of the research in this chapter, I set out to probe for evidence for interaction between *trailer hitch* and *orc5.* In crossing a *trailer hitch* mutant with an *orc5* mutant, a large increase in ventralization defects was observed. Specifically, 29.8% ventralized embryos were obtained, a dramatic increase over *trailer hitch* mutant alone. This suggested a genetic interaction. However, much of the increase in ventralized phenotypes could be attributed to interaction with the balancers (9.7%). The balancers MKRS and Cyo may have multiple unidentified mutations that genetically interact with either *tral* or o*rc5*. Thus, we conclude that only a weak genetic interaction may exist between *tral* and *orc5.* Further study will be necessary to define the nature of the interaction and if it is truly specific. Considering that the balancer chromosomes played a significant role in the analysis, I propose experiment be repeated using different balancers and using different alleles of *tral* and *orc5,* using the same crossing methods to obtain a double mutant and method of egg ventralization scoring phenotype. This should better test for a potential genetic interaction between *tral* and *orc5.*

In the second portion of the research in this chapter, I conducted a broad genetic screen to attempt to locate a deficiency that would enhance *tral* phenotypes. My screen of 60 deficiencies yielded no interactions, i.e., no increase in ventralization phenotypes, suggesting that dominant interactions with *tral* are not at all common. Since this screen was limited to Chromosome III, further screening may identify regions of the *Drosophila* genome that genetically interact with *tral*.

Table 2: *orc5* and *tral* interaction. The table displays the results of egg counts performed on the double mutant and controls. The double mutant w; *tral*^{KG8052}/MKRS; *orc5*^{CG7833}/cyo has a high percentage of egg defects; however, controls also have a high number of defects. This reveals that the balancers are interfering with the genes and doubling defective rates.

Tested Alleles	Number of Normal Phenotypes	Number of Defective Phenotypes	Total	Percentage of Deficiency
$W; \frac{KG8052}{MKRS}; \frac{orc5}{cyo}$	345	147	492	29.8%
$W; \frac{KG8052}{+}; \frac{orc5}{+}$	250	27	277	9.7%
$\frac{orc5}{cyo}$	304	32	336	9.5%
$\frac{orc5}{+}$	992	53	1045	5%
$\frac{KG8052}{TM3,Sb}$	300	5	300	0.02%
$\frac{KG8052}{+}$	300	8	300	0.03%

Table 3: Broad genetic screen with *tral* allele. The table represents every deficiency tested with the *tral* allele e03082. Fused base appendages were not observed while collapsed eggs were found rarely. This chart strongly suggests that an interaction does not exist amongst these deficiencies.

Deficiency Genotype	Total # Scored	# normal appendages	# collapsed appendages	# fused appendage	# 0 appendages	% defect
Df(3R)ED2	190	180	10	0	0	0.052
Df(3R)ED5577	201	201	0	0	0	0
Df(3L)ED4079	144	144	0	0	0	0
Df(3L)ED201	58	58	0	0	0	0
Df(3L)ED4177	119	119	0	0	0	0
Df(3L)ED202	49	49	0	0	0	0
Df(3L)ED4238	64	64	0	0	0	0
Df(3L)ED4256	182	170	12	0	0	0.065
Df(3L)ED4284	89	89	0	0	0	0
Df(3L)ED4288	129	129	0	0	0	0
Df(3L)ED4293	292	282	10	0	0	0.034
Df(3L)ED208	264	248	16	0	0	0.060
Df(3L)ED4341	215	207	8	0	0	0.037
Df(3L)ED210	176	176	0	0	0	0
Df(3L)ED4342	107	103	4	0	0	0.037
Df(3L)ED4408	252	247	5	0	0	0.019
Df(3L)ED4470	149	149	0	0	0	0
Df(3L)ED4475	246	246	0	0	0	0
Df(3L)ED215	360	353	7	0	0	0.019
Df(3L)ED217	338	338	0	0	0	0
Df(3L)ED218	223	223	0	0	0	0
Df(3L)ED220	146	146	0	0	0	0
Df(3L)ED223	75	75	0	0	0	0
Df(3L)ED224	195	189	6	0	0	0.030
Df(3L)ED225	287	277	10	0	0	0.034
Df(3L)ED4786	413	410	3	0	0	0.007
Df(3L)ED4789	261	258	3	0	0	0.011
Df(3L)ED4799	355	353	2	0	0	0.005
Df(3L)ED228	107	107	0	0	0	0
Df(3L)ED4858	96	96	0	0	0	0
Df(3L)ED230	405	402	3	0	0	0.007
Df(3L)ED231	84	84	0	0	0	0
Df(3R)ED5066	104	104	0	0	0	0
Df(3R)ED5095	215	215	0	0	0	0

Table 3 continued.

Df(3R)ED5558	150	150	0	0	0	0
Df(3L)ED4287	126	126	0	0	0	0
Df(3L)ED4502	149	149	0	0	0	0
Df(3L)ED4674	83	83	0	0	0	0
Df(3L)ED4685	24	24	0	0	0	0
Df(3R)ED6090	84	84	0	0	0	0
Df(3R)ED6091	40	40	0	0	0	0
Df(3R)ED10257	46	46	0	0	0	0
Df(3R)ED5021	87	87	0	0	0	0
Df(3R)ED5785	47	47	0	0	0	0
Df(3R)ED5815	29	29	0	0	0	0
Df(3R)ED6255	226	226	0	0	0	0
Df(3R)ED6220	31	31	0	0	0	0
Df(3L)ED4536	30	30	0	0	0	0
Df(3L)ED4416	62	62	0	0	0	0
Df(3R)ED5301	393	393	0	0	0	0
Df(3R)ED5634	180	180	0	0	0	0
Df(3R)ED6237	180	180	0	0	0	0
Df(3R)ED5296	110	110	0	0	0	0
Df(3R)ED5197	249	249	0	0	0	0
Df(3R)ED6187	200	200	0	0	0	0
Df(3L)ED4457	148	148	0	0	0	0
Df(3R)ED6235	358	358	0	0	0	0
Df(3R)ED6052	121	121	0	0	0	0
Df(3R)ED10639	100	100	0	0	0	0

References

Abdu, U., Brodsky, M., and Schüpbach, T. 2002. Activation of a meiotic checkpoint during *Drosophila* oogenesis regulates the translation of Gurken through Chk2/Mnk. Current Biology. 12:1645-19.

Bastock, R., and Johnston, D. S. 2008. *Drosophila* Oogenesis. Current Biology. 18:R1082-23.

Cha, B.J., Koppetsch, B.S., and Theurkauf, W.E. 2001. In vivo analysis of *Drosophila* bicoid mRNA localization reveals a novel microtubule-dependent axis specification pathway. Cell. 106:35-1.

Chekulaeva, M., Hentze, M.W., and Ephrussi, A. 2006. Bruno acts as a dual repressor of oskar translation, promoting mRNA oligomerization and formation of silencing particles. Cell. 124:521-3.

Chesnokov I., Remus D., and Botchan M. 2001. Functional analysis of mutant and wild-type *Drosophila* origin recognition complex. Proceedings of the National Academy of Sciences of the United States of America. 98:11997–21.

Clouse, K.N., Ferguson, S.B., and Schüpbach, T. 2008. Squid, Cup and PABP 55B function together to regulate gurken translation in *Drosophila* . Developmental Biology. 313:713-2.

Dayan, A., Bertrand, R., Beauchemin, M., Chahla, D., Mamo, A., Filion, M., Skup, D., Massie, B., and Jolivet, J. 1995. Cloning and characterization of the human 5,10-methenyltetrahydrofolate synthetase-encoding cDNA. Gene. 165:307-2.

Dillin, A. and Rine, J. 1997. Separable functions of *ORC5* in replication initiation and silencing in Saccharomyces cerevisiae. Genetics. 147:1053-3.

Dreyfuss, G., Kim, V.N., and Kataoka, N. 2002. Messenger-RNA-Binding Proteins and the Messages They Carry. Nature Reviews. Molecular Cell Biology. 3:195-3.

Duncan, J.E., and Warrior, R. 2002. The cytoplasmic dynein and kinesin motors have interdependent roles in patterning the *Drosophila* oocyte. Current Biology. 12:1982-23.

Falnes, P. O. 2005. RNA repair--the latest addition to the toolbox for macromolecular maintenance. RNA Biology. 2:14-1.

Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., and St Johnston, D. 1994. Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell. 79:1221-7.

Fischer, N., and Weis, K. 2002. The DEAD box protein Dhh1 stimulates the decapping enzyme Dcp1. EMBO Journal. 21:2788-11.

Ghabrial, A., and Schüpbach, T. 1999. Activation of a meiotic checkpoint regulates translation of Gurken during *Drosophila* oogenesis. Nature Cell Biology. 1:354-6.

Hou, Y.C., Chittaranjan, S., Barbosa, S.G., McCall, K., and Gorski, S.M. 2008. Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during *Drosophila* melanogaster oogenesis. The Journal of Cell Biology. 182:1127-6.

Huang , Z., Zang, K., and Reichardt, L.F. 2005. The origin recognition core complex regulates dendrite and spine development in postmitotic neurons. The Journal of Cell Biology. 170:527-4.

Huang, Z., Zang, K., and Reichardt, L.F. 2005. The origin recognition core complex regulates dendrite and spine development in postmitotic neurons. The Journal of Cell Biology. 170:527-4.

Imataka, H., and Sonenberg, N. 1997. Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. Molecular and Cellular Biology. 17:6940-12.

Jolivet, J., Dayan, A., Beauchemin, M., Chahla, D., Mamo, A., and Bertrand, R. 1996. Biochemical and Molecular Studies of Human Methenyltetrahydrofolate Synthetase. The Oncologist. 1:248-4.

Kato, I., Matsunaga, F., Miyazaki, S., Yin, L., D'Urso, G., Tanaka, K., and Murakami, Y. 2008. Schizosaccharomyces pombe *Orc5* plays multiple roles in the maintenance of genome stability throughout the cell cycle. Cell Cycle. 7:1083-8.

Kuersten, S., Goodwin, E.B. 2005. Linking nuclear mRNP assembly and cytoplasmic destiny. Biology of the Cell. 97:469-6.

Lie, Y.S., and Macdonald, P.M. 1999. Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in *Drosophila* ovarian extracts. Development. 126:4989-22.

Mansfield, J. H., Wilhelm, J.E., Hazelrigg, T., 2001. Ypsilon Schachtel, a *Drosophila* Y-box protein, acts antagonistically to Orb in the *oskar* mRNA localization and translation pathway. Developmental. 129: 197-209.

Martin, K.C., and Ephrussi, A. 2009. mRNA localization: gene expression in the spatial dimension. Cell. 136:719-4.

Myster, D.L., Bonette, P.C., and Duronio, R.J., 2000. A role for the DP subunit of the E2F transcription factor axis determination during *Drosophia* oogenesis. Development. 127:3249-3261.

Nelson, M.R., Leidal, A.M., and Smibert, C.A. 2004. *Drosophila* Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression. EMBO Journal. 23:150-1.

Nielsen, F.C., Nielsen, J., Kristensen, M.A., 2002. Cytoplasmic trafficking of IGF-II mRNA-binding protein by conserved KH domains. Journal of Cell Science 115: 2087-2097.

Palacios, I.M. 2007. How does an mRNA find its way? Intracellular localisation of transcripts. Seminars in Cell & Developmental Biology. 18:163-2.

Smibert, C.A., Wilson, J.E., Kerr, K., and Macdonald, P.M. 1996. smaug protein represses translation of unlocalized nanos mRNA in the *Drosophila* embryo. Genes and Development. 10:2600-20.

Snee, M.J., and Macdonald, P.M. 2009. Dynamic organization and plasticity of sponge bodies. Development Dynamics. 238:918-4.

Sokac, A.M., and Wieschaus, E. 2008. Local actin-dependent endocytosis is zygotically controlled to initiate *Drosophila* cellularization. Developmental Cell. 14:775-5.

Squirrell, J.M., Eggers, Z.T., Luedke, N., Saari, B., Grimson, A., Lyons, G.E., Anderson, P., and White, J.G. 2006. CAR-1, a Protein That Localizes with the mRNA Decapping Component DCAP-1, Is Required for Cytokinesis and ER Organization in Caenorhabditis elegans Embryos. Molecular Biology of the Cell. 17:336-1.

Teixeira, D., Sheath, U., Valencia-Sanchez, M., Brengues, M., and Parker, R. 2005. Processing bodies require RNA for assembly and contain nontranslating mRNAs. RNA. 11:371-4.

Tharun, S., and Parker, R. 1999. Analysis of mutations in the yeast mRNA decapping enzyme. Genetics. 151:1273-4.

Thio, G.L., Ray, R.P., Barcelo, G. and Schüpbach, T. 2000. Localization of gurken RNA in *Drosophila* Oogenesis Requires Elements in the 5′ and 3′ Regions of the Transcript. Developmental Biology. 221:435-2.

Thio, G.L., Ray, R.P., Barcelo, G., and Schu pbach, T. 2000. Localization of gurken RNA in *Drosophila* Oogenesis Requires Elements in the 5’ and3’ Regions of the Transcript. Developmental Biology. 221:435-2.

Volpe, A.M., Horowitz, H., Grafer, C.M., Jackson, S.M., and Berg, C.A. 2001. *Drosophila* rhino encodes a female-specific chromo-domain protein that affects chromosome structure and egg polarity. Genetics. 159:1117-3.

Wang, S. and Hazelrigg, T. 1994. Implications for *bcd* mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. Nature. 369:400-6479.

Wilhelm, J.E., Buszczak, M., and Sayles, S. 2005. Efficient protein trafficking requires trailer hitch, a component of a ribonucleoprotein complex localized to the ER in *Drosophila* . Developmental Cell. 9:675-5.

Wurtmann, E. J., and Wolin, S. L. 2009. RNA Under Attack: Cellular Handling of RNA Damage. Critical Reviews in Biochemistry and Molecular Biology. 44:34-1.

Zaessinger, S., Busseau, I., and Simonelig, M. 2006. Oskar allows nanos mRNA translation in *Drosophila* embryos by preventing its deadenylation by Smaug/CCR4. Development. 133:4573-22.

Zappavigna, V., Piccioni, F., Villaescusa, J.C., Verrotti, A.C., 2004. Cup is nucleoplasmic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate *Drosophila* ovary development. PNAS. 101: 14800-14805.