Characterization of parcas in Drosophila Melanogaster S2 cells supports role in mitochondria morphology & proper particle assembly of trailer hitch and cup, components of a RNP complex involved in RNA localization, is microtubule dependent.

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Publication Date
2008

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Characterization of parcas in Drosophila Melanogaster S2 Cells Supports Role in Mitochondria Morphology & Proper Particle Assembly of Trailer Hitch and Cup, Components of a RNP Complex Involved in RNA Localization, is Microtubule Dependent

A Thesis submitted in partial satisfaction of the requirement for the degree Master of Science in Biology by Morgan Aleese Roth

Committee in Charge;
Professor Michael Yaffe, Chair
Professor Douglas Forbes
Professor James Wilhelm

2008
The Thesis of Morgan Aleese Roth is approved and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2008
DEDICATION

This Thesis is dedicated to my family for their support, motivation, and willingness to listen. The first chapter is dedicated to the members of the Yaffe lab for their helpful critiques despite the early hour, and especially Heath Balcer for answering countless questions, helping me through several research catastrophes, and guiding me through the early months of my research. The second chapter is dedicated to the members of the Wilhelm lab for their all their help.
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>RNAi</td>
<td>interfering ribonucleic acid</td>
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<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
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<tr>
<td>N term.</td>
<td>amino terminus</td>
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<td>C term.</td>
<td>carboxyl terminus</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<td>μL</td>
<td>micro liter</td>
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<td>kilodaltons</td>
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<td>mM</td>
<td>milimolar</td>
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<td>v</td>
<td>volts</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
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<tr>
<td>UV</td>
<td>ultra violet light</td>
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</tbody>
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Mitochondria morphology is a dynamic process regulated by competing fission and fusion components. In an attempt to identify novel proteins responsible for mitochondria morphology, an RNAi screen was performed using Drosophila S2 cells (Yaffé, unpublished). One candidate produced from this screen is parcas. S2 cells
treated with dsRNA for multiple regions of *parcas* display a significant increase in either general mitochondrial fragmentation or mitochondria that are fragmented into aggregated balls. Microscopy and biochemical characterization of the Parcas protein indicates that the protein localizes to the mitochondria. The effect on the mitochondria in the RNAi experiments supports a role in mitochondria morphology, most likely fusion due to the fragmented phenotype, while the association of Parcas with the mitochondria suggests that this role is fairly direct. The nature of this role, however, remains to be seen.

RNA localization is an important regulator of protein expression during *Drosophila* development. Trailer Hitch and Cup are protein components of a RNP complex that mediates RNA localization events during embryogenesis. These RNP complexes localize in particles that line the surface of the ER, or are occasionally seen in small, mobile particles. A previous study has determined that assembly of Exu, another component of this complex, into particles requires intact microtubules. Here, we demonstrate that disruption of the microtubules also results in a reduction of Tral particles, as well as completely immobilizing all Tral particles within live egg chambers. Abnormal Cup particle formation is also observed.
Chapter One

Characterization of *parcas* in *Drosophila Melanogaster* S2 Cells Supports Role in Mitochondria Morphology

I. Introduction

Mitochondria are membrane bound organelles found in all Eukaryotic cells. They primarily function to produce chemical energy in the form of ATP, but are also involved in the regulation of other processes such as apoptosis, metabolism, development, the cellular redox state, and the cell cycle (McBride et al., 2006).

Mitochondria are composed of a double membrane, with the inner membrane highly folded into structures called cristae. These cristae increase the surface area of the inner membrane, thus facilitating increased ATP production via oxidative phosphorylation (Alberts, 2002). Partly due to their structure, mitochondria are believed to have evolved from aerobic cells engulfed by anaerobic cells. This symbiotic relationship was evolutionarily stable due to dramatic increase in ATP available for the cell.

Mitochondria contain a separate circular genome, which encodes about ten percent of the proteins that function in the mitochondria. Mitochondria also have their own protein synthesis machinery, including ribosomes and tRNAs, which provides further support for the Endosymbiosis theory (Alberts, 2002).

Mitochondria associate with the microtubule cytoskeleton, which facilitates their distribution throughout the cell. The number, size, shape, and mobility of the mitochondria is dependent on cell type, and often varies based on the metabolic needs of the cell (Alberts, 2002). In most cell types, the mitochondria are very dynamic, with constantly competing fusion and fission processes responding to multiple biological...
signals. Although the regulators of mitochondria morphology have yet to be worked out, a few key players have already been identified. Among these are the Mitofusins (Fzo1p in yeast) and OPA1 (Mgm1p in yeast), which have been shown to play a role in outer and inner membrane fusion respectively (Cerveny et al., 2007). Yeast fzo1Δ mutants, as well as Mfn1-null or Mfn2-null mouse embryonic fibroblasts, display highly fragmented mitochondria due to a deficiency in mitochondrial fusion (Chen et al., 2003; Hermann et al., 1998). Mouse embryonic fibroblast that were treated with RNAi against OPA1 also showed a significantly fragmented mitochondrial phenotype, while research on yeast containing temperature sensitive mgm1 alleles displayed abnormal fusion intermediates (Chen et al., 2005; Meeusen et al., 2006). In vivo mitochondrial fusion assays confirmed that these yeast mutants were capable of outer membrane fusion, but were not able to fuse fully (Meeusen et al., 2006). Conversely, Drp1 (Dnm1p in yeast) has been shown to play an important role in mitochondrial fission (Cerveny et al., 2007). C. elegans, mammalian cells, and yeast deficient in Drp1/ Dnm1 display an abnormally clumped mitochondrial morphology, indicating an inability for the mitochondria to divide (Bleazard et al., 1999; Labrousse et al., 1999; Smirnova et al., 2001).

In order to ascertain additional genes responsible for maintenance of mitochondrial morphology and distribution, an RNAi screen was performed using Drosophila S2 cells (Yaffe, unpublished). About 7200 genes, enriched for significant homology to either C. elegan or human genes, were targeted for RNAi-mediated depletion, incubated for five days, and then examined for abnormal mitochondrial morphology or distribution. Of the 7200 genes in the screen, 260 genes displayed
altered mitochondrial phenotypes. Most of these genes are involved in processes such as gene expression, RNA splicing, metabolism, nuclear transport, signaling and regulation, or protein turnover. These proteins are likely to affect the levels or activity of proteins involved in mitochondria morphology, and therefore the observed affect on mitochondria would be indirect (Yaffe, unpublished). Several genes with established roles in mitochondria dynamics were also revealed by the screen, including the mitofusin Marf, an OPA1-like gene, and Drp1. Additionally, 18 novel genes with an affect on mitochondria morphology or distribution were identified. The function of these genes remains to be determined.

Among the 18 novel genes identified in the screen was cg7761, named parcas, which had a mitochondrial phenotype of fragmented aggregated balls in the initial screen. This protein contains SH3 binding and coiled-coiled domains, and is an orthologue of the mammalian protein SH3 Binding Protein 5 (Sab). Sab has been shown to interact with c-Jun N-terminal Kinase (JNK), stress-activated protein kinase-3 (SAPK3), and Bruton’s tyrosine kinase (Btk). It is also associated with the mitochondria (Court et al., 2004; Matsushita et al., 1998; Wiltshire et al., 2004). In *Drosophila*, parcas has been implicated in various stages of development, including anterior-posterior patterning during embryogenesis and somatic muscle development, where it acts via regulation of Btk29A and possibly other non-receptor tyrosine kinases (Beckett and Baylies, 2006). The involvement of parcas in mitochondrial dynamics, however, has yet to be explored. Here, I utilized microscopy and biochemical techniques to identify and characterize the role of parcas in mitochondria morphology and distribution in *Drosophila Melanogaster* S2 cells.
II. Materials and Methods

Buffers and Reagents

Concanavalin A - Sigma-Aldrich, St. Louis, MO

MitoTraker Red CMXRos - Invitrogen, Carlsbad, CA

Paraformaldehyde, 32% solution - Electron Microscopy Sciences, Hatfield, PA

HL3 Buffer - 70 mM NaCl, 5 mM KCl, 1.5 mM CaCl2-2H2O, 20 mM MgCl2-6H2O, 10 mM NaHCO3, 5 mM trehalose, 115 mM sucrose, 5 mM Hepes, pH 7.2

DakoCytomation Mounting Medium- Carpinteria, CA

Cellfectin, Invitrogen, Carlsbad, CA

Lysis Buffer - 20mM Hepes-KOH, pH 7.5, 50 mM KCl, 250 mM sucrose, 1mM DTT (added fresh), 1mM PMSF (added fresh)

Sample Buffer - 100mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol

Western Lighting ™ Chemiluminescence Reagent – PerkinElmer, Boston, MA

RNAi and Microscopy

Cultures of Drosophila Schneider (S2) cells (Invitrogen) were propagated as described previously (Clemens et al., 2000; Rogers et al., 2002). Drosophila S2 cells were incubated for five days with dsRNA targeted for the open reading frame, or both the 5’ and 3’ untranslated regions, of the parcas gene. 10-15μL of cells were plated along with 100μL of M3 S2 media on slides previously coated with concanavalin A, and allowed to sit for 1.5 hours. Cells were then incubated with .2μM of Mitotacker Red CMXRos for 30 minutes, and then fixed with 3.2% paraformaldehyde in HL3
buffer for 20 minutes. Microtubules were stained as previously described, except 3% BSA was added to the antibody solutions as a blocking agent (Rogers et al., 2003). Finally, the cells were mounted in DakoCytomation mounting medium, and viewed with a confocal microscope.

**Parcas-GFP Transfection into S2 Cells**

Constructs fusing GFP to the amino or carboxyl terminus of *parcas* were generated using the Gateway system (Invitrogen) and the pMT plasmid as the destination vector (Yaffe, unpublished). The GFP plasmids were transfected into S2 cells by adding .5μg of plasmid, plus 5μL of Cellfectin, to cells sitting in serum free media. After 3-4 hours, the serum free media was replaced with media containing 10% serum, and expression was induced for 24 hours with 25μM CuSO₄. Cells were plated, stained with Mitotracker Red, and viewed using a confocal microscope as described above.

Stable cell lines expressing *parcas*- N terminus GFP or *parcas*- C terminus GFP were generated by transfecting S2 cells (as described above) with these plasmids in addition to a plasmid containing a gene that confers hygromyosin resistance. The S2 cells were then positively selected with hygromyosin for several passages. Of the remaining population, about 20-30% express the *parcas*- GFP plasmids stably.

**Time-lapse Analysis**

S2 cells expressing *parcas*- GFP were plated on glass bottom microwell dishes (MatTek corporation, Ashland, MA) pre-treated with Concanavalin A for 1.5 hours,
stained with Mitotracker Red CMXRos for 30 minutes, then washed three times with M3 S2 Media to remove excess Mitotracker. The cells were then viewed with a confocal microscope, where a picture was taken every minute for 1 hour, or every 30 seconds for 20 minutes, as indicated. Images provided in figures 5-7 include only the indicated portions of these time-lapse image stacks.

Visual Quantification of Protein localization

Z stacks with slices at increments of .5 μm were taken of 100 fixed wild type S2 cells expressing parcas-GFP using a confocal microscope. The total number of protein puncta per cell was counted, as well as the number of puncta that appeared to associate or did not appear to associate with the mitochondria. A protein puncta was considered to associate with the mitochondria if it either overlapped or touched the mitochondria in a least one of the z stack slices. For each cell, a percentage was generated for the number of puncta that associated or did not associate with the mitochondria. Figure 3C portrays an average percent for each category. The process was repeated for 50 cells that had been treated with dsRNA against Drp1 as just described (see figure 3C).

Biochemical Analysis of Protein localization

For subcellular fractionation, Drosophila S2 cells were pelleted, resuspended in cold lysis buffer, and homogenized on ice with an electric homogenizer. The cells were then spun in a centrifuge at 4°C for the following speeds and time:
Table 1. Centrifuge time and speeds for the subcellular fractionation process.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Speed (xg)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Speed Pellet</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>Mitochondria (washed once)</td>
<td>10,000</td>
<td>15</td>
</tr>
<tr>
<td>Intermediate</td>
<td>20,000</td>
<td>20</td>
</tr>
<tr>
<td>High Speed Pellet</td>
<td>100,000</td>
<td>30</td>
</tr>
<tr>
<td>High Speed Supernatant</td>
<td>--</td>
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Each fraction was then resuspended in sample buffer, boiled for a minute, and spun at 14,000 rpm for a minute. A western blot was performed by loading 10μL of each fraction into a 10% or 6% acrylamide gel, and then transferring the proteins onto a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) at 70v for 2 hours. The membrane was blocked for 30 minutes with 5% dry milk in TBST, then incubated overnight with goat anti-GFP, rabbit anti- F1β, or rabbit anti-Parcas diluted 1:1000 in 3% dry milk in TBST. After three washes with TBST, membranes were incubated in anti-goat HRP or anti-rabbit HRP (at 1:2000) for an hour, washed 3 more times with TBST, and then exposed to Western Lighting ™ Chemiluminescence Reagents to view the signal.
III. Results

*Drosophila* S2 cells were treated for five days with RNAi targeted for the open reading frame of *parcas*, stained for mitochondria and microtubules, then viewed with a confocal microscope. These cells displayed a significant increase in abnormal mitochondria phenotypes, specifically fragmented aggregated balls or general fragmentation, as compared to untreated cells (see figures 1 and 2). The same phenotypes were observed for S2 cells treated with RNAi against the 5’ and 3’ untranslated regions of *parcas* (see figures 1 and 2). Immunostaining for tubulin confirmed that the microtubule cytoskeleton structures were still fully intact for all these cells, and that the observed phenotypes were not simply due to a break down of the microtubule structure (see figure 1). The intact microtubule structure also suggests that these cells are otherwise healthy, since a dismantling of the microtubule structure is an indicator of cellular stress and activation of the apoptotic pathway.

200 cells per slide were counted for three slides (600 total) for the untreated and ORF-RNAi samples, and 100 cells per slide were counted for three slides (300 total) for the UTR-RNAi samples. This quantification of phenotypes showed an average decrease in wild type mitochondrial phenotype of 52% for the ORF-RNAi samples, as compared to the untreated samples, along with an average increase of 21.7% in fragmentation and 31% in fragmented aggregated balls (see figure 2). The UTR-RNAi samples showed a similar trend, with an average decrease of 52.8% in wild type phenotypes, and an average increase over the untreated cells in fragmentation and aggregated balls of 25.8% and 27.7% respectively (see figure 2). There was no significant change in clumped mitochondria phenotypes (see figure 3B for an example
of a clumped mitochondria phenotype) between the untreated and RNAi treated samples, with only an average decrease of .7% for both the RNAi treated samples (see figure 2).

Constructs containing parcas fused to GFP on either the amino or carboxyl terminus were stably transfected into Drosophila S2 cells. When expression was induced with CuSO₄ for 24 hours, the Parcas protein forms distinct punctate structures that appear to associate with the outer mitochondria membrane (see figure 3A; Yaffe, unpublished). The expression pattern is the same between the N terminus and C terminus GFP constructs (see figure 3A). Also, the expression pattern remains the same when induced with various concentrations of CuSO₄, including 0μM, 25μM, 50μM, and 100μM, although the number of cells expressing Parcas- GFP is very low and the fluorescence is very dim when no CuSO₄ is added (data not shown). A concentration of 25μM, the lowest concentration with good expression, was used for the remainder of the experiments. There is great variation in the size and number of Parcas punctate structures per cell. When the number of protein structures was counted for 100 wild type cells, there was a range of 4 to 90 puncta per cell, with a average of 20.1 and a median of 10 (see figure 4A).

To further examine the localization of the Parcas protein, 100 wild type cells were examined to determine the percentage of protein puncta that appeared to associate or did not appear to associate with the mitochondria (see materials and methods section for a more detailed explanation of how this was performed). Of the 100 cells counted, an average of 95.6% appeared to associate with the mitochondria, and an average of 4.4 % did not (see figure 3C). Because the mitochondria in Drosophila S2 cells densely
occupy the cell cytoplasm, the question was asked whether the Parcas protein structures would continue to appear to associate with the mitochondria if the mitochondria was collapsed onto one side of the cell. To this end, S2 cells were treated with RNAi against Drp1, which has been previously demonstrated to produce a clumped mitochondria phenotype in *Drosophila* S2 cells (Yaffe, unpublished). The RNAi depletion of Drp1 was very efficient, with about 90% of the cells possessing collapsed mitochondria (see figure 3B, quantification data not shown). 50 Drp1 depleted S2 cells were examined in the same way as described above, and an average of 89.7% of the protein puncta were determined to associate with the mitochondria (see figure 3C). These cells also displayed variation in the number and size of protein structures per cell. The fifty cells examined had a somewhat smaller range of 5-37 protein puncta per cell, as well as a lower average of 15.6, but a slightly higher median of 11 structures per cell (see figure 4B). The considerable variation seen in both the wild type and Drp1 depleted cell populations may account for the rather large standard deviations calculated for the average percentages shown in figure 3C.

To examine the movement of the Parcas protein in live cells, time-lapse images were acquired of S2 cells expressing Parcas-GFP. Images were taken either every minute for one hour or every 30 seconds for 20 minutes. Figure 5 is 19 minutes of an hour time-lapse stack, in increments of 1 minute, which shows enlarged images of a Parcas protein puncta. As expected, the mitochondria are very dynamic, and can be seen fusing, dividing, and moving within the cytoplasm (see figures 5-7 and data not shown). Although the mobility of the punctuate structures varies, most of them are also very dynamic, as evident by the protein followed in figure 5 (see arrow). Here, the
protein spot moves among the mitochondria tubules in a circular motion, associating with the mitochondria in every frame. Figure 6 depicts the first 38 minutes of a one hour time lapse, in increments of two minutes, of a cell that has been treated with dsRNA against Drp1. The clumping of most of the mitochondria onto one side of the cell provides the opportunity to follow the movement of a single puncta along distinct tubules of mitochondria. This protein spot is far less mobile than the one depicted in figure 5, although other protein spots within the same cell moved at a similar pace (data not shown). Again, the protein spot remains associated with the mitochondria in every frame, even during the apparent jump from the lower tubule to the upper tubule seen between minutes 4 to 6. When each minute increment is examined, one can see that the mitochondria tubule descends down until it touches the protein spot, then moves back up with the protein spot associated with it (data not shown). These protein structures also sometimes change in morphology as they move among the mitochondria. Figure 7 depicts 7 minutes of a 20 minute time-lapse stack in increments of 30 seconds. The protein spot indicated by the arrow elongates along an extending mitochondria tubule, and then returns to a more spherical structure. This elongation along the tubule also provides compelling evidence that the Parcas protein has a close interaction with the moving mitochondria tubule.

To supplement the visual data provided by microscopy, biochemical techniques were next employed to determine the localization of the Parcas protein. Wild type S2 cells or S2 cells expressing the Parcas protein fused to GFP were subjected to subcellular fraction as described in the materials and methods section, resulting in low speed, mitochondria, intermediate, high speed pellet, and high speed supernatant
fractions. A western blot using these fractions was performed using antibodies against GFP (see figure 8A). Although there are many non-specific bands, the band that likely contains the Parcas-GFP protein was determined by comparing the wild type and Parcas-GFP lanes for a band that is only present in the Parcas-GFP fractions, and is the appropriate size (see arrows). The band likely containing Parcas-GFP is only present in the low speed, which contains whole cell extract, and the mitochondria fractions. This indicates that the Parcas protein localizes to the mitochondria. The low intensity of the band may be due to the fact that only a portion of the S2 cell population actually expresses the Parcas-GFP construct. To ensure that the subcellular fraction was efficient in isolating mitochondria to the expected fractions, a western blot for F1β was performed using the same fractions as above (see figure 8B). F1β is a mitochondrial protein involved in ATP synthesis, and would therefore be expected to appear only in the low speed and mitochondria fractions. Although some signal is seen in the other fractions, the low speed and mitochondria fractions are heavily enriched for F1β, indicating a decent fractionation process for both the wild type and Parcas-GFP samples.

In an attempt to gain more conclusive biochemical evidence that the Parcas protein is associated with the mitochondria, a western blot was performed on fractionated S2 cells using an antibody against the Parcas protein itself. Unfortunately, the resulting blot contains several bands with strong signals, making it difficult to determine which band contains the Parcas protein. To help determine the correct band, S2 cells were treated with dsRNA that targets the open reading frame of parcas, and then the low speed pellet for these cells was included on a western blot along with the
untreated fractions (see figure 9A). The arrows in figure 9A indicate the two bands that display a significant reduction in signal compared to the other bands in the RNAi lane. One or both of these bands likely contains the Parcas protein. The lower band runs at the expected size of the protein (53.6 kDa), but it is possible that the protein does not run at the expected size, or that the upper band contains an altered form of the protein. Both of these bands, however, are enriched in the low speed and mitochondria fractions, which supports the idea that the Parcas protein is associated with the mitochondria. A western blot for F1β was also performed using the untreated S2 fractions (see figure 9B). The arrow indicates the band containing F1β. Although there is signal present in the high speed supernatant fraction, there is significantly more signal present in the low speed and mitochondria fractions, indicating a satisfactory fractionation of the cells.
IV. Discussion/ Future Experiments

Depletion of Parcas from *Drosophila* S2 cells resulted in a fairly significant effect on mitochondrial morphology. An average of 62.5% of the cells treated with dsRNA against the ORF of *parcas* displayed abnormal phenotypes of either fragmentation or fragmented aggregated balls. This was supported further with an additional set of RNAi experiments that targeted the 5’ and 3’ UTR of *parcas*, for which an average of 63.3% of the cells possessed either completely fragmented mitochondria or mitochondria that had fragmented into aggregated balls (figure 2). The mitochondria phenotype in these RNAi experiments suggests that *parcas* is involved in mitochondria morphology. The fragmentation phenotype indicates a role in fusion, however much work is needed before specific conclusions can be made.

There are also several pieces of evidence that indicate that the Parcas protein is localized at the mitochondria, which supports the hypothesis that parcas is involved in mitochondria morphology. Microscopy of S2 cells expressing Parcas fused to GFP on either the amino or carboxyl terminus shows that the Parcas protein localizes in punctate structures that appear to line the mitochondria membrane. A quantification of these protein puncta indicates that almost all (an average of 95.6%) of these puncta are either on or touching the mitochondria (figure 3C). Furthermore, treatment with RNAi against Drp1 to collapse the mitochondria onto one side of the cell only reduces the average percent of puncta that associates with the mitochondria slightly (figure 3C). This indicates that the protein puncta are specifically localizing with the mitochondria, and do not just appear to associate simply because the mitochondria occupy the cytoplasm so densely. The slight decrease in association with the mitochondria may be
due to the decreased surface area of the collapsed mitochondria, which may interfere with its interaction with the Parcas protein. Further visual evidence that Parcas is associated with the mitochondria comes from time lapse images of live *Drosophila S2* cells that depict punctate structures of the Parcas protein moving and interacting with the mitochondria tubules (see figures 5-7). This visual evidence is supported by two separate western blots. Western blots for the GFP fused to Parcas as well as for the Parcas protein itself suggest that the Parcas protein localizes in the mitochondria fractions, although the many non-specific bands present on both blots makes it impossible to state this conclusively (figures 8-9).

For further verification, a few more experiments could also be performed concerning localization of the Parcas protein to mitochondria. Since treatment of the S2 cells with RNAi against Drp1 did not disrupt the association of Parcas with the mitochondria significantly, it would be interesting to see if the same is true when the cells are treated with RNAi that targets Mitofusin, which is known to cause a severely fragmented mitochondria phenotype. If so, this would provide additional evidence for the mitochondrial localization of Parcas, while also avoiding the issue of decreased surface area caused by clumping the mitochondria. Additionally, co-localization with other organelles, such as Golgi and lysosomes, should be tested to rule out these possibilities. Quantification using other organelles that produce significantly lower percentages of associating structures would provide additional support that the Parcas protein localizes to the mitochondria. More work is also needed on the biochemical data. It may be possible to obtain fewer non-specific bands on the western blots given more time to adjust the procedure and more specific antibodies. A monoclonal antibody
against Parcas may be more effective than the polyclonal antibody used in this procedure.

While the evidence gathered supports a role in mitochondria morphology, most likely fusion, the nature of this role can not yet be determined. Previous research on the function of parcas in Drosophila development suggests that parcas plays a regulatory role as a signaling protein. The Parcas protein has been shown to regulate the Drosophila Tec kinase Btk29A during oogenesis, and is hypothesized to regulate other non-receptor tyrosine kinases during muscle development ((Beckett and Baylies, 2006). Also, the mammalian orthologue, Sab, is known to act as a signaling protein that is a substrate for both JNK and SAPK3, as well as a negative regulator of Btk (Court et al., 2004; Matsushita et al., 1998; Wiltshire et al., 2004; Wiltshire et al., 2002). Furthermore, the Sab protein also localizes to the mitochondria, which would seem to indicate that parcas functions similarly in Drosophila as Sab does in mammalian cells. It would be interesting to see whether depletion of Sab in mammalian cells has the same effect on mitochondria morphology.

If Parcas does function in signal transduction, exactly how this translates into regulation of mitochondria morphology remains to be seen. The association of the Parcas protein with the mitochondria, however, does suggest a fairly direct role. Much more information about the biochemical activity of Parcas, including the identification and characterization of interacting proteins, is needed before any conclusions can be made about how parcas affects mitochondria morphology. It may still be possible that Parcas has an alternate function in mitochondria morphology than that seen in Drosophila development.
RNAi mediated depletion of Parcas expression results in aberrant mitochondrial phenotypes in *Drosophila* S2 cells. Cells were stained with Mitotracker Red, the first column, then incubated with antibodies against tubulin, the last column. The middle column is a merge of the two images. RNAi against the open reading frame of *parcas* (ORF RNAi) resulted in two aberrant mitochondrial phenotypes: fragmentation and fragmented aggregated balls. RNAi against both the 5’ and 3’ untranslated regions of *parcas* (UTR RNAi) results in similar phenotypes. Only the aggregated ball phenotype is shown for the UTR RNAi sample.

**Figure 1.** RNAi mediated depletion of Parcas expression results in aberrant mitochondrial phenotypes in *Drosophila* S2 cells. Cells were stained with Mitotracker Red, the first column, then incubated with antibodies against tubulin, the last column. The middle column is a merge of the two images. RNAi against the open reading frame of *parcas* (ORF RNAi) resulted in two aberrant mitochondrial phenotypes: fragmentation and fragmented aggregated balls. RNAi against both the 5’ and 3’ untranslated regions of *parcas* (UTR RNAi) results in similar phenotypes. Only the aggregated ball phenotype is shown for the UTR RNAi sample.
Figure 2. Phenotype Quantification for S2 cells treated with dsRNA against *parcas* shows increased percentage of cells with mitochondria that are fragmented or in fragmented aggregated balls. Three separate slides were counted for each sample (200 cells per slide for the wild type and ORF RNAi samples, and 100 cells per slide for the UTR RNAi sample), and then an average percent was calculated for each observed phenotype.
Figure 3. The Parcas protein localizes in punctate structures that appear to associate with the mitochondria. A. Parcas tagged with GFP on either the amino or carboxyl terminus was stably transfected into S2 cells, and then these cells were stained with MitoTracker Red. Both the N terminus and C terminus GFP constructs displayed the same expression pattern. B. Stable cell lines expressing Parcas-GFP were treated with RNAi against Drp1, which results in a robust clumped mitochondria phenotype. C. 100 wild type or 50 Drp1 RNAi cells expressing Parcas-GFP were examined (see materials and methods section) to determine the average percentage of protein puncta that appeared to associate with the mitochondria.
Figure 4. The number of protein spots per cell varies greatly. 100 wild type cells (A) or 50 Drp1 RNAi cells (B) expressing Parcas-GFP were counted. The range, average, and median is shown for both the wild type and Drp1 RNAi samples.
Figure 5. Time lapse images show dynamic movement of Parcas protein structures in association with mitochondria tubules. S2 cells expressing Parcas- GFP were stained with Mitotracker Red to visualize the mitochondria. Lower image includes 19 minutes of a 1 hour time lapse, in increments of 1 minute. The yellow box in the top image indicates the area of the original stack that was enlarged (2x scale) below it to better view the movement of the protein spots. The yellow arrow follows the movement of a single protein spot.
**Figure 6.** Collapsing most of the mitochondria onto one side of the cell provides a clearer visual of Parcas moving in association with the mitochondria. S2 cells expressing Parcas-GFP were treated with RNAi against Drp1, and then stained with Mitotracker Red to visualize the mitochondria. Lower image includes 38 minutes of a 1 hour time lapse, in increments of 2 minute. The yellow box in the top image indicates the area of the original stack that was enlarged (2x scale) below it to better view the movement of the protein puncta.
Figure 7. Parcas protein structures are occasionally seen to elongate along a mitochondria tubule, demonstrating a close association. S2 cells expressing Parcas-GFP were stained with Mitotracker Red to visualize the mitochondria. Lower image includes 7 minutes of a 20 minute time lapse, in increments of .5 minute. The yellow box in the top image indicates the area of the original stack that was enlarged (2x scale) below it to better view the movement of the protein spots. The yellow arrow follows the movement of a single protein spot.
**Figure 8.** The Parcas protein localizes to the mitochondrial fractions in an immunoblot for Parcas-GFP A. A western blot for GFP using S2 cells that were either wild type (WT) or expressing a Parcas-GFP construct. A size ladder in KDa is included on the left-hand side. The indicated lanes include the following subcellular fractions:

1) WT-low speed pellet  
2) WT-mitochondria  
3) WT-intermediate  
4) WT-high speed pellet  
5) WT-high speed supernatant  
6) GFP-low speed pellet  
7) GFP-mitochondria  
8) GFP-intermediate  
9) GFP-high speed pellet  
10) GFP-high speed supernatant

Note the presence of additional bands in the Parcas-GFP expressing lanes that are not present in the wild type lanes. These bands are at the appropriate size for the Parcas-GFP protein, and are only seen in the low speed and mitochondria fractions (see arrows).

**B.** A western blot for F1β using S2 cells that were either wild type or expressing a Parcas-GFP construct. The size ladder is in KDa, and the numbered lanes correspond to the subcellular fractions listed above. Arrow indicates the F1β band, according to the appropriate size. Note the enrichment of the F1β protein in the low speed and mitochondria fractions, indicating an effective subcellular fractionation for both the wild type and Parcas-GFP samples.
A. Anti-GFP

B. Anti-F1β
Figure 9. The Parcas protein localizes to the mitochondrial fractions in an immunoblot for the Parcas protein. A. A western blot for the Parcas protein using S2 cells that were either treated with RNAi against parcas or untreated. A size ladder in KDa is included on the left-hand side. The indicated lanes include the following subcellular fractions:
1) RNAi- low speed pellet 4) Untreated – intermediate
2) Untreated- low speed pellet 5) Untreated – high speed pellet
3) Untreated – mitochondria 6) Untreated – high speed supernatant
The two arrows indicate the bands most likely containing the Parcas protein, as indicated by a relative reduction observed in the lane treated with RNAi (lane 1). The lower arrow indicates the band that runs at the expected size for the protein (53.6 kDa). Note the enrichment of both possible bands in the low speed and mitochondria fractions.

B. A western blot for F1β using the untreated fractions. The indicated lanes include the following subcellular fractions
1) low speed pellet 4) high speed pellet
2) mitochondria 5) high speed supernatant
3) intermediate
The arrow indicates the band containing F1β. Note the enrichment of the protein in the low speed and mitochondria fractions, indicating an efficient subcellular fractionation.
Chapter Two

Proper Particle Assembly of Trailer Hitch and Cup, Components of a RNP Complex Involved in RNA Localization, is Microtubule Dependent

I. Introduction

RNA localization is one method to achieve asymmetric protein expression patterns within the cytoplasm of a cell. This involves repression of the mRNA transcript until it is anchored at the proper location within the cytoplasm, thereby ensuring that the protein is only expressed at that location (Wilhelm and Vale, 1993). An asymmetric distribution of RNA molecules can occur several ways. In some instances, the RNA molecule is diffused throughout the cytoplasm, but accumulates at a particular location via trapping the RNA with binding sites specific for the transcript. Alternatively, the transcript can be stabilized only at the intended location, and simply degraded everywhere else in the cell. In other cases, the mRNA is actively transported to the correct location, generally along actin or microtubule filaments (Wilhelm et al., 2005).

Localization of mRNA transcripts plays an important role during Drosophila embryogenesis. In Drosophila, the oocyte develops within an egg chamber that consists of 15 nurse cells interconnected with the oocyte via cytoplasmic bridges called ring canals, all encased in a monolayer of somatic follicle cells (Spradling, 1993). The ring canals provide passage for several proteins and mRNAs that are synthesized in the nurse cells and actively transported to the developing oocyte, which is transcriptionally inactive through much of oogenesis (Theurkauf and Hazelrigg, 1998). Among these are two mRNAs that are important for anterior-posterior patterning of the oocyte: bicoid
(bcd) and oskar (osk). The bcd transcript is transported in a microtubule dependent manner to the anterior cortex. The result is an anterior-posterior gradient of the Bcd protein, which is important for establishment of the anterior pattern of the developing embryo (Theurkauf and Hazelrigg, 1998; Wilhelm et al., 2000). The osk mRNA, on the other hand, is transported to the posterior of the oocyte, and is important for formation of the abdomen and germ cells (Wilhelm et al., 2000).

The osk and bcd mRNAs associate with a ribonuceloprotein (RNP) complex that includes several proteins that are required for the translational regulation or transport of these and other localized transcripts. One such protein is Ypsilon Schachtel (Yps), which is a Y-box protein involved in repression of the osk mRNA via negative regulation of its localization and translation factor Oo18 RNA-binding protein (Orb) (Mansfield et al., 2002). There are also several other translational repressors of osk mRNA, including Me31B. One of these proteins, Cup, is required for both the translational repression and localization of osk mRNA (Wilhelm et al., 2003). Also found in this complex is the protein Exuperantia (Exu). This protein is required for the transport of both the osk and bcd mRNA transcripts to their respective poles (Theurkauf and Hazelrigg, 1998; Wilhelm et al., 2000).

Another member of this ribonucleoprotein complex, Trailer Hitch (Tral), is involved in dorsal-ventral patterning, although not quite so directly. A primary step for the formation of the dorsal-ventral axis is the localization of the mRNA gurken (grk) to the dorsal-anterior region of the oocyte. This results in the specific secretion of the secretory protein Grk from that region of the oocyte, and eventually leads to the formation of the dorsal appendages from the follicle cells in that area (Wilhelm et al.,
Tral is required for the proper secretion of the Grk protein. The *tral* gene was originally identified in a screen for components involved in embryonic axis formation. Eggs from *Drosophila* containing mutation in the *tral* gene either have no dorsal appendages or a single fused dorsal appendage. Tral was determined to be necessary for the correct formation of ER exit sites. Further analysis of this lead to the discovery that the mRNAs *sar I* and *sec 13*, which are both involved in ER exit site formation, are components of the ribonucleoprotein complexes containing Tral. It is believed that Tral regulates Sar I expression, which in turn affects the formation of ER exit sites. Although this also affects other secretory proteins, it seems to have a pronounced affect on Grk secretion, which may be the rate limiting step of that pathway (Wilhelm et al., 2005).

When components of the ribonucleoprotein complex are fluorescently labeled, they localize in either larger particles that seem to line the surface of ER protrusions or occasionally in smaller mobile particles. It is not clear whether the larger particles are accumulations of smaller particles at the ER surface, or whether they are more defined structures. Experiments involving Exu labeled with GFP show that Exu assembly into these particles requires an intact microtubule cytoskeleton (Cha et al., 2001). Here, we try to verify that other members of this ribonucleoprotein complex, specifically Trailer Hitch and Cup, are also dependent on microtubules to assemble into these visible particles. Using time-lapse images of live egg chambers, we also hope to gain visual evidence that will help to determine if these large particles are deposits of smaller particles that accumulate on the surface of the ER via movement along the microtubule cytoskeleton.
**II. Materials and Methods**

**Buffers and Reagents**

Demecolcine- Sigma Alderich, St. Louis, MO

Grace’s Insect Medium, unsupplemented- Invitrogen, Grand Island, NY

Formaldehyde Solution, 37% - Fisher Scientific, Fair Lawn, NJ

Heptane- EMD Biosciences, Darmstadt, Germany

**Microtubule Depolymerization Treatment and Immunofluorescence**

Demecolcine was added to sterile water at a final concentration of 20μg/mL or 50 μg/mL, and then dry yeast was mixed in until it formed a thick paste. Yellow White *Drosophila* or *Drosophila* expressing GFP tagged KDEL receptor (Wilhelm et al., 2005) were starved for two hours in a glass bottle containing a wet Kimwipe. The flies were then transferred to a clean vial containing either a smear of the yeast paste treated with demecolcine (see above) or untreated yeast paste, and allowed to feed overnight. Ovaries were hand dissected and immunostained as previously described, except they were fixed in a Grace’s Insect Medium solution containing 3.7% Formaldehyde and 5% Heptane (Wilhelm et al., 2005). Primary antibodies were used at the following concentrations: rabbit anti- Tral, 1:2000; rat anti-Cup, 1:5000; goat anti-GFP, 1:2000; mouse anti-Tubulin-FITC, 1:200. Secondary antibodies were used at the following concentrations: Donkey anti- rabbit 568, 1:200; Donkey anti-Goat FITC, 1:200. Egg chambers were viewed with a confocal microscope.
**Time lapse Analysis**

Drosophila flies expressing GFP tagged Trialer Hitch were starved for two hours, and then fed either yeast paste containing demecolcine (see above) or untreated yeast paste overnight. The ovaries were dissected as described above, and mounted on a petriPerm 50 hydrophilic membrane (In Vitro Systems and Services, Germany). Egg chambers were viewed with a confocal microscope, where an image was taken every second for 1-3 minutes. Figures 10-11 include the indicated portions of these time-lapse stacks.
III. Results

Yellow White Flies were fed either untreated yeast paste or yeast paste containing 20μg/mL demecolcine, a drug that results in depolymerization of the microtubule cytoskeleton, and allowed to feed overnight. Egg chambers dissected from these flies were immunostained with antibodies against Tral and Tubulin, and viewed with a confocal microscope (figure 10). The tubulin staining revealed fully intact microtubule cytoskeletons in the untreated egg chambers. These egg chambers also possessed intact Tral particles, although these particles could only be seen along the perimeter of the egg chambers stage 7 and older (figure 10, untreated). The younger stages displayed more Tral particles towards the interior of the egg chamber (data not shown for untreated samples). This is presumably due to a failure of the antibody to fully penetrate the older egg chambers. The effect of the demecolcine drug on the treated egg chambers was also not uniform. It seems to only have a significant effect on the older stages, while the younger stages still had clearly visible microtubule structures (figure 10). The egg chambers that were most affected by the drug treatment also displayed a drastic reduction in Tral particles, even along the perimeter. The egg chambers that continued to have an intact microtubule cytoskeleton also had abundant Tral particles throughout (figure 10).

Since the Tral antibody was unable to fully penetrate the older egg chambers, even when multiple concentrations of the antibody were used, we turned next to another member of the RNP complex. *Drosophila* expressing KDEL receptor-GFP were fed either untreated or treated yeast paste, and the egg chambers were immunostained with antibodies against Cup and GFP. The concentration of the demecolcine drug was
increased to 50μg/mL in an attempt to increase the effect of the drug on the younger egg chambers. The younger stages displayed some evidence of the drug treatment, but the effect remained pronounced in the older stages (data not shown). The Cup particle phenotype in the treated samples, however, differed drastically from that seen in the egg chambers stained for Tral. While the Tral particles dispersed so that little or no particles were observed upon treatment with demecolcine, the Cup particles seemed to aggregate into large spherical structures (see figure 12A). These egg chambers possess fewer particles than the untreated egg chambers, but the particles present are much larger. Both the untreated particles and the particles in the drug treated egg chambers co-localize with the ER (see figure 12B). The KDEL receptor- GFP staining revealed that portions of the ER were aggregated into clumps in the drug treated samples. The majority of these ER clumps co-localized with the large Cup particles, but a few did not (figure 12B, see arrow).

We also attempted to determine the effect of the depolymerized microtubules on RNP movements within the nurse cells. *Drosophila* expressing GFP tagged Trailer Hitch were fed either untreated yeast paste or yeast paste containing 50μg/mL demecolcine. The ovaries were then dissected and time-lapse images were taken every second for 1-3 minutes. For the untreated samples, egg chambers of all stages fluoresced brightly with many Tral-GFP particles filling the cytoplasm of both the nurse cells and oocyte (data not shown). Figure 11A includes 8 seconds of a time-lapse stack taken for one of these untreated samples. The image includes a portion of a nurse cell nucleus and the adjacent cytoplasm. The majority of the Tral particles move in a circular, floating-type motion, and are assumed to be associated with the moving ER
(Wilhelm et al., 2005). Unfortunately these movements are difficult to capture in the still pictures of a montage. Occasionally, particles are observed moving rapidly in a single direction, although they tend go out of focus before a destination can be determined (figure 11A, see arrow). These mobile particles are typically on the smaller side of the particle size range. Time-lapse images were also taken for the egg chambers treated with demecolcine. Figure 11B includes 8 seconds of a time-lapse stack for one of these egg chambers. As with the untreated samples, these images include a portion of a nurse cell nucleus and the adjacent cytoplasm. The demecolcine treated egg chambers displayed a dramatic reduction in the number of visible Tral-GFP particles, with only a single bright particle seen in figure 11B. There is also a complete lack of particle movement seen in these egg chambers. This includes both the directional movement of mobile particles and the floating movement of the particles along the ER surface.
**IV. Discussion/Future Experiments**

The proper formation of the RNP particles involved in RNA localization is microtubule dependent. Depolymerization of nurse cell microtubules via treatment with demecoline led to a significant reduction in the number of Tral particles seen in both fixed and live egg chambers (figure 10, 11). Although the effect of the drug was not universal for all of the egg chamber stages, there was a definite correlation between the presence of intact microtubules and the presence of visible Tral particles. While the same particle dispersion was not seen when treated egg chambers were stained for Cup, these egg chambers still experienced a profound effect on particle formation (figure 12). There was a definite reduction in Cup particles, and the particles that remained were abnormally large.

Although the phenotypes observed for the Tral and Cup particles were very different, they are not necessarily contradicting. The dispersal of the Tral particles in the treated egg chambers supports the idea that these particles are accumulations of smaller Tral particles that are transported to the surface of the ER along microtubules. If these particles were fixed defined structures they would be expected to remain after the demecolcine treatment. Cup particles did remain in the treated egg chambers, but they did so in reduced numbers. Also, the presence of the large particles may represent another aspect of this same particle accumulation model. Perhaps the depolymerization of the microtubules affected the dispersal of smaller Cup particles from the ER, causing them to form large aggregates. This would only be the case if the particles were composed of smaller particles.
On the other hand, it is possible that these Cup stained egg chambers were simply in a state of general cellular stress. The ER also experienced a clumping phenotype, which leads to the question of whether the ER or the Cup particles were first to aggregate (figure 12). It is possible that these large Cup particles are due to a clumping of the ER surface of which they are associated, especially since ER clumps can be found, although rarely, that are not co-localized with Cup particles. Even if the Cup particles were first to aggregate, it could still not be a specific effect of the depolymerized microtubules, but a general response to the toxic drug. Lower concentrations of the demecolcine drug should be tried to determine if these phenotypes continue.

Many other experiments remain to be done before any real conclusions can be drawn. The major area to focus on next would be additional time-lapse analysis of live egg chambers. The aggregated particles model would be further supported if evidence could be gained that showed smaller complexes being deposited onto the ER surface. While the time-lapse images of egg chambers expressing Tral-GFP did include small mobile particles moving in a single direction, we were unable to determine the destination of these particles. Perhaps time-lapse images of egg chambers expressing both Tral-GFP (or Cup-GFP) and KDEL receptor-RFP would be helpful in this regard. This would allow us to see if these mobile Tral particles are moving towards the ER, as well as confirm that the larger Tral particles are indeed associated with the ER. This would also be very helpful in determining whether the aggregated Cup particles or the ER were first to clump. Additionally, it would be useful if we could take advantage of the fact that demecolcine is supposed to be deactivated with UV light. We attempted to
deactivate the drug treated egg chambers with the DAPI exposure on the confocal microscope, which should contain UV light, for 15 seconds to 1 minute, but no recovery was observed. Perhaps the DAPI setting did not contain enough UV light, the exposure time was not long enough, or the egg chambers were simply too damaged to recover. If we could successful recover the microtubules in these treated egg chambers, we might be able to visualize the reformation of the Tral particles or the division of the large Cup particles. This would provide additional support that these particles are formed in a microtubule dependent manner, and help distinguish between the possible models for particle formation.
Figure 10. Trailer Hitch assembly into particles requires microtubule cytoskeleton. *Drosophila* were fed either untreated yeast paste or yeast paste containing 20μg/mL demecolcine, then dissected and immunostained for Trailer Hitch (Tral) and microtubules (Tubulin). Note the presence of Tral particles along the perimeter of the untreated egg chamber, stage 8. The demecolcine treated images include egg chambers at stage 6 (left) and stage 7 (right) to demonstrate the differential effect of the drug treatment.
Figure 11. Trailer Hitch particle assembly and movement is dependent on microtubules. Images depict a portion of a nurse cell nucleus (N) and adjacent cytoplasm, and include 8 seconds of a time-lapse stack in increments of 1 second. A. *Drosophila* flies expressing Tral-GFP fed with untreated yeast paste. Note the abundance of Tral particles, as well as the presence of a directionally moving particle (arrow). The rest of the particles appear to float in a circular pattern, and are assumed to be associated with the ER. B. *Drosophila* flies expressing Tral-GFP fed with yeast paste treated with demecolcine. Note the reduction in Tral particles, as well as the complete lack of any particle movement.
Figure 12. Proper Cup particle formation is microtubule dependent. A. *Drosophila* expressing KDEL Receptor -GFP were fed either untreated or demecolcine treated (50μg/mL) yeast paste, and egg chambers were immunostained for Cup and KDEL Receptor-GFP. Stage 8 shown. B. An enlarged portion of the untreated and treated egg chambers. The Cup particles co-localize with the ER in both cases. Not all of the ER aggregates in the treated samples co-localize with a Tral particle (see arrow).
References


