POLYMERASE-MEMBRANE INTERACTIONS IN VIRAL RNA REPLICATION COMPLEX ASSEMBLY

by

Kenneth Arthur Stapleford

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cellular and Molecular Biology)
in The University of Michigan
2009

Doctoral Committee:

Assistant Professor David Miller, Chair
Associate Professor Amy Chang
Associate Professor Kathleen L. Collins
Associate Professor Alice Telesnitsky

2009

ACKNOWLEDGEMENTS

I'd like to thank everyone who made this part of my life possible. I'd like to give special thanks to David Miller for his encouragement and mentoring throughout this long process. His help has without a doubt pushed me to become the scientist that I am today. In addition, I'd like to thank my thesis committee for helpful discussions and suggestions throughout the years.

Finally, I have to thank my friends and family for shaping me into the person I am today. I have met countless amazing individuals who have all been influential in this process and without you this would not be possible. In particular, I want to give thanks to my family for their continued support and always being there for me. I would not be where I am in my life without you all.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

POLYMERASE-MEMBRANE INTERACTIONS IN VIRAL RNA REPLICATION COMPLEX ASSEMBLY

All characterized positive-strand RNA viruses associate with host intracellular membranes to facilitate viral genome RNA replication, yet the molecular mechanisms required for these essential interactions are poorly understood. To study positive-strand RNA virus replication complex assembly and function I used the established model alphanodavirus, Flock House virus (FHV). FHV establishes robust viral RNA replication in the budding yeast *Saccharomyces cerevisiae* where it assembles functional viral RNA replication complexes on the mitochondrial outer membrane. Using this powerful host-pathogen system I took complementary in vitro, genetic, and biochemical approaches to understand the polymerase-membrane interactions necessary for replication complex assembly and function.

To investigate the initial steps in replication complex assembly, I established an in vitro FHV replicase membrane association assay with which we were able to recapitulate many of the in vivo characteristics of FHV biology in vitro. We found the FHV replicase to be a lipid-binding protein that associated with the mitochondrial outer membrane in a TOM complex independent manner via specificity for mitochondrial-enriched anionic phospholipids. In addition, we

preformed a targeted genomic analysis to address the role of yeast mitochondrial outer membrane components in FHV RNA replication. We identified a deletion of the mitochondrial outer membrane protein Mim1 that led to a significant reduction in FHV RNA accumulation, and subsequent biochemical studies revealed a role for Mim1 in FHV replication complex assembly. Finally, we have begun to explore the composition and structure of FHV RNA replication complexes. We have developed a blue-native agarose gel electrophoresis system to identify the presence of two functionally distinct FHV RNA replication complexes in wildtype yeast. Furthermore, we have employed a lipidomic approach to investigate dynamic cellular and membrane-specific phospholipid changes associated with FHV infection and RNA replication. Thus far, we have identified significant FHV induced changes in global and membrane-specific phospholipid levels further implicating an important role for phospholipids in viral RNA replication. Taken together, this thesis provides significant and novel advances in the field of positive-strand RNA virus biology and identifies new avenues of focus for the development of anti-viral therapies targeted towards these viral pathogens.

CHAPTER I

INTRODUCTION

Positive-strand RNA viruses have the ability to inflict devastating damage to their host, which in many cases includes humans. However, the ability to develop effective vaccines and anti-viral therapies targeted towards these viral pathogens is hindered by our poor understanding of the molecular mechanisms involved in viral replication and pathogenesis. Viruses, being obligate intracellular pathogens, maintain an intimate relationship with their host and thus exploit various cellular components and processes to their advantage. One common feature of all positive-strand RNA viruses is the use of host intracellular membranes for the assembly of viral RNA replication complexes, an essential yet poorly understood process in the viral life cycle. Fortunately, the development of a powerful host-pathogen system making use of the budding yeast *Saccharomyces cerevisiae* and the alphanodavirus Flock House virus provides the proper molecular tools required to study these specific virus-membrane interactions. This chapter will provide a detailed background on the biochemistry, cellular, and molecular biology of this host-pathogen system which will be sufficient to begin to understand the

polymerase-membrane interactions involved in RNA virus replication complex assembly.

Clinical Relevance of Positive-Strand RNA Viruses

The world of viral pathogens is large, complex, and ever-expanding. In general, viruses can be divided based on their genetic material which has come to include double-strand and single-strand, DNA and RNA viruses. Among the RNA viruses, one group of increasing clinical and economic importance are those which contain a positive-sense RNA genome. This group of viruses consists of a long list of clinically relevant viral pathogens including; poliovirus, hepatitis C virus (HCV), severe acute respiratory syndrome (SARS)-coronavirus, West Nile virus, dengue virus, and the encephalitic alphaviruses; all of which are capable of causing significant disease in humans.

More importantly, many of these viruses are still prevalent throughout the world today. The Center of Disease Control and Prevention (CDC) estimates that 3.2 million people in the United States are chronically infected with hepatitis C virus with roughly 170 million people infected worldwide (160). In addition, SARS and West Nile virus, capable of causing severe respiratory disease and encephalitis respectively, have emerged in the last decade leading to devastating effects in Asia and the US. Dengue virus has been endemic in tropical and subtropical regions of the world for hundreds of years, and the World Health Organization (WHO) estimates 2.5 billion people live in endemic areas leading to roughly 50 million dengue infections each year (11, 14). The alphavirus, Chikungunya virus, has re-emerged in Southeast Asia over the last decade

leading to a large epidemic in India in 2006 that subsequently spread to Europe and northern Italy in 2007. Finally, although poliovirus was eradicated from the US in 1994 it is still endemic to countries in Africa and the Indian subcontinent where it can cause problems in unvaccinated individuals.

As one can see, these viruses are significant threats and the necessity for proper anti-viral therapies is required for their control. Unfortunately, to date vaccines and anti-viral treatments targeted towards a majority of positive-strand RNA viruses are not available or are inefficient in the complete clearance of the virus allowing for persistant infections to continue. Taken together, it is clear that positive-strand RNA viruses present a severe health risk to many people around the globe and future work is needed to develop effective therapies to control and remove these viral threats.

General Biology of Positive-Strand RNA Viruses

Positive-strand RNA viruses are made up of a single-stranded RNA molecule oriented in the coding sense with a 5' to 3' polarity, similar to cellular messenger RNAs (mRNA). However, unlike cellular mRNA molecules which contain a 5' 7-methylguanosine cap and polyadenylated 3' tail for stablity and efficient translation, viral RNAs do not always require such modifications. Individual viruses have been found to use these cellular modifications or combinations of them as well as specific viral 5' RNA structural motifs called internal ribosome entry sites (IRES) for proper translation of viral proteins (39, 60).

Upon infection of the host cell, the viral genome is released from the infectious virion into the host cytosol where it can quickly make use of the cellular

translational machinery and begin translating viral proteins (Figure 1.1). Positive-strand RNA viruses encode two general types of viral proteins; non-structural (required for genomic replication) and structural (required for virion assembly). The non-structural or replicase proteins encode the viral RNA-dependent RNA polymerase, the viral enzyme required for efficient viral RNA synthesis, as well as any accessory proteins the virus may need for replication. Once translated, the non-structural proteins along with the viral genome are trafficked via what are thought to be host chaperones and cellular co-factors to a host intracellular membrane where they are assembled into a viral RNA replication complex. Once established, the viral replication complex synthesizes a negative-strand RNA template complementary to the positive-strand RNA genome, producing a double-strand RNA intermediate from which genomic replication can proceed. During active viral replication, viruses induce changes in the membrane ultrastructure where they are then able to efficiently replicate multiple copies of the positive-strand genome that can be encapsided by the viral structural proteins to produce more infectious virions.

The association of positive-strand RNA viruses with host intracellular membranes is an essential step for a successful viral life cycle, yet little is known of how or why this process occurs. Host intracellular membranes play a variety of roles within the cell which may include: (i) spatial separation of cellular processes, (ii) concentration of cellular components for dynamic functions, (iii) structural roles to maintain cellular shape and form, (iv) cellular signalling, and (v)

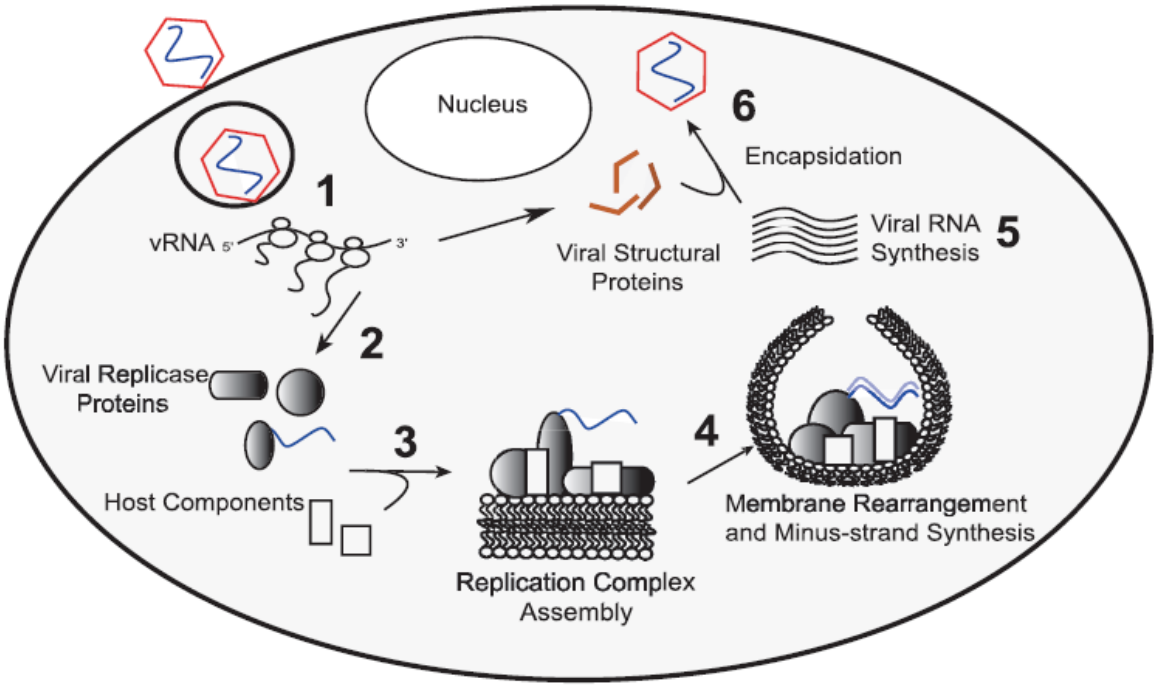

Figure 1.1: Positive-strand RNA virus lifecycle. **(1)** Viral entry and encoating release the viral RNA into the cytosol. **(2)** The viral RNA is translated into nonstructural (replicase) and structural (capsid) proteins. **(3)** Once translated, viral replicase proteins along with the viral genome are trafficked via host components to an intracellular membrane to assemble a viral RNA replication complex. **(4)** The assembly of viral replication complexes leads to large scale membrane rearrangements to produce "viral replication factories" where a minus-strand template can be synthesized. **(5)** Upon the synthesis of a minus-strand template, robust genome and sub-genome replication can occur. **(6)** Finally, viral plus-strand RNA genomes can be packaged and encapsided by the viral structural proteins to produce new infectious virions.

function as co-factors for a variety of biological functions. Thus it is not unreasonable to think that positive-strand RNA viruses have exploited these same cellular features to facilitate viral RNA replication within the host cell.

Although all positive-strand RNA viruses use host intracellular membranes for viral replication, the membranes used by specific viruses are diverse (Table 1.1). Individual viruses have been found associated with the endoplasmic reticulum (26, 56, 122), Golgi apparatus (63, 123) lysosomes (121), peroxisomes (56), vacuoles (144), chloroplasts (114), and the mitochondria (52, 90), and these membranes are necessary for the establishment of viral RNA replication complexes and subsequent viral genomic replication. This essential link between cellular membranes and viral RNA replication came from initial studies conducted with the picornavirus, poliovirus (46, 47). These experiments found that viral RNA replication could be inhibited by treating poliovirus infected cells with the pharmacological inhibitor of cellular fatty acid synthetase, cerulenin, or an inhibitor of ER to Golgi transport, brefeldin A, thus directly implicating the role of host intracellular membranes in poliovirus replication. Since then, substantial work has been done pharmacologically and genetically to define the link between viral RNA replication and cellular phospholipids. In particular, cerulenin has been used efficiently to inhibit viral replication of semliki forest virus (106), Flock House virus (58), and coxsackievirus B3 (117) whereas pharmacological inhibitors of lipid and cholesterol synthesis have been shown to inhibit hepatitis C virus RNA replication (7, 59, 119). In addition, the genetic deletion of the fatty acid desaturase, Ole1, has been shown to inhibit brome mosaic virus replication in

TABLE 1.1: Cellular Membranes used by Positive-strand RNA viruses

Family	Virus	Membranes	Host
Flaviviridae	Hepatitis C virus	ER derived	Human
	West Nile virus	ER-golgi	Human, insect
	Dengue virus	ER	Human, insect
	Yellow Fever virus	ER derived	Human, insect
Picornaviridae	Poliovirus	ER derived	Human
Coronaviridae	SARS	ER-golgi derived	Human
Tombusviridae	Tomato Bushy Stunt Virus**	Peroxisome, ER*	Plant
	Carnation Italian Ringspot virus**	Mitochondria	Plant
	Cucumber Necrosis virus**	ER	Plant
Nodaviridae	Flock House virus**	Mitochondria	Insect
Bromoviridae	Bromo mosaic virus**	ER	Plant
	Alfalfa mosaic virus	Vacuole	Plant
Tombavirus	Tobacco mosaic virus	ER	Plant
Togaviridae	Rubella virus	Endosomes	Human
	Semliki Forest virus	Lysosomes	Human, rodent, insect
Tymoviridae	Turnip Yellow Mosaic virus	Chloroplast	Plant

* TBSV is able to replicate on the endoplasmic reticulum in the absense of peroxisomes.
** Designants viruses that are able to replicate in yeast.

yeast (73, 74) whereas a deletion of acid sphingomyelinase leads to defects in Sindbis virus replication (100). Taken together, these studies provide valuable evidence into the necessity of cellular lipid metabolism and host intracellular membranes for viral RNA replication.

Positive-strand RNA viruses have been shown to use intracellular membranes for a variety of functions during the complex and dynamic process of viral RNA replication. Initially, viral replication complexes must be targeted to specific host intracellular membranes implying the presence of viral and host compatable targeting signals. Individual viral non-structural proteins encode membrane-specific targeting signals which have been well characterized (34, 89, 93, 161) and only recently have host membrane components been identified for proper membrane targeting (105). In most cases, deletion or mutation of these targeting signals can severely impact viral RNA replication (26, 89), however there is evidence that functional viral replication complexes can be retargeted to alternative membranes by changing the viral membrane-specific targeting signal (21, 91) or by removing the proper host membrane-specific receptor (56). These studies bring up a few interesting observations; 1) viral targeting signals act as intracellular addresses and may not play functional roles in viral RNA replication, and more importantly 2) this suggests the presence of common host components shared between cellular membranes that are capable of supporting viral RNA replication. One common component of all intracellular membranes are the glycerophospholipids which have been shown to be required co-factors for the function of the Semliki Forest virus nsp1 replicase protein (1), Sindbis virus

subgenomic promoter gene expression (120), and Flock House virus replicase activity (168).

In concert with establishing viral replication complexes, positive-strand RNA viruses induce dramatic changes in intracellular membrane structure (Figure 1.2) (67, 90-92, 130, 167). Hepatitis C virus for example (Figure 1.2A), which associates with the endoplasmic reticulum, causes the induction of what are termed “membraneous webs” where the ER is turned into small spherical membrane compartments (93, 167). These membrane rearrangments are thought to play important structural and protective effects against cellular nucleases (146) and the host cell innate immune pathways (67, 115) which can be triggered by double-strand RNA intermediates produced during viral RNA replication. However, these changes in membrane structure are not unique to HCV and are induced by a variety of positive-strand RNA viruses (27, 45, 70, 90, 110) yet the mechanisms involved are poorly understood.

In addition, host intracellular membranes play vital roles in positive-strand RNA virus genome packaging and virion production (153). It is thought that intracellular membranes provide a fixed platform at which viral RNA replication and protein production can occur in close proximity (8, 9) as to facilitate a “replication-coupled packaging” mechanism and ensure efficient genome packaging (9, 152). Finally, enveloped positive-strand RNA viruses require the use of host cellular membranes for virion production and budding from the host cell (77, 100, 140).

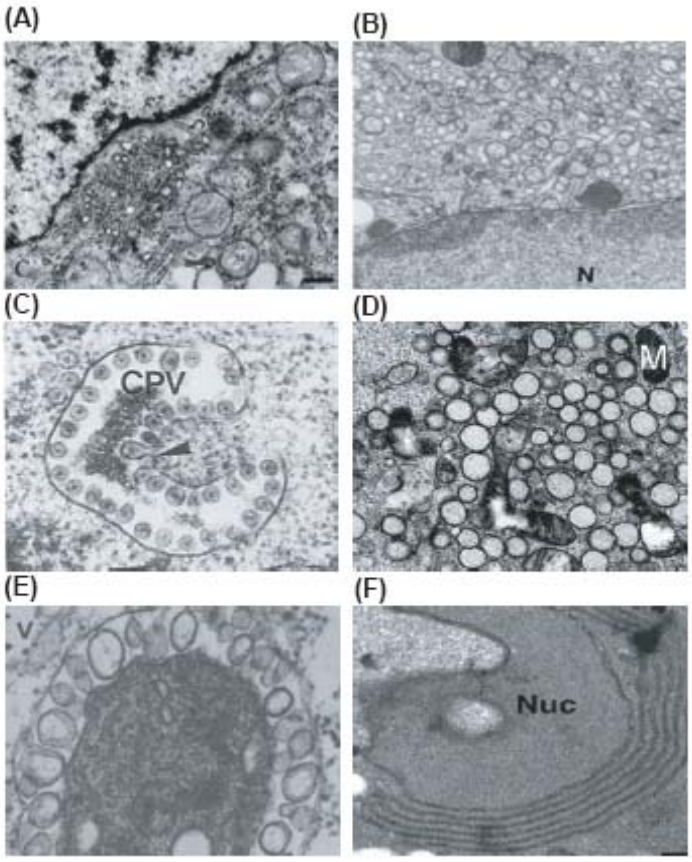

Figure 1.2: Electron micrographs of viral induced membrane changes. **(A)** Hepatitis C virus (HCV) replicon in Huh-7 cells (93). **(B)** Poliovirus infected COS-1 cells (143). **(C)** Semliki Forest virus (SFV) infected BHK cell (41). **(D)** SARS-coronavirus infected Vero E6 cells (63). **(E)** Carnation Italian Ringspot virus infected Nicotiana benthamiana leaves (29) . **(F)** Yeast expressing brome mosaic virus components (130).

Given this, there are a variety of unanswered questions regarding the use of host intracellular membranes for viral RNA replication. To begin, the viral components required for viral replication have been studied in detail, yet what role do cellular components, both protein or lipid play in replication complex assembly and function? In addition, RNA viruses must assemble complex macromolecular structures and induce dramatic changes in membrane architecture, forcing the question; what are the molecular mechanisms and dynamic processes involved in the assembly of viral RNA replication complexes within the host cell? Answering these questions will give valuable insight into not only positive-strand RNA virus biology but also host cellular biology and membrane function. We will address these questions in the coming chapters, but first an introduction into the host-pathogen system we use to study viral RNA replication complex assembly.

Flock House virus

Flock House virus (FHV) is an alphanodavirus and a member of the *Nodaviridae* family of positive-strand RNA viruses. FHV is an insect pathogen and was originally isolated from the grass grub *Costelytra zealandica* in New Zealand (131, 153). Due to its simple genome and ability to replicate in a variety of host cell types it has become a powerful tool to study many aspects of positive-strand RNA virus biology including; viral RNA replication (54, 55, 58, 111-113, 162), virion structure and assembly (129, 151, 152, 158), and innate immunity (42, 79, 80, 139).

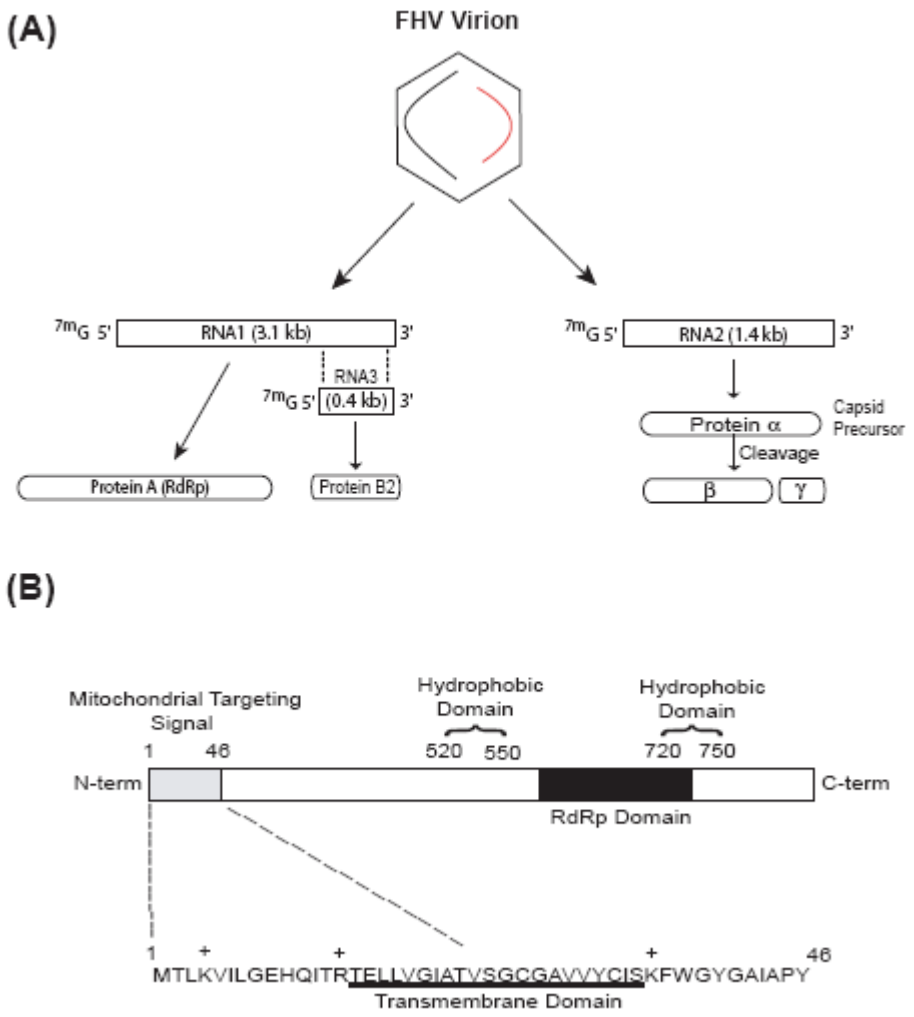

Figure 1.3: Schematic of FHV Genome and Protein A Organization. (A) Flock House virus virions contain a co-packaged bipartite genome consisting of RNA1 (3.1 kb) and RNA 2 (1.4 kb). During active FHV RNA replication a subgenomic RNA3 is produced from the 3' end of RNA1. All FHV RNAs contain a 5'-methylguanosine cap and are non-polyadenylated. RNA1 encodes the nonstructural viral RNA-dependent RNA polymerase, protain A whereas RNA2 encodes the structural capsid precursor protein a which is later cleaved to the mature capsid subunits proteins b and g. RNA3 encodes the RNAi inhibitor protein B2. **(B)** Protein A contains an amino-terminal mitochondrial targeting signal located between amino acids 1-46. The targeting signal is comprised of a central transmembrane domain flanked by positively charged amino acids. Additional predicted hydrophobic domains are located between amino acids 520-550 and 720-750. Protein A also contains a highly conserved RNA-dependent RNA polymerase domain (RdRp Domain) which contains the catalytic resides glycine-aspartic acid-aspartic acid (GDD) at amino acids 690-692.

FHV virions are nonenveloped, icosahedral, and contain a co-packaged bipartite genome consisting of equimolar amounts of RNA1 (3.1 kb) and RNA2 (1.4 kb) (Figure 1.3A). Both RNAs contain a 5'-methlyguanosine cap and are non-polyadenylated. 5' and 3' of the viral open reading frame contain untranslated cis-elements which are required for the proper replication of the genome (5, 13, 76), proper recruitment of RNA1 to the site of viral replication (148), and genome packing (177). During active RNA replication, FHV also encodes a subgenomic RNA3 (0.4 kb) from the 3' end of RNA1 which is not packaged into mature virions.

FHV RNA1 encodes the non-structural viral RNA-dependent RNA polymerase (RdRp), protein A (89, 90). Protein A is a large multi-domain protein of 112 kDa that contains an amino-terminal transmembrane domain and mitochondrial targeting signal as well as a conserved RdRp catalytic domain containing the amino acids glycine-aspartic acid-aspartic acid (GDD) located towards the carboxy-terminus of the protein (108) (Figure 1.3B). In addition, the transmembrane domain of protein A as well other regions have been shown to mediate protein A-protein A interactions in vivo (31) as well as viral RNA recognition (149) and membrane targeting (89). Deletion or mutation of the mitochondrial targeting signal or catalytic GDD residues abolish protein A mitochondrial localization (89, 91) and viral RNA replication, respectively (112, 149). Interestingly, the deletion of the mitochondrial targeting signal does not inhibit FHV RNA replication suggesting it does not play a functional role in FHV RNA synthesis (91). One possible explanation for this is that although a deletion

of the targeting signal disrupts mitochondrial localization, protein A still remains partially membrane-associated in vivo (89), and these protein-membrane interactions may be sufficient for RNA replication. Additionally, protein A contains a variety of predicted hydrophobic regions as well as amphipathic alpha helices located upstream of the mitochondrial targeting signal which may play roles in membrane targeting yet have not been characterized in detail (89). Further speculation on the involvement of other protein A structural motifs in viral RNA replication will be discussed in Chapter V.

FHV RNA2 encodes the 43 kDa structural capsid precursor protein, protein alpha, which is arranged into sixty triangular asymmetric subunits to give rise to the roughly 35 nm capsid (153, 158) . Immature provirions are composed of 180 protein alpha subunits which then undergo a maturation step cleaving protein alpha into two capsid subunits, protein beta and gamma, generating the mature and infectious viral particle (129, 153). In addition, the capsid proteins play key roles in not only the production of virion structure but also in specific packaging of RNA1 and RNA2 into infectious virions. The carboxy-terminus of protein alpha has been shown to specifically recognize FHV RNA1 and RNA2 (128) and the amino-terminus of protein alpha contains regions for RNA2 packaging (82). Deletions of these portions of the protein lead to the packaging of nonspecific cellular RNAs into virions. In addition to studies on virion assembly, significant work with RNA2 has been done to address the dynamic interactions between FHV RNA replication and RNA packaging. These studies demonstrated that only capsid proteins that were translated from replicating RNA2 molecules were able

to package FHV RNAs into virions (150, 152), suggesting that viral RNA packaging and RNA replication are functionally linked. Finally, FHV RNA2 plays key regulatory roles in RNA replication by inhibiting subgenomic RNA3 synthesis during FHV infection (32, 33).

FHV subgenomic RNA3 encodes protein B2, a potent inhibitor of the RNA interference innate immune pathway. Protein B2 is a small protein of approximately 10 kDa that is expressed at high levels early in infection and is necessary for viral RNA replication and virion production in organisms that contain the RNA silencing machinery such as insects, plants, and nematodes yet it is dispensable for replication yeast. Protein B2 has been shown to interact with dsRNAs in vitro and is thought to function by binding to viral dsRNA intermediates and short dsRNA products protecting them from degradation and processing by the cellular RNAi machinery (79). Lastly, similar to RNA2, RNA3 plays regulatory roles in RNA replication by transactivating RNA2 synthesis during infection (33).

FHV has been used extensively to study the molecular mechanisms involved in positive-strand RNA virus replication in part due to the relative simplicity of the virus and development of in vitro and in vivo FHV RNA replication systems. The study of FHV biology using infectious virions is limited by the cellular tropism of the virus. Thus in an alternative approach, initial studies identified the ability to establish robust FHV RNA replication and virion production by introducing virion free, FHV RNA1 and RNA2 into insect, plant, mammalian, and yeast cells (12, 113, 132). The ability of FHV to replicate in this wide array of host cells brought

forth the hypothesis that the host requirements for FHV viral RNA replication must be conserved between organisms yet the complexity of these requirements is currently unclear. It was later revealed that the expression of RNA1 alone was sufficient for the establishment of robust FHV RNA1 replication in insect and mammalians cells, and the discovery of inducible plasmid-based viral replicons (self-replicating viral RNAs) quickly advanced the FHV replication field and allowed for the availability of a variety of host systems in which to study FHV host-pathogen interactions including *Saccharomyces cerevisiae* (58, 162). Finally, the development of whole animal transgenic *Drosophila* (42) and *C. elegans* (80) FHV replication systems within the last few years have proved important in understanding the role of cellular innate immunity and antiviral pathways, and will be helpful in understanding other aspects of positive-strand RNA virus biology in the future.

Host intracellular membranes are essential for FHV RNA replication (58). The first reports of FHV RNA replication in association with intracellular membranes began nearly two decades ago with the observation that subcellular membrane fractions isolated from FHV infected insect cells were able to synthesize viral dsRNA and ssRNAs in vitro (169). This initial report made two key findings: 1) FHV replication complexes retained the ability to synthesize dsRNA products after treatment with the detergent dodecyl β-D-maltoside; and 2) after nuclease removal of endogenous templates replicase activity can be restored with the addition of an exogenous template. These two points indicated that viral dsRNA synthesis is in part independent of membrane structure and that

viral RNAs may function in trans for viral RNA synthesis. The results that replicase function was retained after detergent treatment led to a report in which specific neutral glycerophospholipids were able to restore ssRNA synthesis when added back to detergent solubilized replication complexes (168). Taken together, these two initial reports concluded that FHV RNA replicase activity is membrane-associated and depends in part on specific glycerophospholipids.

Years later it was discovered that FHV assembles and establishes viral replication complexes on the mitochondrial outer membrane in insect and yeast cells where protein A is the only viral protein necessary for RNA replication in yeast (31, 89, 90, 149). FHV protein A is targeted to the mitochondrial outer membrane by an amino-terminal membrane targeting signal where it assembles as an integral membrane protein with the majority of its carboxy-terminus extended into the cytosol (89). Protein A self-associates in vivo and specifically interacts with viral RNA1 to target the FHV genome to the mitochondria during replication complex assembly (31, 149). Interestingly, it has been established that functional FHV RNA replication can be retargeted to alternative cellular membranes by exchanging the mitochondrial targeting signal, and in some cases such as the ER, FHV replication is more efficient than when targeted to the mitochondria (91). These studies bring forth the possibility the FHV replication is not membrane specific but the requirements for RNA synthesis may be common cellular membrane components.

In conjunction with viral RNA replication FHV has been seen, by both global protein mass spectroscopy (43) and electron microscopy (67, 90), to induce

dramatic cellular and mitochondrial changes leading to production of invaginations in the mitochondrial outer membrane and the creation of what are now termed “viral replication factories” (67, 90) (Figure 1.4). It is thought that within these membrane-bound vesicles is the site of active FHV RNA synthesis. Three-demensional reconstructions of these membrane vesicles by cyro-electron microscopy have allowed for the detailed visualization of replication complex containing membrane compartments (67), yet the mechanisms underlying the formation of these membrane changes or the assembly of the viral RNA replication complexes are unclear. Thus we took advantage of using the well-studied virus FHV in combination with the genetically tractable host *Saccharomyces cerevisiae* to study the host-pathogen interactions involved in FHV RNA replication complex assembly and function.

Yeast as a model system for Positive-strand RNA virus replication

The budding yeast has been used as a model eukaryotic system to study many aspects of cellular and molecular biology, and with the discovery of plasmid based replicons and viral expression plasmids it has now become a host model system for the study of positive-strand RNA virus biology as well (95, 96, 104). The yeast research community (www.yeastgenome.org) and the facile genetics of the organism has provided the availability of an array of genetic deletion libraries along with well worked out biochemical and cellular biology techniques. Using these tools, large scale genomic and proteomic screens have been performed to identify host factors required for positive-strand RNA virus

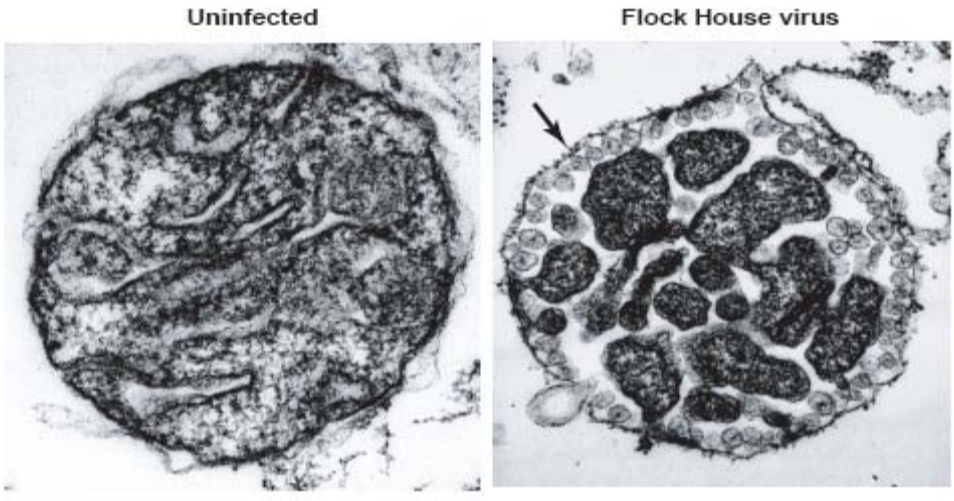

Figure 1.4: Ultrastructural membrane changes induced by Flock House virus infection. Electron micrograph of purified mitochondria isolated from uninfected and FHV infected insect cells. Arrow indicates FHV induced spherules along the mitochondrial outer membrane. Images are courtesy of Dr. David Miller.

RNA replication (71, 74, 75, 105, 133, 134, 159, 162). However, although there has been significant work done using global cellular analyses to identify host factors necessary for viral replication, there has yet to be a targeted, membrane-specific approach to understand the role of host components and processes in positive-strand RNA virus replication complex assembly and function.

Yeast Mitochondrial Biology

FHV assembles functional RNA replication complexes on the mitochondrial outer membrane in yeast and thus we were interested in understanding the role of yeast mitochondrial biology in FHV RNA replication. The mitochondria play essential roles in cellular function which include; energy production, amino acid metabolism, phospholipid biosynthesis, and apoptosis (30). In order to faciliate these processes, the mitochondria have become efficient compartmentalized double-membrane organelles consisting of the inner matrix surrounded by the mitochondrial inner and outer membranes. The mitochondrial matrix is the major enzymatic center required for the metabolism of cellular lipids, carbohydrates, and amino acids; and works in conjunction with the protein-rich mitochondrial inner membrane which contains cellular proteins necessary for metabolism and ATP production such as the electron transport chain and ATP synthase. The mitochondrial outer membrane creates the outer physical barrier of the mitochondria and is required for protein import, mitochondrial morphology, and metabolite exchange (30). The intermembrane space is the compartment

between the outer and inner membrane and is required for protein folding as well as metabolism (72, 166).

Yeast Mitochondrial Protein Import

It is estimated that there are roughly 1,000 proteins present in the mitochondria of the budding yeast (18, 86, 137). Many of these proteins are encoded by nuclear genes and must be properly targeted to their appropriate submitochondrial environment. To facilitate this process, mitochondrial proteins contain a variety of mitochondrial targeting signals (Figure 1.5) (16, 18, 116). Proteins destined for the mitochondrial matrix contain an amino-terminal amphipathic α-helix which is cleaved by the mitochondrial peptidase during import through the mitochondrial outer and inner membranes whereas those proteins taking residence in the mitochondrial inner membrane contain internal targeting signals located throughout the protein (145). However, the proteins of the mitochondrial outer membrane, such as FHV protein A, contain a variety of mitochondrial targeting signals depending on their structure and orientation. Mitochondrial outer membrane proteins that contain a more complex structure and topology, such as β-barrel proteins, contain mitochondrial targeting signals located within the last transmembrane segment of the protein (72). On the other hand, there are two types of simple single-transmembrane proteins embedded in the mitochondrial outer membrane (4, 99, 116, 156). Those with their carboxy-terminus embedded in the bilayer, or tail-anchored proteins (48, 51, 61), and

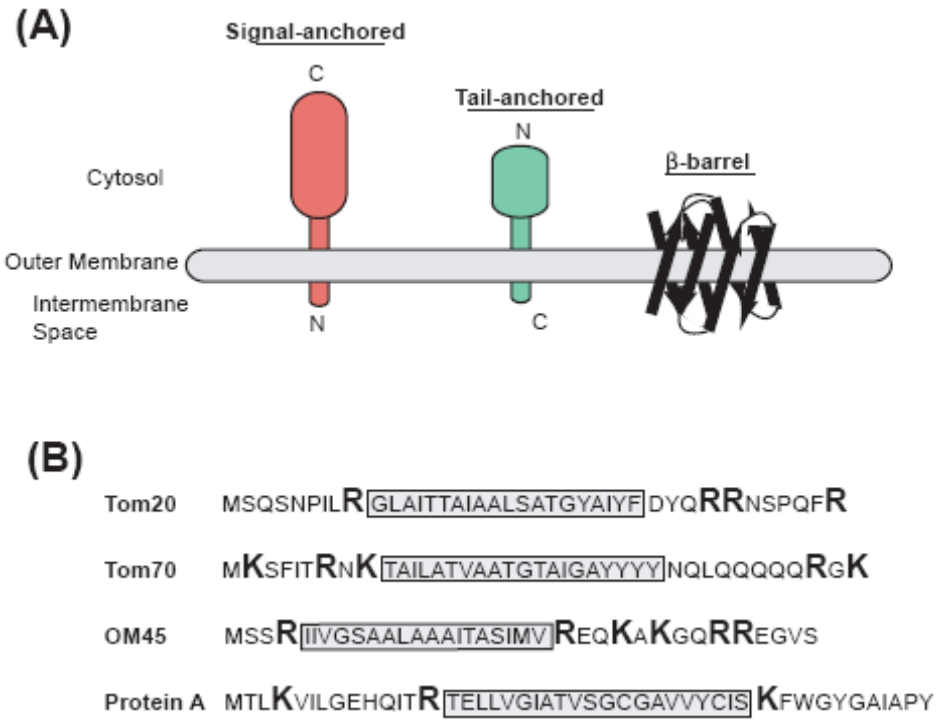

Figure 1.5: Mitochondrial targeting signals of amino-terminal anchored mitochondrial outer membrane proteins. (A) Schematic of mitochondrial outer membrane protein topology. Signal-anchored and tail-anchored proteins contain mitochondrial targeting signals encoded in their amino- and carbox-terminus respectively. These targeting signals contain a single transmembrane domain flanked by positively charged amino acids. β-barrel proteins contain a more complex topolgy and are targeted via internal targeting signals. **(B)** Amino acid sequences of mitochondrial targeting signals of yeast mitochondrial outer membrane proteins Tom20, Tom70, OM45, and Flock House virus protein A. Transmembrane domains are in gray and positively charged amino acids are bold.

those with the amino-terminus embedded in the bilayer, or signal-anchored proteins (Figure 1.5A) (15, 156). The mitochondrial targeting signals for these two types of proteins do not share any primary sequence similarity as the targeting information is contained by structural determinants within the protein (156). The targeting signal contains a transmembrane domain of roughly 18-20 amino acids flanked on both sides by an array of positively charged amino acids. FHV protein A is a signal-anchored protein and contains a mitochondrial targeting signal similar to those encoded by resident yeast mitochondrial outer membrane proteins (Figure 1.5B) (89).

Proteins encoded from nuclear genes must first come into contact with the mitochondrial outer membrane to be imported into the mitochondria or mitochondrial membranes. To facilitate the import of mitochondrial proteins, the mitochondrial outer membrane contains a variety of macromolecular protein import complexes (16, 18, 99) (Table 1.2, Figure 1.6). The translocase of the mitochondrial outer membrane (TOM complex) is a large multicomponent protein structure that functions as the major import pore of the mitochondria (3, 107, 165). The TOM complex consists of three main receptors Tom20, Tom22, and Tom70, three accessory proteins Tom5, Tom6, and Tom7, and the pore structure Tom40. The TOM complex is engineered to import the many varieties of mitochondrial proteins via the individual mitochondrial receptors. Tom20 and Tom70 are the two main import receptors and are both signal-anchored proteins similar to FHV protein A (19, 35, 156). Tom20 is the major import receptor and sits on the periphery of the TOM complex with its amino-terminus embedded in

Table 1.2: Yeast Mitochondrial Outer Membrane Protein Import Machinery

Component	Orientation	Mitochondrial Function
Translocase of the Mitochondrial Outer Membrane (TOM Complex)		
Tom70	Signal-anchored	Receptor for import of carrier proteins
Tom20	Signal-anchored	Major mitochondrial protein import receptor
Tom22	Tail-anchored	Co-receptor for protein import
Tom40	β-barrel	Pore forming subunit
Tom5	Tail-anchored	TOM complex stability/assembly
Tom6	Tail-anchored	TOM complex stability/assembly
Tom7	Tail-anchored	TOM complex stability/assembly
Sorting and Assembly Machinery (SAM Complex)		
Sam50	Integral, β-barrel	Pore forming subunit
Sam37	Peripheral	SAM complex subunit
Sam35	Peripheral	SAM complex subunit
Mim1	single transmembrane	TOM complex and signal-anchored protein biogenesis

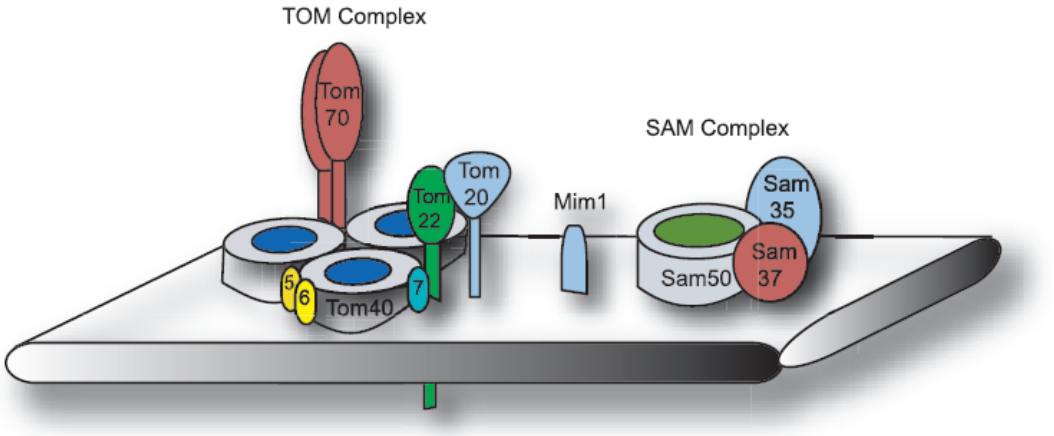

Figure 1.6: Schematic of yeast mitochondrial outer membrane protein import machinery. The translocase of the outer membrane (TOM complex) is composed of the major import pore Tom40, three receptors Tom20, Tom22, and Tom70, and the accessory proteins Tom5, Tom6, and Tom7. The sorting and assembly machinery (SAM complex) is composed of a major pore component Sam50 and two accessory proteins Sam35 and Sam37. Mim1 is a small lipid bilayer protein that has been shown to interact with the SAM complex.

the outer membrane (49). The cytosolic tail of Tom20 is highly negatively charged (172) and provides the physical interactions for the import of complex mitochondrial outer membrane protein such as the β-barrel proteins porin and Tom40 (20, 69, 157). On the other hand, Tom70 functions as a dimer for the import of the carrier domain proteins that contain internal targeting signals such as the ATP/ADP carrier and phosphate carrier proteins via physical interactions with tricopeptide repeats present in the cytosolic arm of Tom70 (17, 66, 125, 174). In addition to Tom70, yeast encode a similar protein, Tom71, which has been shown to play redundant roles with Tom70 as a minor component of the TOM complex (65, 124) and regulate mitochondrial morphology (66). Tom22 is a member of the core general import pore and is thought to function along with Tom20 (171) as a multifunctional organizer of the TOM complex. Tom22, is a tail-anchored protein with cytosolic and intermembrane space acidic domains that are thought to function with chaperone-like activity to escort proteins from the main receptor Tom20, through the import pore, and into the intermembrane space (97, 171). However, although the import of β-barrel and carrier proteins require the TOM complex and import receptors this is not the case for single transmembrane proteins of the mitochondrial outer membrane. It has recently been shown that both tail-anchored and signal-anchored proteins can be imported via a TOM complex independent mechanism which has yet to be identified (61, 84). Tom40 is a β-barrel protein embedded in the mitochondrial outer membrane and forms the major component and pore of the TOM complex (2). Tom5 (28, 126), Tom6 (22), and Tom7 (50) are three accessory proteins

that are important for TOM complex structural stability as well as maintenance of mitochondrial morphology (87). The import pore Tom40 is the only component of the TOM complex that is essential for cellular function, suggesting the TOM complex receptors and accessory proteins play redundant roles in mitochondrial function .

In addition to the TOM complex, the mitochondrial outer membrane also contains the sorting and assembly machinery (SAM complex) (Figure 1.6, Table 1.2) (16, 72, 141, 165). This structure was only recently identified and has been shown to be required for the proper import and assembly of complex proteins and protein structures such as the β-barrel protein porin and the TOM complex (72). Similar to the TOM complex, the SAM complex is a multicomponent protein complex that consists of a conserved core component Sam50 (Tob55) (23, 68) and two subunits Sam35 (Tob38) (23) and Sam37 (44, 165) Sam50 is an integral β-barrel protein whereas Sam35 and Sam37 are peripheral membrane proteins which are though to function in SAM complex stability. Sam37 was originally identified in a screen for mutant yeast strains that were defective in phospholipid biosynthesis and has been found to play many roles within the cell (44). It was originally thought to be a protein import receptor based on genetic and physical interactions with Tom70 (44, 125), yet it was later shown to be necessary for β-barrel protein import and recently it has been implicated in the import of TOM complex proteins. It has also been implicated in a screen for genes required for peroxisome function suggesting it may have roles in other cellular functions (78). Furthermore, the SAM complex has been shown to

cooperate with other mitochondrial outer membrane proteins such as Mdm10, Mdm12, and Mmm1 for the assembly of β-barrel proteins, all of which have been shown to influence mitochondrial morphology (6, 85). Finally, the small mitochondrial outer membrane protein Mim1 was recently shown to physically interact with the SAM complex (15) and has been shown to be required for the specific import of the signal-anchored receptors Tom20 and Tom70 as well as the assembly of the TOM complex (155). Of the two major protein import complexes, the SAM complex seems to play a much more essential role in mitochondrial biogenesis as only a deletion of the peripheral membrane protein Sam37 is viable (6, 68). One possible explanation for this is the similarity between the SAM complex and its bacterial equivalent Omp85, suggesting that this complex is evolutionarily conserved for cellular function (107). The deletion of many of the components of the SAM and TOM complexes leads to severe defects in mitochondrial protein import as well as mitochondrial morphology suggesting a link between protein import and the maintenance of mitochondrial shape and structure (142). The deletion of Mdm10 in particular, leads to the formation of giant-mitochondria which is similar to what is seen in the presence of active FHV RNA replication (85), yet unfortunately work has not been done to solidify the link between import components and mitochondrial morphology.

Yeast Mitochondrial Fusion and Fission

Yeast mitochondrial morphology is maintained by the two evolutionarily conserved opposing forces of mitochondrial fusion and fission (88, 101, 170).

The budding yeast has been a valuable host system in which to study these two processes. The development of yeast deletion strains along with the availability of molecular biology techniques have led to the identification of the mitochondrial fusion and fission machinery which is located on the mitochondrial outer membrane (Figure 1.7). The machinery responsible for mitochondrial fusion consists of the mitochondrial outer membrane proteins Fzo1 and Ugo1 along with the mitochondrial inner membrane protein Mgm1 (175). Fzo1 and Mgm1 are integral membrane GTPase proteins which perform the GTP dependent fusion of the mitochondrial membranes whereas Ugo1 is an adaptor protein that physically connects Dnm1 and Mgm1 through the intermembrane space (135, 136). The mitochondrial fission machinery is composed of the integral mitochondrial outer membrane protein Fis1 and the peripheral membrane proteins Dnm1 and Mdv1 (109, 163). Dnm1 is a dynamin-related GTPase and the major component of the mitochondrial fission machinery. Fis1 is a tail-anchored protein that is required for interactions between Mdv1 and Dnm1 and anchoring of the fission machinery to the mitochondrial outer membrane (98, 163). In addition to normal mitochondrial fission, this pathway plays key roles in programmed cell death (36, 62) and peroxisome fission (64, 94). Similar to the mitochondrial protein import machinery, a deletion of these components leads to dramatic defects in mitochondrial morphology.

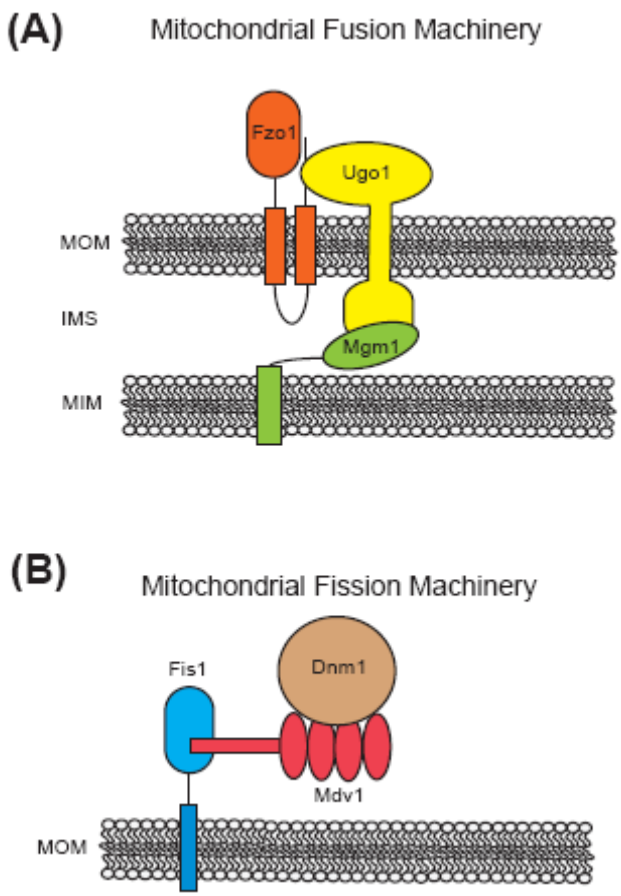

Figure 1.7: Yeast mitochondrial fusion and fission machinery. **(A)** The mitochondrial fusion machinery is composed of the mitochondrial outer membrane (MOM) proteins Fzo1 and Ugo1 and and the mitochondrial inner membrane (MIM) protein Mgm1 with span the intermembrane space (IMS). **(B)** The mitochondrial fission machinery is composed of the intergral membrane protein Fis1 and peripheral membrane proteins Mdv1 and Dnm1.

Yeast Mitochondrial Phospholipid Biology

Although the mitochondria contain roughly 1,000 proteins, the mitochondrial outer membrane contains only a small fraction of this total. A proteomic analysis has identified a handful of resident proteins present in the mitochondrial outer membrane leaving the remaining space to be filled by phospholipids (30, 127, 173). The mitochondrial outer membrane is made primarily of the glycerophospholipids (Figure 1.8) phosphatidlycholine (PC) (~45%) and phosphatidlyethanolamine (PE) (~35%), the minor phospholipid components phosphatidylserine (PS) (~2%) and phosphatidylinositol (PI) (~10%), and the mitochondrial specific phospholipids phosphatidlyglycerol (PG) and cardiolipin (CL) (~4%) (25, 147). In particular, the anionic phospholipids PS, PG, PA, and CL have been shown to be important cellular phospholipids to mediate protein import and protein-lipid interactions within the cell (37, 83, 103, 176)

Cellular and membrane phospholipid biosynthesis has been studied in great detail in yeast due to the availability of yeast deletion strains and an easily manipulatable cellular biology system. The endoplasmic reticulum is the prime location for the biosynthesis of the major glycerophospholipids. However the mitochondria contains the enzymatic machinery for the synthesis of PE as well as the mitochondrial specific phospholipids PG and CL (53, 147) suggesting a key role for this organelle in phospholipid biosynthesis (147, 154). The mitochondrial specific phospholipid, cardiolipin, is a diphosphatidylglycerol molecule that is synthesized from PG by the enzyme cardiolipin synthase (Crd1) (57). Cardiolipin is located primarily in the mitochondrial inner membranes where

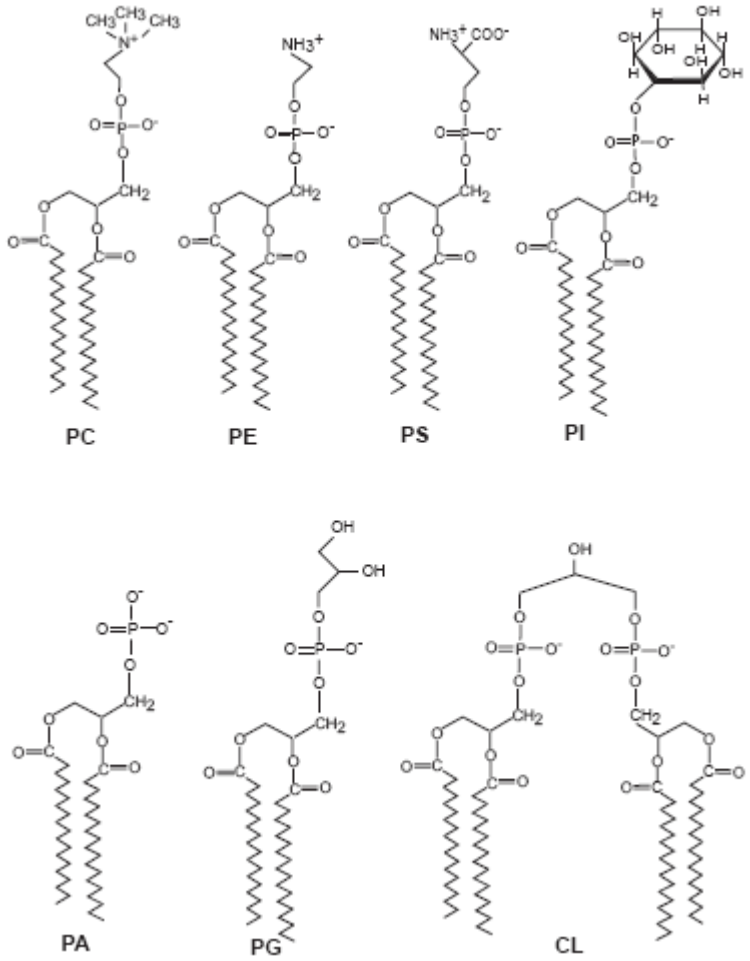

Figure 1.8: Schematic of the major cellular glycerophospholipids. Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylserine (PS), Phosphatidylinositol (PI), Phosphatidic Acid (PA), Phosphatidylglycerol (PG), and Cardiolipin (CL).

it makes up roughly 15% of the total phospholipid composition, and has been implicated in a variety of mitochondrial functions including mitochondrial protein import, maintainence of protein-protein interactions, apoptosis, and mitochondrial morphology (24, 57, 81, 102). Interestingly, although CL makes up roughly 5% of the mitochondrial outer membrane phospholipids, it can reach concentrations of up to 20% at “contacts sites” where the mitochondrial outer and inner membranes come into physical contact (10, 118, 138). Mitochondrial contact sites are thought to be localized areas where proteins and phospholipids destined for trafficking into the mitochondrial matrix can easily pass through the double membrane structure (118, 138). In addition, contacts sites contain a variety of proteins that are required for protein import and mitochondrial morphology (40, 164), suggesting that these sites play essential roles in yeast mitochondrial biogenesis (118).

The mitochondrion is a complex organelle that is vital to cellular function. Mutations or deletions of mitochondrial proteins can give rise to a variety of mitochondrial disorders such as Barth Syndrome and dominant optic atrophy which are caused by defects in cardiolipin synthesis and mitochondrial fusion respectively (38). Finally, it should be noted that although the study of the individual processes of protein import, mitochondrial morphology, and phospholipid biosynthesis can be separated in the laboratory, one has to keep in mind that all of these processes are intimately linked during mitochondrial biogenesis and defects in one process can have multiple effects on others.

Summary

Positive-strand RNA viruses comprise a long list of viral pathogens capable of causing both health and economic problems around the world, yet to date there is little available to fight these pathogens. To aid in the development of anti-viral therapies, the understanding of essential molecular processes required for positive-strand RNA virus replication is necessary, and the association of all positive-strand RNA viruses with host intracellular membranes provides this crucial step in the viral life cycle on which to focus our studies.

In my thesis we made use of the established model positive-strand RNA virus, Flock House virus (FHV), and the budding yeast *Saccharomyces cerevisiae* to utilize a powerful host-pathogen system with which to study positive-strand RNA virus replication complex assembly and function. FHV assembles functional viral RNA replication complexes on the mitochondrial outer membrane in yeast where protein A is the only viral protein necessary for this process. Protein A contains a mitochondrial targeting signal similar to those of host mitochondrial outer membrane proteins and we hypothesize that similar to yeast mitochondrial outer membranes, FHV uses the yeast mitochondrial import machinery for the assembly of mitochondrial associated replication complexes. In order to address this hypothesis we proposed the following three specific aims:

Aim1: Establish an FHV in vitro membrane association system (Chapter II).

Aim2: Investigate the role of individual mitochondrial outer membrane proteins in FHV RNA replication (Chapter III).

Aim 3. Investigate the composition and structure of FHV RNA replication Complexes (Chapter IV)

REFERENCES

1. **Ahola, T., A. Lampio, P. Auvinen, and L. Kaariainen.** 1999. Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. EMBO J **18:**3164-72.

2. **Ahting, U., M. Thieffry, H. Engelhardt, R. Hegerl, W. Neupert, and S. Nussberger.** 2001. Tom40, the pore-forming component of the protein-conducting TOM channel in the outer membrane of mitochondria. J Cell Biol **153:**1151-60.

3. **Ahting, U., C. Thun, R. Hegerl, D. Typke, F. E. Nargang, W. Neupert, and S. Nussberger.** 1999. The TOM core complex: the general protein import pore of the outer membrane of mitochondria. J Cell Biol **147:**959-68.

4. **Ahting, U., T. Waizenegger, W. Neupert, and D. Rapaport.** 2005. Signal-anchored proteins follow a unique insertion pathway into the outer membrane of mitochondria. J Biol Chem **280:**48-53.

5. **Albarino, C. G., L. D. Eckerle, and L. A. Ball.** 2003. The cis-acting replication signal at the 3' end of Flock House virus RNA2 is RNA3-dependent. Virology **311:**181-91.

6. **Altmann, K., and B. Westermann.** 2005. Role of essential genes in mitochondrial morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell **16:**5410-7.

7. **Amemiya, F., S. Maekawa, Y. Itakura, A. Kanayama, A. Matsui, S. Takano, T. Yamaguchi, J. Itakura, T. Kitamura, T. Inoue, M. Sakamoto, K. Yamauchi, S. Okada, A. Yamashita, N. Sakamoto, M. Itoh, and N. Enomoto.** 2008. Targeting lipid metabolism in the treatment of hepatitis C virus infection. J Infect Dis **197:**361-70.

8. **Annamalai, P., and A. L. Rao.** 2006. Packaging of brome mosaic virus subgenomic RNA is functionally coupled to replication-dependent transcription and translation of coat protein. J Virol **80:**10096-108.

9. **Annamalai, P., F. Rofail, D. A. Demason, and A. L. Rao.** 2008. Replication-coupled packaging mechanism in positive-strand RNA viruses: synchronized coexpression of functional multigenome RNA components of an animal and a plant virus in Nicotiana benthamiana cells by agroinfiltration. J Virol **82:**1484-95.

10. **Ardail, D., J. P. Privat, M. Egret-Charlier, C. Levrat, F. Lerme, and P. Louisot.** 1990. Mitochondrial contact sites. Lipid composition and dynamics. J Biol Chem **265:**18797-802.

11. **Asia, W. H. O. R. O. f. S.-E.** 2006, posting date. Situation of Dengue/Dengue Haemorrhagic Fever in the South-East Asia Region. [Online.]

12. **Ball, L. A., J. M. Amann, and B. K. Garrett.** 1992. Replication of nodamura virus after transfection of viral RNA into mammalian cells in culture. J Virol **66:**2326-34.

13. **Ball, L. A., and Y. Li.** 1993. cis-acting requirements for the replication of flock house virus RNA 2. J Virol **67:**3544-51.

14. **Bartenschlager, R., and S. Miller.** 2008. Molecular aspects of Dengue virus replication. Future Microbiol **3:**155-65.

15. **Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenkovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann.** 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. J Biol Chem **283:**120-7.

16. **Becker, T., F. N. Vogtle, D. Stojanovski, and C. Meisinger.** 2008. Sorting and assembly of mitochondrial outer membrane proteins. Biochim Biophys Acta **1777:**557-63.

17. **Beddoe, T., S. R. Bushell, M. A. Perugini, T. Lithgow, T. D. Mulhern, S. P. Bottomley, and J. Rossjohn.** 2004. A biophysical analysis of the tetratricopeptide repeat-rich mitochondrial import receptor, Tom70, reveals an elongated monomer that is inherently flexible, unstable, and unfolds via a multistate pathway. J Biol Chem **279:**46448-54.

18. **Bolender, N., A. Sickmann, R. Wagner, C. Meisinger, and N. Pfanner.** 2008. Multiple pathways for sorting mitochondrial precursor proteins. EMBO Rep **9:**42-9.

19. **Brix, J., K. Dietmeier, and N. Pfanner.** 1997. Differential recognition of preproteins by the purified cytosolic domains of the mitochondrial import receptors Tom20, Tom22, and Tom70. J Biol Chem **272:**20730-5.

20. **Brix, J., S. Rudiger, B. Bukau, J. Schneider-Mergener, and N. Pfanner.** 1999. Distribution of binding sequences for the mitochondrial import receptors Tom20, Tom22, and Tom70 in a presequence-carrying preprotein and a non-cleavable preprotein. J Biol Chem **274:**16522-30.

21. **Burgyan, J., L. Rubino, and M. Russo.** 1996. The 5'-terminal region of a tombusvirus genome determines the origin of multivesicular bodies. J Gen Virol **77 (Pt 8):**1967-74.

22. **Cao, W., and M. G. Douglas.** 1995. Biogenesis of ISP6, a small carboxyl-terminal anchored protein of the receptor complex of the mitochondrial outer membrane. J Biol Chem **270:**5674-9.

23. **Chan, N. C., and T. Lithgow.** 2008. The peripheral membrane subunits of the SAM complex function codependently in mitochondrial outer membrane biogenesis. Mol Biol Cell **19:**126-36.

24. **Chen, S., M. Tarsio, P. M. Kane, and M. L. Greenberg.** 2008. Cardiolipin mediates cross-talk between mitochondria and the vacuole. Mol Biol Cell **19:**5047-58.

25. **de Kroon, A. I., M. C. Koorengevel, S. S. Goerdayal, P. C. Mulders, M. J. Janssen, and B. de Kruijff.** 1999. Isolation and characterization of highly purified mitochondrial outer membranes of the yeast Saccharomyces cerevisiae (method). Mol Membr Biol **16:**205-11.

26. **den Boon, J. A., J. Chen, and P. Ahlquist.** 2001. Identification of sequences in Brome mosaic virus replicase protein 1a that mediate association with endoplasmic reticulum membranes. J Virol **75:**12370-81.

27. **Denison, M. R.** 2008. Seeking membranes: positive-strand RNA virus replication complexes. PLoS Biol **6:**e270.

28. **Dietmeier, K., A. Honlinger, U. Bomer, P. J. Dekker, C. Eckerskorn, F. Lottspeich, M. Kubrich, and N. Pfanner.** 1997. Tom5 functionally links mitochondrial preprotein receptors to the general import pore. Nature **388:**195-200.

29. **DiFrano, A. R., M., and G.P. Martelli.** 1984. Ultrastructure and Origin of Cytoplasmic Multivesicular Bodies Induced by Carnation Italian Ringspot Virus. J Gen Virol **65:**1233-1237.

30. **Dimmer, K. S., and D. Rapaport.** 2008. Proteomic view of mitochondrial function. Genome Biol **9:**209.

31. **Dye, B. T., D. J. Miller, and P. Ahlquist.** 2005. In vivo self-interaction of nodavirus RNA replicase protein a revealed by fluorescence resonance energy transfer. J Virol **79:**8909-19.

32. **Eckerle, L. D., C. G. Albarino, and L. A. Ball.** 2003. Flock House virus subgenomic RNA3 is replicated and its replication correlates with transactivation of RNA2. Virology **317:**95-108.

33. **Eckerle, L. D., and L. A. Ball.** 2002. Replication of the RNA segments of a bipartite viral genome is coordinated by a transactivating subgenomic RNA. Virology **296:**165-76.

34. **Elazar, M., K. H. Cheong, P. Liu, H. B. Greenberg, C. M. Rice, and J. S. Glenn.** 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. J Virol **77:**6055-61.

35. **Endo, T., and D. Kohda.** 2002. Functions of outer membrane receptors in mitochondrial protein import. Biochim Biophys Acta **1592:**3-14.

36. **Fannjiang, Y., W. C. Cheng, S. J. Lee, B. Qi, J. Pevsner, J. M. McCaffery, R. B. Hill, G. Basanez, and J. M. Hardwick.** 2004. Mitochondrial fission proteins regulate programmed cell death in yeast. Genes Dev **18:**2785-97.

37. **Fernandez-Murray, J. P., and C. R. McMaster.** 2006. Identification of novel phospholipid binding proteins in Saccharomyces cerevisiae. FEBS Lett **580:**82-6.

38. **Foury, F., and M. Kucej.** 2002. Yeast mitochondrial biogenesis: a model system for humans? Curr Opin Chem Biol **6:**106-11.

39. **Fraser, C. S., J. W. Hershey, and J. A. Doudna.** 2009. The pathway of hepatitis C virus mRNA recruitment to the human ribosome. Nat Struct Mol Biol.

40. **Fritz, S., D. Rapaport, E. Klanner, W. Neupert, and B. Westermann.** 2001. Connection of the mitochondrial outer and inner membranes by Fzo1 is critical for organellar fusion. J Cell Biol **152:**683-92.

41. **Froshauer, S., J. Kartenbeck, and A. Helenius.** 1988. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. J Cell Biol **107:**2075-86.

42. **Galiana-Arnoux, D., C. Dostert, A. Schneemann, J. A. Hoffmann, and J. L. Imler.** 2006. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nat Immunol **7:**590-7.

43. **Go, E. P., W. R. Wikoff, Z. Shen, G. O'Maille, H. Morita, T. P. Conrads, A. Nordstrom, S. A. Trauger, W. Uritboonthai, D. A. Lucas, K. C. Chan, T. D. Veenstra, H. Lewicki, M. B. Oldstone, A. Schneemann, and G. Siuzdak.** 2006. Mass spectrometry reveals specific and global molecular transformations during viral infection. J Proteome Res **5:**2405-16.

44. **Gratzer, S., T. Lithgow, R. E. Bauer, E. Lamping, F. Paltauf, S. D. Kohlwein, V. Haucke, T. Junne, G. Schatz, and M. Horst.** 1995. Mas37p, a novel receptor subunit for protein import into mitochondria. J Cell Biol **129:**25-34.

45. **Grimley, P. M., J. G. Levin, I. K. Berezesky, and R. M. Friedman.** 1972. Specific membranous structures associated with the replication of group A arboviruses. J Virol **10:**492-503.

46. **Guinea, R., and L. Carrasco.** 1991. Effects of fatty acids on lipid synthesis and viral RNA replication in poliovirus-infected cells. Virology **185:**473-6.

47. **Guinea, R., and L. Carrasco.** 1990. Phospholipid biosynthesis and poliovirus genome replication, two coupled phenomena. EMBO J **9:**2011-6.

48. **Habib, S. J., A. Vasiljev, W. Neupert, and D. Rapaport.** 2003. Multiple functions of tail-anchor domains of mitochondrial outer membrane proteins. FEBS Lett **555:**511-5.

49. **Harkness, T. A., F. E. Nargang, I. van der Klei, W. Neupert, and R. Lill.** 1994. A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria. J Cell Biol **124:**637-48.

50. **Honlinger, A., U. Bomer, A. Alconada, C. Eckerskorn, F. Lottspeich, K. Dietmeier, and N. Pfanner.** 1996. Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import. EMBO J **15:**2125-37.

51. **Horie, C., H. Suzuki, M. Sakaguchi, and K. Mihara.** 2003. Targeting and assembly of mitochondrial tail-anchored protein Tom5 to the TOM complex depend on a signal distinct from that of tail-anchored proteins dispersed in the membrane. J Biol Chem **278:**41462-71.

52. **Hwang, Y. T., A. W. McCartney, S. K. Gidda, and R. T. Mullen.** 2008. Localization of the Carnation Italian ringspot virus replication protein p36 to the mitochondrial outer membrane is mediated by an internal targeting signal and the TOM complex. BMC Cell Biol **9:**54.

53. **Jiang, F., M. T. Ryan, M. Schlame, M. Zhao, Z. Gu, M. Klingenberg, N. Pfanner, and M. L. Greenberg.** 2000. Absence of cardiolipin in the crd1 null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. J Biol Chem **275:**22387-94.

54. **Johnson, K. L., and L. A. Ball.** 1999. Induction and maintenance of autonomous flock house virus RNA1 replication. J Virol **73:**7933-42.

55. **Johnson, K. L., and L. A. Ball.** 1997. Replication of flock house virus RNAs from primary transcripts made in cells by RNA polymerase II. J Virol **71:**3323-7.

56. **Jonczyk, M., K. B. Pathak, M. Sharma, and P. D. Nagy.** 2007. Exploiting alternative subcellular location for replication: tombusvirus replication switches to the endoplasmic reticulum in the absence of peroxisomes. Virology **362:**320-30.

57. **Joshi, A. S., J. Zhou, V. M. Gohil, S. Chen, and M. L. Greenberg.** 2008. Cellular functions of cardiolipin in yeast. Biochim Biophys Acta.

58. **Kampmueller, K. M., and D. J. Miller.** 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. J Virol **79:**6827-37.

59. **Kapadia, S. B., and F. V. Chisari.** 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci U S A **102:**2561-6.

60. **Kauder, S. E., and V. R. Racaniello.** 2004. Poliovirus tropism and attenuation are determined after internal ribosome entry. J Clin Invest **113:**1743-53.

61. **Kemper, C., S. J. Habib, G. Engl, P. Heckmeyer, K. S. Dimmer, and D. Rapaport.** 2008. Integration of tail-anchored proteins into the mitochondrial outer membrane does not require any known import components. J Cell Sci **121:**1990-8.

62. **Kitagaki, H., Y. Araki, K. Funato, and H. Shimoi.** 2007. Ethanol-induced death in yeast exhibits features of apoptosis mediated by mitochondrial fission pathway. FEBS Lett **581:**2935-42.

63. **Knoops, K., M. Kikkert, S. H. Worm, J. C. Zevenhoven-Dobbe, Y. van der Meer, A. J. Koster, A. M. Mommaas, and E. J. Snijder.** 2008. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. PLoS Biol **6:**e226.

64. **Koch, A., Y. Yoon, N. A. Bonekamp, M. A. McNiven, and M. Schrader.** 2005. A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell **16:**5077-86.

65. **Koh, J. Y., P. Hajek, and D. M. Bedwell.** 2001. Overproduction of PDR3 suppresses mitochondrial import defects associated with a TOM70 null mutation by increasing the expression of TOM72 in Saccharomyces cerevisiae. Mol Cell Biol **21:**7576-86.

66. **Kondo-Okamoto, N., J. M. Shaw, and K. Okamoto.** 2008. Tetratricopeptide repeat proteins Tom70 and Tom71 mediate yeast mitochondrial morphogenesis. EMBO Rep **9:**63-9.

67. **Kopek, B. G., G. Perkins, D. J. Miller, M. H. Ellisman, and P. Ahlquist.** 2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. PLoS Biol **5:**e220.

68. **Kozjak, V., N. Wiedemann, D. Milenkovic, C. Lohaus, H. E. Meyer, B. Guiard, C. Meisinger, and N. Pfanner.** 2003. An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. J Biol Chem **278:**48520-3.

69. **Krimmer, T., D. Rapaport, M. T. Ryan, C. Meisinger, C. K. Kassenbrock, E. Blachly-Dyson, M. Forte, M. G. Douglas, W. Neupert, F. E. Nargang, and N. Pfanner.** 2001. Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. J Cell Biol **152:**289-300.

70. **Kujala, P., A. Ikaheimonen, N. Ehsani, H. Vihinen, P. Auvinen, and L. Kaariainen.** 2001. Biogenesis of the Semliki Forest virus RNA replication complex. J Virol **75:**3873-84.

71. **Kushner, D. B., B. D. Lindenbach, V. Z. Grdzelishvili, A. O. Noueiry, S. M. Paul, and P. Ahlquist.** 2003. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. Proc Natl Acad Sci U S A **100:**15764-9.

72. **Kutik, S., D. Stojanovski, L. Becker, T. Becker, M. Meinecke, V. Kruger, C. Prinz, C. Meisinger, B. Guiard, R. Wagner, N. Pfanner, and N. Wiedemann.** 2008. Dissecting membrane insertion of mitochondrial beta-barrel proteins. Cell **132:**1011-24.

73. **Lee, W. M., and P. Ahlquist.** 2003. Membrane synthesis, specific lipid requirements, and localized lipid composition changes associated with a positive-strand RNA virus RNA replication protein. J Virol **77:**12819-28.

74. **Lee, W. M., M. Ishikawa, and P. Ahlquist.** 2001. Mutation of host delta9 fatty acid desaturase inhibits brome mosaic virus RNA replication between template recognition and RNA synthesis. J Virol **75:**2097-106.

75. **Li, Z., J. Pogany, T. Panavas, K. Xu, A. M. Esposito, T. G. Kinzy, and P. D. Nagy.** 2009. Translation elongation factor 1A is a component of the tombusvirus replicase complex and affects the stability of the p33 replication co-factor. Virology **385:**245-60.

76. **Lindenbach, B. D., J. Y. Sgro, and P. Ahlquist.** 2002. Long-distance base pairing in flock house virus RNA1 regulates subgenomic RNA3 synthesis and RNA2 replication. J Virol **76:**3905-19.

77. **Lindenbach, B. D. a. R. C. M.** 2001. *Flaviviridae*: The Viruses and Their Replication, p. 991-1041. *In* K. D. M. a. H. P.M. (ed.), Fields Virology, Fourth ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.

78. **Lockshon, D., L. E. Surface, E. O. Kerr, M. Kaeberlein, and B. K. Kennedy.** 2007. The sensitivity of yeast mutants to oleic acid implicates the peroxisome and other processes in membrane function. Genetics **175:**77-91.

79. **Lu, R., M. Maduro, F. Li, H. W. Li, G. Broitman-Maduro, W. X. Li, and S. W. Ding.** 2005. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature **436:**1040-3.

80. **Lu, R., E. Yigit, W. X. Li, and S. W. Ding.** 2009. An RIG-I-Like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in Caenorhabditis elegans. PLoS Pathog **5:**e1000286.

81. **Lutter, M., M. Fang, X. Luo, M. Nishijima, X. Xie, and X. Wang.** 2000. Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat Cell Biol **2:**754-61.

82. **Marshall, D., and A. Schneemann.** 2001. Specific packaging of nodaviral RNA2 requires the N-terminus of the capsid protein. Virology **285:**165-75.

83. **Meglei, G., and G. A. McQuibban.** 2009. The Dynamin-Related Protein Mgm1p Assembles into Oligomers and Hydrolyzes GTP To Function in Mitochondrial Membrane Fusion (dagger). Biochemistry **48:**1774-84.

84. **Meineke, B., G. Engl, C. Kemper, A. Vasiljev-Neumeyer, H. Paulitschke, and D. Rapaport.** 2008. The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. FEBS Lett **582:**855-60.

85. **Meisinger, C., M. Rissler, A. Chacinska, L. K. Szklarz, D. Milenkovic, V. Kozjak, B. Schonfisch, C. Lohaus, H. E. Meyer, M. P. Yaffe, B. Guiard, N. Wiedemann, and N. Pfanner.** 2004. The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Dev Cell **7:**61-71.

86. **Meisinger, C., A. Sickmann, and N. Pfanner.** 2008. The mitochondrial proteome: from inventory to function. Cell **134:**22-4.

87. **Meisinger, C., N. Wiedemann, M. Rissler, A. Strub, D. Milenkovic, B. Schonfisch, H. Muller, V. Kozjak, and N. Pfanner.** 2006. Mitochondrial protein sorting: differentiation of beta-barrel assembly by Tom7-mediated segregation of Mdm10. J Biol Chem **281:**22819-26.

88. **Merz, S., M. Hammermeister, K. Altmann, M. Durr, and B. Westermann.** 2007. Molecular machinery of mitochondrial dynamics in yeast. Biol Chem **388:**917-26.

89. **Miller, D. J., and P. Ahlquist.** 2002. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J Virol **76:**9856-67.

90. **Miller, D. J., M. D. Schwartz, and P. Ahlquist.** 2001. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J Virol **75:**11664-76.

91. **Miller, D. J., M. D. Schwartz, B. T. Dye, and P. Ahlquist.** 2003. Engineered retargeting of viral RNA replication complexes to an alternative intracellular membrane. J Virol **77:**12193-202.

92. **Miller, S., and J. Krijnse-Locker.** 2008. Modification of intracellular membrane structures for virus replication. Nat Rev Microbiol **6:**363-74.

93. **Moradpour, D., R. Gosert, D. Egger, F. Penin, H. E. Blum, and K. Bienz.** 2003. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. Antiviral Res **60:**103-9.

94. **Motley, A. M., G. P. Ward, and E. H. Hettema.** 2008. Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p. J Cell Sci **121:**1633-40.

95. **Nagy, P. D.** 2008. Yeast as a model host to explore plant virus-host interactions. Annu Rev Phytopathol **46:**217-42.

96. **Nagy, P. D., and J. Pogany.** 2006. Yeast as a model host to dissect functions of viral and host factors in tombusvirus replication. Virology **344:**211-20.

97. **Nargang, F. E., D. Rapaport, R. G. Ritzel, W. Neupert, and R. Lill.** 1998. Role of the negative charges in the cytosolic domain of TOM22 in the import of precursor proteins into mitochondria. Mol Cell Biol **18:**3173-81.

98. **Naylor, K., E. Ingerman, V. Okreglak, M. Marino, J. E. Hinshaw, and J. Nunnari.** 2006. Mdv1 interacts with assembled dnm1 to promote mitochondrial division. J Biol Chem **281:**2177-83.

99. **Neupert, W., and J. M. Herrmann.** 2007. Translocation of proteins into mitochondria. Annu Rev Biochem **76:**723-49.

100. **Ng, C. G., I. Coppens, D. Govindarajan, J. Pisciotta, V. Shulaev, and D. E. Griffin.** 2008. Effect of host cell lipid metabolism on alphavirus replication, virion morphogenesis, and infectivity. Proc Natl Acad Sci U S A **105:**16326-31.

101. **Okamoto, K., and J. M. Shaw.** 2005. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet **39:**503-36.

102. **Osman, C., M. Haag, C. Potting, J. Rodenfels, P. V. Dip, F. T. Wieland, B. Brugger, B. Westermann, and T. Langer.** 2009. The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. J Cell Biol **184:**583-96.

103. **Ostrander, D. B., M. Zhang, E. Mileykovskaya, M. Rho, and W. Dowhan.** 2001. Lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain. A yeast genetic model system for the study of anionic phospholipid function in mitochondria. J Biol Chem **276:**25262-72.

104. **Panavas, T., E. Serviene, J. Pogany, and P. D. Nagy.** 2008. Genome-wide screens for identification of host factors in viral replication. Methods Mol Biol **451:**615-24.

105. **Pathak, K. B., Z. Sasvari, and P. D. Nagy.** 2008. The host Pex19p plays a role in peroxisomal localization of tombusvirus replication proteins. Virology **379:**294-305.

106. **Perez, L., R. Guinea, and L. Carrasco.** 1991. Synthesis of Semliki Forest virus RNA requires continuous lipid synthesis. Virology **183:**74-82.

107. **Pfanner, N., N. Wiedemann, C. Meisinger, and T. Lithgow.** 2004. Assembling the mitochondrial outer membrane. Nat Struct Mol Biol **11:**1044-8.

108. **Poch, O., I. Sauvaget, M. Delarue, and N. Tordo.** 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J **8:**3867-74.

109. **Praefcke, G. J., and H. T. McMahon.** 2004. The dynamin superfamily: universal membrane tubulation and fission molecules? Nat Rev Mol Cell Biol **5:**133-47.

110. **Prentice, E., W. G. Jerome, T. Yoshimori, N. Mizushima, and M. R. Denison.** 2004. Coronavirus replication complex formation utilizes components of cellular autophagy. J Biol Chem **279:**10136-41.

111. **Price, B. D., P. Ahlquist, and L. A. Ball.** 2002. DNA-directed expression of an animal virus RNA for replication-dependent colony formation in Saccharomyces cerevisiae. J Virol **76:**1610-6.

112. **Price, B. D., M. Roeder, and P. Ahlquist.** 2000. DNA-Directed expression of functional flock house virus RNA1 derivatives in Saccharomyces cerevisiae, heterologous gene expression, and selective effects on subgenomic mRNA synthesis. J Virol **74:**11724-33.

113. **Price, B. D., R. R. Rueckert, and P. Ahlquist.** 1996. Complete replication of an animal virus and maintenance of expression vectors derived from it in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **93:**9465-70.

114. **Prod'homme, D., A. Jakubiec, V. Tournier, G. Drugeon, and I. Jupin.** 2003. Targeting of the turnip yellow mosaic virus 66K replication protein to the chloroplast envelope is mediated by the 140K protein. J Virol **77:**9124-35.

115. **Quinkert, D., R. Bartenschlager, and V. Lohmann.** 2005. Quantitative analysis of the hepatitis C virus replication complex. J Virol **79:**13594-605.

116. **Rapaport, D.** 2003. Finding the right organelle. Targeting signals in mitochondrial outer-membrane proteins. EMBO Rep **4:**948-52.

117. **Rassmann, A., A. Henke, N. Jarasch, F. Lottspeich, H. P. Saluz, and T. Munder.** 2007. The human fatty acid synthase: a new therapeutic target for coxsackievirus B3-induced diseases? Antiviral Res **76:**150-8.

118. **Reichert, A. S., and W. Neupert.** 2002. Contact sites between the outer and inner membrane of mitochondria-role in protein transport. Biochim Biophys Acta **1592:**41-9.

119. **Sagan, S. M., Y. Rouleau, C. Leggiadro, L. Supekova, P. G. Schultz, A. I. Su, and J. P. Pezacki.** 2006. The influence of cholesterol and lipid metabolism on host cell structure and hepatitis C virus replication. Biochem Cell Biol **84:**67-79.

120. **Saito, K., M. Nishijima, and O. Kuge.** 2006. Phosphatidylserine is involved in gene expression from Sindbis virus subgenomic promoter. Biochem Biophys Res Commun **345:**878-85.

121. **Salonen, A., L. Vasiljeva, A. Merits, J. Magden, E. Jokitalo, and L. Kaariainen.** 2003. Properly folded nonstructural polyprotein directs the semliki forest virus replication complex to the endosomal compartment. J Virol **77:**1691-702.

122. **Schaad, M. C., P. E. Jensen, and J. C. Carrington.** 1997. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J **16:**4049-59.

123. **Schlegel, A., T. H. Giddings, Jr., M. S. Ladinsky, and K. Kirkegaard.** 1996. Cellular origin and ultrastructure of membranes induced during poliovirus infection. J Virol **70:**6576-88.

124. **Schlossmann, J., R. Lill, W. Neupert, and D. A. Court.** 1996. Tom71, a novel homologue of the mitochondrial preprotein receptor Tom70. J Biol Chem **271:**17890-5.

125. **Schlossmann, J., and W. Neupert.** 1995. Assembly of the preprotein receptor MOM72/MAS70 into the protein import complex of the outer membrane of mitochondria. J Biol Chem **270:**27116-21.

126. **Schmitt, S., U. Ahting, L. Eichacker, B. Granvogl, N. E. Go, F. E. Nargang, W. Neupert, and S. Nussberger.** 2005. Role of Tom5 in maintaining the structural stability of the TOM complex of mitochondria. J Biol Chem **280:**14499-506.

127. **Schmitt, S., H. Prokisch, T. Schlunck, D. G. Camp, 2nd, U. Ahting, T. Waizenegger, C. Scharfe, T. Meitinger, A. Imhof, W. Neupert, P. J. Oefner, and D. Rapaport.** 2006. Proteome analysis of mitochondrial outer membrane from Neurospora crassa. Proteomics **6:**72-80.

128. **Schneemann, A., and D. Marshall.** 1998. Specific encapsidation of nodavirus RNAs is mediated through the C terminus of capsid precursor protein alpha. J Virol **72:**8738-46.

129. **Schneemann, A., W. Zhong, T. M. Gallagher, and R. R. Rueckert.** 1992. Maturation cleavage required for infectivity of a nodavirus. J Virol **66:**6728-34.

130. **Schwartz, M., J. Chen, W. M. Lee, M. Janda, and P. Ahlquist.** 2004. Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. Proc Natl Acad Sci U S A **101:**11263-8.

131. **Scotti, P. D., S. Dearing, and D. W. Mossop.** 1983. Flock House virus: a nodavirus isolated from Costelytra zealandica (White) (Coleoptera: Scarabaeidae). Arch Virol **75:**181-9.

132. **Selling, B. H., R. F. Allison, and P. Kaesberg.** 1990. Genomic RNA of an insect virus directs synthesis of infectious virions in plants. Proc Natl Acad Sci U S A **87:**434-8.

133. **Serva, S., and P. D. Nagy.** 2006. Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. J Virol **80:**2162-9.

134. **Serviene, E., Y. Jiang, C. P. Cheng, J. Baker, and P. D. Nagy.** 2006. Screening of the yeast yTHC collection identifies essential host factors affecting tombusvirus RNA recombination. J Virol **80:**1231-41.

135. **Sesaki, H., and R. E. Jensen.** 2004. Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. J Biol Chem **279:**28298-303.

136. **Sesaki, H., S. M. Southard, M. P. Yaffe, and R. E. Jensen.** 2003. Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. Mol Biol Cell **14:**2342-56.

137. **Sickmann, A., J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H. E. Meyer, B. Schonfisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, and C. Meisinger.** 2003. The proteome of Saccharomyces cerevisiae mitochondria. Proc Natl Acad Sci U S A **100:**13207-12.

138. **Simbeni, R., L. Pon, E. Zinser, F. Paltauf, and G. Daum.** 1991. Mitochondrial membrane contact sites of yeast. Characterization of lipid components and possible involvement in intramitochondrial translocation of phospholipids. J Biol Chem **266:**10047-9.

139. **Singh, G., S. Popli, Y. Hari, P. Malhotra, S. Mukherjee, and R. K. Bhatnagar.** 2009. Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. Faseb J.

140. **Stertz, S., M. Reichelt, M. Spiegel, T. Kuri, L. Martinez-Sobrido, A. Garcia-Sastre, F. Weber, and G. Kochs.** 2007. The intracellular sites of early replication and budding of SARS-coronavirus. Virology **361:**304-15.

141. **Stojanovski, D., B. Guiard, V. Kozjak-Pavlovic, N. Pfanner, and C. Meisinger.** 2007. Alternative function for the mitochondrial SAM complex in biogenesis of alpha-helical TOM proteins. J Cell Biol **179:**881-93.

142. **Stojanovski, D., M. Rissler, N. Pfanner, and C. Meisinger.** 2006. Mitochondrial morphology and protein import--a tight connection? Biochim Biophys Acta **1763:**414-21.

143. **Suhy, D. A., T. H. Giddings, Jr., and K. Kirkegaard.** 2000. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J Virol **74:**8953-65.

144. **Van Der Heijden, M. W., J. E. Carette, P. J. Reinhoud, A. Haegi, and J. F. Bol.** 2001. Alfalfa mosaic virus replicase proteins P1 and P2 interact and colocalize at the vacuolar membrane. J Virol **75:**1879-87.

145. **van der Laan, M., M. Rissler, and P. Rehling.** 2006. Mitochondrial preprotein translocases as dynamic molecular machines. FEMS Yeast Res **6:**849-61.

146. **van Hemert, M. J., S. H. van den Worm, K. Knoops, A. M. Mommaas, A. E. Gorbalenya, and E. J. Snijder.** 2008. SARS-coronavirus replication/transcription complexes are membrane-protected and need a host factor for activity in vitro. PLoS Pathog **4:**e1000054.

147. **van Meer, G., D. R. Voelker, and G. W. Feigenson.** 2008. Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol **9:**112-24.

148. **Van Wynsberghe, P. M., and P. Ahlquist.** 2009. 5' cis elements direct nodavirus RNA1 recruitment to mitochondrial sites of replication complex formation. J Virol.

149. **Van Wynsberghe, P. M., H. R. Chen, and P. Ahlquist.** 2007. Nodavirus RNA replication protein a induces membrane association of genomic RNA. J Virol **81:**4633-44.

150. **Venter, P. A., N. K. Krishna, and A. Schneemann.** 2005. Capsid protein synthesis from replicating RNA directs specific packaging of the genome of a multipartite, positive-strand RNA virus. J Virol **79:**6239-48.

151. **Venter, P. A., D. Marshall, and A. Schneemann.** 2009. Dual roles for an arginine-rich motif in specific genome recognition and localization of viral coat protein to RNA replication sites in flock house virus-infected cells. J Virol **83:**2872-82.

152. **Venter, P. A., and A. Schneemann.** 2007. Assembly of two independent populations of flock house virus particles with distinct RNA packaging characteristics in the same cell. J Virol **81:**613-9.

153. **Venter, P. A., and A. Schneemann.** 2008. Recent insights into the biology and biomedical applications of Flock House virus. Cell Mol Life Sci **65:**2675-87.

154. **Voelker, D. R.** 2003. New perspectives on the regulation of intermembrane glycerophospholipid traffic. J Lipid Res **44:**441-9.

155. **Waizenegger, T., S. Schmitt, J. Zivkovic, W. Neupert, and D. Rapaport.** 2005. Mim1, a protein required for the assembly of the TOM complex of mitochondria. EMBO Rep **6:**57-62.

156. **Waizenegger, T., T. Stan, W. Neupert, and D. Rapaport.** 2003. Signal-anchor domains of proteins of the outer membrane of mitochondria: structural and functional characteristics. J Biol Chem **278:**42064-71.

157. **Walther, D. M., and D. Rapaport.** 2008. Biogenesis of mitochondrial outer membrane proteins. Biochim Biophys Acta.

158. **Walukiewicz, H. E., J. E. Johnson, and A. Schneemann.** 2006. Morphological changes in the T=3 capsid of Flock House virus during cell entry. J Virol **80:**615-22.

159. **Wang, R. Y., J. Stork, and P. D. Nagy.** 2009. A key role for heat shock protein 70 in the localization and insertion of tombusvirus replication proteins to intracellular membranes. J Virol **83:**3276-87.

160. **Wasley, A., S. Grytdal, and K. Gallagher.** 2008. Surveillance for acute viral hepatitis--United States, 2006. MMWR Surveill Summ **57:**1-24.

161. **Weber-Lotfi, F., A. Dietrich, M. Russo, and L. Rubino.** 2002. Mitochondrial targeting and membrane anchoring of a viral replicase in plant and yeast cells. J Virol **76:**10485-96.

162. **Weeks, S. A., and D. J. Miller.** 2008. The heat shock protein 70 cochaperone YDJ1 is required for efficient membrane-specific flock house virus RNA replication complex assembly and function in Saccharomyces cerevisiae. J Virol **82:**2004-12.

163. **Wells, R. C., L. K. Picton, S. C. Williams, F. J. Tan, and R. B. Hill.** 2007. Direct binding of the dynamin-like GTPase, Dnm1, to mitochondrial dynamics protein Fis1 is negatively regulated by the Fis1 N-terminal arm. J Biol Chem **282:**33769-75.

164. **Westermann, B.** 2008. Molecular machinery of mitochondrial fusion and fission. J Biol Chem **283:**13501-5.

165. **Wiedemann, N., V. Kozjak, A. Chacinska, B. Schonfisch, S. Rospert, M. T. Ryan, N. Pfanner, and C. Meisinger.** 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature **424:**565-71.

166. **Wiedemann, N., N. Pfanner, and A. Chacinska.** 2006. Chaperoning through the mitochondrial intermembrane space. Mol Cell **21:**145-8.

167. **Wolk, B., B. Buchele, D. Moradpour, and C. M. Rice.** 2008. A dynamic view of hepatitis C virus replication complexes. J Virol **82:**10519-31.

168. **Wu, S. X., P. Ahlquist, and P. Kaesberg.** 1992. Active complete in vitro replication of nodavirus RNA requires glycerophospholipid. Proc Natl Acad Sci U S A **89:**11136-40.

169. **Wu, S. X., and P. Kaesberg.** 1991. Synthesis of template-sense, single-strand Flockhouse virus RNA in a cell-free replication system. Virology **183:**392-6.

170. **Yaffe, M. P.** 1999. Dynamic mitochondria. Nat Cell Biol **1:**E149-50.

171. **Yamano, K., Y. Yatsukawa, M. Esaki, A. E. Hobbs, R. E. Jensen, and T. Endo.** 2008. Tom20 and Tom22 share the common signal recognition pathway in mitochondrial protein import. J Biol Chem **283:**3799-807.

172. **Yano, M., K. Terada, and M. Mori.** 2004. Mitochondrial import receptors Tom20 and Tom22 have chaperone-like activity. J Biol Chem **279:**10808-13.

173. **Zahedi, R. P., A. Sickmann, A. M. Boehm, C. Winkler, N. Zufall, B. Schonfisch, B. Guiard, N. Pfanner, and C. Meisinger.** 2006. Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. Mol Biol Cell **17:**1436-50.

174. **Zara, V., A. Ferramosca, L. Capobianco, K. M. Baltz, O. Randel, J. Rassow, F. Palmieri, and P. Papatheodorou.** 2007. Biogenesis of yeast dicarboxylate carrier: the carrier signature facilitates translocation across the mitochondrial outer membrane. J Cell Sci **120:**4099-106.

175. **Zhang, Y., and D. C. Chan.** 2007. New insights into mitochondrial fusion. FEBS Lett **581:**2168-73.

176. **Zhong, Q., and M. L. Greenberg.** 2005. Deficiency in mitochondrial anionic phospholipid synthesis impairs cell wall biogenesis. Biochem Soc Trans **33:**1158-61.

177. **Zhong, W., R. Dasgupta, and R. Rueckert.** 1992. Evidence that the packaging signal for nodaviral RNA2 is a bulged stem-loop. Proc Natl Acad Sci U S A **89:**11146-50.

CHAPTER II

MITOCHONDRIAL-ENRICHED ANIONIC PHOSPHOLIPIDS FACILITATE FLOCK HOUSE VIRUS RNA POLYMERASE MEMBRANE ASSOCIATION

Positive-strand RNA viruses make up a large and diverse group of microbial pathogens responsible for significant clinical diseases in humans. The availability of vaccines and antiviral therapies targeted towards these viruses is limited, and thus the study of common molecular mechanisms involved in positive-strand RNA virus pathogenesis is important for the identification of novel candidate antiviral targets. One universal characteristic of all positive-strand RNA viruses is the necessity to assemble viral RNA replication complexes on host intracellular membranes. Viral replication complexes have been found associated with a variety of intracellular membranes, including the endoplasmic reticulum (ER), endosomes, peroxisomes, chloroplasts, and the mitochondria (32). To facilitate the formation of viral RNA replication complexes, individual viruses encode replicase proteins containing membrane-specific targeting signals, several of which have been characterized in detail (6, 29, 31, 45, 47). However, to date the host components present on organelle-specific membranes that facilitate targeting and assembly are poorly defined, and only a limited

number of cellular membrane components have been shown to be important in viral RNA replication complex assembly (16, 35, 37). The identification of host factors necessary for this essential step in the positive-strand RNA virus life cycle will enhance our understanding of the molecular mechanisms of replication complex assembly, and may highlight possible targets for future antiviral therapies.

To investigate the role of host factors in viral RNA replication complex assembly, we use the established and versatile model alphanodavirus *Flock House virus* (FHV). FHV is the best studied member of the *Nodaviridae* family and has been used extensively to investigate positive-strand RNA virus replication (7, 14, 15, 30, 31, 40, 41), host innate immunity (9, 24), and virus assembly (26, 48). FHV virions contain a co-packaged bipartite genome consisting of RNA1 (3.1 kb) and RNA2 (1.4 kb), which encode protein A, the viral RNA-dependent RNA polymerase, and the capsid protein precursor, respectively. Additionally, during active RNA replication a small subgenomic RNA3 (0.4 kb) co-linear with the 3' end of RNA1 is produced, which encodes protein B2, a potent inhibitor of the innate immunity RNA interference pathway (9, 24). FHV RNA replication is supported in cells derived from a wide variety of genetically tractable hosts, including *Drosophila melanogaster* (30), *Caenorhabditis elegans* (24), and the budding yeast *Saccharomyces cerevisiae* (31, 40, 41), which makes FHV a unique model virus to study the molecular mechanisms of host-pathogen interactions that control RNA replication complex assembly.

FHV protein A is the only viral protein necessary for RNA replication complex assembly, a process that takes place on the mitochondrial outer membrane (29-31). Protein A is targeted to the mitochondrial outer membrane via an amino-terminal targeting signal (29). This signal resembles those present in signal-anchored proteins that are targeted to the mitochondrial outer membrane (4, 20, 27, 56), suggesting that FHV may use established mitochondrial targeting and trafficking machinery for assembly. Mitochondrial protein import has been studied extensively in yeast in part due to the facile genetics of this model organism and the development of an easily manipulated in vitro import system (23, 46, 54, 58). The majority of mitochondrial proteins are translated from nuclear-encoded genes within the cytosol and therefore must be properly trafficked and targeted to their final mitochondrial compartments. The import of most proteins into mitochondria is facilitated by the translocase of the mitochondrial outer membrane (TOM) complex, a large macromolecular structure consisting of the main import component, Tom40; three receptors, Tom20, Tom22, and Tom70; and three accessory proteins, Tom5, Tom6, and Tom7 (8, 38, 42). However, it has recently been shown that some mitochondrial outer membrane proteins containing a signal-anchor amino-terminal domain, including the NADH-cytochrome *b5* reductase isoform encoded by the yeast *MCR1* gene, can use an undefined TOM complex-independent mechanism for membrane targeting and insertion (27).

In this report, we investigated the role of host mitochondrial outer membrane components in the binding and insertion of protein A to mitochondria, an initial

step of FHV RNA replication complex assembly. To facilitate these studies we established an in vitro protein A translation and membrane association system with which we were able to recapitulate many of the in vivo biochemical characteristics of protein A. We found that protein A rapidly and specifically associated with yeast, insect, and mammalian mitochondria after in vitro translation, and furthermore acquired resistance to alkaline extraction, consistent with its characteristic as an integral membrane protein in vivo (29, 30). Protein A membrane insertion was independent of protease-sensitive outer membrane components and the main component of the TOM complex, Tom40. However, we found that protein A associated efficiently with anionic phospholipids, and in particular the mitochondrial-specific anionic phospholipid cardiolipin. These studies provide insight into the early steps of FHV RNA replication complex assembly, and implicate organelle membrane lipids in the specific intracellular targeting of positive-strand RNA virus replication.

MATERIALS AND METHODS

Yeast strains, Drosophila cells, and culture conditions. Yeast strains used in this study are listed in Table 1. For isolation of mitochondria, yeast were grown in YPG (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 3% glycerol) or YPD (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% dextrose) media. *Drosophila* S2 cells were grown in Schneider's *Drosophila* media with 10% heat-inactivated fetal bovine serum at 25°C as previously described (18). Mammalian baby hamster kidney (BHK) cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's media (DMEM) supplemented with 5% bovine growth serum, 10 units per ml penicillin, and 10 µg per ml streptomycin.

Plasmids and antibodies. Standard molecular biology techniques were used for the production of all plasmids. The protein A in vitro expression plasmid, pIVT-PA, was generated by placing the open reading frame from pS2FA (18) into the EcoRI/XbaI sites of pCMV-TnT (Promega, Madison, WI). The β-galactosidase (β-gal) in vitro expression plasmid, pIVT-LacZ, was generated by placing the SpeI/AgeI fragment from pMT/V5/LacZ (BD Biosciences, San Jose, CA) into the AvrII/BspEI sites of pIVT-PA. The protein A-green fluorescent protein (GFP) chimera in vitro expression plasmid, pIVT-PA1-46/GFP, was generated by placing the PstI/BamHI fragment from pPA1-46/GFP (29) into the same sites of pGEM-4Z (Promega). The GFP in vitro expression plasmid, pIVT-GFP, was generated by first placing the PstI/HindIII fragment from pGal-YFP into the same sites of pGEM-4Z to create pIVT-YFP, followed by placing the

TABLE 2.1: *S. cerevisiae* Strains Used in this Study

Strain/Source	Genotype
BY4743 (WT) (ATCC)	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0*
Δtom70 (ATCC)	*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0 tom70Δ/tom70Δ*
YPH499 (WT) (58)	*MATα ade2-101 his3*Δ200 *leu2Δ1 ura3-52 trp1-Δ63 lys-801*
MM112-C (*Δtom20*) (58)	*MATα ade2-101 his3*Δ200 *leu2Δ1 ura3-52 trp1-Δ63* lys-801 tom20::URA3 (Yep13-TOM22)
KKY3.7 (WT -TK8) (58)	*MATα his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3- 52* tom40::HIS3 (pRS314-Tom40)
KKY3.3 (TK4) *tom40-3* (2)	*MATα his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52* tom40::HIS3 (pRS314-Tom40-3)
KKY3.6 (TK7) *tom40-6* (58)	*MATα his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52* tom40::HIS3 (pRS314-Tom40-6)

BspEI/HindIII fragment from pPA1-46/GFP (29) into the same sites of pIVT-YFP. The in vitro expression plasmid encoding the ATP/ADP carrier protein (AAC) was generously provided by F. Ulrich Hartl (Max-Planck-Institute of Biochemistry, Germany). In vitro expression plasmids encoding Tom20, porin, or His6-tagged pSu9-dihydrofolate reductase (DHFR) have been previously described (2, 27). We expressed and purified pSu9-DHFR from *E. coli* BL21 cells by nickel affinity chromatography as previously described (2, 27). Mouse monoclonal antibodies against yeast porin were purchased from Molecular Probes (Carlsbad, CA). Rabbit polyclonal antibodies against yeast Tim44 or Tom40 were generously provided by Donna M. Gordon (University of Pennsylvania) or Nikolaus Pfanner (University of Freiburg, Germany), respectively. Rabbit polyclonal antibodies against yeast Tom20 and Tom70 have been previously described (2, 27).

Mitochondrial isolation and in vitro membrane association. Yeast mitochondria were isolated as previously described (28) and stored in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM 3-morpholinopropanesulphonic acid [MOPS]-KOH, pH 7.2) at -80°C. *Drosophila* S2 cell and BHK mitochondria were isolated by mechanical disruption and differential centrifugation as previously described (11, 18). mRNAs were generated by in vitro transcription with SP6 or T7 RNA polymerase (Epicentre, Madison, WI) from pGEM-4Z- or pCMV-TnT-based vectors, respectively. Initial in vitro translation experiments with wheat germ, *S. cerevisiae*, and *D. melanogaster* cell-free lysates failed to produce detectable full length protein A (data not shown), and thus we used commercially available nuclease-treated rabbit reticulocyte lysates (RRL)

(Promega) for all subsequent experiments. Proteins for in vitro assays were translated using RRLs in the presence of 400 µCi/ml [^{35}S] methionine + cysteine (Amersham) with optimized amounts of in vitro transcribed mRNAs for 1 h at 25°C. Translation mixtures (5 µl per 100 µl reaction) were incubated with isolated mitochondria (50 µg total protein per 100 µl reaction) in import buffer (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS-KOH [pH 7.2], 0.25 mg/ml bovine serum albumin, 2 mM ATP, 2 mM NADH) at 25°C. Import reactions were diluted with 4 volumes of SEM buffer, mitochondria were pelleted by centrifugation at 12,000 x *g* for 15 min, resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl; pH 6.8, 2% SDS, 10% [w/v] glycerol, 0.001 mg/ml bromophenol blue, 5% 2-mercaptoethanol), and heated to 95°C for 5 min. For carbonate extractions, pelleted membranes were resuspended in 0.1 M sodium carbonate (pH 11.5) and incubated on ice for 30 min. Extracted membranes were reisolated by centrifugation at 55,000 x *g* for 30 min at 4°C, and resuspended in sample buffer. Porin and AAC protein import were analyzed by incubating mitochondria with 100 µg per ml proteinase K for 5 min on ice followed by the addition 1 mM phenylmethanesulphonyl fluoride (PMSF). Mitochondria were reisolated by centrifugation at 12,000 x *g* for 15 min and resuspended in sample buffer. Denatured samples were separated by SDS-PAGE, gels were fixed in 25% isopropanol:10% acetic acid, incubated in 1 M sodium salicylate for 1 h, dried under vacuum, and exposed to autoradiography film. Digital images were obtained using an Alpha Innotech Fluorchem 8900, quantified with AlphaEase

FC software, and final images were prepared with Adobe Photoshop software. All contrast adjustments to the final images were done prior to cropping.

Flotation of membranes and liposomes. In vitro membrane association reactions were brought to 300 µl with flotation buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 50 mM KCl, 2 mM $MgCl_2$, pH 7.5) and mixed with 900 µl 50% Nycodenz solution to yield a final concentration of 37.5% Nycodenz. Samples were loaded under 3.2 ml 35% and 0.2 ml 5% Nycodenz solutions, and centrifuged at 134,000 x *g* for 20 h at 4°C in a Beckman MLS-50 swing bucket rotor. Fractions of equal volume were collected and analyzed by SDS-PAGE and fluorography as described above. *E. coli* and porcine brain total lipid extracts as well as purified 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS), L-a-phosphatidylinositol (PI), 1,2-dioleoyl-*sn*-glycero-3-phosphate (PA), 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (PG), 1,1'2,2'-tetraoleoyl cardiolipin (CL), and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Chloroform solvent was removed under vacuum centrifugation for 1 h and lipids were resuspended in HEPES buffer (20 mM HEPES-KOH, pH 7.2) by vortexing at 4°C for 1 h. Lipids (250 µg per 100 µl reaction) were incubated with in vitro translation mixtures in protein import buffer for 30 min at 25°C, diluted to 1 ml with 85% sucrose in HEPES buffer to a final sucrose concentration of 71.25%, overlaid with 2.5 ml 65% sucrose and 1 ml 10% sucrose in HEPES buffer, and

centrifuged at 134,000 x *g* for 20 h at 4°C in a Beckman MLS-50 swing bucket rotor. Gradients were fractionated and analyzed as described above.

Statistics. We used a two-tailed Student's t-test assuming equal variances for comparative statistical analyses and considered a *p*-value < 0.05 as significant. Unless otherwise indicated all quantitative data represent results from at least 3 independent experiments and are presented as the means ± standard errors of the means.

RESULTS

In vitro translated FHV protein A associates specifically with mitochondria. FHV assembles RNA replication complexes on the mitochondrial outer membrane (29, 30). To investigate the initial steps of replication complex assembly and identify host components responsible for protein A membrane association we developed an in vitro mitochondrial binding assay, similar to the in vitro system used to study the import and assembly of endogenous mitochondrial proteins (23, 46, 58). We initially attempted to purify full length FHV RNA polymerase produced in *E. coli* for in vitro experiments, but protein A produced with this heterologous system was highly insoluble (D. Miller, unpublished data). Thus, we explored an in vitro translation system, where FHV protein A was synthesized from a synthetic mRNA template in a nuclease-treated RRL. Initial studies with a carboxy-terminal epitope-tagged construct and subsequent immunoblotting with epitope tag-specific antibodies demonstrated that full length protein A was produced (data not shown). For all subsequent experiments ^{35}S-radiolabeled protein was used to enhance detection sensitivity.

We incubated in vitro translation mixtures with mitochondria isolated from cells capable of supporting robust FHV RNA replication, including wildtype YPH499 yeast (31, 40, 41), *Drosophila* S2 cells (30), or mammalian BHK cells (15). Mitochondria were subsequently pelleted by centrifugation and membrane association was analyzed by SDS-PAGE and fluorography of pelleted fractions (Figure 2.1). We found protein A rapidly sedimented in the presence of

mitochondria from yeast, insect, or mammalian cells, whereas β-gal, a similar sized (~100 kDa) control protein that was translated as efficiently as protein A showed negligible mitochondrial association and sedimentation (Figure 2.1A). In the absence of mitochondria neither protein A nor β-gal were found in significant amounts in the pellet fractions, indicating that the in vitro translated proteins were not forming aggregates that rapidly sedimented with centrifugation (Fig. 1A, bottom gel). Maximal binding of protein A was 70-80% of input protein and was seen after 10 to 20 min. Protein A association with mitochondria was independent of ATP levels, and extended incubations past 30 min did not significantly increase the fraction of bound protein A (data not shown). Furthermore, 60% of protein A translated in vitro remained associated with mitochondrial membranes even after alkaline extraction with 0.1 M sodium carbonate, suggesting that protein A behaved as an integral membrane protein after in vitro translation and mitochondrial association, similar to results obtained in vivo with both yeast and cultured *Drosophila* cells (29, 30). Based on the comparable results with yeast, insect, and mammalian mitochondria, and the ready availability of yeast strains with deletions or mutations in specific mitochondrial outer membrane components, we used yeast mitochondria for all subsequent studies unless otherwise indicated.

To examine the biochemical characteristics of in vitro translated protein A and further exclude the possibility of aggregation due to the multiple hydrophobic regions within the protein (29), we used membrane flotation assays (Figure 2.1B). In vitro translation or membrane association reactions were mixed with

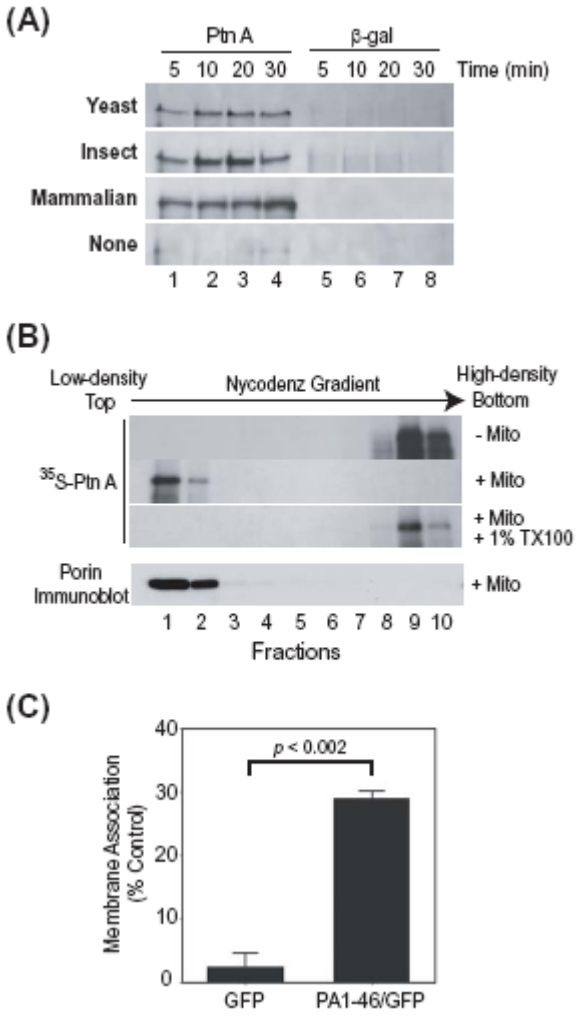

Figure 2.1: FHV protein A specifically associates with mitochondrial membranes in vitro. (A) Equal amounts of in vitro translated ^{35}S-labelled protein A (PtnA) (lanes 1-4) or β-gal (lanes 5-8) were incubated in the absence of mitochondria or with isolated yeast (*S. cerevisiae*), insect (*D. melanogaster*), or mammalian (BHK cells) mitochondria at 25°C for the indicated time period. Mitochondrial source is shown in bold on the left. Mitochondria were pelleted by centrifugation and membrane association was analyzed by SDS-PAGE and fluorography. **(B)** In vitro translated protein A was incubated in the presence or absence of purified yeast mitochondria and detergent, subjected to equilibrium centrifugation in nycodenz gradients, and equal volume fractions were analyzed as described above. The bottom image represents an immunoblot for porin to identify membrane containing fractions from yeast mitochondria. **(C)** In vitro translated GFP or a GFP chimera fused to the first 46 amino acids of protein A (PA1-46/GFP) was incubated with isolated wildtype yeast mitochondria for 20 min at 25°C, separated into pellet and supernatant fractions by centrifugation, and membrane association was quantified as the percent of total in vitro translated protein present in the pellet fraction.

nycodenz, subjected to equilibrium centrifugation, and fractionated to analyze protein A distribution in low-density and high-density regions, which correspond to membrane-associated and soluble or aggregated proteins, respectively (29, 30). Protein A was recovered in high-density fractions in the absence of mitochondria (Figure 2.1B, first gel), whereas with the addition of mitochondria protein A became membrane associated and floated to the low-density fractions (Figure 2.1B, second gel), indicating that protein A was not aggregating after in vitro translation and mitochondrial association. Similar results were obtained when mitochondria were extracted with 0.1 M sodium carbonate prior to flotation, further confirming the protein A was inserted as an integral membrane protein (data not shown). As a control, the addition of the detergent Triton X-100 shifted protein A distribution back to the high-density fractions (Figure 2.1B, third gel). Immunoblotting against the mitochondrial outer membrane protein porin was used as a control to identify membrane containing fractions (Figure 2.1B, bottom blot).

To explore membrane specificity of in vitro translated protein A, we initially used constructs with defined amino-terminal deletions that disrupt mitochondrial targeting and membrane association in vivo (29). Deletions of amino acids 9-45 or 9-135 reduced protein A membrane association with mitochondria in vitro by 20% or 40%, respectively, and there was a direct correlation between the levels of membrane association in vitro with purified mitochondria and in vivo with intact yeast ($R = 0.999$, $p < 0.02$). We also conducted gain-of-function studies and found that the amino-terminal region containing 46 amino acids from protein A,

which includes a mitochondrial targeting signal (29), was sufficient to increase the in vitro mitochondrial association of a GFP fusion construct ten-fold (Figure 2.1C). To further test membrane specificity, we examined the ability of wildtype protein A to associate with canine microsomes in vitro. As a control for these experiments, we used a chimeric protein A where the amino-terminal mitochondrial targeting signal was replaced with a hepatitis C virus NS5B ER targeting signal (31), and performed the assays cotranslationally to mimic protein import in the ER. We found that 51.9 ± 6.0% of the protein A-ER chimera associated with canine microsomes in vitro compared to only 26.8 ± 6.2% of wildtype protein A ($p < 0.004$). We concluded from these data that FHV protein A translated in vitro recapitulated important membrane specificity and biochemical characteristics of protein produced in vivo.

The in vitro membrane insertion of protein A is independent of Tom20, Tom70, and the general import pore. The majority of host mitochondrial proteins utilize the TOM complex for transport to their appropriate mitochondrial compartments (8, 38, 42). Thus, we examined the role of this macromolecular complex in FHV protein A mitochondrial membrane association and insertion using both biochemical and genetic approaches. Several TOM components, such as the primary receptors Tom20 and Tom70 are exposed to the cytosol (5, 19, 23) and therefore susceptible to protease digestion. We treated mitochondria isolated from YPH499 yeast with proteinase K to degrade accessible outer membrane components and analyzed protein A membrane association and subsequent bilayer insertion in vitro by assessing resistance to alkaline extraction

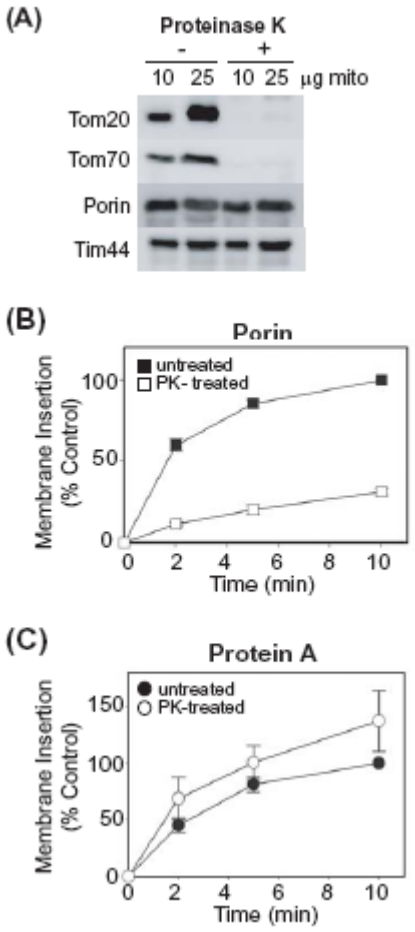

Figure 2.2: Protease-sensitive outer membrane components are not required for protein A mitochondrial membrane association and insertion in vitro. (A) Immunoblot analysis of yeast mitochondrial membrane proteins after proteinase K digestion of purified mitochondria. **(B)** In vitro translated porin was incubated with untreated (closed squares) or proteinase K (PK)-treated (open squares) yeast mitochondria for the indicated times. Mitochondria were isolated by centrifugation and porin membrane insertion was analyzed by resistance to protease-digestion followed by SDS-PAGE and fluorography. Data are presented as percentage of porin insertion at 10 min in untreated mitochondria. **(C)** In vitro translated protein A (PtnA) was incubated with untreated (closed circles) or proteinase K (PK)-treated (open circles) mitochondria for the indicated times. Mitochondria were isolated by centrifugation and protein A membrane insertion was analyzed by resistance to alkaline extraction followed by SDS-PAGE and fluorography. Data are presented as percentage of protein A insertion at 10 min in untreated mitochondria.

(Figure 2.2). Immunoblot examination of protease-treated mitochondria revealed the near complete loss of Tom20 and Tom70, whereas a pore-forming protein wholly embedded in the mitochondrial outer membrane (porin) or an inner membrane protein (Tim44) were relatively unaffected by protease treatment (Figure 2.2A). Protease-treated mitochondria showed a marked defect in the insertion of porin (Figure 2.2B), an endogenous host protein previously shown to use the Tom20 receptor for proper membrane insertion (23). In contrast, protein A showed a slight increase in insertion efficiency upon the removal of protease-sensitive outer membrane components (Figure 2.2C*).* These results suggested that protease-sensitive components were not required for efficient protein A mitochondrial membrane association and insertion.

To further examine the role of specific mitochondrial outer membrane components in protein A insertion, we conducted in vitro assays with mitochondria purified from yeast strains with deletions or mutations in individual TOM components (Table 1 and Figure 2.3). We verified the genotype of deletion strains by PCR (data not shown) and immunoblotting (Figure 2.3A), and used purified mitochondria for protein A insertion assays. Protein A insertion was not significantly reduced using mitochondria from *Δtom20* or *Δtom70* compared to wildtype yeast, whereas insertion or import of porin or AAC, which have previously been shown to depend primarily on Tom20 or Tom70, respectively (23, 61), was reduced in mitochondria from the appropriate deletion strain (Figure 2.3B). We also examined the role of Tom40, the main component of the general

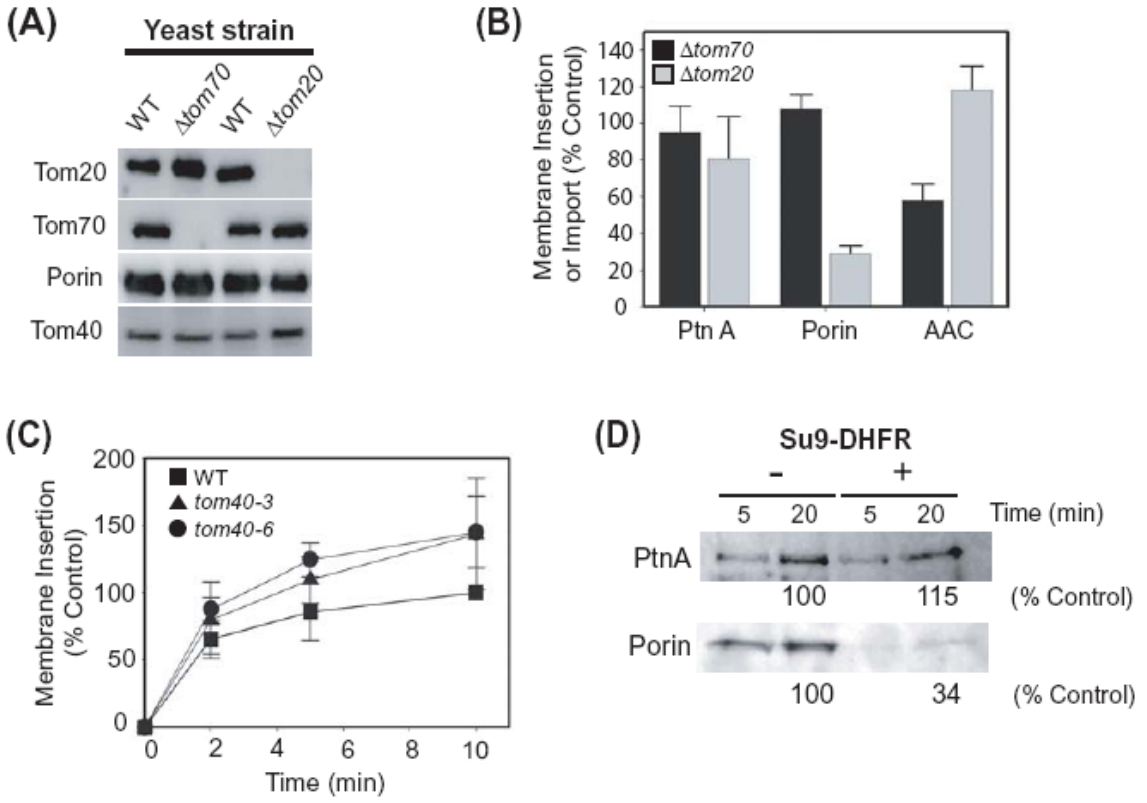

Figure 2.3: Mitochondrial import machinery is not required for protein A membrane association and insertion in vitro. (A) Immunoblot analysis of purified mitochondria from Δ*tom20*, Δ*tom70*, and corresponding wildtype (WT) yeast strains (see Table 1). **(B)** In vitro translated protein A (PtnA), porin, or AAC were incubated with mitochondria from Δ*tom70* (black bars), and Δ*tom20* (grey bars) yeast for 10 min at 25°C. Protein A and porin membrane insertion were analyzed as described in Fig. 2. AAC import was analyzed by resistance to protease-digestion followed by SDS-PAGE and fluorography. Data are presented as percent of mitochondrial insertion or import compared to control wildtype yeast. **(C)** Protein A insertion into mitochondria from wildtype (squares), *tom40-3* (triangles), or *tom40-6* (circles) yeast. Mitochondrial membrane insertion was analyzed as described in Fig. 2 and data are presented as percentage of control mitochondrial from wildtype yeast at 10 min. **(D)** Wildtype yeast mitochondria were incubated with excess recombinant Su9-DHFR for 5 min prior to incubation with in vitro translation mixtures. Protein A and porin insertion were analyzed at 5 and 20 min as described in Fig. 2. Quantitative data are presented as the percentage of protein A or porin inserted at 20 min in untreated samples and are the averages of two independent experiments.

import pore, in protein A membrane insertion. Since Tom40 is an essential protein, we made use of two temperature-sensitive (ts) strains, *tom40-3* and *tom40-6*. Mitochondria from *tom40-3* have a defect in Tom20 import (2), whereas mitochondria from *tom40-6* have not been shown to have any defects in protein import but do have reduced levels of assembled TOM complex (58). Wildtype and ts Tom40 strains were grown at 25°C for mitochondrial isolation, and prior to insertion assays mitochondria were incubated at 37°C for 15 min to induce the ts phenotype through temperature-dependent destabilization of Tom40. Mitochondria from *tom40-3* or *tom40-6* yeast showed no reduction in protein A insertion but rather showed a trend toward increased insertion efficiency (Figure 2.3C), similar to results with protease-treated mitochondria (Figure 2.2C). To further examine the role of the TOM complex in protein A mitochondrial insertion we conducted competition experiments with purified pSu9-DHFR, a chimeric protein that competes for protein insertion through the main import pore (2, 23, 27). Protein A insertion into wildtype mitochondria was not inhibited by Su9-DHFR, whereas porin insertion was substantially reduced (Figure 2.3D). Taken together, these data suggested that protein A association and insertion into the mitochondrial membrane did not require Tom20, Tom70, and the general import pore.

Protein A is a lipid binding protein with affinity for specific anionic phospholipids. The observation that protein A membrane association and insertion was independent of protease-sensitive outer membrane components and Tom40 (Figures 2.2 and 2.3) suggested that non-protein components, such

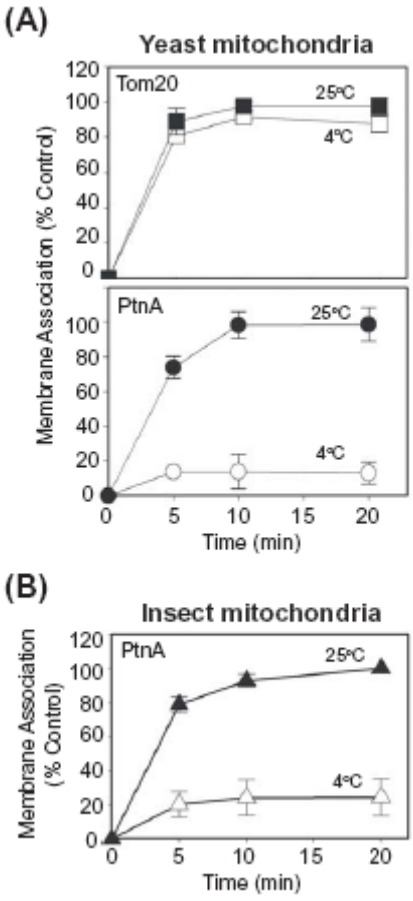

Figure 2.4: Protein A membrane association is temperature-dependent. (A) In vitro translated Tom20 (upper graph) or protein A (lower graph) was incubated with purified yeast mitochondria at 4°C (open symbols) or 25°C (closed symbols) for the indicated time period. Mitochondria were isolated by centrifugation and protein membrane association was analyzed as described in Fig. 1. Data are presented as the percentage of control membrane association at 20 min and 25°C. **(B)** In vitro translated protein A was incubated with purified *Drosophila* mitochondria at 4°C (open symbols) or 25°C (closed symbols) for the indicated time period and membrane association was analyzed as described above.

as membrane lipids, may be involved. To initially test this hypothesis we examined the temperature dependence of protein A-membrane interactions in vitro (Figure 2.4). Protein-protein interactions are often temperature independent, and this characteristic is frequently used to study processes such as initial receptor-ligand binding at low temperatures to avoid receptor internalization or remodeling after ligand-mediated activation. We found that Tom20 binding to purified mitochondria occurred equally well at 4°C and 25°C (Figure 2.4A, upper graph), consistent with its known interaction with the import pore Tom40 during mitochondrial membrane binding and insertion (2, 4). We obtained similar temperature-independent results with porin (data not shown). In contrast, protein A membrane association with purified wildtype yeast (Figure 2.4A, bottom graph) and *Drosophila* (Figure 2.4B) mitochondria was significantly reduced at 4°C to less than 20% of the levels seen at 25°C.

To directly examine potential protein A-lipid interactions, we used lipid extracts from *E. coli* and brain with in vitro binding assays. We selected these extracts due in part to their differences in phospholipid composition. For example, *E. coli* lipid extracts contain a lipid composition similar to that of eukaryotic mitochondria with large quantities of the anionic phospholipids PG (15.1%) and CL (9.8%) (Avanti Polar Lipids product data). In contrast, brain extracts contain a more diverse group of phospholipids with low concentrations of anionic phospholipids and a larger quantity of neutral phospholipids. We incubated in vitro translated protein A or β-gal with liposomes generated from individual lipid extracts for 30 min, and subsequently examined liposome binding

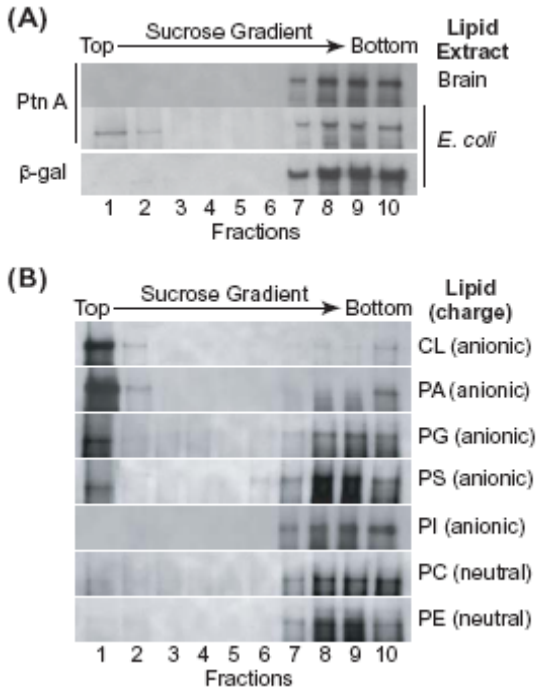

Figure 2.5: Protein A is a lipid-binding protein with affinity for specific anionic phospholipids. (A) In vitro translated protein A or β-gal was incubated with liposomes generated from *E. coli* or brain lipid extracts, subjected to equilibrium centrifugation in sucrose gradients, and fractions were analyzed by SDS-PAGE and fluorography. **(B)** In vitro translated protein A was incubated with liposomes generated from equal amounts of the indicated purified lipids and analyzed as described above.

by equilibrium gradient centrifugation (Figure 2.5A). The control protein β-gal did not bind to liposomes generated with either lipid extract (Figure 2.5A, lower gradient, and data not shown). In contrast, protein A associated with liposomes generated from *E. coli* lipid extracts, where approximately 25% partitioned into the low density fractions (Figure 2.5A, middle gradient). Only minimal protein A binding was seen with liposomes generated from brain extracts (Figure 2.5A, upper gradient).

To further explore potential phospholipid selectivity, we analyzed the in vitro binding capabilities of protein A with liposomes generated from equal amounts of purified individual membrane phospholipids (Figure 2.5B). We observed substantial protein A binding to the anionic phospholipids CL and PA, moderate binding to the anionic phospholipids PG and PS, and negligible binding to the anionic phospholipids PI and the neutral lipids PC and PE. The absence of significant protein A binding to PC was not due to decreased flotation, as control experiments with fluorescent PC demonstrated almost complete partitioning of this neutral lipid into upper low-density gradient fractions (data not shown). Of interest with the purified lipid experiments was protein A binding to the mitochondrial-enriched anionic phospholipids CL, PA, and PG (17, 55), and in particular the mitochondrial-specific CL, where protein A was recovered almost exclusively in the low-density fractions (Figure 2.5B, top gradient, fractions 1 and 2).

The diphosphatidyl-based lipid CL is found in small concentrations in the mitochondrial outer membrane, with the notable exception of the contact sites

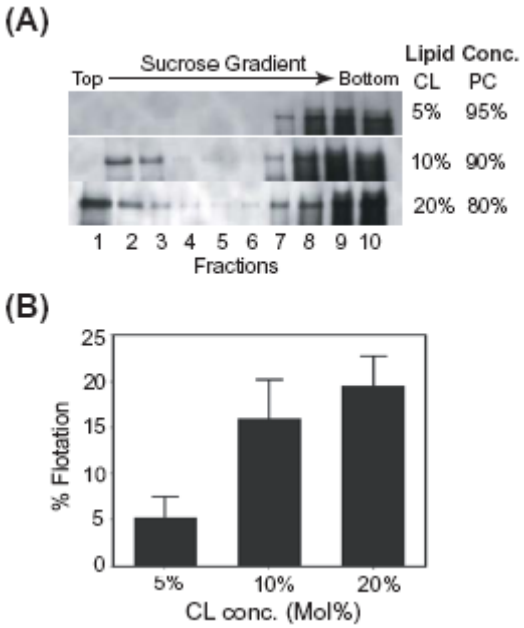

Figure 2.6: Protein A binding to liposomes is correlated with CL content. **(A)** In vitro translated protein A was incubated with liposomes containing the indicated CL and PC concentrations (Mol %) and analyzed as described in Fig. 5. **(B)** Correlation of CL content and protein A-liposome binding. Percent flotation was calculated as the ratio of the amount of protein A in the upper four gradient samples (fractions 1-4) compared to the total signal (fractions 1-10).

between mitochondrial outer and inner membranes, where it can increase to roughly 20% of total membrane phospholipids (3, 51). To further confirm the CL-binding capabilities of protein A, we generated liposomes containing increasing concentrations of CL relative to PC and assayed protein A in vitro binding by gradient flotation assays (Figure 2.6). Protein A showed CL concentration-dependent flotation, where approximately 20% of protein A partitioned to low density fractions when the total CL concentration was 20% (Figure 2.6A, bottom gradient, and 2.6B). The positive correlation between CL concentration and protein A flotation (Fig. 6B, R = 0.92) was not due to changes in liposome partitioning within sucrose gradients, as control experiments with fluorescent PC-CL liposomes demonstrated equivalent flotation characteristics regardless of CL concentration (data not shown).

To examine the role of CL in protein A association with intact mitochondria, we initially conducted in vitro membrane association assays with mitochondria purified from Δ*crd1* yeast, which lack the biosynthetic enzyme CL synthase and hence produce no CL (12). Protein A membrane association in vitro was not significantly different between mitochondria from Δ*crd1* and wildtype yeast (data not shown). However, the deletion of CL synthase results in the increased accumulation of PG (12), which along with PA is a precursor in the CL biosynthetic pathway and an anionic phospholipid to which protein A also significantly bound in flotation assays (Figure 2.5B). To avoid the potential confounding effects of altered phospholipid composition in yeast strains with deletions or mutations of specific phospholipid biosynthesis genes, we

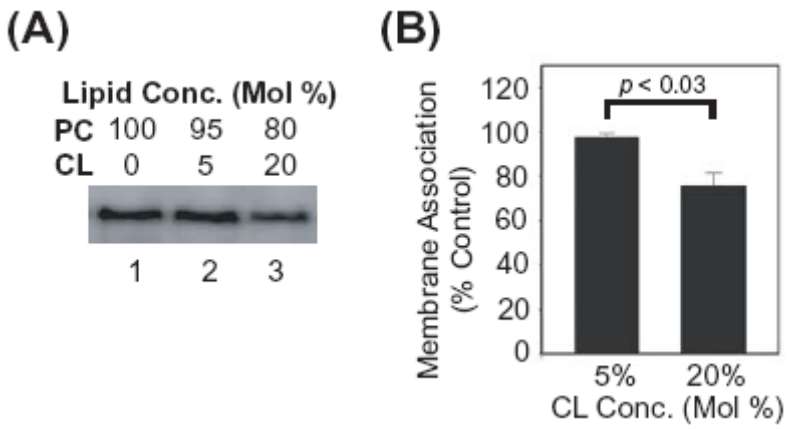

Figure 2.7: CL containing liposomes disrupt protein A binding to wildtype mitochondria. (A) Mitochondria from YPH499 yeast (50 μg total protein) were incubated with in vitro translated protein A and liposomes (50 μg) containing 100% PC (lane 1), 80% PC:20% CL (lane 2), or 95% PC:5% CL (lane 3) for 10 min at 25°C. Samples were separated by differential centrifugation at 1,000 x g for 5 min at 4°C, pellets were resuspended in SDS-PAGE buffer, and samples were analyzed by fluorography. **(B)** Quantitative analysis of liposome competition assays. Data are presented as the percentage of protein A present in the mitochondrial pellet compared to competition with 100% PC liposomes.

subsequently conducted differential centrifugation competition experiments with mitochondria from wildtype yeast and CL-containing liposomes (Figure 2.7). We initially used fluorescent PC-CL liposomes to optimize differential centrifugation conditions and minimize liposome fusion or co-sedimentation with intact mitochondria, and achieved conditions under which approximately 15% of the input lipid-associated fluorescence signal pelleted with mitochondria regardless of the CL liposome concentration (data not shown). Under these optimized conditions, liposomes containing 20% CL reduced protein A association with wildtype mitochondria by approximately 25% compared to PC-only liposomes, whereas minimal competition was seen with 5% CL liposomes (Figure 2.7A and 2.B). Similar results were obtained with mitochondria from *Drosophila* cells (data not shown). Taken together, these data indicated that FHV protein A is a lipid-binding protein with affinity for specific anionic phosopholipids, and in particular the mitochondrial-specific phospholipid CL.

DISCUSSION

In this study, we investigated the interactions between FHV protein A and intracellular membranes, an initial and important step in the assembly of viral RNA replication complexes. Based on our results we conclude that protein A is a lipid-binding protein with particular affinity for specific anionic phospholipids. Furthermore, both biochemical and genetic studies indicated that protease-accessible membrane proteins, and in particular the TOM complex components Tom20, Tom70, and Tom40, were not required for protein A interactions with the mitochondrial membrane. However, we cannot exclude a role for protease-resistant and possibly membrane-embedded non-TOM complex mitochondrial outer membrane proteins in FHV protein A-membrane interactions. Indeed, although we saw almost complete association of in vitro translated protein A with intact mitochondria, we obtained only partial association with lipid extracts or liposomes, with the exception of 100% CL liposomes. Furthermore, changes in membrane-embedded protein conformation may have accounted for the dramatic temperature dependence we observed in protein A mitochondrial binding. Nonetheless, the results with protein A in this study are consistent with those published for some endogenous mitochondrial outer membrane proteins that contain an amino-terminal signal-anchor transmembrane domain that resemble the targeting sequence of FHV protein A, suggesting a potentially similar pathway for membrane insertion (27, 57). The published study with Mcr1 used mitochondrial import assays similar to the system described in this manuscript,

including the use of protease digestion, TOM receptor component deletion strains, and Su9-DHFR competition experiments, but did not explore lipid binding (27). Further experiments with both cellular mitochondrial proteins and FHV protein A will be required to fully define this pathway.

The observation that protein A has lipid-binding capabilities is consistent with the well described association between positive-strand RNA virus replication complexes and intracellular membranes (32, 49). Host membranes likely play multiple roles in viral RNA replication, which may include: (i) serving as a scaffold for assembly of a macromolecular structure such as an RNA replication complex; (ii) shielding viral RNA replication intermediates such as double-stranded RNA from cellular innate antiviral pathways; and (iii) providing co-factors for optimal enzymatic activity of viral replicase proteins. These functions could be mediated by either protein or lipid constituents within particular organelle membranes. Thus far the predominant emphasis in the field has been placed on identifying either membrane-resident proteins or cytosolic proteins that become membrane associated upon viral RNA replication complex assembly (13, 34). The results presented in this report indicate that lipids, and in particular anionic phospholipids, may also play important roles in viral RNA replication complex assembly.

The speculative role of lipids in replication complex assembly is consistent with the observation that the replicase protein nsP1 from Semliki Forest, a positive-strand RNA virus that assembles its replication complexes on membranes derived from endosomes and lysosomes (52), also binds anionic

phospholipids (1). FHV protein A binding to predominantly anionic phospholipids suggests that ionic forces may mediate in part the interactions with phospholipids. Indeed, for nsP1 the anionic phospholipid interaction domain was mapped to an amphipathic α-helix with a cluster of positively charged residues (1). Sequence and predicted secondary structural analyses has revealed the presence of several similar amphipathic α-helices within the FHV protein A coding region that may be involved in anionic phospholipid interactions (K. Stapleford and D. Miller, unpublished data). However, the selectivity of protein A for certain anionic phospholipids suggests that non-ionic forces also contribute to protein A-lipid interactions.

The ability of protein A to interact with several different anionic phospholipids suggests a level of “promiscuity” that may help explain the robustness of FHV RNA replication in cells derived from multiple organisms from several kingdoms (24, 30, 41) and the relative ease with which FHV RNA replication complexes can be retargeted to alternative intracellular membranes such as the ER (31). However, the ubiquitous nature of anionic phospholipids and the substantial amounts of PI and PS present in many cellular membranes, including the ER (62), indicates that net membrane charge cannot be the sole determinant of replication complex targeting. Nonetheless, protein A did not bind all anionic phospholipids, but rather showed preferential interactions with CL, PA, and PG, which are enriched in mitochondrial membranes (55), suggesting that specific anionic phospholipids can influence FHV replication complex targeting. Furthermore, we cannot exclude a role for local charge clusters within membrane

microdomains or the impact of protease-resistant endogenous membrane protein interactions with anionic phospholipids in the membrane-specific targeting of FHV RNA replication complexes.

The hypothesis that anionic phospholipids play targeting or structural roles in FHV RNA replication complex assembly is particularly interesting given the results with CL, which we identified as a significant interaction partner of protein A. CL is a cellular phospholipid whose distribution is almost entirely limited to the mitochondria (17). Although the majority of CL is found in the inner mitochondrial membrane, it is also present in high localized concentrations in the outer membrane at contacts sites between inner and outer membranes (3, 44). One might speculate that FHV RNA replication complexes may initially assemble at or near these contact sites via interactions between protein A and CL. This proposed mechanism resembles the targeting of the pro-apoptotic promoter tBID to CL that can initiate cytochrome c-mediated apoptotic cell death (10, 25). The potential similarity between protein A-CL and tBID-CL interaction is intriguing given the recent demonstration that FHV infection induces apoptosis in cultured *Drosophila* cells (50), although the delayed onset of FHV-induced apoptosis until approximately 12 h post-infection suggests that a threshold of protein A-CL interactions may be required. In addition, it is possible that protein A-mediated disruption of the inner-outer membrane contact sites, an essential substructure needed to maintain mitochondrial shape (44), may lead to some of the morphological alterations that are seen in mitochondria from yeast or *Drosophila* cells that contain active FHV RNA replication complexes (30, 31). This is

consistent with the observation that components of the endogenous mitochondrial fission and fusion machinery can localize to these contact sites (44, 53), and that disruption of the normal fission-fusion processes can lead to abnormal mitochondrial morphology (36, 43) that also resemble mitochondria in cells with active FHV RNA replication (29, 31). Furthermore, the dimeric nature of CL, which consists of four acyl chains attached to diphosphatidylglycerol, imparts a unique conical structure that favors a hexagonal H_{II} phase that may play a role in the membrane curvatures necessary to produce the spherules associated with FHV RNA replication complexes (22, 30).

Although the focus of this study was to identify host components involved in protein A-membrane interactions, an initial step in FHV replication complex assembly, we found that protein A translated in RRLs had RNA polymerase activity when provided with an excess of exogenous virion template RNA template (data not shown). However, we did not observe the FHV RNA replication complex activity that has been described with membrane preparations from FHV-infected *Drosophila* cells (59, 60) and replicon-expressing yeast (31), which includes the production of single-stranded products, with in vitro translated protein A in the presence of whole mitochondria or liposomes. The particularly difficult feat of de novo assembly of fully functional viral RNA replication complexes using a cell-free in vitro translation system has only been accomplished for a select few positive-strand RNA viruses, including poliovirus, which requires the use of uninfected mammalian cell extracts (33), the plant pathogens tomato mosaic virus, brome mosaic virus, and turnip crinkle virus,

which require the use of evacuolated plant cell extracts (21), and tomato bushy stunt virus, which uses a related system that employs a yeast cell-free extract and purified recombinant viral proteins (39). Further studies with FHV will be required to identify the optimal conditions under which fully functional viral RNA replication complexes can be formed in vitro, and both the results presented in this report and others (59, 60) indicate that the inclusion of specific phospholipids may be a particularly important aspect of these studies.

In summary, the studies presented in this report demonstrate that FHV protein A mitochondrial association and membrane insertion is mediated by a TOM-complex independent mechanism, similar to what has been seen previously for mitochondrial outer membrane signal-anchored proteins, and provide evidence for the importance of host membrane-specific phospholipids in positive-strand RNA virus replication complex assembly. Future in vitro and in vivo studies using this established host-pathogen system will give further insight into the role of phospholipids in membrane-specific targeting as well as the biochemical mechanisms involved in positive-strand RNA virus replication complex assembly and function.

REFERENCES

1. **Ahola, T., A. Lampio, P. Auvinen, and L. Kaariainen.** 1999. Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. EMBO J. **18:**3164-3172.

2. **Ahting, U., T. Waizenegger, W. Neupert, and D. Rapaport.** 2005. Signal-anchored proteins follow a unique insertion pathway into the outer membrane of mitochondria. J. Biol. Chem. **280:**48-53.

3. **Ardail, D., J. P. Privat, M. Egret-Charlier, C. Levrat, F. Lerme, and P. Louisot.** 1990. Mitochondrial contact sites. Lipid composition and dynamics. J. Biol. Chem. **265:**18797-18802.

4. **Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenkovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann.** 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. J. Biol. Chem. **283:**120-127.

5. **Brix, J., K. Dietmeier, and N. Pfanner.** 1997. Differential recognition of preproteins by the purified cytosolic domains of the mitochondrial import receptors Tom20, Tom22, and Tom70. J. Biol. Chem. **272:**20730-20735.

6. **den Boon, J. A., J. Chen, and P. Ahlquist.** 2001. Identification of sequences in Brome mosaic virus replicase protein 1a that mediate association with endoplasmic reticulum membranes. J. Virol. **75:**12370-12381.

7. **Eckerle, L. D., C. G. Albarino, and L. A. Ball.** 2003. Flock House virus subgenomic RNA3 is replicated and its replication correlates with transactivation of RNA2. Virology **317:**95-108.

8. **Endo, T., and D. Kohda.** 2002. Functions of outer membrane receptors in mitochondrial protein import. Biochim. Biophys. Acta **1592:**3-14.

9. **Galiana-Arnoux, D., C. Dostert, A. Schneemann, J. A. Hoffmann, and J. L. Imler.** 2006. Essential function in vivo for Dicer-2 in host defense against RNA viruses in *Drosophila*. Nat. Immunol. **7:**590-597.

10. **Gonzalvez, F., J. J. Bessoule, F. Rocchiccioli, S. Manon, and P. X. Petit.** 2005. Role of cardiolipin on tBid and tBid/Bax synergistic effects on yeast mitochondria. Cell Death Differ. **12:**659-667.

11. **Graham, J. M.** 2001. Isolation of mitochondria from tissues and cells by differential centrifugation. Curr. Protoc. Cell. Biol. **Chapter 3:**Unit 3 3.

12. **Jiang, F., M. T. Ryan, M. Schlame, M. Zhao, Z. Gu, M. Klingenberg, N. Pfanner, and M. L. Greenberg.** 2000. Absence of cardiolipin in the *crd1* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. J. Biol. Chem. **275:**22387-22394.

13. **Jiang, Y., E. Serviene, J. Gal, T. Panavas, and P. D. Nagy.** 2006. Identification of essential host factors affecting tombusvirus RNA replication based on the yeast Tet promoters Hughes Collection. J. Virol. **80:**7394-7404.

14. **Johnson, K. L., and L. A. Ball.** 1999. Induction and maintenance of autonomous Flock House virus RNA1 replication. J. Virol. **73:**7933-7942.

15. **Johnson, K. L., and L. A. Ball.** 1997. Replication of Flock House virus RNAs from primary transcripts made in cells by RNA polymerase II. J. Virol. **71:**3323-3327.

16. **Jonczyk, M., K. B. Pathak, M. Sharma, and P. D. Nagy.** 2007. Exploiting alternative subcellular location for replication: tombusvirus replication switches to the endoplasmic reticulum in the absence of peroxisomes. Virology **362:**320-330.

17. **Joshi, A. S., J. Zhou, V. M. Gohil, S. Chen, and M. L. Greenberg.** 2009. Cellular functions of cardiolipin in yeast. Biochim. Biophys. Acta. **1793:**212-218.

18. **Kampmueller, K. M., and D. J. Miller.** 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in *Drosophila* cells. J. Virol. **79:**6827-6837.

19. **Kemper, C., S. J. Habib, G. Engl, P. Heckmeyer, K. S. Dimmer, and D. Rapaport.** 2008. Integration of tail-anchored proteins into the mitochondrial outer membrane does not require any known import components. J. Cell Sci. **121:**1990-1998.

20. **Komiya, T., S. Rospert, G. Schatz, and K. Mihara.** 1997. Binding of mitochondrial precursor proteins to the cytoplasmic domains of the import receptors Tom70 and Tom20 is determined by cytoplasmic chaperones. EMBO J. **16:**4267-4275.

21. **Komoda, K., S. Naito, and M. Ishikawa.** 2004. Replication of plant RNA virus genomes in a cell-free extract of evacuolated plant protoplasts. Proc. Natl. Acad. Sci. U.S.A. **101:**1863-1867.

22. **Kopek, B. G., G. Perkins, D. J. Miller, M. H. Ellisman, and P. Ahlquist.** 2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. PLoS Biol. **5:**e220.

23. **Krimmer, T., D. Rapaport, M. T. Ryan, C. Meisinger, C. K. Kassenbrock, E. Blachly-Dyson, M. Forte, M. G. Douglas, W. Neupert, F. E. Nargang, and N. Pfanner.** 2001. Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. J. Cell Biol. **152:**289-300.

24. **Lu, R., M. Maduro, F. Li, H. W. Li, G. Broitman-Maduro, W. X. Li, and S. W. Ding.** 2005. Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. Nature **436:**1040-1043.

25. **Lutter, M., M. Fang, X. Luo, M. Nishijima, X. Xie, and X. Wang.** 2000. Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat. Cell Biol. **2:**754-761.

26. **Marshall, D., and A. Schneemann.** 2001. Specific packaging of nodaviral RNA2 requires the N-terminus of the capsid protein. Virology **285:**165-175.

27. **Meineke, B., G. Engl, C. Kemper, A. Vasiljev-Neumeyer, H. Paulitschke, and D. Rapaport.** 2008. The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. FEBS Lett. **582:**855-860.

28. **Meisinger, C., T. Sommer, and N. Pfanner.** 2000. Purification of *Saccharomcyes cerevisiae* mitochondria devoid of microsomal and cytosolic contaminations. Anal. Biochem. **287:**339-342.

29. **Miller, D. J., and P. Ahlquist.** 2002. Flock House virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J. Virol. **76:**9856-9867.

30. **Miller, D. J., M. D. Schwartz, and P. Ahlquist.** 2001. Flock House virus RNA replicates on outer mitochondrial membranes in *Drosophila* cells. J. Virol. **75:**11664-11676.

31. **Miller, D. J., M. D. Schwartz, B. T. Dye, and P. Ahlquist.** 2003. Engineered retargeting of viral RNA replication complexes to an alternative intracellular membrane. J. Virol. **77:**12193-12202.

32. **Miller, S., and J. Krijnse-Locker.** 2008. Modification of intracellular membrane structures for virus replication. Nat. Rev. Microbiol. **6:**363-374.

33. **Molla, A., A. V. Paul, and E. Wimmer.** 1991. Cell-free, de novo synthesis of poliovirus. Science **254:**1647-1651.

34. **Nagy, P. D.** 2008. Yeast as a model host to explore plant virus-host interactions. Annu. Rev. Phytopathol. **46:**217-242.

35. **Nishikiori, M., K. Dohi, M. Mori, T. Meshi, S. Naito, and M. Ishikawa.** 2006. Membrane-bound tomato mosaic virus replication proteins participate in RNA synthesis and are associated with host proteins in a pattern distinct from those that are not membrane bound. J. Virol. **80:**8459-8468.

36. **Otsuga, D., B. R. Keegan, E. Brisch, J. W. Thatcher, G. J. Hermann, W. Bleazard, and J. M. Shaw.** 1998. The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. J. Cell Biol. **143:**333-349.

37. **Pathak, K. B., Z. Sasvari, and P. D. Nagy.** 2008. The host Pex19p plays a role in peroxisomal localization of tombusvirus replication proteins. Virology **379:**294-305.

38. **Pfanner, N., N. Wiedemann, C. Meisinger, and T. Lithgow.** 2004. Assembling the mitochondrial outer membrane. Nat. Struct. Mol. Biol. **11:**1044-1048.

39. **Pogany, J., J. Stork, Z. Li, and P. D. Nagy.** 2008. In vitro assembly of the tomato bushy stunt virus replicase requires the host heat shock protein 70. Proc. Natl. Acad. Sci. U.S.A. **105:**19956-19961.

40. **Price, B. D., P. Ahlquist, and L. A. Ball.** 2002. DNA-directed expression of an animal virus RNA for replication-dependent colony formation in *Saccharomyces cerevisiae*. J. Virol. **76:**1610-1616.

41. **Price, B. D., M. Roeder, and P. Ahlquist.** 2000. DNA-Directed expression of functional Flock House virus RNA1 derivatives in *Saccharomyces cerevisiae*, heterologous gene expression, and selective effects on subgenomic mRNA synthesis. J. Virol. **74:**11724-11733.

42. **Rapaport, D.** 2002. Biogenesis of the mitochondrial TOM complex. Trends Biochem. Sci. **27:**191-197.

43. **Rapaport, D., M. Brunner, W. Neupert, and B. Westermann.** 1998. Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae*. J. Biol. Chem. **273:**20150-20155.

44. **Reichert, A. S., and W. Neupert.** 2002. Contact sites between the outer and inner membrane of mitochondria-role in protein transport. Biochim. Biophys. Acta **1592:**41-49.

45. **Schaad, M. C., P. E. Jensen, and J. C. Carrington.** 1997. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J. **16:**4049-4059.

46. **Schlossmann, J., and W. Neupert.** 1995. Assembly of the preprotein receptor MOM72/MAS70 into the protein import complex of the outer membrane of mitochondria. J. Biol. Chem. **270:**27116-27121.

47. **Schmidt-Mende, J., E. Bieck, T. Hugle, F. Penin, C. M. Rice, H. E. Blum, and D. Moradpour.** 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. **276:**44052-44063.

48. **Schneemann, A., W. Zhong, T. M. Gallagher, and R. R. Rueckert.** 1992. Maturation cleavage required for infectivity of a nodavirus. J. Virol. **66:**6728-6734.

49. **Schwartz, M., J. Chen, W. M. Lee, M. Janda, and P. Ahlquist.** 2004. Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. Proc. Natl. Acad. Sci. U.S.A. **101:**11263-11268.

50. **Settles, E. W., and P. D. Friesen.** 2008. Flock House virus induces apoptosis by depletion of *Drosophila* inhibitor-of-apoptosis protein DIAP1. J. Virol. **82:**1378-1388.

51. **Simbeni, R., L. Pon, E. Zinser, F. Paltauf, and G. Daum.** 1991. Mitochondrial membrane contact sites of yeast. Characterization of lipid components and possible involvement in intramitochondrial translocation of phospholipids. J. Biol. Chem. **266:**10047-10049.

52. **Spuul, P., A. Salonen, A. Merits, E. Jokitalo, L. Kaariainen, and T. Ahola.** 2007. Role of the amphipathic peptide of Semliki forest virus replicase protein nsP1 in membrane association and virus replication. J. Virol. **81:**872-883.

53. **Stojanovski, D., M. Rissler, N. Pfanner, and C. Meisinger.** 2006. Mitochondrial morphology and protein import--a tight connection? Biochim. Biophys. Acta **1763:**414-421.

54. **Stuart, R. A., and C. M. Koehler.** 2007. In vitro analysis of yeast mitochondrial protein import. Curr. Protoc. Cell Biol. **Chapter 11:**Unit 11 19.

55. **van Meer, G., D. R. Voelker, and G. W. Feigenson.** 2008. Membrane lipids: where they are and how they behave. Nat. Rev. Mol. Cell. Biol. **9:**112-124.

56. **Waizenegger, T., T. Stan, W. Neupert, and D. Rapaport.** 2003. Signal-anchor domains of proteins of the outer membrane of mitochondria: structural and functional characteristics. J. Biol. Chem. **278:**42064-42071.

57. **Walther, D. M., and D. Rapaport.** 2009. Biogenesis of mitochondrial outer membrane proteins. Biochim. Biophys. Acta. **1793:**42-51.

58. **Wiedemann, N., V. Kozjak, A. Chacinska, B. Schonfisch, S. Rospert, M. T. Ryan, N. Pfanner, and C. Meisinger.** 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature **424:**565-571.

59. **Wu, S. X., P. Ahlquist, and P. Kaesberg.** 1992. Active complete in vitro replication of nodavirus RNA requires glycerophospholipid. Proc. Natl. Acad. Sci. U.S.A. **89:**11136-11140.

60. **Wu, S. X., and P. Kaesberg.** 1991. Synthesis of template-sense, single-strand Flock House virus RNA in a cell-free replication system. Virology **183:**392-396.

61. **Young, J. C., N. J. Hoogenraad, and F. U. Hartl.** 2003. Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. Cell **112:**41-50.

62. **Zinser, E., and G. Daum.** 1995. Isolation and characterization of organelles from the yeast, Saccharomyces cerevisiae. Yeast **11:**494-536.

CHAPTER III

THE ROLE OF MITOCHONDRIAL OUTER MEMBRANE PROTEINS IN FLOCK HOUSE VIRUS RNA REPLICATION: UNCOUPLING POLYMERASE TRANSLATION AND RNA SYNTHESIS IN MIM1 DEFICIENT YEAST

All characterized positive-strand RNA viruses employ the use of host intracellular membranes to facilitate viral replication complex assembly and subsequent genomic replication (7, 33). Individual viruses have been found associated with a wide variety of host membranes including the endoplasmic reticulum (ER) (6, 46), endosomes (45), Golgi appartatus (18, 47), peroxisomes (16), chloroplasts (43), and the mitochondria (10, 31). This membrane specificity is facilitated by membrane-specific targeting signals encoded by viral replicase components (6, 30, 43, 46, 48), which in turn ensure the proper assembly of the viral replication complex on the appropriate membrane. To date, many of the viral determinants required for viral RNA replication complex assembly have been described in detail whereas the study of required membrane-specific host components has received only minor attention (16, 37, 39).

To study the role of host intracellular membranes and membrane components in positive-strand RNA virus RNA replication we use the well-studied insect pathogen, Flock House virus (FHV). The FHV viral life cycle shares many

common features seen among positive-strand RNA viruses. These include the membrane-specific targeting and assembly of functional membrane-associated replication complexes (19, 30, 31), the exploitation of various cellular processes and host factors for viral replication (5, 17, 59), and the induction of large-scale membrane rearrangements (19, 31, 32), thus making it an ideal model system to study many aspects of positive-strand RNA virus biology. FHV contains a simple bipartite genome consisting of RNA1 (3.1 kb) and RNA2 (1.4 kb) which encode the nonstructural RNA-dependent RNA polymerase, protein A (14, 15, 42), and structural capsid proteins respectively (27, 49). During active FHV genomic replication a subgenomic RNA3 (0.4 kb) is produced encoding the RNAi inhibitor, protein B2, a viral component required for FHV RNA replication in cells that contain an RNA interference innate immune pathway (20, 25, 53). Furthermore, FHV is able to establish robust genomic replication in a wide variety of well-studied host cell types including *Drosophila melanogaster* (17, 31), *Caenorhabditis elegans* (25, 26) , mammalian cells (15), plants cells (50), and *Saccharomyces cerevisiae* (30, 42, 59), suggesting the presence of common molecular mechanisms used for FHV RNA replication.

The budding yeast has been used extensively as a model eukaryotic system to study not only cellular processes but also viral RNA replication. The facile genetics of the organism along with the vast array of available cellular and molecular biology techniques make it an ideal model system for the identification of host factors required for positive-strand RNA virus replication (13, 21, 35, 52). Recent yeast genomic and proteomic analyses have identified a variety of

cytosolic host components required for viral replication including the yeast Hsp70 family of chaperones and co-chaperones (13, 51, 59), transcription and translation factors (12, 24, 51), and factors influencing membrane dynamics (23, 39). However, with few exceptions (16, 37, 39), little is known regarding the role of host membrane-specific components in viral RNA replication.

Flock House virus assembles functional RNA replication complexes on the mitochondrial outer membrane in yeast, and thus we were interested in exploring the role of yeast mitochondrial outer membrane proteins in FHV RNA replication. Yeast mitochondrial biogenesis has been studied in great detail, leading to the identification of the cellular machinery required for various complex processes involved in mitochondrial function. A proteomic analysis of the yeast mitochondrial outer membrane (64) has identified roughly 30 resident proteins which function to facilitate a variety of cellular processes including proper mitochondrial protein import and sorting (36, 40) and the maintenance of mitochondrial morphology (38, 60). The translocase of the mitochondrial outer membrane (TOM complex) as well as the sorting and assembly machinery (SAM complex) are used to ensure the proper assembly of many of the mitochondrial outer membrane proteins in yeast (22, 36) and thus may play an important role in FHV RNA replication complex assembly and function.

In this study we used a targeted genomic approach utilizing yeast strains harboring deletions of mitochondrial outer membrane proteins to explore the role of membrane-specific host components in FHV RNA replication. Of the deletion strains studied, we found that a majority had no significant effect on FHV RNA

replication whereas several deletion strains led to significant increases or decreases in viral replication. In particular, the deletion of the small mitochondrial outer membrane protein Mim1 led to a significant reduction in FHV RNA accumulation yet did not influence protein A translation or membrane association. A biochemical analysis of membrane-associated FHV replication complexes isolated from Mim1 deficient yeast revealed a significant reduction in replicase activity as well as mitochondrial-associated FHV specific RNAs. Further blue-native agarose gel electrophoresis (BN-AGE) analysis indicated that this defect was in part due to the absence of the functional component of the FHV RNA replication complex suggesting a possible role for Mim1 in FHV replication complex assembly and function. Taken together, these studies reveal the host and RNA synthesis dependent assembly of two functionally distinct FHV RNA replication complexes.

MATERIALS AND METHODS

Yeast strains, transformations, and culture conditions. Yeast strains used in this study were purchased from the American Type Culture Collection (ATCC) in the homozygous diploid BY4743 wildtype background (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0*) unless otherwise indicated in Table 3.1. BY4743 deletions were generated by the insertion of a KanMX cassette in place of the indicated ORF. Yeast were transformed using the E-Z Transformation Kit (Zymo Research, Orange, CA). Individual clones were grown to early stationary phase in selectable liquid media containing 2% [wt/vol] dextrose at 30°C. For the induction of galactose-inducible plasmids, yeast were washed once with distilled water, resuspended in selectable liquid media containing 2% [wt/vol] galactose at an OD_{600} of 0.2, and grown for 24 h at 25°C for all experiments. Wildtype YPH499 and *Δmim1* strains were grown to early stationary phase in selectable liquid media containing 2% [wt/vol] raffinose at 25°C followed by dilution to an OD_{600} of 0.2 in selectable liquid media containing 2% [wt/vol] raffinose and 2% [wt/vol] galactose for 24 h at 25°C unless otherwise indicated.

Plasmids and antibodies. The FHV RNA1 expression plasmid pF1 has been previously described (42). pF1 contains a full-length FHV RNA1 cDNA containing authentic viral 5' and 3' ends under the control of the yeast galactose-inducible *GAL1* promoter. Upon induction of the plasmid in the presence of galactose, FHV RNA1 can be translated and act as a template for protein A-

Table 3.1. *S. cerevisiae* Strains Used in this Study not in the ATCC Deletion Library

Strain/Source	Genotype
ADM548 (WT) (34)	*MAT*α *ura3-52 his3*Δ200 *trp1-Δ63*
ADM575 (*Δfzo1*) (34)	*MAT*α *ura3-52 his3*Δ200 *leu2*Δ*1 fzo1::HIS3*
YPH499 (WT) (41)	*MAT*α *ade2-101 his3*Δ200 *leu2*Δ*1 ura3-52* trp1-Δ63 lys-801
Δmim1 (41)	*MAT*α *ade2-101 his3*Δ*200 leu2*Δ*1 ura3-52* trp1Δ63 lys2-801 mim1::his3
MM112-C (*Δtom20*) (61)	*MAT*α *ade2-101 his3*Δ200 *leu2*Δ*1 ura3-52* trp1-Δ63 lys-801tom20::URA3 (Yep13-TOM22)
OL223 (WT) (61)	*MAT*α *his3-Δ200 leu2-Δ1 ura3-52 trp1*Δ*63 rho(o/-)*
OL201 (*Δtom22*) (61)	*MAT*α *his3-Δ200 leu2-Δ1 ura3-52 trp1*Δ*63* tom22::HIS3 rho(o/-)
KKY3.7 (WT -TK8) (61)	*MAT*α *his3-Δ200 leu2-3,112 ade2-101* suc2-Δ9 trp1-Δ901 ura3- 52 tom40::HIS3 (pRS314-Tom40)
KKY3.3 (TK4) *tom40-3* (1)	*MAT*α *his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52* tom40::HIS3 (pRS314-Tom40-3)
KKY3.6 (TK7) *tom40-6* (61)	*MAT*α *his3-Δ200 leu2-3,112 ade2-101 suc2-Δ9 trp1-Δ901 ura3-52* tom40::HIS3 (pRS314-Tom40-6)

dependent viral RNA1 and sub-genomic RNA3 replication (Figure 3.1). The replication incompetent FHV RNA1 expression plasmid, pF1(GNN), was generated by PCR and confirmed by sequencing. The Mim1 expression plasmid encoding yeast Mim1 under its endogenous promoter (pMim1) was previously described (41). Rabbit polyclonal antibodies against FHV protein A and protein B2 were previously described (30, 31, 59). Mouse monoclonal antibodies against yeast porin and 3-phosphoglycerate kinase (PGK1) were purchased from Molecular Probes (Carlsbad, CA) and rabbit polyclonal antibodies against the hemagglutinin (HA) epitope were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies against Mim1 and Tom70 were previously described (41, 58).

RNA and protein analysis. For total protein isolation, equal amounts of cells were isolated 24 hours after plasmid induction and resuspended in protein extraction buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1:50 dilution of yeast protease inhibitor cocktail (Sigma)). Cell walls were removed by vortexing in the presence of glass beads for 10 min at 4°C followed by the addition of 1% SDS and heating at 100°C for 10 min. Samples were pelleted by centrifugation at 10,000 x g for 5 min and supernatant aliquots were removed, mixed with 2X SDS-PAGE sample buffer (125 mM Tris-HCl; pH 6.8, 4% SDS, 20% (w/v) glycerol, 0.002 mg/ml bromophenol blue, 10% 2-mercaptoethanol), and stored at -20°C for further analysis. Total protein extractions were separated by SDS-PAGE, transferred in polyvinylidene fluoride (PVDF) membranes, and immunoblotted as previously described (31). For total

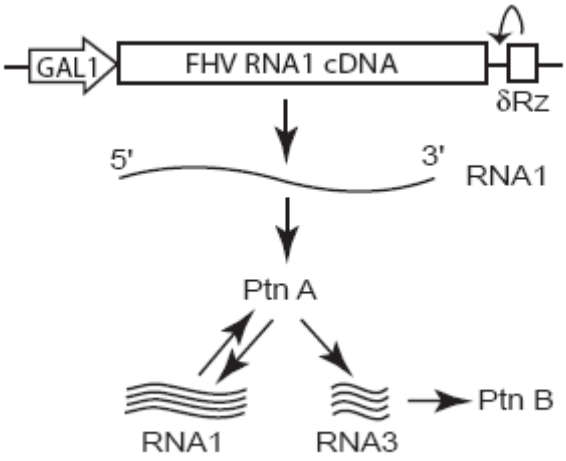

Figure 3.1: Schematic of the FHV Replicon, pF1. The FHV replicon is a plasmid-based system containing a cDNA copy of FHV RNA1 with authentic viral 5' and 3' ends driven by the galactose-inducible GAL1 promoter. Upon induction of the plasmid in the presence of galactose, RNA1 is transcribed and cleavaged by the hepatitis δ ribozyme to ensure the proper 3' end of the viral RNA. RNA1 can then be translated into protein A, the viral RNA-dependent RNA polyermase, and establish a mitochondrial-associated replication complex to initiate robust replication of RNA1 and subgenomic RNA3.

RNA isolation, equal amounts of cells were subject to hot acidic phenol extraction as previously described (30, 32, 59) and stored at -80°C until northern blot analysis. RNA was separated on a 1.2% agarose-formaldehyde gel, blotted onto nylon membranes, and probed for positive- and negative-strand RNA1 and RNA3 as previously described (31).

Mitochondrial isolation, carbonate extraction and RNA analysis. Yeast mitochondria were isolated as previously described (29) and stored in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM 3-morpholinopropanesulphonic acid (MOPS)-KOH, pH 7.2) at -80°C. For carbonate extraction, mitochondria were pelleted at 10,000 x g for 15 min at 4°C, resuspended in 0.1 M sodium carbonate (pH 11.5), and incubated on ice for 30 min. Mitochondria were reisolated by centrifugation at 55,000 x g for 1 h in a TLA-55 rotor (Beckman), resuspended in SDS-PAGE buffer and subject to immunoblot analysis as described above. For mitochondrial RNA isolated, RNA was extract using an RNeasy Mini Kit (Qiagen) following manufacturers' instructions, and 0.5 µg total RNA was analyzed by northern blotting as described above.

In vitro RdRp activity. Mitochondria (20 µg total protein) were incubated in RdRp buffer (50 mM Tris, pH 8.0, 50 mM potassium acetate, 15 mM magnesium acetate, 40 units RNAsin (Promega), 5 µg/ml Actinomycin D, 1 mM ATP, 1 mM CTP, 1 mM GTP, 25 µM UTP, 10 µCi [^{32}P]UTP (Fisher Scientific) at 25°C for 3 h. Total RNA was extracted with one volume phenol-chloroform (pH 5.2) and unincorporated nucleotides were removed from the aqueous phase by a

Micro Bio-Spin Column (BioRad) following the manufacturer's instructions. Nucleic acid dye buffer (0.05% bromophenol blue, 0.05% xylene cyanol FF, 5% glycerol) was added to each sample and RNAs were separated by 1.4% agarose-TBE gel. Gels were dried under vacuum and subject to autoradiography.

Blue-Native Agarose Gel Electrophoresis (BN-AGE). Membrane fractions (100 µg total protein) were resuspended in solublization buffer (0.5% n-dodecyl β-D-maltoside (DDM) 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF), incubated on ice for 30 min, and centrifuged at 100,000 x g for 15 min at 4°C. The supernatant was removed and mixed with loading dye (0.5% coomassie brilliant blue G-250, 50 mM aminocaproic acid, 10 mM Bis-Tris, pH 7.0). Samples were then loaded on a 1.5% BN-agarose gel containing 66.7 mM aminocaproic acid, 50 mM Bis-Tris pH 7.0, ran at 100 V for 2 hour in 50 mM Bis-Tris pH 7.0 as a running buffer. For immunoblotting, gels were incubated in SDS-PAGE running buffer for 20 min followed by transfer to PVDF membranes and immunoblot analysis as described (31). To analyze radiolabeled viral RNAs, gels were tried under vacuum, and analyzed by autoradiography.

Statistics. We used a two-tailed t-test assuming equal variance for comparative statistical analysis and considered a *p*-value of < 0.05 as significant. Unless otherwise indicated all quantitative data represent results from at least 3 independent experiments and are presented as the means ± standard errors of the mean.

RESULTS

Flock House virus RNA replication in yeast strains harboring mitochondrial outer membrane protein deletions. Flock House virus utilizes the mitochondrial outer membrane for RNA replication complex assembly and subsequent genome replication. To investigate the role of host mitochondrial outer membrane proteins in FHV RNA replication we took advantage of a well-established plasmid based FHV replicon system (Figure 3.1) in combination with yeast strains harboring individual deletions or mutations of mitochondrial outer membrane proteins (Table 3.2). Yeast deletion strains were transformed with the FHV replicon pF1 and FHV RNA replication was initiated by growing yeast in the presence of galactose for 24 hours at 25°C. Total viral RNA production was analyzed by northern blotting with probe specific for positive-strand RNA1 and sub-genomic RNA3 (Table 3.2). We found that a majority of the deletion strains had no significant change in FHV RNA production, yet interestingly there were several strains that led to significant increases or decreases in FHV RNA accumulation. In particular we found that two temperature-sensitive mutants of the major import pore of the mitochondrial outer membrane, Tom40, lead to significant increases in RNA production, whereas deletions of *POR1*, *TOM6*, *DNM1*, and *MIM1* led to significant defects FHV RNA accumulation.

One possible explanation for these changes in FHV RNA accumulation may be due to differences in yeast deletion and mutant strain growth rates, whereas slow growing yeast may accumulate more RNA during the 24 hour period as

Table 3.2: Flock House virus RNA Replication in Yeast Strains with Mitochondrial Outer Membrane Protein Deletions or Mutations.

Cellular Function	Gene	Description	Growth	(+)RNA3[a] Replication
Mitochondrial Fission	***DNM1***	Dynamin-related GTPase	1.0 ± 0.0	**0.3 ± 0.0**
	FIS1	Localization of Dnm1p and Mdv1p	1.1 ± 0.0	1.1 ± 0.0
	MDV1	WD repeat protein, interacts with Dnm1p and Fis1p	1.0 ± 0.0	0.9 ± 0.2
Mitochondrial Fusion	*FZO1**	Mitofusin, GTPase	1.2 ± 0.0	1.0 ± 0.1
	UGO1	Links GTPases Fzo1p and Mgm1p during fusion	1.1 ± 0.0	1.6 ± 0.4
Mitochondrial Protein Import and Assembly	*TOM5*	TOM complex, Import and stability	1.2 ± 0.0	0.8 ± 0.2
	TOM6	TOM complex, Assembly and stability	1.2 ± 0.0	**0.4 ± 0.0**
	TOM7	TOM complex, Assembly and stability	1.3 ± 0.1	0.8 ± 0.3
	TOM70	TOM complex, carrier proteins	0.9 ± 0.0	0.8 ± 0.2
	TOM72	TOM complex, similar to Tom70	0.9 ± 0.0	1.2 ± 0.3
	*TOM20**	TOM complex, Main receptor	1.0 ± 0.1	1.6 ± 0.1
	*TOM22**	TOM complex, Co-receptor	2.4 ± 0.3	1.2 ± 0.3
	TOM40-3*	TOM complex, Translocation channel	1.3 ± 0.0	**2.1 ± 0.3**
	TOM40-6*	TOM complex, Translocation channel	1.0 ± 0.1	**3.8 ± 0.4**
	SAM37	SAM complex, β-barrel protein import	1.1 ± 0.1	1.2 ± 0.2
	MDM12	B-barrel protein import and assembly	1.2 ± 0.0	1.2 ± 0.3
	MIM1*	Assembly of TOM complex	1.6 ± 0.1	**0.2 ± 0.0**
Other	*MCR1*	NADH-cytochrome b5 reductase	1.0 ± 0.0	0.7 ± 0.3
	UTH1	Mitochondrial autophagy	1.1 ± 0.0	1.5 ± 0.5
	MMR1	Mitochondrial distribution	1.0 ± 0.0	0.5 ± 0.2
	MSP1	Protein sorting	1.1 ± 0.0	0.9 ± 0.2
	OM45	Unknown function	1.1 ± 0.0	1.9 ± 0.5
	POR1	Voltage-dependent Anion Channel1	1.4 ± 0.0	**0.4 ± 0.0**
	POR2	Voltage-dependent Anion Channel2	1.2 ± 0.0	0.8 ± 0.2
	MDM34	Mitochondrial morphology and inheritance	1.3 ± 0.0	2.3 ± 1.1

[a]RNA3 replication is represented as percent of wildtype. *Not in BY4743 background (see Table 3.1). Tom40-3 and Tom40-6 are temperature-sensitive strains. Bold = $p < 0.05$

compared to a dividing wildtype cell. However, we found that there was no significant differences in yeast growth rates in those strains which lead to increased accumulation of FHV RNA. In contrast, the Mim1 deletion strain grew roughly 1.5 times slower then its wildtype strain yet FHV RNA accumulation was significantly impaired. Given this and the role of Mim1 in mitochondrial protein import and TOM complex assembly we chose to further explore the role of Mim1 in FHV RNA replication.

FHV RNA replication is impaired in Mim1 deficient yeast. Mim1 is a small mitochondrial outer membrane protein of approximately 13 kDa that has been shown to be required for proper mitochondrial protein import and the assembly of the yeast TOM complex and therefore may play a vital role in FHV RNA replication complex assembly and function (3, 9, 11, 41, 58). To investigate the role of Mim1 in FHV RNA replication we first addressed the ability of exogenously expressed Mim1 to complement the defect in FHV RNA replication. Yeast strains were transformed with the FHV replicon plasmid and Mim1 deficient yeast received either a vector control or plasmid expressing Mim1 under its endogenous promoter. We confirmed that the Mim1 expression plasmid was functional by its ability to complement growth of Mim1 deficient yeast at 37ºC (Figure 3.2A). Yeast were grown in the presence of galactose to induce FHV RNA replication and we analyzed total FHV positive- and negative-strand RNA1 and RNA3 levels by northern blotting. Mim1 deficient yeast showed a 5-fold reduction in both positive- and negative strand RNA1 and RNA3 levels compared

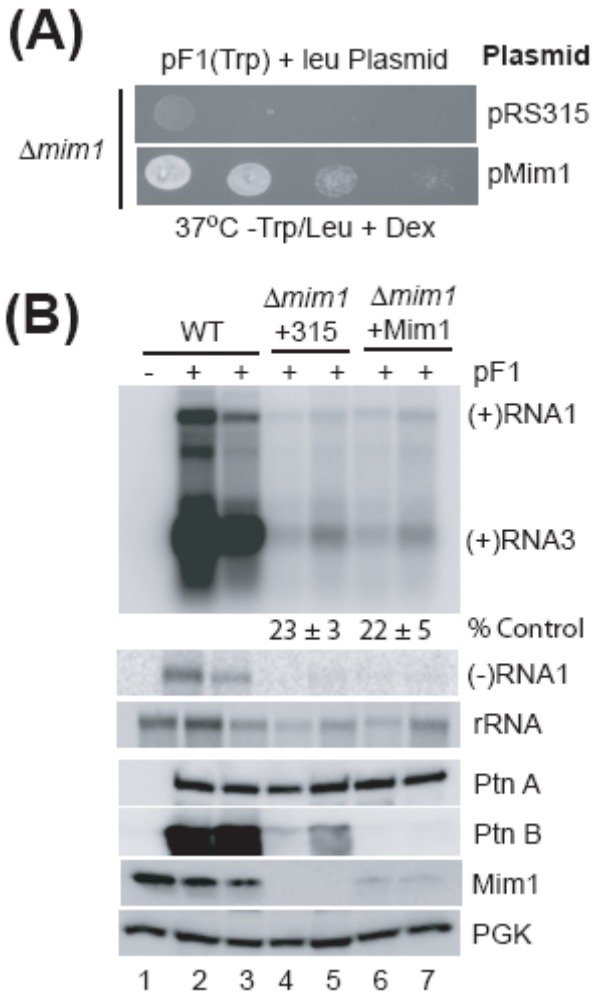

Figure 3.2: Complementation of FHV RNA Replication in Mim1 Deficient Yeast. (A) Mim1 deleted yeast were transformed with pF1 and either an empty plasmid (pRS315) or a plasmid expressing Mim1 under the control of the yeast Mim1 promoter (pMim1), grown to saturation in selectable media with 2% raffinose, diluted to an OD of 0.5, and diluted in 10-fold increments. Yeast were spotted on plates containing selectable media with dextrose and grown at 37°C. **(B)** Wildtype, Mim1 deleted (315), and Mim1 complemented yeast (Mim1) containing pF1 were induced in 2% galactose for 24 hours at 25°C. Equal numbers of cells were isolated and total RNA and protein were analyzed by northern blotting and immunoblot as described previously.

to wildtype yeast (Figure 3.2B, lanes 4 and 5), yet we were unable to rescue this defect to any degree with the exogenous expression of Mim1 (Figure 3.2B, lanes 6 and 7). This phenotype correlated with a reduction in protein B2 translation from RNA3 but was not due to defects in the translation of the polymerase as protein A levels were found to be equal between all samples. One possible explanation for this could be that Mim1 expression levels were not sufficient to complement the FHV RNA replication defect as we found total Mim1 levels to be approximately 10% of wildtype by immunoblot (Figure 3.2B, compare lanes 2 and 3 with lanes 6 and 7). However, when we looked at membrane fractions isolated from wildtype and Mim1 complemented yeast we did not see this dramatic difference in membrane-associated Mim1 levels (Figure 3.3A). Additionally, corresponding with the ability to rescue the temperature-sensitive phenotype, we found that expression of Mim1 was able to restore Tom70 and porin protein levels to that of wildtype (Figure 3.3A) indicating that although Mim1 is expressed at a low levels it is able to rescue a variety of yeast phenotypes. Nonetheless, given the significant defect in FHV RNA accumulation in Mim1 deficient yeast and the role of Mim1 in mitochondrial protein import and assembly we chose to further investigate the role of Mim1 in FHV RNA replication using the endogenous expression system.

FHV protein A is membrane-associated and behaves as an integral membrane protein in Mim1 deficient yeast. The observation that protein A is translated properly in Mim1 deficient yeast suggest that the defect may occur at a post-translational step in replication complex assembly. After translation, FHV

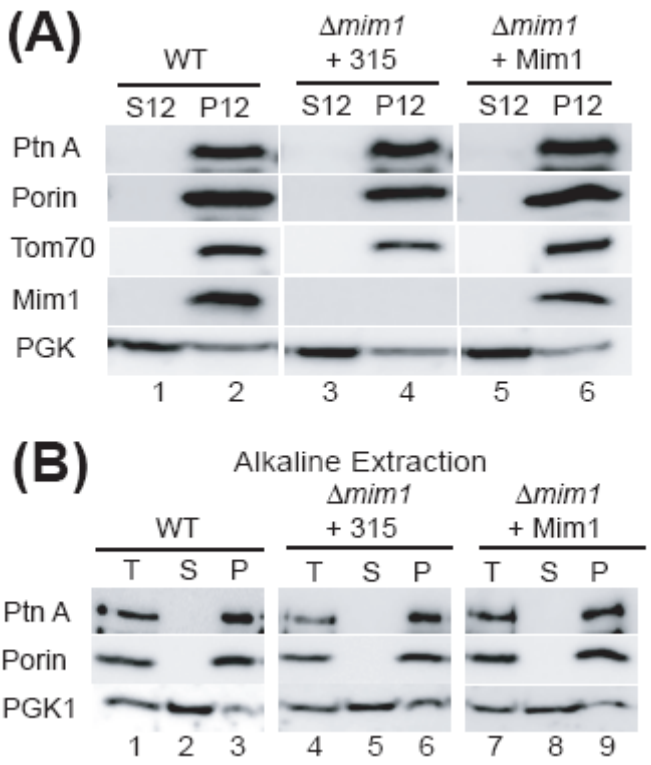

Figure 3.3: Protein A is membrane-associated in Mim1 deficient yeast. (A) Yeast expressing pF1 were fractionated into supernatant (S12) and crude membrane (P12) fractions. 20 µg of total protein was separated by SDS-PAGE and proteins were analyzed by immunblotting. **(B)** 100 µg of total crude membrane fractions (T) were treated with 0.1 M sodium carbonate, pH 11.5 for 30 min on ice. Samples were then separated by centrifugation at 55,000 x g for 30 min to yield a supernatant (S) and pellet (P) fraction. Proteins were separated by SDS-PAGE and analyzed as previously described.

protein A is targeted to the mitochondrial outer membrane in wildtype yeast (30) and thus it is possible that the RNA replication defect is due to improper membrane association of protein A. To address if protein A was membrane associated, we fractionated pF1 expressing wildtype, Mim1 deficient, and Mim1 complemented yeast by differential centrifugation to obtain supernatant (S12) and crude membrane fractions (P12). In all strains, protein A was found to fractionate in the membrane fraction similar to the mitochondrial outer membrane proteins Tom70, porin, and Mim1 (Figure 3.3A). To rule out the possibility that protein A is aggregating after lysis in Mim1 deficient and Mim1 complemented yeast strains we performed membrane flotation assays with each strain and confirmed that protein A was membrane-associated (data not shown). Additionally, given the role of Mim1 in mitochondrial protein import, we addressed whether membrane bound protein A behaved as an integral membrane protein as seen in wildtype yeast (30). We found that in all strains, protein A remained membrane associated after alkaline treatment similar to the β-barrel protein porin whereas we were able to remove portions of soluble protein PGK into the supernatant fraction. These data indicate that the membrane bound replicase is inserted as an integral membrane protein (Figure 3.3B) in Mim1 deficient yeast. Taken together, these studies show that protein A is tightly membrane-associated and inserted as an integral membrane protein as seen in wildtype yeast.

FHV membrane-associated RNA replication complexes are less active in Mim1 deficient yeast. Given that protein A is translated and membrane-

associated in Mim1 deficient yeast we hypothesized that the defect in FHV RNA replication is at the level of replicase function. To address the specific activity of FHV RNA replication complexes we incubated normalized amounts of protein A containing membrane-associated viral replication complexes in an in vitro RdRp assay and subsequently analyzed the ability of the replicase to synthesize viral RNAs in vitro. As a control, membrane fractions not expressing protein A showed very little background whereas wildtype yeast expressing pF1 synthesized distinct FHV RNA products (Figure 3.4A, lane 2-3). However, with both the Mim1 deficient and Mim1 complemented membrane fractions there was a significant reduction in all FHV RNA species (Figure 3.4A, lanes 4-7), suggesting a defect in FHV replication complex function.

The FHV replication complex is composed of two major viral components, the viral RNA-dependent RNA polymerase and the viral RNA template. One reason for reduced activity could be the lack of an RNA template associated with the membrane bound replicase. It has been shown that during replication complex assembly, protein A promotes the membrane association of RNA1 and this interaction is required for efficient RNA replication (55, 56). We addressed membrane-associated FHV RNA by extracting RNA from equal amounts of isolated membrane fractions and probing for both positive- and negative-strand FHV RNAs. Interestingly compared to wildtype, we found a significant reduction in both membrane-associated FHV positive- and negative-strand RNA1 and RNA3 in both Mim1 deficient and Mim1 complemented yeast (Figure 3.4B).

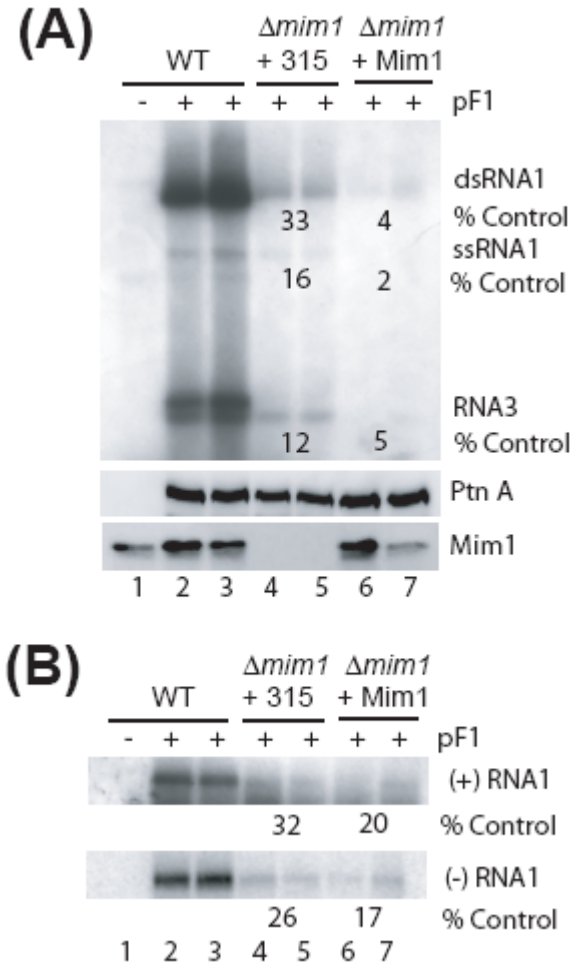

Figure 3.4: FHV in vitro replicase activity and membrane-associated RNA levels are reduced in *Δmim1* yeast. (A) Membrane fractions (20 µg total protein) were incubated in the presence of RdRp buffer for 3 hours at 25° followed by the addition of an equal volume of phenol:cholorform (pH 5.2). Unincorporated nucleotides were removed from the aqueous phase, RNAs were separated on a 1.2% TBE-agarose gel, and analyzed by autoradiography. **(B)** Total RNA was isolated from membrane fractions (100 µg total protein) using the RNeasy Kit and 0.5 µg total RNA was analyzed by northern blotting as previously described.

Taken together, these data suggest that FHV RNA replication complexes isolated from Mim1 deficient yeast are defective at the level of replicase activity.

Mim1 deficient yeast lack a large FHV protein A complex capable of RNA synthesis. Given the role of Mim1 in mitochondrial protein import and protein complex assembly, it is possible that protein A is translated and membrane-associated but not assembled properly into a native FHV RNA replication complex. The assembly of yeast native mitochondrial outer membrane complexes has been studied in detail in part due to the development of blue-native polyacrylamide gel electrophoresis (BN-PAGE). This technique has been used to separate large macromolecular membrane-protein complexes based on size, however it is unable to efficiently resolve much larger protein-RNA complexes. In an alternative approach, we used blue-native agarose gel electrophoresis (BN-AGE) followed by immunoblotting to visualize protein A containing complexes (Figure 3.5). FHV RNA replication complexes were solubilized with the mild detergent n-dodecyl β-D-maltoside (DDM) which has been used previously to study FHV replicase function in vitro (62, 63). When we analyzed FHV RNA replication complexes from wildtype yeast we found there to be two distinct protein A containing complexes (Figure 3.5A). Interestingly, we found that membrane-associated protein A complexes from Mim1 deficient yeast were missing the larger band but retained equal amounts of the faster migrating smaller complex.

To further examine these two distinct protein complexes we were interested in which of the complexes retained FHV RNA replicase activity. We first subjected

membrane fractions to an in vitro RdRp assay to label actively synthesized viral RNAs followed by solublization in DDM and separation of protein-RNA complexes by BN-AGE. We hypothesized that the fraction of protein A that retained replicase activity would incorporate radiolabeled nucleotides into the newly synthesized RNA strands allowing us to visualize these complexes. We found that in wildtype yeast the slower migrating protein A complex was capable of RNA synthesis as it was the only complex to incorporate nucleotides into the synthesized RNAs (Figure 3.5B, lane 2). This complex was not present in Mim1 deficient yeast (Figure 3.5B) suggesting that the FHV RNA replication defect in Mim1 deficient yeast is due to a defect in the assembly of large protein A containing complexes capable of RNA synthesis.

The assembly of wildtype FHV replication complexes is dependent on active viral RNA synthesis. These data with Mim1 deficient yeast suggest the presence of two functionally distinct FHV protein A containing complexes which are required for efficient FHV RNA replication. In addition, the FHV replicon system is designed to establish robust protein A-dependent RNA replication, and we hypothesized that in using this system protein A levels would be correlated with (+)RNA1 such that a defect in FHV RNA1 production would yield a similar defect in protein levels. This effect as been seen previously in yeast deficient in YDJ1 (59), however although we observed significant differences in positive-strand RNA1 levels there were no differences in protein A accumulation between wildtype and Mim1 deficient yeast (Figure 3.2B). One possible explanation for

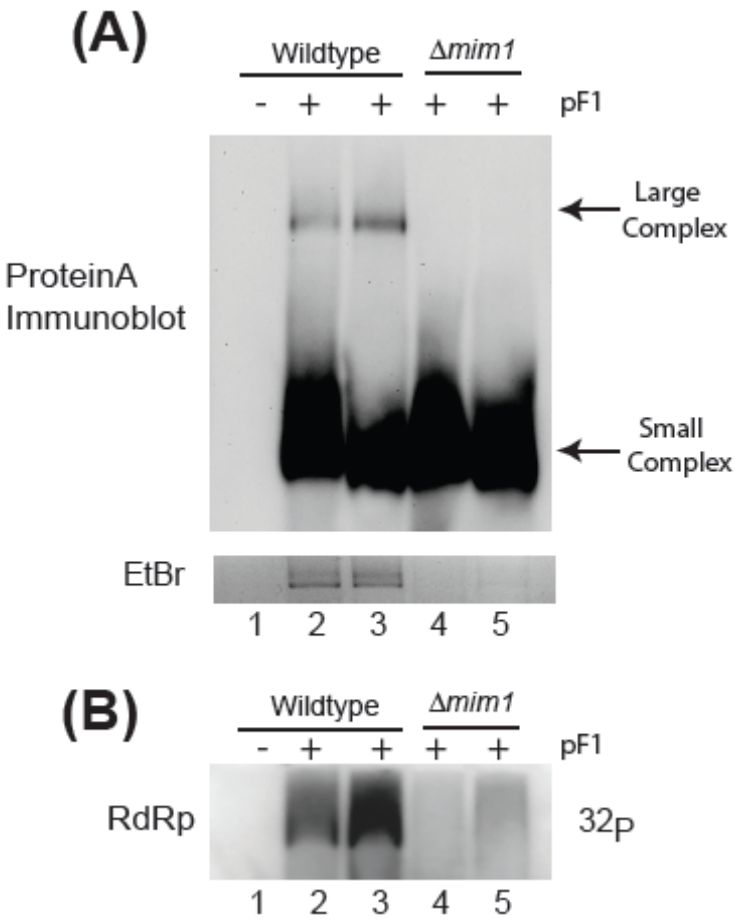

Figure 3.5: *Δmim1* yeast lack large protein A complex containing nucleotides and replicase activity (A) Membrane fractions isolated from wildtype and *Δmim1* were solubilized in 0.5% DDM, separated on a 1.5% BN-AGE gel, and immunoblotted for protein A. Lower panel is a UV exposure of BN-AGE gel before immunoblot **(B)** Membrane fractions were first incubated in an RdRp assay followed by BN-AGE electrophoresis. Gels were dried and visualized by autoradiography.

this is that plasmid based transcription of RNA1 from pF1, which is a 2-micron plasmid, is sufficient to make up for the lack of RNA1 produced from FHV replication. However, this is unlikely as we have obtained similar results using a variety of pF1 constructs expressed from centromeric plasmids (data not shown).

This suggests that protein A translation is independent of active protein A-dependent (+)RNA1 production. We chose to address this directly in wildtype yeast by separating protein A translation from protein A-dependent RNA replication with the introduction of an active site mutation into the FHV replicon (Figure 3.6A). This would allow RNA1 to be transcribed and translated into protein A as before, but this protein A (containing a GDD to GNN mutation) would be unable to further amplify more (+)RNA1 to be used for translation. Using this system, we compared protein A and (+)RNA1 production between the replication incompetent replicon and the wildtype replicon. We found that similar to what is seen in Mim1 deficient yeast, equal amounts of protein A were produced between the wildtype and replication incompetent replicons yet the amount of the (+)RNA1 was significantly different (Figure 3.6B). This data confirms that RNA1 dependent polymerase translation and genome replication can be separated in vivo. If this is the case, then this may explain the two protein A containing complexes we see with BN-AGE. To address this, we investigated the assembly of protein A containing complexes using the replication incompetent replicon by BN-AGE. Interestingly, we found that both protein complexes which were present with the wildtype replicon were missing in yeast expressing the replication incompetent replicon (Figure 3.6C). These data suggest a possible

stepwise assembly mechanism for FHV RNA replication complexes which is dependent on functional viral RNA synthesis.

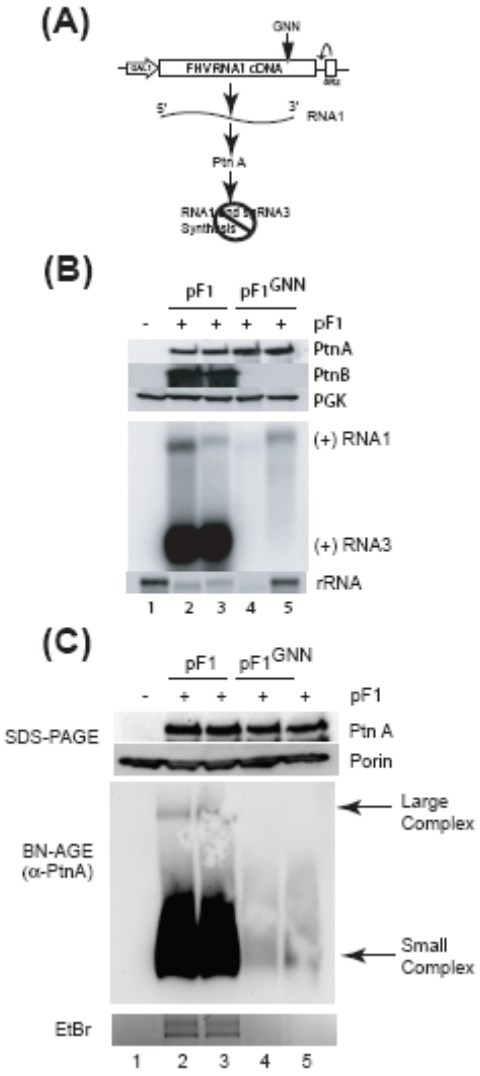

Figure 3.6: Assembly of protein A complexes is dependent on viral RNA synthesis. (A) Schematic of replication incompetent replicon containing GDD to GNN mutation. **(B)** Immunoblot and northern blot of FHV RNA replication with wildtype and replication competent replions. **(C)** BN-AGE analysis of membrane associated FHV replication complexes in yeast expressing the wildtype and replication incompontent replicons.

DISCUSSION

In this report we have taken a targeted genomic approach to investigate the role of yeast mitochondrial outer membrane proteins in Flock House virus RNA replication. We have identified a variety of mitochondrial outer membrane deletion strains that lead to increases or decreases in FHV RNA replication which include members of the protein import and fission machinery. In addition, we identified Mim1 which led to a significant reduction in positive- and negative-strand FHV RNA1 and RNA3 accumulation. This defect is independent of FHV polymerase translation or membrane-association as we found equal amounts of protein A to behave as an integral membrane protein in Mim1 deficient yeast. However, we found membrane-associated FHV RNA replication complexes from Mim1 deficient yeast to have reduced in vitro activity and an analysis of membrane-associated viral RNAs yielded lower levels of positive- and negative-strand viral RNAs associated with membrane fractions. Lastly, we found *Δmim1* yeast to lack the functional subunit of the FHV RNA replication complex suggesting a potential role for Mim1 in FHV replication complex assembly. These data provide evidence for the role of viral RNA synthesis in the assembly of functional FHV RNA replication complexes, and suggest an important role for host components in this process.

Of particular interest is the observation that protein A translation and RNA1 production are uncoupled using the FHV replicon. It is thought that during active FHV RNA replication, RNA1 that is produced from protein A-dependent RNA synthesis can then be used as a template for protein A translation and

subsequent replication complex assembly, and that defects in FHV RNA replication yield similar defects in polymerase production, a phenotype that has been observed with FHV RNA replication in YDJ1 deficient yeast (59). However, the deletion of Mim1 sheds light on the fact that these two processes may be mutually exclusive. Positive-strand RNA viruses in particular must begin with one genomic template which is used for a variety of processes including; the initial translation of viral proteins, membrane trafficking and replication complex assembly, genomic replication, and finally packaging of the genome to produce new infectious virions. It is thus not unreasonable that these processes may be temporally or spatially linked during the viral life cycle. Studies with FHV RNA2 have shown that RNA replication, translation, and packaging are linked during FHV replication (57) yet the molecular mechanisms involved in these processes are unknown. One possible mechanism came from work with Brome Mosaic virus where viral RNAs localize with P-bodies during replication complex assembly and these discrete cellular compartments may separate translating from replicating viral RNAs (4). Future studies of FHV RNA replication with various yeast deletions such as Mim1 will provide insight into the complex and dynamic process of viral replication complex assembly and RNA replication.

Furthermore, the presence of two functionally distinct FHV RNA replication complexes is intriguing. It is currently hypothesized, based on quantitative analyses of protein and RNA levels, that viral RNA replication complexes are composed primarily of non-functional subunits whereas the functional subunits make up a small portion which contain the viral RNAs (19, 44). Our data are in

line with this hypothesis indicating that not only are there two protein complexes but one complex contains RNA and replicase activity, thus providing biochemical evidence to support this theory. Furthermore, we found that FHV RNA synthesis and replication complex assembly are intimately linked providing another example of the coupling of viral processes during RNA replication. It will be interesting to further explore the assembly of these complexes as well as the structure and composition that is required for RNA synthesis.

In addition to Mim1 we found a variety of other yeast mitochondrial outer membrane deletions which led to changes in FHV RNA replication. Of particular interest were members of the TOM complex, which is important for the import and assembly of the majority of mitochondrial proteins in yeast, and the mitochondrial fission machinery. We have previously shown that the TOM complex is not require for the proper in vitro membrane association of protein A (Chapter II) yet functional studies presented in this chapter indicate that deletions or mutations in TOM complex components can significantly influence FHV RNA replication. Similar to Mim1, deletions of protein import and fission components lead to changes in not only protein import but also changes in mitochondrial morphology (2, 28, 54) suggesting possible changes in membrane phospholipid components as secondary effects in these deletion strains. Mim1 has been shown to physically interact with members of the SAM complex (3). One membrane protein in particular Sam37 was originally identified in a screen looking for genes regulating cellular phospholipids biosynthesis (8). We did not find any changes in FHV RNA replication in yeast lacking Sam37 using the

replicion system however, we did find that when using the trans-replication system a deletion of this protein leads to roughly an 8-fold increase in FHV RNA replication (K. Stapleford and D. Miller, unpublished data). The reason for these differences in FHV RNA replication between replication systems is currently unclear but future work exploring this may provide valuable information regarding viral replication. Nonetheless, these data indicate a possible functional role for host protein and lipid components in FHV RNA replication. Unfortunately, work with Sam37 and phospholipid biosynthesis was not followed up but a detailed phospholipid analysis of the mitochondrial outer membrane from many of the deletion strains used in this studied would yield valuable information not only for FHV RNA replication but for yeast mitochondrial biology as well.

In summary these studies identify several mitochondrial outer membrane deletion yeast strains that have dramatic changes in FHV RNA replication. In particular, a deletion of Mim1 leads to significant reductions in RNA synthesis in part due a defect in the assembly of functional viral RNA replication complexes, a process which is dependent on active RNA synthesis and host processes. Mim1 deficient yeast have provided a system with which viral RNA replication can be dissected in vivo to better understand the molecular mechanisms involved in these processes. Future studies with Mim1 deficient yeast and other mitochondrial outer membrane deletion yeast strains will provide valuable insight into the cellular components and processes which govern positive-strand RNA virus RNA replication.

REFERENCES

1. **Ahting, U., T. Waizenegger, W. Neupert, and D. Rapaport.** 2005. Signal-anchored proteins follow a unique insertion pathway into the outer membrane of mitochondria. J Biol Chem **280:**48-53.

2. **Altmann, K., and B. Westermann.** 2005. Role of essential genes in mitochondrial morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell **16:**5410-7.

3. **Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenkovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann.** 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. J Biol Chem **283:**120-7.

4. **Beckham, C. J., H. R. Light, T. A. Nissan, P. Ahlquist, R. Parker, and A. Noueiry.** 2007. Interactions between brome mosaic virus RNAs and cytoplasmic processing bodies. J Virol **81:**9759-68.

5. **Castorena, K. M., S. A. Weeks, K. A. Stapleford, A. M. Cadwallader, and D. J. Miller.** 2007. A functional heat shock protein 90 chaperone is essential for efficient flock house virus RNA polymerase synthesis in Drosophila cells. J Virol **81:**8412-20.

6. **den Boon, J. A., J. Chen, and P. Ahlquist.** 2001. Identification of sequences in Brome mosaic virus replicase protein 1a that mediate association with endoplasmic reticulum membranes. J Virol **75:**12370-81.

7. **Denison, M. R.** 2008. Seeking membranes: positive-strand RNA virus replication complexes. PLoS Biol **6:**e270.

8. **Gratzer, S., T. Lithgow, R. E. Bauer, E. Lamping, F. Paltauf, S. D. Kohlwein, V. Haucke, T. Junne, G. Schatz, and M. Horst.** 1995. Mas37p, a novel receptor subunit for protein import into mitochondria. J Cell Biol **129:**25-34.

9. **Hulett, J. M., F. Lueder, N. C. Chan, A. J. Perry, P. Wolynec, V. A. Likic, P. R. Gooley, and T. Lithgow.** 2008. The transmembrane segment of Tom20 is recognized by Mim1 for docking to the mitochondrial TOM complex. J Mol Biol **376:**694-704.

10. **Hwang, Y. T., A. W. McCartney, S. K. Gidda, and R. T. Mullen.** 2008. Localization of the Carnation Italian ringspot virus replication protein p36 to the mitochondrial outer membrane is mediated by an internal targeting signal and the TOM complex. BMC Cell Biol **9:**54.

11. **Ishikawa, D., H. Yamamoto, Y. Tamura, K. Moritoh, and T. Endo.** 2004. Two novel proteins in the mitochondrial outer membrane mediate beta-barrel protein assembly. J Cell Biol **166:**621-7.

12. **Jaag, H. M., J. Stork, and P. D. Nagy.** 2007. Host transcription factor Rpb11p affects tombusvirus replication and recombination via regulating the accumulation of viral replication proteins. Virology **368:**388-404.

13. **Jiang, Y., E. Serviene, J. Gal, T. Panavas, and P. D. Nagy.** 2006. Identification of essential host factors affecting tombusvirus RNA replication based on the yeast Tet promoters Hughes Collection. J Virol **80:**7394-404.

14. **Johnson, K. L., and L. A. Ball.** 1999. Induction and maintenance of autonomous flock house virus RNA1 replication. J Virol **73:**7933-42.

15. **Johnson, K. L., and L. A. Ball.** 1997. Replication of flock house virus RNAs from primary transcripts made in cells by RNA polymerase II. J Virol **71:**3323-7.

16. **Jonczyk, M., K. B. Pathak, M. Sharma, and P. D. Nagy.** 2007. Exploiting alternative subcellular location for replication: tombusvirus replication switches to the endoplasmic reticulum in the absence of peroxisomes. Virology **362:**320-30.

17. **Kampmueller, K. M., and D. J. Miller.** 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. J Virol **79:**6827-37.

18. **Knoops, K., M. Kikkert, S. H. Worm, J. C. Zevenhoven-Dobbe, Y. van der Meer, A. J. Koster, A. M. Mommaas, and E. J. Snijder.** 2008. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. PLoS Biol **6:**e226.

19. **Kopek, B. G., G. Perkins, D. J. Miller, M. H. Ellisman, and P. Ahlquist.** 2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. PLoS Biol **5:**e220.

20. **Korber, S., P. Shaik Syed Ali, and J. C. Chen.** 2009. Structure of the RNA-Binding Domain of Nodamura Virus Protein B2, a Suppressor of RNA Interference (dagger) , (double dagger). Biochemistry.

21. **Kushner, D. B., B. D. Lindenbach, V. Z. Grdzelishvili, A. O. Noueiry, S. M. Paul, and P. Ahlquist.** 2003. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. Proc Natl Acad Sci U S A **100:**15764-9.

22. **Kutik, S., D. Stojanovski, L. Becker, T. Becker, M. Meinecke, V. Kruger, C. Prinz, C. Meisinger, B. Guiard, R. Wagner, N. Pfanner, and N. Wiedemann.** 2008. Dissecting membrane insertion of mitochondrial beta-barrel proteins. Cell **132:**1011-24.

23. **Lee, W. M., and P. Ahlquist.** 2003. Membrane synthesis, specific lipid requirements, and localized lipid composition changes associated with a positive-strand RNA virus RNA replication protein. J Virol **77:**12819-28.

24. **Li, Z., J. Pogany, T. Panavas, K. Xu, A. M. Esposito, T. G. Kinzy, and P. D. Nagy.** 2009. Translation elongation factor 1A is a component of the tombusvirus replicase complex and affects the stability of the p33 replication co-factor. Virology **385:**245-60.

25. **Lu, R., M. Maduro, F. Li, H. W. Li, G. Broitman-Maduro, W. X. Li, and S. W. Ding.** 2005. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature **436:**1040-3.

26. **Lu, R., E. Yigit, W. X. Li, and S. W. Ding.** 2009. An RIG-I-Like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in Caenorhabditis elegans. PLoS Pathog **5:**e1000286.

27. **Marshall, D., and A. Schneemann.** 2001. Specific packaging of nodaviral RNA2 requires the N-terminus of the capsid protein. Virology **285:**165-75.

28. **Meisinger, C., M. Rissler, A. Chacinska, L. K. Szklarz, D. Milenkovic, V. Kozjak, B. Schonfisch, C. Lohaus, H. E. Meyer, M. P. Yaffe, B. Guiard, N. Wiedemann, and N. Pfanner.** 2004. The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. Dev Cell **7:**61-71.

29. **Meisinger, C., T. Sommer, and N. Pfanner.** 2000. Purification of Saccharomcyes cerevisiae mitochondria devoid of microsomal and cytosolic contaminations. Anal Biochem **287:**339-42.

30. **Miller, D. J., and P. Ahlquist.** 2002. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J Virol **76:**9856-67.

31. **Miller, D. J., M. D. Schwartz, and P. Ahlquist.** 2001. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J Virol **75:**11664-76.

32. **Miller, D. J., M. D. Schwartz, B. T. Dye, and P. Ahlquist.** 2003. Engineered retargeting of viral RNA replication complexes to an alternative intracellular membrane. J Virol **77:**12193-202.

33. **Miller, S., and J. Krijnse-Locker.** 2008. Modification of intracellular membrane structures for virus replication. Nat Rev Microbiol **6:**363-74.

34. **Mozdy, A. D., J. M. McCaffery, and J. M. Shaw.** 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J Cell Biol **151:**367-80.

35. **Nagy, P. D.** 2008. Yeast as a model host to explore plant virus-host interactions. Annu Rev Phytopathol **46:**217-42.

36. **Neupert, W., and J. M. Herrmann.** 2007. Translocation of proteins into mitochondria. Annu Rev Biochem **76:**723-49.

37. **Nishikiori, M., K. Dohi, M. Mori, T. Meshi, S. Naito, and M. Ishikawa.** 2006. Membrane-bound tomato mosaic virus replication proteins participate in RNA synthesis and are associated with host proteins in a pattern distinct from those that are not membrane bound. J Virol **80:**8459-68.

38. **Okamoto, K., and J. M. Shaw.** 2005. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet **39:**503-36.

39. **Pathak, K. B., Z. Sasvari, and P. D. Nagy.** 2008. The host Pex19p plays a role in peroxisomal localization of tombusvirus replication proteins. Virology **379:**294-305.

40. **Pfanner, N., N. Wiedemann, C. Meisinger, and T. Lithgow.** 2004. Assembling the mitochondrial outer membrane. Nat Struct Mol Biol **11:**1044-8.

41. **Popov-Celeketic, J., T. Waizenegger, and D. Rapaport.** 2008. Mim1 functions in an oligomeric form to facilitate the integration of Tom20 into the mitochondrial outer membrane. J Mol Biol **376:**671-80.

42. **Price, B. D., M. Roeder, and P. Ahlquist.** 2000. DNA-Directed expression of functional flock house virus RNA1 derivatives in Saccharomyces cerevisiae, heterologous gene expression, and selective effects on subgenomic mRNA synthesis. J Virol **74:**11724-33.

43. **Prod'homme, D., A. Jakubiec, V. Tournier, G. Drugeon, and I. Jupin.** 2003. Targeting of the turnip yellow mosaic virus 66K replication protein to the chloroplast envelope is mediated by the 140K protein. J Virol **77:**9124-35.

44. **Quinkert, D., R. Bartenschlager, and V. Lohmann.** 2005. Quantitative analysis of the hepatitis C virus replication complex. J Virol **79:**13594-605.

45. **Salonen, A., L. Vasiljeva, A. Merits, J. Magden, E. Jokitalo, and L. Kaariainen.** 2003. Properly folded nonstructural polyprotein directs the semliki forest virus replication complex to the endosomal compartment. J Virol **77:**1691-702.

46. **Schaad, M. C., P. E. Jensen, and J. C. Carrington.** 1997. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J **16:**4049-59.

47. **Schlegel, A., T. H. Giddings, Jr., M. S. Ladinsky, and K. Kirkegaard.** 1996. Cellular origin and ultrastructure of membranes induced during poliovirus infection. J Virol **70:**6576-88.

48. **Schmidt-Mende, J., E. Bieck, T. Hugle, F. Penin, C. M. Rice, H. E. Blum, and D. Moradpour.** 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J Biol Chem **276:**44052-63.

49. **Schneemann, A., W. Zhong, T. M. Gallagher, and R. R. Rueckert.** 1992. Maturation cleavage required for infectivity of a nodavirus. J Virol **66:**6728-34.

50. **Selling, B. H., R. F. Allison, and P. Kaesberg.** 1990. Genomic RNA of an insect virus directs synthesis of infectious virions in plants. Proc Natl Acad Sci U S A **87:**434-8.

51. **Serva, S., and P. D. Nagy.** 2006. Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. J Virol **80:**2162-9.

52. **Serviene, E., Y. Jiang, C. P. Cheng, J. Baker, and P. D. Nagy.** 2006. Screening of the yeast yTHC collection identifies essential host factors affecting tombusvirus RNA recombination. J Virol **80:**1231-41.

53. **Singh, G., S. Popli, Y. Hari, P. Malhotra, S. Mukherjee, and R. K. Bhatnagar.** 2009. Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. Faseb J.

54. **Stojanovski, D., M. Rissler, N. Pfanner, and C. Meisinger.** 2006. Mitochondrial morphology and protein import--a tight connection? Biochim Biophys Acta **1763:**414-21.

55. **Van Wynsberghe, P. M., and P. Ahlquist.** 2009. 5' cis elements direct nodavirus RNA1 recruitment to mitochondrial sites of replication complex formation. J Virol.

56. **Van Wynsberghe, P. M., H. R. Chen, and P. Ahlquist.** 2007. Nodavirus RNA replication protein a induces membrane association of genomic RNA. J Virol **81:**4633-44.

57. **Venter, P. A., and A. Schneemann.** 2007. Assembly of two independent populations of flock house virus particles with distinct RNA packaging characteristics in the same cell. J Virol **81:**613-9.

58. **Waizenegger, T., S. Schmitt, J. Zivkovic, W. Neupert, and D. Rapaport.** 2005. Mim1, a protein required for the assembly of the TOM complex of mitochondria. EMBO Rep **6:**57-62.

59. **Weeks, S. A., and D. J. Miller.** 2008. The heat shock protein 70 cochaperone YDJ1 is required for efficient membrane-specific flock house virus RNA replication complex assembly and function in Saccharomyces cerevisiae. J Virol **82:**2004-12.

60. **Westermann, B.** 2008. Molecular machinery of mitochondrial fusion and fission. J Biol Chem **283:**13501-5.

61. **Wiedemann, N., V. Kozjak, A. Chacinska, B. Schonfisch, S. Rospert, M. T. Ryan, N. Pfanner, and C. Meisinger.** 2003. Machinery for protein sorting and assembly in the mitochondrial outer membrane. Nature **424:**565-71.

62. **Wu, S. X., P. Ahlquist, and P. Kaesberg.** 1992. Active complete in vitro replication of nodavirus RNA requires glycerophospholipid. Proc Natl Acad Sci U S A **89:**11136-40.

63. **Wu, S. X., and P. Kaesberg.** 1991. Synthesis of template-sense, single-strand Flockhouse virus RNA in a cell-free replication system. Virology **183:**392-6.

64. **Zahedi, R. P., A. Sickmann, A. M. Boehm, C. Winkler, N. Zufall, B. Schonfisch, B. Guiard, N. Pfanner, and C. Meisinger.** 2006. Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. Mol Biol Cell **17:**1436-50.

APPENDIX

PRELIMINARY STUDIES ON THE ROLE OF YEAST MIM1 IN FHV RNA REPLICATION

We have identified a deletion of the mitochondrial outer membrane protein Mim1 that leads to a significant reduction in FHV RNA accumulation. We found this defect to be in part due to the absence of a functional FHV replication complex which leads to a decrease in membrane-associated replicase activity and membrane-associated viral RNA. We attempted to rescue this defect with the exogenous expression of Mim1 under the control of its endogenous promoter but have thus far been unsuccessful. These results were particularly interesting in that we confirmed that Mim1 was functional by its ability to complement protein defects and temperature-sensitive phenotypes associated with the deletion of Mim1. Taken together, these data suggest that FHV RNA replication may depend on other cellular processes in which the exogenous expression of Mim1 can not complement. To this end, the molecular role of Mim1 in FHV RNA replication complex assembly and function is currently unclear.

Mim1 is a small mitochondrial outer membrane protein of approximately 13 kDa that was originally identified, along with Sam35, in a screen for yeast proteins involved in mitochondrial β-barrel protein import and assembly (9).

Since then, additional studies have implicated Mim1 in the import of the signal-anchored receptor proteins, Tom20 and Tom70, as well as the assembly of the TOM complex (2, 8, 22). Furthermore, Mim1 has been shown to physically interact with the mitochondrial outer membrane SAM complex although a functional interaction between these two complexes has yet to be shown (2). Finally, the deletion of Mim1 has been shown to lead to dramatic changes in mitochondrial morphology similar to those seen in the deletion of various mitochondrial protein import machinery components (1). Given this, we were interested in identifying the specific role of Mim1 in FHV RNA replication complex assembly and function.

MATERIALS AND METHODS

Yeast strains, transformations, and culture conditions. The wildtype yeast strain YPH499 (*MAT*α *ade2-101 his3*Δ200 *leu2*Δ*1 ura3-52* trp1-Δ63 lys-801) and *Δmim1* (*MAT*α *ade2-101 his3*Δ*200 leu2*Δ*1 ura3-52* trp1Δ63 lys2-801 mim1::HIS3) were obtained from Dr. Doron Rapaport (University of Tubingen, Germany). Yeast were transformed using the E-Z Transformation Kit (Zymo Research, Orange, CA). Individual clones were grown to early stationary phase in selectable liquid media containing 2% [wt/vol] raffinose at 30°C. For the induction of galactose-inducible plasmids, yeast were washed once with distilled water, resuspended in selectable liquid media containing 2% raffinose [wt/vol] and 2% [wt/vol] galactose at an OD_{600} of 0.2, and grown for 24 h at 25°C for all experiments. For mitochondrial phospholipid extractions, yeast were grown in YPDGal (1% yeast extract [wt/vol], 2% peptone [wt/vol], 1% dextrose [wt/vol], and 1% galactose [wt/vol] at 25°C for 48 hours.

Plasmids and antibodies. The FHV RNA and protein expression plasmids pF1, pF1fs, pFA-C/HA, and pFA-HCV-HA have been previously described (16, 19, 23). The Mim1 overexpression plasmid, pMIM1-OX, encoding yeast Mim1 under the triosephosphate isomerase (TPI) promoter was previously described (18). Rabbit polyclonal antibodies raised against FHV protein A and protein B2 have been previously described (11, 15). Rabbit polyclonal antibodies against the hemagglutinin (HA) epitope and mouse polyclonal antibodies against 3-phosphoglycerate kinase (PGK1) were purchased from Santa Cruz Biotech

(Santa Cruz, CA) and Molecular Probes (Carlsbad, CA) respectively. Rabbit polyclonal antibodies against Mim1 were previously described (18).

RNA and protein analysis. For total protein isolation, equal amounts of cells were isolated 24 hours after plasmid induction and resuspended in protein extraction buffer (20 mM Tris, pH 8.0, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1:50 dilution of yeast protease inhibitor cocktail (Sigma)). Cell walls were removed by vortexing in the presence of glass beads for 10 min at 4°C followed by the addition of 1% SDS and heating at 100°C for 10 min. Samples were pelleted by centrifugation at 10,000 x g for 5 min and supernatant aliquots were removed, mixed with 2X SDS-PAGE sample buffer (125 mM Tris-HCl; pH 6.8, 4% SDS, 20% (w/v) glycerol, 0.002 mg/ml bromophenol blue, 10% 2-mercaptoethanol), and stored at -20°C for further analysis. Total protein extractions were separated by SDS-PAGE, transferred in polyvinylidene fluoride (PVDF) membranes, and immunoblotted as previously described (15). For total RNA isolation, equal amounts of cells were subject to hot acidic phenol extraction as previously described and stored at -80°C until northern blot analysis. RNA was separated on a 1.2% agarose-formaldehyde gel, blotted onto nylon membranes, and probed for positive- and negative-strand RNA1 and RNA3 as previously described (15).

Phospholipid extraction and analysis. Total mitochondrial phospholipids were extracted as previously described (3). Yeast mitochondria (100 µg total protein) were resuspended in 100 µl SEM buffer followed by the addition of 375 µl chloroform:methanol (1:2) and vortexed vigorously. Samples were extracted

again with 125 µl chloroform and 125 µl H_2O with vortexing in between each step. The aqueous (top) and organic (bottom) layers were separated by centrifugation at 1,000 rpm for 5 min at room temperature. The organic layer was removed and phospholipids were dried by removing the solvent under air stream. Samples were sent to the Kansas Lipidomic Research Center (Kansas State University) for mass spectroscopy analysis.

RESULTS

Complementation of FHV RNA replication with *URA3* auxotrophic marker. We previously attempted to complement the FHV RNA accumulation defect with the exogenous expression of Mim1 under the control of its endogenous promoter, yet we were unable to rescue the defect to any degree. One possible explanation for this could be that although the expression of Mim1 with this system was able to complement the yeast temperature-sensitive phenotypes, it is insufficient to rescue the FHV phenotype. To address this we attempted to rescue the FHV RNA replication defect by introducing a Mim1 overexpression plasmid under the control of the strong triosephosphate isomerase (TPI) promoter. As seen previously, overexpression of Mim1 was able to rescue the temperature-sensitive phenotype (Figure 3.7A), yet interestingly we found FHV RNA replication increased over wildtype levels by the control plasmid alone (Figure 3.7B, lanes 4 and 5) whereas the overexpression of Mim1 brought RNA levels back down towards wildtype (Figure 3.7B, lanes 6 and 7). Protein B2 translation correlated with RNA3 production whereas there was no change in protein A expression between the strains indicating that this defect was not due to increased polymerase accumulation. These results were unexpected as the only differences between the two control plasmids used in the Mim1 endogenous and overexpression systems were the auxotrophic markers (LEU vs URA) and the plasmid copy number (centromeric vs 2-micron).

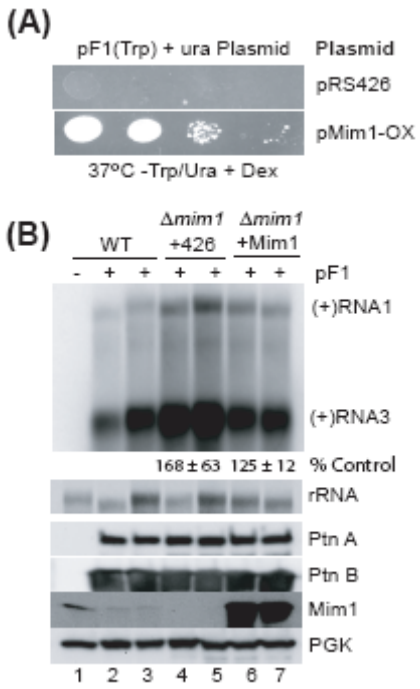

Figure 3.7: Complementation of FHV RNA replication with Mim1 overexpression. (A). Yeast strains were transformed with the control plasmid pRS426 or Mim1 overexpression plasmid pMim1-OX, grown inSpotted selective liquid media, and plated in ten-fold dilutions onto selective plates with 2% dextrose. Strains were grown at 37°C for 4 days. **(B)**. Yeast were induced for 24 hours in selectable liquid media with 2% galactose at 25°C. Equal amounts of cells were isolated, protein and RNA was extracted, and analyzed by northern and western blotting respectively.

However, these initial studies indicate that growth conditions and possible alternative functions of Mim1 may play a significant role in FHV RNA replication as introduction of the control plasmid into Mim1 deficient yeast does not complement the temperature-sensitive phenotype but does rescue FHV RNA replication.

Reduction of ER retargeted FHV RNA replication in Mim1 deficient yeast. The ability to complement FHV RNA replication with the addition of a ura3 plasmid suggests the role of global cellular processes in FHV RNA replication. Thus, we were interested in exploring whether this defect in FHV RNA replication is specific to the mitochondria in Mim1 deficient yeast. To address this we made use of a two plasmid trans-replication system in which a translationally optimized protein A expression plasmid is co-expressed with a plasmid encoding an RNA1 template that contains a 5' frameshift and is thus unable to be translated into protein A (Figure 3.8A). Expressed together protein A is targeted to the mitochondrial outer membrane and initiates RNA1 and RNA3 synthesis (14, 16). Additionally this system allows for the manipulation of protein A such that by exchanging the amino-terminal mitochondrial targeting signal with that of the hepatitis C virus NS5B ER targeting signal one can establish robust FHV RNA replication on the ER (16). We introduced both the mitochondrial and ER targeted systems into wildtype and Mim1 deficient yeast, induced FHV RNA replication, and analyzed positive- and negative-strand RNA1 and RNA3 levels by northern blotting. Interestingly in contrast to what was seen previously with the replicon system, we found no significant defect in viral replication when

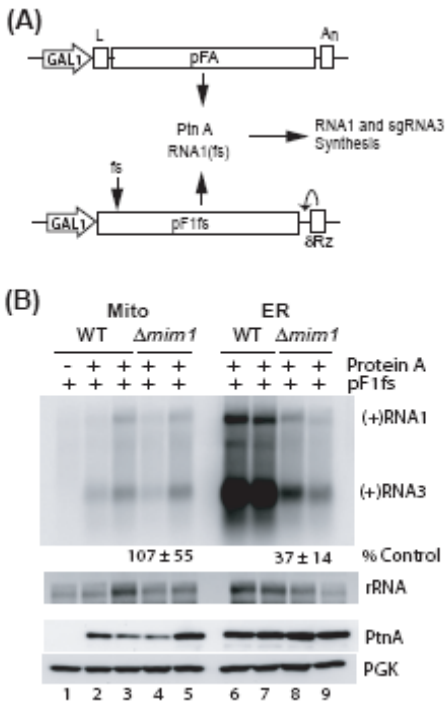

Figure 3.8: ER targeted FHV RNA replication is inhibited in Mim1 deficient yeast. (A). Schematic of FHV trans replication system. The translationally optimized plasmid (pFA) and replication competent RNA1 plasmid (pF1fs) are co-expressed in yeast and lead to RNA1 and RNA3 synthesis. **(B).** Wildtype and Mim1 deficient yeast were transformed with mitochondrial or ER targeted protein A molecules, plasmids were induced with galactose, and RNA replication was analyzed by northering and western blotting as previously described.

wildtype protein A was used (Figure 3.8B, lanes 2-5). However, when protein A was targeted to the ER we found a significant reduction in both positive- and negative-strand RNA1 and RNA3 levels in Mim1 deficient yeast (Figure 3.8B, lanes 6-9). These data suggest that the deletion of Mim1 leads to a defect in FHV RNA replication that is not mitochondrial-specific but may play a broader role which influences multiple membranes.

Mim1 deficient yeast have defects membrane phospholipid composition. The observation that ER retargeted FHV replication was reduced in Mim1 deficient yeast suggests the presence of global cellular defects in the absence of Mim1. Cellular phospholipids play key roles in FHV RNA replication and thus we hypothesize there may be defects in phospholipid biosynthesis in Mim1 deficient yeast. To address this directly we isolated mitochondria from the wildtype and Δ*mim1* yeast, extracted the total mitochondrial phospholipids, and analyzed the phospholipid content by mass spectroscopy. We found there to be dramatic changes in mitochondrial phospholipid composition in Mim1 deficient yeast (Figure 3.9A), and identified reductions in total PC and PE levels which corresponded with increases in mitochondrial PI content (Figure 3.9A). In particular, we found decreases in unsaturated PC species which have been implicated in FHV replicase function in vitro (Figure 3.9B) (24). In addition to these preliminary experiments preformed by our laboratory, similar results were obtained using thin-layer chromatography (TLC) (personal communication from Dr. Doron Rapaport). Taken together, these data provide initial evidence that Mim1 may play a role in maintanence of mitochondrial phospholipid composition.

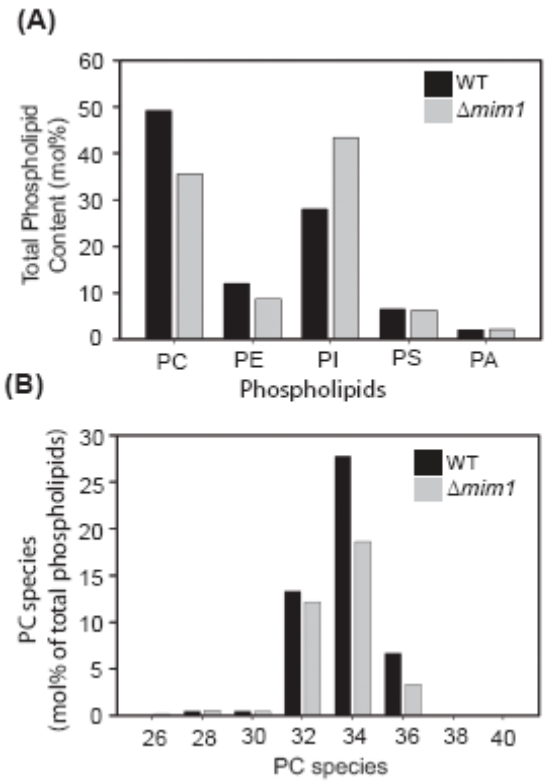

Figure 3.9. Total mitochondrial phospholipid composition of Δ*mim1* yeast. (A). Changes in total mitochondrial phospholipid species from wildtype and Δ*mim1* yeast. **(B).** Changes in phosphatidylcholine species in wildtype and Δ*mim1* yeast.

DISCUSSION

In these preliminary studies we have begun to address the molecular deficiencies in Δ*mim1* yeast responsible for the defect in FHV RNA replication and replication complex assembly. We attempted to complement the FHV RNA accumulation defect by the overexpression of Mim1, and found unexpectedly that FHV RNA accumulation could be rescued by the expression of a control plasmid expressing the *URA3* auxotrophic marker. These data suggest a possible role for pyrimidine biosynthesis in FHV RNA replication. Furthermore, using a trans replication system we found that ER targeted FHV RNA replication was also defective in Mim1 deficient yeast, indicating that Mim1 may play a global role within the cell influencing multiple membranes. Finally, we addressed the mitochondrial phospholipid composition of Δ*mim1* yeast and found large changes in total mitochondrial phospholipids content including unsaturated species of PC. These data provide initial insights into a possible molecular role of Mim1 in FHV RNA replication and suggests that membrane phospholipid composition may be a key determinant in FHV RNA replication.

This notion of Mim1 being involved in phospholipid biology is not unreasonable as Mim1 is a small protein composed mostly of a highly conserved transmembrane domain thus allowing for close physical contact with the lipid bilayer. The transmembrane itself is sufficient for Mim1 function in vivo (18), and a deletion of Mim1 leads to various defects influencing mitochondrial morphology (1). We found that by using the Mim1 overexpression system, Mim1 was able to complement the temperature-sensitive phenotype but to our surprise FHV RNA

replication was complemented by only expressing a control plasmid encoding a *URA3* auxotrophic marker. It is possible that although the protein defects are not complemented in order to rescue growth at 37°C, other cellular processes are complemented leading to increased FHV RNA replication. The *URA*3 gene plays an essential role in the production of the nucleotides UTP and CTP, two molecules which play multiple roles within the cell including nucleic acid and phospholipid biosynthesis. This idea of a global cellular process is further confirmed by the defect in FHV replication when replication complexes are targeted to the ER. In addition, we have confirmed that Mim1 deficient yeast contain changes in total mitochondrial phospholipid content that correlate with reductions in viral RNA synthesis. It will be interesting to repeat these analyses with ER membranes to confirm these observations.

These data suggest that membrane phospholipid content may be an important determinant in viral RNA replication. Initial studies on the retargeting of FHV RNA replication complexes made the observation that FHV RNA replication was more efficient when targeted to the ER as opposed to the mitochondria (16). In addition, other viruses have been shown to use alternative membranes for viral replication (10). One possible explanation for this phenotype may involve the different lipid compositions of cellular membranes which can act to facilitate viral RNA replication. It is currently unclear as to what specific role Mim1 plays in these processes as there is a possibility of defects in phospholipid biosynthesis directly or in lipid trafficking, both processes which could lead to defects in FHV RNA replication.

The synthesis of many of the major phospholipids occurs in the ER and thus they must be trafficked to other organelles (20, 21). The mitochondria is capable of synthesizing its own phospholipids such as phosphatidylethanolamine (PE), and the mitochondrial specific phospholipids cardiolipin and phosphatidyglycerol, yet it is unable to synthesize phosphatidylcholine (PC). PC, along with PE, make up the majority of phospholipids found in cellular membranes suggesting that proper trafficking of these particular phospholipids is important for a variety of cellular functions (21). It is well known that phospholipids play important roles in positive-strand RNA virus membrane targeting and replication (5, 17). The addition of the fatty acid synthetase inhibitor cerulinen has been shown to reduce viral replication in FHV (4) and poliovirus (6, 7) in vitro and deletion of the fatty acid saturase Ole1 leads to defects in bromo mosaic virus RNA replication in yeast (12, 13). Additionally, membrane-associated FHV replication complex activity can be stimulated in vitro with the addition of exogenous PE and PC (24), indicating an important role for specific phospholipids in viral RNA replication. One may speculate that in Mim1 deficient yeast there is a defect in phospholipid biosynthesis or trafficking which ultimately leads to defects in both mitochondrial and ER targeted FHV RNA replication.

In summary, we have identified a potential role for Mim1 in cellular phospholipid biosynthesis and a possible mechanism which regulates FHV RNA replication and replication complex assembly in yeast. It will be interesting to further investigate the role of Mim1 in these cellular and FHV related processes. The observation that *URA3* expression can complement FHV RNA replication is

intriguing and begs the question as to what specific role pyrimidine biosynthesis plays in viral RNA replication? Studies are currently in progress to investigate the role of a variety of plasmid encoded auxotrophic makers on FHV RNA replication. In addition, we would like to confirm the defects in total mitochondrial phospholipids levels as well as phospholipid changes unique to the mitochondrial outer membrane where FHV RNA replication takes place. Future studies will provide further insight into the role of membrane phospholipids in positive-strand RNA virus replication.

REFERENCES

1. **Altmann, K., and B. Westermann.** 2005. Role of essential genes in mitochondrial morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell **16:**5410-7.

2. **Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenkovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann.** 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. J Biol Chem **283:**120-7.

3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:**911-7.

4. **Castorena, K. M., S. A. Weeks, K. A. Stapleford, A. M. Cadwallader, and D. J. Miller.** 2007. A functional heat shock protein 90 chaperone is essential for efficient flock house virus RNA polymerase synthesis in Drosophila cells. J Virol **81:**8412-20.

5. **Denison, M. R.** 2008. Seeking membranes: positive-strand RNA virus replication complexes. PLoS Biol **6:**e270.

6. **Guinea, R., and L. Carrasco.** 1991. Effects of fatty acids on lipid synthesis and viral RNA replication in poliovirus-infected cells. Virology **185:**473-6.

7. **Guinea, R., and L. Carrasco.** 1990. Phospholipid biosynthesis and poliovirus genome replication, two coupled phenomena. EMBO J **9:**2011-6.

8. **Hulett, J. M., F. Lueder, N. C. Chan, A. J. Perry, P. Wolynec, V. A. Likic, P. R. Gooley, and T. Lithgow.** 2008. The transmembrane segment of Tom20 is recognized by Mim1 for docking to the mitochondrial TOM complex. J Mol Biol **376:**694-704.

9. **Ishikawa, D., H. Yamamoto, Y. Tamura, K. Moritoh, and T. Endo.** 2004. Two novel proteins in the mitochondrial outer membrane mediate beta-barrel protein assembly. J Cell Biol **166:**621-7.

10. **Jonczyk, M., K. B. Pathak, M. Sharma, and P. D. Nagy.** 2007. Exploiting alternative subcellular location for replication: tombusvirus replication switches to the endoplasmic reticulum in the absence of peroxisomes. Virology **362:**320-30.

11. **Kampmueller, K. M., and D. J. Miller.** 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. J Virol **79:**6827-37.

12. **Lee, W. M., and P. Ahlquist.** 2003. Membrane synthesis, specific lipid requirements, and localized lipid composition changes associated with a positive-strand RNA virus RNA replication protein. J Virol **77:**12819-28.

13. **Lee, W. M., M. Ishikawa, and P. Ahlquist.** 2001. Mutation of host delta9 fatty acid desaturase inhibits brome mosaic virus RNA replication between template recognition and RNA synthesis. J Virol **75:**2097-106.

14. **Miller, D. J., and P. Ahlquist.** 2002. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J Virol **76:**9856-67.

15. **Miller, D. J., M. D. Schwartz, and P. Ahlquist.** 2001. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J Virol **75:**11664-76.

16. **Miller, D. J., M. D. Schwartz, B. T. Dye, and P. Ahlquist.** 2003. Engineered retargeting of viral RNA replication complexes to an alternative intracellular membrane. J Virol **77:**12193-202.

17. **Miller, S., and J. Krijnse-Locker.** 2008. Modification of intracellular membrane structures for virus replication. Nat Rev Microbiol **6:**363-74.

18. **Popov-Celeketic, J., T. Waizenegger, and D. Rapaport.** 2008. Mim1 functions in an oligomeric form to facilitate the integration of Tom20 into the mitochondrial outer membrane. J Mol Biol **376:**671-80.

19. **Price, B. D., M. Roeder, and P. Ahlquist.** 2000. DNA-Directed expression of functional flock house virus RNA1 derivatives in Saccharomyces cerevisiae, heterologous gene expression, and selective effects on subgenomic mRNA synthesis. J Virol **74:**11724-33.

20. **van Meer, G., D. R. Voelker, and G. W. Feigenson.** 2008. Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol **9:**112-24.

21. **Voelker, D. R.** 2003. New perspectives on the regulation of intermembrane glycerophospholipid traffic. J Lipid Res **44:**441-9.

22. **Waizenegger, T., S. Schmitt, J. Zivkovic, W. Neupert, and D. Rapaport.** 2005. Mim1, a protein required for the assembly of the TOM complex of mitochondria. EMBO Rep **6:**57-62.

23. **Weeks, S. A., and D. J. Miller.** 2008. The heat shock protein 70 cochaperone YDJ1 is required for efficient membrane-specific flock house virus RNA replication complex assembly and function in Saccharomyces cerevisiae. J Virol **82:**2004-12.

24. **Wu, S. X., P. Ahlquist, and P. Kaesberg.** 1992. Active complete in vitro replication of nodavirus RNA requires glycerophospholipid. Proc Natl Acad Sci U S A **89:**11136-40.

CHAPTER IV

BIOCHEMICAL STUDIES ON THE STRUCTURE AND COMPOSITION OF FHV RNA REPLICATION COMPLEXES

Positive-strand RNA viruses have been associated with a variety of diseases in plants and animals, and as a consequence this has driven the necessity to further understand the molecular mechanisms involved in viral RNA replication and pathogenesis. In order to establish robust genomic replication viruses must assemble functional RNA replication complexes on host intracellular membranes (8, 30, 35). Individual viruses encode a variety of replicase proteins that make up these membrane-bound complexes, which can range in complexity from one viral protein in the *Nodaviridae* family (16, 36) to as many as 16 nonstructural proteins in the *Coronaviridae* family (49, 51). In addition, these complexes are hypothesized to contain various host components required for RNA synthesis (33, 44) as well as the viral RNA genome (38, 54, 62) which for some viruses can include 2 or more segments (34, 57). The identification of viral components has led to biochemical and proteomic characterizations (6, 11, 27, 38, 44, 51) of positive-strand RNA virus replication complexes and has identified a variety of host factors that have been shown to be associated with viral RNA replication complexes (6, 33, 44) . However, to date only a handful of viral replication

complexes have been characterized in detail, and further studies regarding the composition and structure of these macromolecular protein-RNA complexes is necessary to understand their function in viral RNA replication.

To study positive-strand RNA virus replication complex structure and composition we use the well-established model alphanodavirus, Flock House virus (FHV). FHV has been used extensively to study many aspects of positive-strand RNA biology including; viral RNA replication (16, 17, 28, 37, 60), virion assembly (42, 43, 55, 56), innate immunity (22, 46), and host-pathogen interactions (7, 18, 48). The FHV bipartite RNA genome consists of RNA1 (3.1 kb) and RNA2 (1.4 kb) which are both co-packaged into infectious virions. RNA1 encodes protein A, the viral RNA-dependent RNA polymerase and during active FHV RNA replication a subgenomic RNA3 (0.4 kb) is produced from the 3' end of RNA1 which encodes protein B2, a potent inhibitor of RNA silencing (22). FHV RNA2 encodes the structural capsid subunit, protein alpha, which is necessary for the production of mature virions. Furthermore, FHV is capable of robust RNA replication in a variety of well-studied eukaryotic model systems such as *Drosophila melanogaster* (13, 18, 28)*, Caenorhabditis elegans* (22, 23)*,* and *Saccharomyces cerevisiae* (29, 36, 54, 60) providing powerful host systems in which to study viral replication complex assembly and function.

FHV assembles functional viral RNA replication complexes on the mitochondrial outer membrane where protein A is the only viral protein necessary for this process in yeast (27, 28, 54). Protein A is an integral, signal-anchored membrane protein that is directed to the mitochondrial outer membrane via an

amino-terminal targeting signal (27). During replication complex assembly, protein A recruits the viral RNA genome to the mitochondrial outer membrane (53, 54), and self-associates to form protein A oligomers which are necessary for RNA synthesis (11). The initial steps in this process use a TOM complex independent mechanism for mitochondrial import (48), similar to what has been seen for other yeast mitochondrial outer membrane proteins (3, 24). Given this we hypothesized that FHV RNA replication complexes possess biochemical characteristics similar to those of yeast mitochondrial outer membrane protein complexes.

Yeast mitochondrial biogenesis has been studied in detail in part due to the availability of yeast strains harboring deletions of mitochondrial proteins as well as established biochemical and cellular biology techniques. The ability to isolate purified yeast mitochondria and submitochondrial membranes has led to the biochemical characterization and purification of mitochondrial outer membrane protein complexes (2, 4, 31) and complete proteomic analyses of the yeast mitochondria and mitochondrial compartments (10, 41, 45, 64). In addition, the development of blue-native polyacrylamide gel electrophoresis (BN-PAGE), a technique used to separate native protein complexes by size, has aided in visualizing the structure of large macromolecular protein complexes in vivo and in vitro (32, 39, 40, 61).

In this report we addressed the biochemical composition and structure of the FHV RNA replication complex by affinity chromatography, native gel electrophoresis, and lipidomic analysis. We used a crosslinking technique to

isolate protein A complexes by affinity chromatography, however we were unable to reproducibly identify any complex-associated host components by mass spectroscopy. In an additional approach to investigate complex structure, we used blue-native agarose gel electrophoresis to separate functional FHV RNA replication complexes by size. With this technique we were able to efficiently separate FHV replication complexes and identified the presence of two functionally distinct replication complexes in wildtype yeast. Finally, we have taken a lipidomic approach to understand the cellular and membrane-specific phospholipid requirements for replication complex assembly and function. These studies provide insight into the composition and structure of FHV replication complexes and develop techniques with which to study FHV replication complex assembly and function in the future.

MATERIALS AND METHODS

Yeast Strains, transformations, and growth conditions. The wildtype diploid yeast strains BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0*) and YPH499 (*MAT*α *ade2-101 his3*Δ200 *leu2*Δ*1 ura3-52* trp1-Δ63 lys-801) were purchase from the American Type Culture Collection (ATCC) and obtained from Dr. Doron Rapaport respectively. Yeast were transformed as previously described (27). Individual yeast clones were grown to early stationary phase in liquid selectable media with 2% dextrose [wt/vol] for 48 hours at 30°C. Yeast were washed with ddH_2O, resuspended at an OD_{600} of 0.2 in selectable media with 2% galactose [wt/vol]. For the growth of YPH499, yeast were grown in selectable media with 2% raffinose [wt/vol] to early saturation phase, washed with ddH_2O, and diluted to an OD_{600} of 0.2 in selectable liquid media with 2% raffinose and 2% galactose [wt/vol]. All yeast strains were grown for 24 hours at 25°C for all experiments.

Plasmid and antibodies. Standard molecular biology techniques were used for the generation of all plasmids. The yeast FHV expression plasmids pF1, $pF1_{fs}$, pFA, and pFA-C/HA have been previously described (27, 36). The yellow fluorescent protein (YFP)-tagged endoplasmic reticulum targeted protein A, pFA-HCV-YFP, was generated by moving the NheI/HindIII fragment from pFA-YFP into the same sites of pFA-HCV (29). Rabbit polyclonal antibodies against FHV protein A have been previously described (28). Rabbit polyclonal antibodies

against the hemagglutinin (HA)-epitope and green fluorescent protein (GFP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Yeast mitochondrial isolation. Yeast mitochondria were isolated as previously described (26), resuspended at 5 mg/ml in SM buffer (250 mM sucrose, 10 mM MOPS-KOH, pH 7.2), and stored at -80°C. To remove contaminating membranes, crude mitochondria (5 mg/ml) were subject to 10 strokes of dounce homogenization, loaded on a sucrose step gradient (15%, 23%, 32%, 60% (w/v) sucrose in 10 mM MOPS-KOH, pH 7.2), and centrifuged at 134,000 x g for 1 hour at 4°C. Mitochondria were removed from the 32%/60% interface, diluted with one volume SM buffer, and reisolated by centrifugation at 12,000 x g for 15 min at 4°C.

Detergent solubilization and co-immunoprecipitation. Mitochondrial samples (100 µg total protein) were resuspended in 1 ml solublization buffer (TBS with 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml Aprotinin containing one of the following detergent concentrations [w/v]; TritonX-100 (0.5%), dodecylmaltoside (DDM) (0.5%), CHAPS (1%), octyl glucopyranoside (1%), lysophosphatidyl choline (0.5%), taurodeoxycholate (0.5%), Brij 35 (0.5%), Digitonin (0.5%), Saponin (0.5%), Na-deoxycholate (0.5%), nonidet P-40 (0.5%), C16TAB (0.5%), Tween 80 (0.5%)). Samples were solubilized on ice for 30 mins, centrifuged at 17,000 x g for 10 min at 4°C, and 900 µl of the supernatant was removed for immunoprecipitation. Supernatants were precleared with 20 µl αIgG-agarose beads for 1 hour, beads were removed by centrifugation at 10,000 x g for 1 min, and 20 µl α-HA-agarose beads (Sigma Aldrich) were added

overnight at 4°C. Beads were reisolated by centrifugation at 10,000 x g for 1 min, samples were washed 3 times with respective solublization buffer, resuspended in 2X SDS-PAGE buffer, and heated to 100°C for 10 mins. Samples were separated by SDS-PAGE and analyzed by immunoblotting.

Chemical crosslinking and affinity chromatography of FHV replication complexes. Mitochondria isolated from yeast expressing $pF1_{fs}$ and pFA or pFA-C/HA were resuspended at a concentration of 4 mg/ml in PBS and cross-linked with 0.25 mM 3,3´-dithiobis[sulfosuccinimidylpropionate] (DTSSP) (Pierce, Rockford, IL) on ice for 2 hours. To stop crosslinking, 50 mM Tris-HCl (pH 7.4) was added and samples were incubated for an addition 15 min on ice. Mitochondria were reisolated by centrifugation at 12,000 x g for 15 min, resuspended in swelling buffer (10 mM MOPS pH 7.2, 1 mM EDTA), and incubated on ice for 30 min. Membranes were sonicated on ice 3 times for 5 min each and centrifuged at 100,000 x g for 15 min. The mitochondrial pellet was resuspended in solubilization buffer (0.5% TX-100, TBS, 0.5 mM EDTA, 1mM PMSF), placed on ice for 1 hour, and then centrifuged at 100,000 x g for 15 min and diluted into 20 ml solublization buffer. Samples were placed onto columns containing α-HA agarose beads overnight, washed with RIPA buffer (1% TX-100, 1% Na-Deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA), and eluted with 150 mM Tris-HCl, 50 mM NaCl, pH 2.5. Samples were brought to neutral pH with the address of 1 M Tris (pH 8.0). Elution fractions were analyzed by SDS-PAGE as previously described (28). For mass spectroscopy protein analysis, elutions were concentrated with an Ambicon Ultra

centrifugation filter and TCA precipitated. Mass spectroscopy analysis was preformed by the Michigan Proteome Consortium (University of Michigan).

Blue-Native Agarose Gel Electrophoresis (BN-AGE). Membrane fractions (100 µg total protein) were resuspended in solublization buffer (0.5% n-dodecyl β-D-maltoside (DDM) 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 10% glycerol, 1 mM PMSF), incubated on ice for 30 min, and centrifuged at 100,000 x g for 15 min at 4°C. The supernatant was removed and mixed with loading dye (0.5% coomassie brilliant blue G-250, 50 mM aminocaproic acid, 10 mM Bis-Tris, pH 7.0). Samples were then loaded on a 1.5% BN-agarose gel containing 66.7 mM aminocaproic acid, 50 mM Bis-Tris pH 7.0, ran at 100 V for 2 hour in 50 mM Bis-Tris pH 7.0 as a running buffer. For immunoblotting, gels were incubated in SDS-PAGE running buffer for 20 min followed by transfer to PVDF membranes and immunoblot analysis as described (28). To analyze radiolabeled viral RNAs, gels were tried under vacuum, and analyzed by autoradiography.

In vitro RdRp Assay. Mitochondria (20 µg total protein) were incubated in RdRp buffer (50 mM Tris, pH 8.0, 50 mM potassium acetate, 15 mM magnesium acetate, 40 units RNAsin (Promega), 5 µg/ml Actinomycin D, 1 mM ATP, 1 mM CTP, 1 mM GTP, 25 µM UTP, 10 µCi [^{32}P]UTP (Amersham)) at 25°C for 3 h. Total RNA was extracted with one volume phenol-chloroform (pH 5.2) and unincorporated nucleotides were removed from the aqueous phase by a Micro Bio-Spin Column (BioRad) following manufacturers' instructions. Nucleic acid dye buffer (0.05% bromophenol blue, 0.05% xylene cyanol FF, 5% glycerol) was

added to each sample and RNAs were separated by 1.4% agarose-TBE gel. Gels were dried under vacuum and subject to autoradiography. For BN-AGE analysis, 2X solublization buffer was added to RdRp reactions, placed on ice for 30 min, and centrifuged as described previously.

Phospholipid extraction and analysis. Total mitochondrial phospholipids were extracted as previously described (5). Yeast mitochondria (100 µg total protein) were resuspended in 100 µl SEM buffer followed by the addition of 375 µl chloroform:methanol (1:2) and vortexed vigorously. Samples were extracted again with 125 µl chloroform and 125 µl H_2O with vortexing in between each step. The aqueous (top) and organic (bottom) layers were separated by centrifugation at 1,000 rpm for 5 min at room temperature. The organic layer was removed and the phospholipids were dried by removing the solvent under air stream. Samples were sent to the Kansas Lipidomic Research Center (Kansas State University) for mass spectroscopy analysis.

RESULTS

Isolation of protein A membrane complexes by affinity chromatography. Affinity purification of viral RNA replication has been used successfully to identify complex-associated host components (33, 44), and thus we were interested in using this approach for the isolation of FHV RNA replication complexes. Flock House virus assembles functional RNA replication complexes on the mitochondrial outer membrane in yeast (27). The budding yeast has provided a powerful tool to study mitochondrial protein complex composition and structure, and biochemical isolation of native mitochondrial outer membrane protein complexes have been completed (4, 31). To begin, we first used a detergent analysis to identify a detergent that was capable of maintaining the structural integrity and stability of FHV replication complexes. Protein A is the only viral protein required for replication complex assembly and the ability of protein A to self-associate is essential for FHV replication complex function (11). Thus we used protein A self-association as a marker for proper replication complex stability. To address the relative stability of replication complex structure we used a well established FHV trans replication system which utilizes a two plasmid approach to assemble functional membrane-associated replication complexes (see Chapter III) (29, 54). We co-expressed protein A containing a carboxy-terminal HA or YFP tag along with a replication competent viral RNA in the same cell, isolated membrane fractions, solubilized the replication complexes with various detergents, and analyzed the ability to co-immunoprecipitation the two

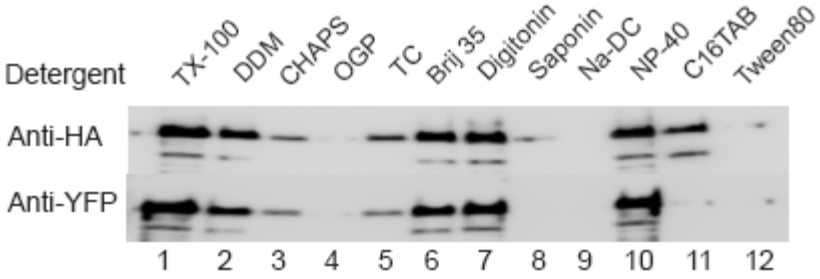

Figure 4.1: Detergent solubilization of FHV replication complexes. Yeast membrane fractions expressing pFA-C/HA and pFA-YFP were solubilized in the indicated detergent, immunopreciptated with anti-HA agarose beads, boiled, and immuno-blotted for HA or YFP.

protein A molecules (Figure 4.1). We found that only the detergents Tx-100, DDM, digitonin, Brij 35, and NP-40 retained the ability to efficiently co-immunoprecipitate protein A whereas anionic and zwitterionic detergents inhibited the ability to immunoprecipitate either protein suggesting dramatic changes in protein structure. Interestingly, the treatment with the cationic detergent C16TAB had no effect on the ability to immunoprecipitate protein A but specifically disrupted protein A-protein A interactions. These data suggest that certain detergents are able to disrupt protein-protein interactions whereas treatment with mild detergents has minimal effects on FHV protein A-protein A self-association.

Given this, we then attempted to isolate membrane-associated protein A replication complexes by affinity chromatography. We expressed wildtype or HA-tagged protein A along with a replication competent viral RNA in trans, isolated crude membrane fractions, and solublized the replication complexes with Tx-100, a detergent shown not to disruption protein A-protein A interactions. We then isolated complexes by affinity chromatography with anti-HA agarose beads and eluted associated proteins by low pH. Interestingly, although we were able to sufficiently immunoprecipitate HA-tagged protein A as seen by boiling the beads before elution, initial studies were unsuccessful at eluting protein complexes from the anti-HA beads. We hypothesized that given the strong interactions between protein A molecules, this may be due to the association of multi-protein A complexes after solubilization.

To address this directly we expressed HA- and YFP-tagged protein A in separate yeast cells, isolated membrane fractions, and solubilized them separately with Tx-100. We then mixed the two solublized membrane fractions together and co-immunoprecipitated protein A as before (Figure 4.2A). We found that we were still able to co-immunoprecipitate the two protein complexes after mixing individually solubilized membrane fractions isolated from different yeast, suggesting the ability of separate protein complexes to interact after solubilization. In addition, we also took advantage of the ability to re-target protein A to the endoplasmic reticulum (ER) by exchanging the amino-terminal mitochondrial targeting signal with that of the ER targeting signal of the hepatitis C virus NS5B protein (29). We hypothesized that if replication complexes could interact in solution then the solublization of membrane fractions isolated from yeast expressing both ER and mitochondrial targeted FHV replication complexes in the same cell would also interact (Figure 4.2B). We co-expressed an HA-tagged mitochondrial targeted protein A along with an YFP-tagged ER targeted protein A and investigated the ability to co-immunoprecipate these two proteins after solublization of isolated membrane fractions (Figure 4.2B). Interestingly, we found that even when FHV RNA replication complexes were targeted to the different membranes in the same cell we could still co-immunoprecipitate the protein complexes after solubilization of the membrane fractions. These data confirm that protein A complexes can associate after solublization and may be inhibiting the ability to elute the complexes after affinity chromatography. An additional explanation for these results may be that although detergent

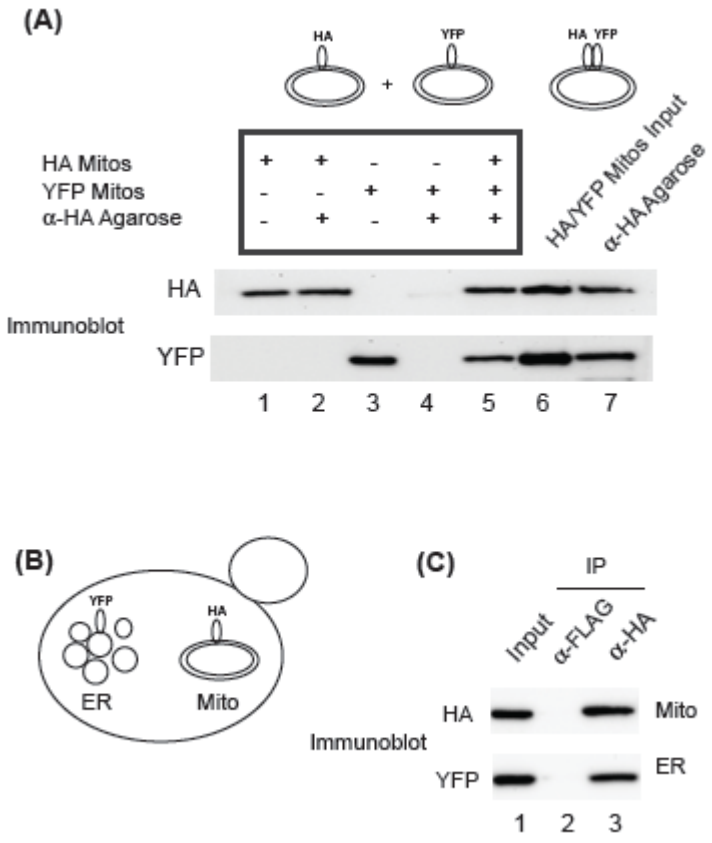

Figure 4.2: Co-immunprecipitation of individually solubilized replication complexes. (A). Individual yeast mitochondrial expressing pFA-C/HA or pFA-YFP were isolated, solubilized with 0.5% Tx-100, mixed together and co-immunoprecipitated as described previously. **(B)** Schmatic of duel membrane targeting protein A in one yeast cell. **(C).** Co-immunoprecipitation of solubilized membrane fraction containing ER and mitochondrial associated replication complexes.

treatment does not disrupt protein A-protein A interactions it does cause other conformational changes within the complex leading to the association of replication complexes in solution. To address this we chose to first chemically crosslink the membrane-associated protein complexes to fix them into their native confirmation and then assay the ability to co-immunoprecipate the differentially tagged protein A molecules. As a control for protein crosslinking we used the detergent C16TAB which we saw previously had no effect on immunprecipitation but disrupted protein A-protein A interactions. We found that after chemical crosslinking, C16TAB was unable to disrupt protein A-protein A interaction confirming that replication complexes were being crosslinked on the membrane (Figure 4.3A). We then individually crosslinked membrane fractions containing HA-tagged or YFP-tagged protein A, solublized these complexes with Tx-100, and looked for the ability of these complexes to interact by co-immunopreciptation as in Figure 4.2. We found that chemical crosslinking membrane bound protein complexes inhibited the ability to co-immunoprecipitate solublized protein complexes (Figure 4.3B) suggesting they were fixed in conformation different than that caused by detergent treatment alone. These data indicate that detergent treatment is able to induce changes in protein structure different from that of the membrane bound complex causing them to associate in solution. We then applied this crosslinking technique before affinity chromatography and found that we were now able to efficiently elute protein A complexes from the HA beads (Figure 4.3C). Silver stain analysis of protein A containing elution fractions revealed that protein A was the prominent protein

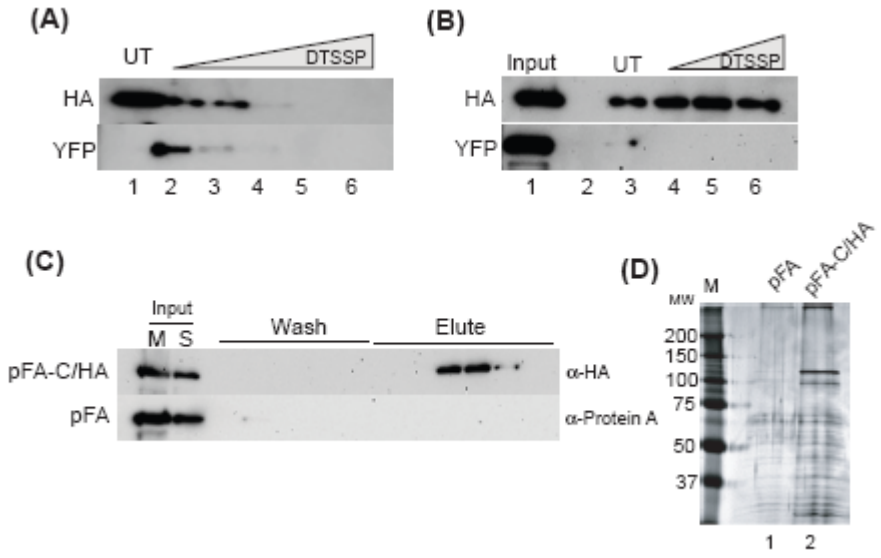

Figure 4.3: Affinity chromatography of protein A complexes. (A). Yeast mitochondria expressing HA- and YFP tagged protein A were incubated with increasing amounts of the crosslinker DTSSP, solubilized with C16TAB and co-immunoprecipitated as described previously. **(B).** individual yeast mitochondrial containing HA- or YFP-tagged protein A were crosslinked with increasing amounts of DTSSP, solubilized with Tx-100, mixed together, and co-immunoprecipitated as before. **(C).** Yeast mitochondria expressing wildtype or HA-tagged protein A were isolated by affinity chromatography with anti-HA beads, washed with RIPA buffer, and eluted with low pH (2.5). M = membrane, S = start solubilization. **(D).** Silver stain of combined elution fractions 5-7 separated on 10% SDS-PAGE gel.

species and we found a variety of protein bands that were not present in the control (Figure 4.3D). We concentrated and TCA precipitated the elution fractions, and analyzed the specific bands by mass spectroscopy. However, no host proteins were reproducibly identified using this approach.

Analysis of native FHV replication complexes. In an alternative approach we were interested in analyzing the native structure of FHV RNA replication complexes. Native yeast mitochondrial outer membrane protein complexes have been studied in great detail in part due to the development of blue-native polyacrylamide gel electrophoresis (BN-PAGE) (39, 40). This technique is a modified version of native electrophoresis which was developed to examine protein complexes in their native cellular conformation. However, native electrophoresis separates protein complexes based on a mass to charge ratio which may vary between complexes. To avoid this potential problem, BN-PAGE utilizes the negatively charged dye coomassie brilliant blue G250 which coats protein complexes and allows them to be efficiently separated by size.

In particular, BN-PAGE has been used to investigate the large protein complexes present in the mitochondria and mitochondrial membranes such as those necessary for protein import and assembly (25, 50, 58, 59). We thus chose to use this technique to investigate the relative size and structure of the FHV RNA replication complex. Membrane fractions isolated from yeast containing FHV RNA replication complexes were solubilized with digitonin, a mild detergent used to solublized mitochondrial complexes, and separated on a 4-12% bis-tris acrylamide gel. We found FHV replication complexes to be large

protein complexes in comparison to the mitochondrial outer membrane TOM complex of approximately 440 kDa (Figure 4.4A). In addition we compared membrane fractions containing FHV RNA replication complexes isolated from yeast and insect cells. We found that the replication complexes were of similar size between species and between infected cells and cells expressing an FHV replicon, suggesting that functional FHV RNA replication complexes are large protein structures (Figure 4.4B).

However, although we were able to visualize native protein A complexes by BN-PAGE, these complexes were large and only entered the upper 4% portion of the acrylamide gel after running the gel for approximately 24 hours. It is possible that although BN-PAGE is efficient for complexes which contain only protein components it is not sufficient for larger protein-RNA complexes such as viral RNA replication complexes. The FHV RNA replication complex is hypothesized to contain large RNA molecules in RNA1 and much larger in the case of double-stranded RNA1 intermediates which are formed during active FHV RNA replication. We thus developed a modified version of BN-PAGE by using agarose in place of acrylamide to increase the pore size of the gel and the ability to separate large protein-RNA complexes (15, 20). In this case, we used the detergent DDM for solublization as it found previously to not interfere with replicase function (63). Using blue-native agarose gel electrophoresis (BN-AGE) we could easy visualize large protein A complexes, and more importantly, we found that BN-AGE revealed two protein A containing complexes which contained nucleotides when visualized with ethidium bromide (Figure 4.4C).

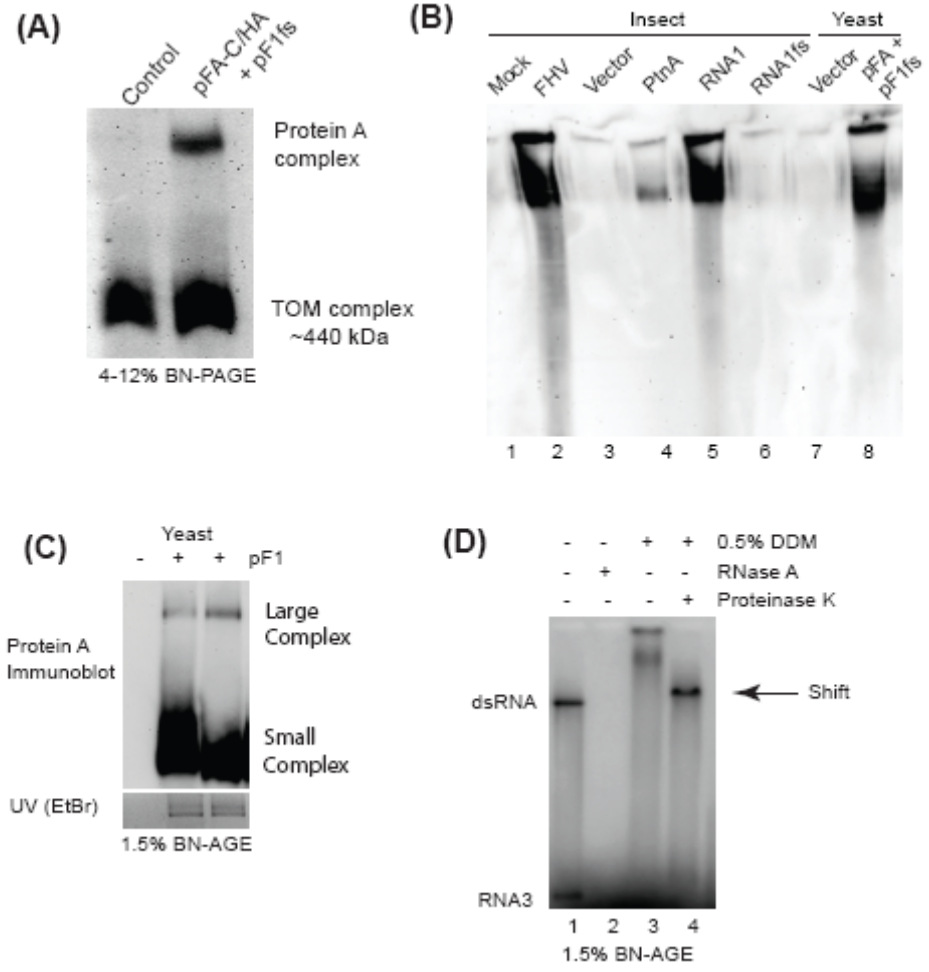

Figure 4.4: Blue-native analysis of FHV replication complexes. (A). Blue-Native analysis of isolated yeast mitochondria expressing pFA-C/HA and pF1fs. Mitochondria were solubilized in 1% digitonin and separated on a 4-12% Bis-Tris acrylamide gel. Gels were immunoblotted for HA and yeast Tom40. **(B).** Membrane fractions isolated from insect and yeast cells were solubilize and separated as described previously. **(C).** Isolated yeast mitochondria were solubilized in 0.5% dodecylmaltoside and separated on a 1.5% blue-native agarose gel. Gels were immunoblotted with antibodies to protein A. **(D).** Yeast mitochondria harboring FHV replication complexes were incubated in a RdRp assay and separated by BN-AGE. Lane 1 and 2 are phenol:cholorform extraction of RdRp assay.

We additionally addressed which of the two protein A species retained replicase function by first incubating membrane fractions in an RdRp assay to label complex associated RNAs with radiolabled nucleotides followed by BN-AGE analysis. We found there only to be one product which retained replicase activity as seen by autoradiography (Figure 4.4D, lane 3) and we confirmed this to be mediated in part by a protein component as proteinase K treatment causes a shift in the radiolabled band (Figure 4.4C, lane 4). These data indicate that BN-AGE can efficiently separate FHV replication complexes and that the FHV replication complex consists of two functionally distinct protein A containing complexes.

Lipidomic analysis of mitochondria harboring FHV RNA replication complexes. Viral infection and RNA replication are dynamic processes likely involving many cellular components and processes. A global mass spectroscopy analysis of FHV infected insect cells has identified dramatic changes in cellular proteins and metabolites (14), yet an analysis of cellular and membrane specific phospholipid changes has not been completed. Cellular phospholipids have been shown to play important structural and functional roles in positive-strand RNA virus replication (1, 48, 63). Flock House virus induces large scale mitochondrial membrane changes (Figure 1.5, Chapter I) during replication and thus we were interested in exploring the role of cellular phospholipids in these processes (28). Recently, cellular lipidomics has emerged as a new area of cellular biology to investigate cellular lipid compositions (9, 12, 52), and we used this approach to analyze the phospholipid composition of total cellular and

mitochondrial phospholipids in the presence of FHV infection and RNA replication. Initially, we extracted lipids from whole FHV infected insect cells and analyzed phospholipid composition by mass spectroscopy. We found that in both infected cells and cells expressing an FHV replicon that there was an increase in total phosphatidylcholine (PC) levels and more specifically PC molecules containing unsaturated acyl side chains (Figure 4.5A). We performed similar analyses with purified yeast mitochondria expressing the FHV replicon and obtained similar results (Figure 4.5B-D). Taken together, these data indicate that FHV induces dramatic phospholipid changes during infection and RNA replication.

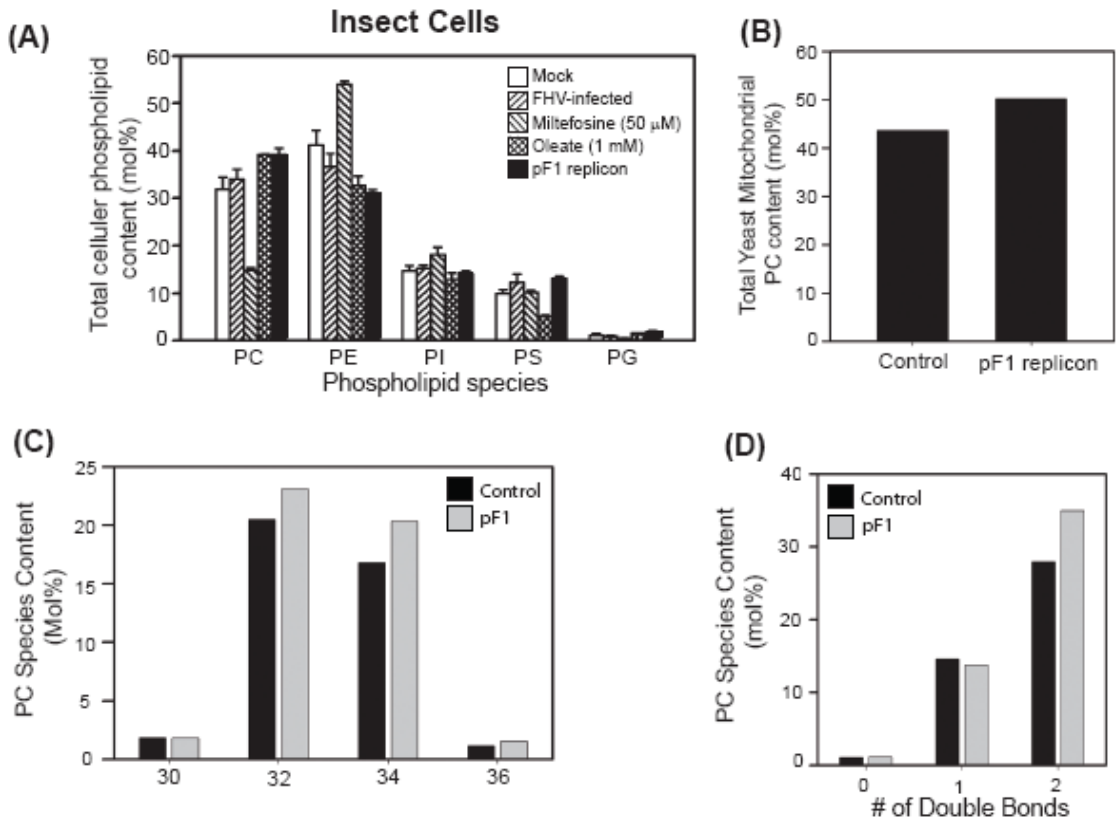

Figure 4.5: Phospholipid analysis of insect cells and yeast mitochondria during viral RNA replication. (A). Total phospholipid composition of whole insect cell extracts. **(B).** Total PC content from yeast mitochondria. **(C).** Specific PC species from yeast mitochondria. **(D).** Specific changes in unsaturated PC species.

DISCUSSION

These studies have investigated the structure and composition of Flock House virus RNA replication complexes. We have shown that treatment with specific detergents was able to disrupt replication complex protein-protein interactions whereas other detergents may not disrupt these interactions but do induce conformational changes in complex structure. These conformational changes led to the association of solublized replication complexes and the inability to elute the complexes after affinity chromatography. To avoid this, we used a chemical crosslinking approach to fix FHV replication complexes on the mitochondrial outer membrane before solubilization, and found we were now able to isolate protein A complexes from yeast mitochondria and analyze associated proteins by mass spectroscopy. However, thus far we have been unable to confidently identify any host proteins in these analyses. In addition, we have addressed native FHV replication complexes by blue-native agarose gel electrophoresis and have identified two high molecular weight and functionally distinct protein A containing complexes. Lastly, we have taken a lipidomic approach to analyze cellular and membrane-specific phospholipid changes during FHV RNA replication and FHV infection. These data provide initial insight into the biochemical characterization of the Flock House virus RNA replication complexes and establish a starting point for the future studies of replication complex structure and composition.

The observation that FHV protein-protein interactions were not disrupted by specific detergent treatment is consistent with initial studies in which the in

vitro FHV replicase activity was not disrupted by treatment with dodecylmaltoside (DDM) (63), however these studies were not followed up with other detergents. A biochemical analysis of the Tomato Mosaic virus (ToMV) 180K and 130K replicase proteins revealed that treatment with various detergents had differential effects on both in vitro replicase activity and protein structure further supporting our results with FHV (33). Detergents can have varying effects on both protein and lipid structure, and one possible explanation for these differences in replicase function could be due to the ability of detergents to remove phospholipid co-factors which are required for RNA synthesis. Previous studies exploring the native structure of the yeast TOM complex revealed that there is roughly a 40-fold difference in the concentration of TOM complex associated phospholipids simply between the detergents DDM and digitonin leading to major effects in complex function (47). Thus one may speculate that detergents can have many effects on protein associated phospholipid content and protein structure leading to variety of biochemical and functional consequences.

These data are particularly important when choosing a detergent for the study of viral RNA replication complex structure and function. We found that the commonly used detergent Triton X-100 was able to alter replication complex structure and function suggesting that this may not be the best detergent for these studies and data obtained using different detergents should be analyzed and interpreted carefully. Future studies will need to be completed using a variety of detergents in order to be able to make accurate conclusions on FHV replication complex biology

With this in mind, there are many reasons why we were unable to identify complex associated host proteins by affinity chromatography. It is possible that detergent treatment removed loosely associate proteins or altered the protein structure leading to their release. However, we attempted to get around this by first crosslinking the membrane bound complexes before solubilization. This approach was successful at inhibiting the detergent C16TAB to disrupt protein A-protein A interactions and inhibiting proteins complex to associate in solution indicating that FHV RNA replication complexes were fixed in one confirmation. Using this approach, we were able to identify several yeast host proteins in one analysis whereas a second analysis only identified protein A. One protein of interest was Isn1, the Inosine 5'-monophosphate (IMP)-specific 5'-nucleotidase which is necessary for the breakdown of IMP to inosine in the cell. Interestingly, Isn1 was identified in a proteomic screen for proteins that interact with the tomato bushy stunt virus p33 replicase protein suggesting that this protein may be involved in viral RNA replication (21). As this gene is necessary for nucleotide metabolism it may also be involved in specific metabolism of nucleotides necessary for viral RNA synthesis or related processes yet additional experiments will have to be done to confirm these interactions.

In addition, the observation that we were only able to identify protein A in one analysis brings forth the intriguing hypothesis that protein A is the sole protein component of the FHV RNA replication complex. This idea is supported by the fact that FHV can establish RNA replication in many cell types suggesting that the requirements necessary for FHV RNA replication may be minimal.

Furthermore, protein A mitochondrial membrane association has shown to be mediated by membrane phospholipids and not the core protein import machinery suggesting a simplified and common system to establish viral replication complexes (see Chapter II). Finally, although host chaperones have been implicated in FHV RNA replication (7, 18, 60) physical interactions between protein A and chaperones have not been seen yet.

An alternative approach to identifying host components is to use BN-AGE which we have developed as a technique to efficiently separate FHV replication complexes (15, 20). The observation that FHV replication complexes consist of two functionally distinct complexes is consistent with proposed theories of viral replication complex structure (19, 38). The study of viral component stoichiometry within FHV infected cells has estimated there to be roughly 100 protein A molecules per spherule yet only one negative-strand RNA1 and two negative-strand RNA2 templates (19). This suggests that within a given spherule there is only one replication complex containing a negative-strand RNA and thus capable of RNA synthesis, and would imply that the remainder of protein A molecules are used for structural or undetermined purposes in RNA synthesis. Our data indicate the presence of a large complex which contains replicase activity and a smaller complex that is not functional but contains much more protein A. It will be interesting to isolate these individual complexes and investigate their content to identify viral and host factors in native FHV replication complexes.

Finally, although the composition of the FHV replication complex may be simple, the changes we saw by lipidomic analysis indicate FHV infection and RNA replication are dynamic and complicated processes. The observation that FHV infection and RNA replication lead to increases in phosphatidycholine levels in both insect and yeast cells was particularly interesting. These data are consistent with previous in vitro studies which identified PC and other specific phospholipids as required for FHV replicase activity suggesting a functional role for PC in RNA synthesis (62). In addition, the specific increase in unsaturated PC species was consistent with in vitro finding indicating that long unsaturated acyl side chains are important for FHV RNA replication (62). However, although these analyses looked at global cellular and mitochondrial changes it will be equally important to investigate mitochondrial outer membrane specific changes as this is the proposed site of FHV RNA replication.

In summary, this report provides initial studies on FHV RNA replication complex structure and composition. In addition, it establishes a variety of biochemical techniques in which to study the biochemical and functional characteristics of viral RNA replication complexes. These data provide the first biochemical evidence of two functionally distinct FHV RNA replication complexes by BN-AGE and implicate important roles for host cellular phospholipids in replication complex assembly and function. Future studies are in progress to further characterize the FHV RNA replication complex structure and composition.

REFERENCES

1. **Ahola, T., A. Lampio, P. Auvinen, and L. Kaariainen.** 1999. Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. EMBO J **18:**3164-72.

2. **Ahting, U., C. Thun, R. Hegerl, D. Typke, F. E. Nargang, W. Neupert, and S. Nussberger.** 1999. The TOM core complex: the general protein import pore of the outer membrane of mitochondria. J Cell Biol **147:**959-68.

3. **Ahting, U., T. Waizenegger, W. Neupert, and D. Rapaport.** 2005. Signal-anchored proteins follow a unique insertion pathway into the outer membrane of mitochondria. J Biol Chem 280:48-53.

4. **Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenkovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann.** 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. J Biol Chem **283:**120-7.

5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol **37:**911-7.

6. **Burnham, A. J., L. Gong, and R. W. Hardy.** 2007. Heterogeneous nuclear ribonuclear protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA. Virology **367:**212-21.

7. **Castorena, K. M., S. A. Weeks, K. A. Stapleford, A. M. Cadwallader, and D. J. Miller.** 2007. A functional heat shock protein 90 chaperone is essential for efficient flock house virus RNA polymerase synthesis in Drosophila cells. J Virol **81:**8412-20.

8. **Denison, M. R.** 2008. Seeking membranes: positive-strand RNA virus replication complexes. PLoS Biol **6:**e270.

9. **Dennis, E. A.** 2009. Lipidomics joins the omics evolution. Proc Natl Acad Sci U S A **106:**2089-90.

10. **Dimmer, K. S., and D. Rapaport.** 2008. Proteomic view of mitochondrial function. Genome Biol **9:**209.

11. **Dye, B. T., D. J. Miller, and P. Ahlquist.** 2005. In vivo self-interaction of nodavirus RNA replicase protein a revealed by fluorescence resonance energy transfer. J Virol **79:**8909-19.

12. **Ejsing, C. S., J. L. Sampaio, V. Surendranath, E. Duchoslav, K. Ekroos, R. W. Klemm, K. Simons, and A. Shevchenko.** 2009. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc Natl Acad Sci U S A **106:**2136-41.

13. **Galiana-Arnoux, D., C. Dostert, A. Schneemann, J. A. Hoffmann, and J. L. Imler.** 2006. Essential function in vivo for Dicer-2 in host defense against RNA viruses in drosophila. Nat Immunol **7:**590-7.

14. **Go, E. P., W. R. Wikoff, Z. Shen, G. O'Maille, H. Morita, T. P. Conrads, A. Nordstrom, S. A. Trauger, W. Uritboonthai, D. A. Lucas, K. C. Chan, T. D. Veenstra, H. Lewicki, M. B. Oldstone, A. Schneemann, and G. Siuzdak.** 2006. Mass spectrometry reveals specific and global molecular transformations during viral infection. J Proteome Res **5:**2405-16.

15. **Henderson, N. S., L. G. Nijtmans, J. G. Lindsay, E. Lamantea, M. Zeviani, and I. J. Holt.** 2000. Separation of intact pyruvate dehydrogenase complex using blue native agarose gel electrophoresis. Electrophoresis **21:**2925-31.

16. **Johnson, K. L., and L. A. Ball.** 1999. Induction and maintenance of autonomous flock house virus RNA1 replication. J Virol **73:**7933-42.

17. **Johnson, K. L., and L. A. Ball.** 1997. Replication of flock house virus RNAs from primary transcripts made in cells by RNA polymerase II. J Virol **71:**3323-7.

18. **Kampmueller, K. M., and D. J. Miller.** 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. J Virol **79:**6827-37.

19. **Kopek, B. G., G. Perkins, D. J. Miller, M. H. Ellisman, and P. Ahlquist.** 2007. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. PLoS Biol **5:**e220.

20. **Lee, C. S., R. Das, and R. Reed.** 2003. Agarose gel separation/isolation of RNA-protein complexes. Curr Protoc Mol Biol **Chapter 27:**Unit 27 1.

21. **Li, Z., D. Barajas, T. Panavas, D. A. Herbst, and P. D. Nagy.** 2008. Cdc34p ubiquitin-conjugating enzyme is a component of the tombusvirus replicase complex and ubiquitinates p33 replication protein. J Virol **82:**6911-26.

22. **Lu, R., M. Maduro, F. Li, H. W. Li, G. Broitman-Maduro, W. X. Li, and S. W. Ding.** 2005. Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature **436:**1040-3.

23. **Lu, R., E. Yigit, W. X. Li, and S. W. Ding.** 2009. An RIG-I-Like RNA helicase mediates antiviral RNAi downstream of viral siRNA biogenesis in Caenorhabditis elegans. PLoS Pathog **5:**e1000286.

24. **Meineke, B., G. Engl, C. Kemper, A. Vasiljev-Neumeyer, H. Paulitschke, and D. Rapaport.** 2008. The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. FEBS Lett **582:**855-60.

25. **Meisinger, C., S. Pfannschmidt, M. Rissler, D. Milenkovic, T. Becker, D. Stojanovski, M. J. Youngman, R. E. Jensen, A. Chacinska, B. Guiard, N. Pfanner, and N. Wiedemann.** 2007. The morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria. EMBO J **26:**2229-39.

26. **Meisinger, C., T. Sommer, and N. Pfanner.** 2000. Purification of Saccharomcyes cerevisiae mitochondria devoid of microsomal and cytosolic contaminations. Anal Biochem **287:**339-42.

27. **Miller, D. J., and P. Ahlquist.** 2002. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J Virol **76:**9856-67.

28. **Miller, D. J., M. D. Schwartz, and P. Ahlquist.** 2001. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J Virol **75:**11664-76.

29. **Miller, D. J., M. D. Schwartz, B. T. Dye, and P. Ahlquist.** 2003. Engineered retargeting of viral RNA replication complexes to an alternative intracellular membrane. J Virol **77:**12193-202.

30. **Miller, S., and J. Krijnse-Locker.** 2008. Modification of intracellular membrane structures for virus replication. Nat Rev Microbiol **6:**363-74.

31. **Model, K., T. Prinz, T. Ruiz, M. Radermacher, T. Krimmer, W. Kuhlbrandt, N. Pfanner, and C. Meisinger.** 2002. Protein translocase of the outer mitochondrial membrane: role of import receptors in the structural organization of the TOM complex. J Mol Biol **316:**657-66.

32. **Nijtmans, L. G., N. S. Henderson, and I. J. Holt.** 2002. Blue Native electrophoresis to study mitochondrial and other protein complexes. Methods **26:**327-34.

33. **Nishikiori, M., K. Dohi, M. Mori, T. Meshi, S. Naito, and M. Ishikawa.** 2006. Membrane-bound tomato mosaic virus replication proteins participate in RNA synthesis and are associated with host proteins in a pattern distinct from those that are not membrane bound. J Virol **80:**8459-68.

34. **Noueiry, A. O., and P. Ahlquist.** 2003. Brome mosaic virus RNA replication: revealing the role of the host in RNA virus replication. Annu Rev Phytopathol **41:**77-98.

35. **Novoa, R. R., G. Calderita, R. Arranz, J. Fontana, H. Granzow, and C. Risco.** 2005. Virus factories: associations of cell organelles for viral replication and morphogenesis. Biol Cell **97:**147-72.

36. **Price, B. D., M. Roeder, and P. Ahlquist.** 2000. DNA-Directed expression of functional flock house virus RNA1 derivatives in Saccharomyces cerevisiae, heterologous gene expression, and selective effects on subgenomic mRNA synthesis. J Virol **74:**11724-33.

37. **Price, B. D., R. R. Rueckert, and P. Ahlquist.** 1996. Complete replication of an animal virus and maintenance of expression vectors derived from it in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **93:**9465-70.

38. **Quinkert, D., R. Bartenschlager, and V. Lohmann.** 2005. Quantitative analysis of the hepatitis C virus replication complex. J Virol **79:**13594-605.

39. **Reisinger, V., and L. A. Eichacker.** 2006. Analysis of membrane protein complexes by blue native PAGE. Proteomics **6 Suppl 2:**6-15.

40. **Reisinger, V., and L. A. Eichacker.** 2008. Solubilization of membrane protein complexes for blue native PAGE. J Proteomics **71:**277-83.

41. **Schmitt, S., H. Prokisch, T. Schlunck, D. G. Camp, 2nd, U. Ahting, T. Waizenegger, C. Scharfe, T. Meitinger, A. Imhof, W. Neupert, P. J. Oefner, and D. Rapaport.** 2006. Proteome analysis of mitochondrial outer membrane from Neurospora crassa. Proteomics **6:**72-80.

42. **Schneemann, A., and D. Marshall.** 1998. Specific encapsidation of nodavirus RNAs is mediated through the C terminus of capsid precursor protein alpha. J Virol **72:**8738-46.

43. **Schneemann, A., W. Zhong, T. M. Gallagher, and R. R. Rueckert.** 1992. Maturation cleavage required for infectivity of a nodavirus. J Virol **66:**6728-34.

44. **Serva, S., and P. D. Nagy.** 2006. Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. J Virol **80:**2162-9.

45. **Sickmann, A., J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H. E. Meyer, B. Schonfisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, and C. Meisinger.** 2003. The proteome of Saccharomyces cerevisiae mitochondria. Proc Natl Acad Sci U S A **100:**13207-12.

46. **Singh, G., S. Popli, Y. Hari, P. Malhotra, S. Mukherjee, and R. K. Bhatnagar.** 2009. Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. FASEB J.

47. **Stan, T., U. Ahting, M. Dembowski, K. P. Kunkele, S. Nussberger, W. Neupert, and D. Rapaport.** 2000. Recognition of preproteins by the isolated TOM complex of mitochondria. EMBO J **19:**4895-902.

48. **Stapleford, K. A., D. Rapaport, and D. J. Miller.** 2009. Mitochondrial-Enriched Anionic Phospholipids Facilitate Flock House Virus Rna Polymerase Membrane Association. J Virol.

49. **Tan, Y. J., S. G. Lim, and W. Hong.** 2005. Characterization of viral proteins encoded by the SARS-coronavirus genome. Antiviral Res **65:**69-78.

50. **van der Laan, M., M. Meinecke, J. Dudek, D. P. Hutu, M. Lind, I. Perschil, B. Guiard, R. Wagner, N. Pfanner, and P. Rehling.** 2007. Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. Nat Cell Biol **9:**1152-9.

51. **van Hemert, M. J., S. H. van den Worm, K. Knoops, A. M. Mommaas, A. E. Gorbalenya, and E. J. Snijder.** 2008. SARS-coronavirus replication/transcription complexes are membrane-protected and need a host factor for activity in vitro. PLoS Pathog **4:**e1000054.

52. **van Meer, G.** 2005. Cellular lipidomics. EMBO J **24:**3159-65.

53. **Van Wynsberghe, P. M., and P. Ahlquist.** 2009. 5' cis elements direct nodavirus RNA1 recruitment to mitochondrial sites of replication complex formation. J Virol.

54. **Van Wynsberghe, P. M., H. R. Chen, and P. Ahlquist.** 2007. Nodavirus RNA replication protein a induces membrane association of genomic RNA. J Virol **81:**4633-44.

55. **Venter, P. A., N. K. Krishna, and A. Schneemann.** 2005. Capsid protein synthesis from replicating RNA directs specific packaging of the genome of a multipartite, positive-strand RNA virus. J Virol **79:**6239-48.

56. **Venter, P. A., D. Marshall, and A. Schneemann.** 2009. Dual roles for an arginine-rich motif in specific genome recognition and localization of viral coat protein to RNA replication sites in flock house virus-infected cells. J Virol **83:**2872-82.

57. **Venter, P. A., and A. Schneemann.** 2008. Recent insights into the biology and biomedical applications of Flock House virus. Cell Mol Life Sci **65:**2675-87.

58. **Waizenegger, T., and D. Rapaport.** 2007. Analyzing import intermediates of mitochondrial proteins by blue native gel electrophoresis. Methods Mol Biol **372:**287-95.

59. **Waizenegger, T., S. Schmitt, J. Zivkovic, W. Neupert, and D. Rapaport.** 2005. Mim1, a protein required for the assembly of the TOM complex of mitochondria. EMBO Rep **6:**57-62.

60. **Weeks, S. A., and D. J. Miller.** 2008. The heat shock protein 70 cochaperone YDJ1 is required for efficient membrane-specific flock house virus RNA replication complex assembly and function in Saccharomyces cerevisiae. J Virol **82:**2004-12.

61. **Wittig, I., and H. Schagger.** 2008. Features and applications of blue-native and clear-native electrophoresis. Proteomics **8:**3974-90.

62. **Wu, S. X., P. Ahlquist, and P. Kaesberg.** 1992. Active complete in vitro replication of nodavirus RNA requires glycerophospholipid. Proc Natl Acad Sci U S A **89:**11136-40.

63. **Wu, S. X., and P. Kaesberg.** 1991. Synthesis of template-sense, single-strand Flockhouse virus RNA in a cell-free replication system. Virology **183:**392-6.

64. **Zahedi, R. P., A. Sickmann, A. M. Boehm, C. Winkler, N. Zufall, B. Schonfisch, B. Guiard, N. Pfanner, and C. Meisinger.** 2006. Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. Mol Biol Cell **17:**1436-50.

CHAPTER V

DISCUSSION

Positive-strand RNA viruses comprise a long list of viral pathogens of which the molecular mechanisms required for viral RNA replication and pathogenesis are poorly understood. One characteristic of all positive-strand RNA viruses is the association of viral RNA replication with host intracellular membranes, an essential yet unclear process in the viral life cycle. To study these necessary virus-membrane interactions we used the versatile model system Flock House virus which establishes functional RNA replication complexes on the mitochondrial outer membrane (18, 19). Given this, we hypothesized that mitochondrial-specific host components and processes are required to facilitate the assembly of the FHV RNA replication complex. To address this hypothesis, we employed complementary in vitro, genetic, and biochemical approaches using the well studied host organism *Saccharomyces cerevisiae*. This thesis explores the dynamic processes and host-pathogen interactions involved in positive-strand RNA virus replication complex assembly, and provides novel insight into the molecular mechanisms involved in viral and cellular biology.

To begin, we took an in vitro approach to study the initial steps of replication complex assembly and protein A mitochondrial membrane association and insertion (Chapter II). We hypothesized, based on similarities of protein A with other mitochondrial outer membrane proteins, that FHV utilizes the yeast TOM complex for the insertion of protein A into the mitochondrial outer membrane. To investigate these initial steps, we established an in vitro protein A mitochondrial association system similar to those used for the study of yeast mitochondrial protein import (1, 33). We efficiently translated protein A in a rabbit reticulocyte lysate and incubated in vitro translation mixtures with mitochondrial fractions isolated from various organisms capable of supporting FHV RNA replication. We found protein A to efficiently associate with each mitochondrial fraction as well as exhibit many of the in vivo biochemical characteristics of protein A biology such as mitochondrial specificity and a resistance to alkaline extraction. Using this system, we determined that protein A was inserted into the mitochondrial outer membrane independent of protease-sensitive outer membrane components and the yeast TOM complex. Furthermore, we found protein A membrane association to be temperature-dependent and identified protein A as a lipid-binding protein with specificity for mitochondrial-enriched anionic phospholipids. These data are consistent with the import of yeast signal- and tail-anchored mitochondrial outer membrane proteins which use a TOM complex independent mechanism for mitochondrial insertion (11, 17). However, these data provide novel insight into the role of membrane phospholipids in mitochondrial protein import and positive-strand RNA virus replication complex targeting.

Secondly, we took a targeted genomic approach to investigate the role of host membrane-specific components in FHV RNA replication (Chapter III). We analyzed FHV RNA replication using an established replicon system in conjugation with yeast harboring deletions or mutations in individual mitochondrial outer membrane components. Of the deletions studied, we found that a majority of outer membrane proteins had no effect on FHV RNA replication whereas several strains led to significant increases or decreases in FHV RNA accumulation. A deletion of the mitochondrial outer membrane protein Mim1 led to the greatest defect in FHV RNA accumulation, and given the role of Mim1 in protein import and protein complex assembly we chose to further explore the role of this deletion in FHV RNA replication (3, 10, 37).

To explore the role of Mim1 in FHV RNA accumulation, we attempted to rescue the FHV phenotype with the exogenous expression of Mim1 but have thus far been unsuccessful. There are several possible explanations for this phenotype which include; potential secondary, non-specific effects introduced during the generation of the *Δmim1* yeast strain, as well as expression level effects as the exogenous expression of Mim1p yielded levels much lower then wildtype yeast. We are currently addressing these hypotheses to further understand the role of Mim1p in FHV RNA replication. However, we did find that FHV RNA accumulation was independent of polymerase translation and membrane association suggesting a post-translation defect in *Δmim1* yeast. Further biochemical analysis of membrane-associated FHV RNA replication complexes revealed that Mim1 deficient yeast were defective in the assembly of

a large functional FHV RNA replication complex which led to a decrease in RNA synthesis, FHV replicase activity, and membrane-associated viral RNA levels. The observation that polymerase translation did not correlate with FHV RNA replication in *Δmim1* yeast was interesting and thus we attempted to recapitulate this process in wildtype yeast by introducing an active site mutation into the wildtype FHV replicon. We hypothesized this mutation would disrupt viral RNA replication and as a consequence decrease polymerase translation. However, there was no effect on protein A translation yet FHV RNA replication was severely impaired indicating that polymerase translation is independent of viral RNA replication. Furthermore, BN-AGE studies revealed that active FHV RNA synthesis is required for the formation of viral replication complexes. These data are consistent with reports regarding the coupled processes of positive-strand RNA virus translation and RNA replication and provide the first biochemical evidence of two functionally distinct FHV RNA replication complexes (2, 35). Moreover, these studies with *Δmim1* yeast and the replication incompetent replicon have now allowed us to begin to understand the mechanisms involved in FHV replication complex assembly. Taken together, we hypothesize that FHV replication complexes are assembled in a stepwise fashion that involves both host cell processes and active viral RNA synthesis, allowing us to create a model of the at least three different states of FHV replication complex assembly (Figure 5.1).

Finally, we performed biochemical studies to investigate the structure and composition of FHV RNA replication complexes (Chapter IV). To investigate

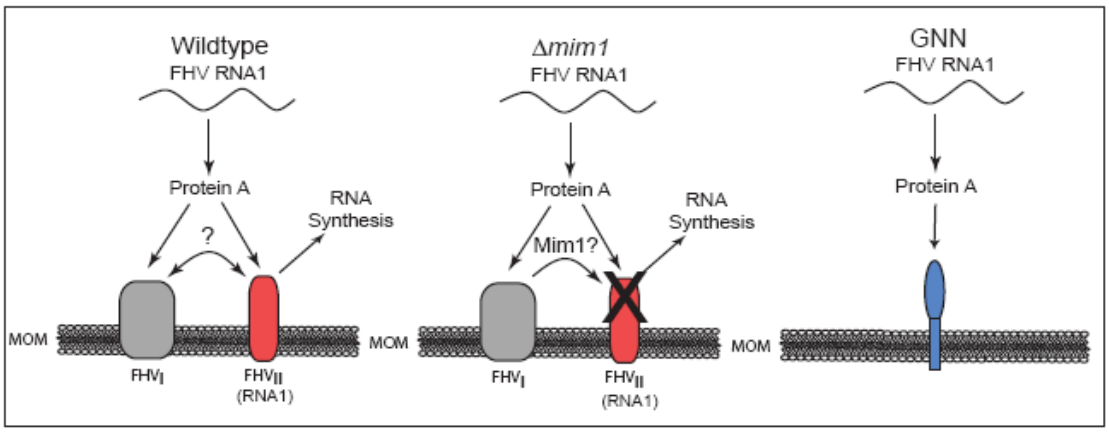

Figure 5.1: Schematic of FHV RNA replication complex states. We have identified three potential replication complex scenarios in these studies. In the first (Wildtype) protein A is assembled into two functionally distinct replication complexes (FHV_I and FHV_{II}) of which FHV_{II} is capable of robust RNA synthesis. In the second (*Δmim1*) protein A is assembled only into FHV_I leading to a defect in RNA synthesis, suggesting a role for Mim1 in this process. In the third (GNN), protein A is translated but neither replication complex is formed indicating a role for RNA synthesis in replication complex assembly.

replication complex associated host components, we established a protein A affinity chromatography system which takes advantage of a crosslinking approach to fix membrane bound protein A complexes before solubilization. We were able to efficiently isolate protein A complexes, yet we were unable to reproducibly identify any host proteins. Furthermore, during these studies we found that various detergents had differential effects on FHV RNA replication complex protein interactions and protein structure providing initial insight into the use of detergents to study FHV biology. In an alternative approach, we employed the use of blue-native gel electrophoresis to explore the native size and structure of FHV RNA replication complexes. In initial studies we primarily used the common technique of blue-native polyacrylamide gel electrophoresis (BN-PAGE) but found that we were not able to efficiently resolve the FHV protein-RNA complexes (36, 38). Given this, we developed a blue-native agarose gel electrophoresis (BN-AGE) system with which were able to efficiently separate large FHV protein-RNA complexes (9, 15). This technique allowed for the visualization of two functionally distinct FHV replication complexes and provides a valuable tool to study positive-strand RNA virus replication complex assembly in the future. Finally, we took a lipidomic approach to investigate the cellular and membrane-specific phospholipid changes involved in FHV RNA replication. We found increases in cellular phosphotidylcholine levels during FHV infection and RNA replication in insect cells as well as similar changes in mitochondrial phospholipid content in yeast.

In summary, we have taken complementary approaches to investigate the polymerase-membrane interactions in FHV RNA replication complex assembly and function. We have established an in vitro membrane association assay which has identified mitochondrial anionic phospholipids as potential host determinants for the initial steps in FHV replication complex assembly. In addition, we have investigated the role of a majority of the yeast mitochondrial outer membrane proteins in FHV RNA replication, and explored the role of Mim1 in detail. Finally, we have used biochemical approaches to investigate the FHV RNA replication complex structure and composition as well as to define a variety of techniques to be used in future experiments. Taken together, these data provide insight into the molecular mechanisms involved in positive-strand RNA virus replication complex membrane targeting and association, coupling of RNA translation and replication, and replication complex structure, allowing us to build a stepwise model for FHV replication complex assembly (Figure 5.2).

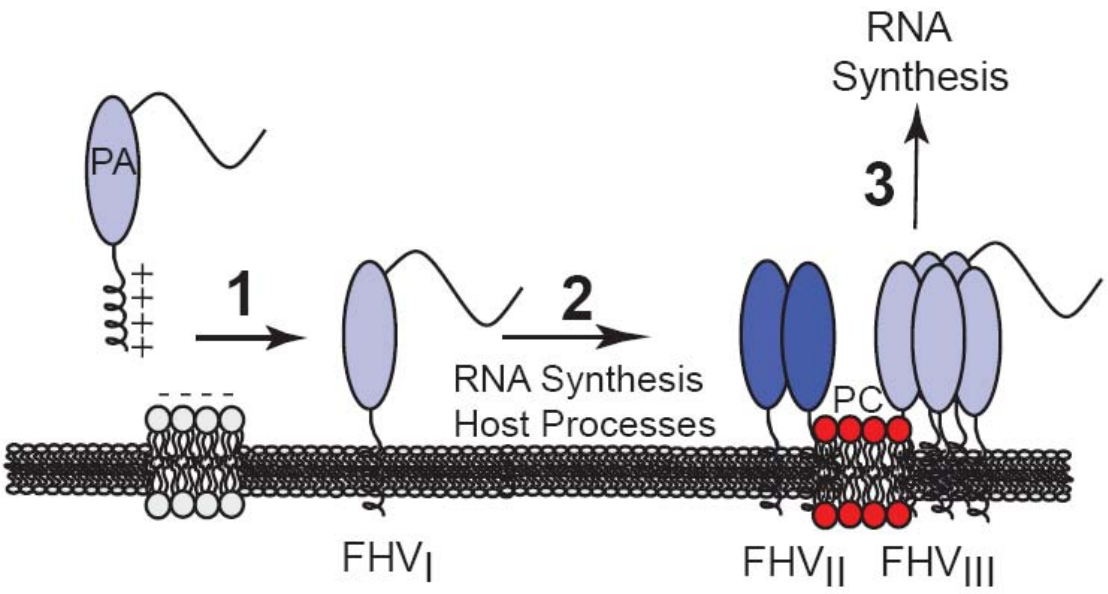

Figure 5.2: Proposed Model for Stepwise FHV RNA replication complex assembly. **(1)** Protein A associates with mitochondrial-enriched anionic phospholipids (Chapter I) for membrane binding and insertion. **(2)** Once inserted into the mitochondrial outer membrane, protein A utilizes host components and viral RNA synthesis to assemble two functionally distinct FHV RNA replication complexes (Chapter III and IV). **(3)** FHV RNA replication complex assembly and RNA synthesis induce increases in phosphatidylcholine levels (PC) allowing for robust genome replication (Chapter III and IV).

Unanswered Questions and Future Studies

These studies give rise to many unanswered questions including:

1. What are the protein A structural determinants that facilitate membrane association and insertion?
2. What are the biophysical characteristics of protein A membrane association?
3. What role does Mim1 and other mitochondrial membrane components play in FHV replication complex assembly and function?
4. What is the composition and structure of functional FHV replication complexes?
5. What are the dynamic processes involved in the assembly of FHV RNA replication complexes?

We will address these questions as well as future directions in the following pages.

What are the protein A structural determinants that facilitate protein A membrane association and insertion?

This question was originally addressed in yeast by expressing a variety of protein A deletion constructs and analyzing protein A membrane association (18). Interestingly, it was found that many of these deletions, including deletions of the mitochondrial-targeting signal, still remained membrane-associated possibly due to the presence of multiple membrane binding domains within protein A. However, these studies looked at protein A-membrane interactions 24 hours after protein A induction, suggesting that a variety of confounding effects influencing membrane association could take place during this long time period. In addition, these studies did not address the specific domains responsible for the initial steps in protein A membrane association directly after translation. These experiments would be difficult in vivo as the identification of a cytosolic pool of protein A has not been seen in insect or yeast cells suggesting that protein A associates rapidly with the mitochondrial membrane in vivo (5).

To investigate these initial steps in replication complex assembly we will use the in vitro FHV membrane association system described in Chapter II. One advantage of this system is the ability to easily manipulate the system to address various aspects of protein A and membrane biology. I have created in vitro protein A deletion constructs which we will use to directly study the role of protein A domains on the initial steps of membrane-association. Furthermore, as protein A is a lipid-binding protein it will be interesting to see what domains are required for the association with specific mitochondrial-enriched anionic phospholipids.

One may speculate that protein A contains multiple domains which are required for the specific interactions with membrane components either protein or lipid, and studies using this in vitro system will allow us to identify these domains.

What are the biophysical characteristics of protein A membrane association?

The observation that protein A membrane-association is temperature-dependent was unexpected as we hypothesized protein A to behave similarly to the yeast mitochondrial outer membrane proteins Tom20 and porin (13). One possible explanation for this phenotype is that protein A membrane-association is energy dependent and thus inhibited at low temperatures. However, we found that exogenous ATP was despensible for protein A membrane-association and preliminary studies enzymatically depleting rabbit reticulocyte lysate ATP levels led to no changes in protein A membrane association (data not shown). These data suggest an alternative mechanism for the temperature-dependence of protein A membrane-association.

However, before we can address these mechanisms we first need to address the temperature-dependence of protein A binding to purified liposomes. These experiments are important to determine if the temperature-sensitive phenotype is specific to mitochondria or all lipid vesicles. One could speculate that the mitochondria contains a component that undergoes a conformational change at low temperature leading to low protein A binding, and experiments using liposomes would address this possibility. Nonetheless, the data we observed

with mitochondria brings up two potential hypotheses for this phenotype; 1) the cold temperature induces structural changes in protein A or membrane proteins inhibiting the ability to bind mitochondria, or 2) protein A membrane-association is dependent on membrane fluidity which is decreased at low temperatures.

To address the first hypothesis, we will take advantage of the multiple cysteine residues located throughout protein A suggesting that disulfide bonds are required for protein folding. We will incubate in vitro translated protein A with increasing concentrations of the reducing agent DTT to disrupt intramolecular disulfide bonds to change protein structure, and address membrane-association at low temperature. We hypothesize that if protein A structure is the key determinant for membrane binding that DTT treatment will lead to changes in membrane association. It is of course possible that the disruption of disulfide bonds will have no effect on in vitro membrane association, and as a complementary approach we will repeat this experiments with increasing amounts of urea, a denaturing agent that has been used for the study of yeast mitochondrial protein import (37).

To address the second hypothesis we will first address the ability of protein A to bind mitochondrial fractions at increasing temperatures. If membrane fluidity plays a role in protein A membrane association we would expect that increasing the temperature would lead to increased membrane binding of protein A. One potential downfall of this approach is that increasing temperature may also affect protein A structure leading to difficult to interrupt results. To address membrane fluidity directly we will introduce purified ergosterol, a molecule similar to

cholesterol, into mitochondria and liposomes to decrease membrane fluidity and look at protein A membrane-association at 25°C. It has been shown that the introduction of ergosterol into purified liposomes is able to decrease the membrane insertion of the yeast mitochondrial outer membrane protein Fis1 suggesting that membrane fluidity may play important roles in protein import (11). The idea of membrane fluidity in protein A mitochondrial import is intriguing as we have shown cellular and mitochondrial-specific phospholipids to be involved in a variety of processes in the FHV RNA replication including membrane binding and RNA synthesis. However, we have not ruled out the possibility that protease-resistant mitochondrial protein components are involved in protein A membrane-association. Taken together, future studies using this in vitro membrane association system will provide further insight into the host components and biophysical mechanisms required for protein A membrane-association.

What role does Mim1 and other mitochondrial membrane components play in FHV replication complex assembly and function?

The Δ*mim1* yeast strain has provided valuable insight into various processes in FHV RNA replication complex assembly and function but still the specific role of Mim1 remains unclear. It is possible that Mim1 is directly responsible for the FHV assembly defects but until we are able to exogenously complement this system with Mim1 we cannot make this conclusion. We have attempted to complement this system using two different expression systems but have thus far

obtained inconclusive results. Of particular interest are the results obtained using the Mim1 overexpression system, in which the control 2-micron plasmid containing a *URA3* marker was able to complement FHV RNA replication in *Δmim1* yeast. In an alternative approach we are generating a similar Mim1 overexpression plasmid using a 2-micro plasmid with a *LEU2* marker instead of the *URA3* in an attempt to exogenously complement the FHV defect.

However, we cannot completely ignore the ability of *URA3* to complement FHV RNA replication in Mim1 deficient yeast. One possible explanation for these results is that FHV RNA replication is dependent on the de novo synthesis of pyrimidines as opposed to salvaging these metabolites from the media. To address this in wildtype yeast we explored the effect of adding increasing amounts of exogenous uracil into the media during FHV RNA replication. Surprisingly, FHV RNA synthesis was enhanced with increasing amounts of uracil suggesting that FHV RNA synthesis is not dependent on de novo synthesis of these metabolites. Given the fact that FHV is an RNA virus it is not unreasonable that pyrimidine biosynthesis is important for genomic synthesis. One may hypothesize that having the *URA3* gene or excess uracil present during viral replication increases the cellular uracil concentration which leads to an increase in FHV RNA replication. This is an interesting idea but may be unlikely as UTP functions as a feedback inhibitor of pyrimidine biosynthesis shutting down this pathway in the presence of extra UTP (27), however we can not completely rule out this hypothesis just yet.

An additional explanation for the ability to complement viral replication with *URA3* or UTP may involve CTP, the product of UTP amination and end product of pyrimidine biosynthesis. UTP and more importantly CTP is not only required for the synthesis of nucleic acids but it also plays major roles in cellular phospholipid biosynthesis (Figure 5.3)(4). *S. cerevisiae* has been used extensively to study phospholipid biosynthesis and in particular the specific role of CTP in these processes (4, 16, 22). It has been shown that increases in cellular CTP concentrations correlate with increases in cellular phosphatidylcholine levels in vivo (16). These data are particularly interesting given the preliminary data analyzing the mitochondrial phospholipid composition of wildtype and *Δmim1* yeast (Chapter III).

Taken together we hypothesize that Mim1 plays an important role in maintaining cellular phospholipid levels and a deletion of this protein leads to decreases in phosphatidylcholine levels and FHV RNA replication. The presence of *URA3* gene in *Δmim1* yeast is able to rescue the defect in phosphatidylcholine levels allowing for a complementation of FHV RNA replication. To address this hypothesis we will need to confirm the role of Mim1 in phospholipid biosynthesis. We will repeat the mass spectroscopy analysis of *Δmim1* yeast mitochondria as well as biochemically altering the intracellular phospholipid composition of *Δmim1* yeast to rescue FHV RNA replication. In particular, we will grow yeast in the absence of inositol, a potent regulator of phospholipid biosynthesis as well as in the presence of choline which can be used for phosphatidylcholine synthesis (4). The growth of wildtype yeast in the presence of inositol has been shown to

increase phosphatidylinositol levels leading to a corresponding decrease in phosphatidylcholine levels (Figure 5.3A) (4). In addition, the supplementation of the media with exogenous choline provides an alternative mechanism to increase phosphatidylcholine levels through the Kennedy pathway (Figure 5.3B). We expect that growth of *Δmim1* yeast in these conditions will increase phosphatidylcholine levels leading to an increase in FHV RNA synthesis. Furthermore given the FHV replication in wildtype yeast induces changes in phospholipids, it will be interesting to investigate the overall role of phospholipid biosynthesis on FHV RNA replication by similar methods as described above.

Although *Δmim1* yeast provide an interesting system to study viral RNA replication it was not the only membrane-specific deletion strain that lead to changes in FHV RNA replication. We observed that two temperature sensitive mutants of Tom40, the major import pore, led to significant increases in RNA synthesis where as deletions of *TOM6*, *DNM1*, and *POR1* reduced RNA replication. Of particular interest are the opposing effects of TOM complex components on FHV RNA replication. It is possible that deletions in these proteins lead to protein import defects that positively and negatively influence FHV RNA synthesis. In addition, a deletion of Dnm1, the major component of the mitochondrial fission machinery, leads to constitutive mitochondrial fusion and severe mitochondrial morphology defects possibly influencing FHV replication as well (20). Lastly, Por1 is the major voltage-dependent anion channel (VDAC)

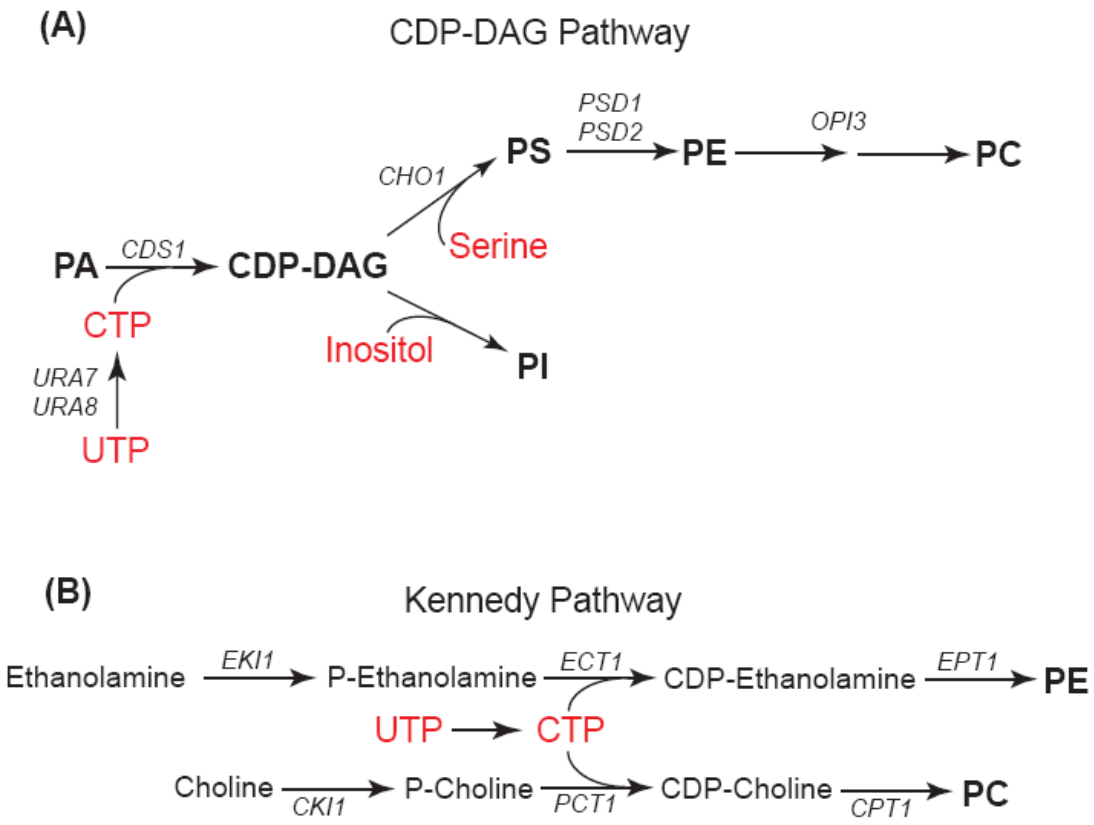

Figure 5.3: Schematic of the yeast phospholipid biosynthetic pathways. **(A)** CDP-DAG pathway for the de novo synthesis of glycerophospholipids. **(B)** Kennedy pathway (salvage pathway) for phospholipid biosynthesis.

and most abundant protein in the mitochondria. In addition, porin has been implicated in apoptosis apart from its normal cellular function in channeling metabolites (39, 41). To facilitate these functions porin has been localized to mitochondrial contact sites, a hypothesized site for protein A-membrane interactions (8, 25). One may speculate that given the abundance of porin in the mitochondria, it is an important determinant in contact site structure and function, and a deletion of porin disrupts these sites leading to defects in FHV replication complex assembly.

Nonetheless it should be noted that one reoccuring theme between all of these deletion strains including Mim1 are their effects on mitochondrial morphology and membrane structure. This idea is intriguing given that FHV induces dramatic changes in membrane morphology, and it is possible that deletions or mutations in certain host proteins governing membrane morphology will have effects on FHV replication. Furthermore, defects in mitochondrial morphology may lead to corresponding defects in phospholipid composition yet little work has been done thus far to link these processes. In the future it will be necessary to address the role of these mitochondrial components in viral RNA replication to better understand the role of host components in these processes.

What is the composition and structure of functional FHV replication complexes?

The identification of two functionally distinct replication complexes begs the questions as to what are their molecular compositions? We have attempted to

explore the composition of FHV replication complexes by affinity chromatography and mass spectroscopy yet we were unable to identify any host components with this technique. However, we have now developed an electrophoresis technique in which to efficiently separate these complexes. BN-AGE has the advantage of removing smaller contaminating proteins to clean up the protein analysis, and in the future we will isolate these complexes by cutting the proteins out of the gel and separating the components by SDS-PAGE or by isolating the native complexes by preparative electrophoresis. Nonetheless, our "unsuccessful" attempts at identifying host components by affinity chromatography provide an interesting suggestion that protein A may be in the only viral component of FHV replication complexes. One may hypothesis that genetically simple viruses, in the case of FHV which encodes only one nonstructural protein, would need more help from the host to facilitate RNA synthesis then a more complex virus such as HCV, which encodes 6 nonstructural proteins. However in fact, the opposite may be true in that genetically simple viruses may need very little from the host, and work with FHV suggests this may be the case. It should also be noted that the complexes we have identified are only composed of the viral components protein A and RNA1.

In a natural infection RNA2 and capsid protein would be present within the cell thus adding to the system. It will be interesting to investigate membrane-associated replication complexes isolated from infected cells as well as introducing RNA2 into the yeast replication system. These future studies will

give a detailed analysis of the FHV RNA replication complex and identify the host components present in these complexes if any.

Flock House virus protein A has been biochemically defined as a signal-anchored, integral membrane protein with its amino-terminus imbedded in the lipid bilayer and carboxy-terminus exposed to the cytosol (Figure 1.6)(18). The results that lead to this predicted protein topology were base on immunofluorescence studies using epitope tagged protein A molecules in combination with a commonly used differential detergent treatment using saponin and TX-100. In this thesis we have shown that treatment of membrane fractions with TX-100 led to protein structural and functional changes and it is possible that similar events occurred during protein topology studies. One possible explanation for the results that were obtained previously is that the epitope tag which was hypothesized to be protected by the mitochondrial outer membrane was instead protected by the protein complex structure. Treatment of these structures with TX-100 induced a conformational change in protein structure and exposed the hidden epitope and immunofluorescent signal. To directly address the topology of signal- or tail-anchored proteins we can take advantage of the fact that a small portion of protein A which contains the amino-terminus and transmembrane domain is embedded in the bilayer and resistant to proteinase degradation (1). We can then treat membrane fractions with protease and separate small protein fragments by urea-acrylamide gel electrphoresis. However, when we used this approach to look at protein A topology we were unable to find small membrane protected fragments, indicating that the amino-

terminus may be exposed to the cytosol and degraded by the protease. Furthermore, both in vivo and in vitro membrane association studies using protein A deletions revealed that large deletions of protein A, which included the amino-terminal transmembrane domain still remained membrane associated. These data suggest that there are multiple membrane interacting regions of protein A which have not been well characterized and future detailed biochemical studies will provide insight into these domains.

In addition to hydrophobic membrane binding domains protein A also contains a variety of highly conserved amino acid sequences (Figure 5.4). To begin, protein A contains a common A/GxxxA/G motif in the amino-terminal transmembrane domain. This structural motif has been shown to mediate transmembrane domain interactions in such proteins as Mim1 and other yeast proteins (24, 28, 31). The idea of transmembrane domain interactions is supported by FRET studies which indicated that deletion of the transmembrane domain inhibited protein A self-association (7). In addition, it is possible that this motif mediates interactions with host proteins as well.

Secondly, protein A contains three highly conserved carrier protein domains with the consenses sequence Px(D/E)xxR/K (14, 26). These domains have been found in metabolite carrier proteins such as the ATP/ADP carrier protein and phosphate carrier protein and are essential for their function (21, 40). In addition, a single carrier domain has been found in the mitochondrial fusion protein Ugo1 which is also required for membrane fusion (6). The presence of

Figure 5.4: Conserved amino acid sequences in FHV protein A

these three conserved domains is very interesting, and based on the known topology of carrier proteins it may give insight to the topology of protein A. In addition, we are curious as to function of these carrier domains in FHV RNA replication. Given that protein A is the only viral protein necessary for FHV RNA synthesis it makes sense that the polymerase contains many functional domains to facilitate this process. It is possible that during RNA replication protein A acts to move metabolites such as nucleotides into and out of the viral replication factories aiding in viral replication, and the fact that one of the carrier domains is located just downstream of the catalytic resides may suggest such a mechanism.

In addition, protein A contains the conserved caspase-8 cleavage site, IETD (34). This sequence is contained within the conserved replicase domains found in a variety of RNA-dependent RNA polymerases, and the aspartic acid provides a constant residue when lined up with the sequence of other viral RdRps (23). Furthermore, the other caspase cleavage sequences LETD and DEVD have been found in a variety of positive-strand RNA viruses including members of the alphavirus family (23). The idea of a caspase cleavage signal is interesting given the link between FHV, cardiolipin, and apoptosis (32). In particular, cardiolipin provides a key membrane determinant for the recruitment of apoptotic proteins including caspase-8, and thus it is possible that cardiolipin provides a platform to localize FHV protein A to these mitochondrial areas as well (30).

Finally, protein A contains a variety of common protein-protein interaction motifs including three CX_5C motifs, indicating that disulfide bonds may play important roles in FHV replication complex structure and function (12), and

several PxxP motifs which are responsible for the binding of adaptor proteins (29). Unfortunately, a specific analysis of these Flock House virus motifs has not been completed. It will be important to specifically mutate key residue in these sequences and explore their role in viral replicase function. However, it must be noted that since the replication complex is composed of two functionally distinct pools of protein A it is not unreasonable to think that there are also two structurally distinct replication complexes as well. Future analysis of these two FHV replication complexes will provide valuable insight into the correct structure and composition of viral replication complexes.

What are the dynamic temporal and spatial processes involved in the assembly of FHV RNA replication complexes?

In this thesis we have looked at two time points in FHV replication complex assembly. The first involves the initial steps in replication complex assembly of protein A-membrane interactions and the second is an end point analysis of functional FHV RNA replication complexes. However, one has to wonder about the dynamic processes occurring between these two points. We have identified dramatic cellular lipid changes induced by FHV RNA replication and evidence with *Δmim1* yeast suggests these lipid changes are necessary for the assembly of functional replication complexes. Future studies will be conducted to explore the temporal and spatial requirements for FHV RNA replication complex assembly. We will take advantage of BN-AGE to analyze the assembly of complexes over time as well as submitochondrial fractionation techniques to

investigate the mitochondrial localization of these complexes during RNA replication. In addition we will analyze the temporal pattern of phospholipid changes in association with replication complex assembly to piece together the involvement of these cellular processes in replication complex assembly. These future studies will offer valuable insight in to the assembly of viral RNA replication complexes and will provide the first complete biochemical analysis of positive-strand RNA virus replication complex assembly.

Overall Significance

In summary, this thesis has provided novel insight into the molecular mechanisms and host processes involved in positive-strand RNA virus replication complex assembly and function. These studies have led to the development of a variety of in vitro and biochemical techniques which we have used to identify key roles for cellular phospholipids in positive-strand RNA virus membrane targeting and replication complex assembly and function. Furthermore, we have provided the first biochemical evidence of the presence of two functionally distinct viral replication complexes of which their assembly is host and viral dependent. This work has opened many avenues of future study in FHV and yeast biology, but more importantly it has provided a starting point to focus on common molecular mechanisms shared by all positive-strand RNA viruses with the hope of understanding the complex processes of viral RNA replication and pathogenesis.

REFERENCES

1. **Ahting, U., T. Waizenegger, W. Neupert, and D. Rapaport.** 2005. Signal-anchored proteins follow a unique insertion pathway into the outer membrane of mitochondria. J Biol Chem **280:**48-53.

2. **Annamalai, P., and A. L. Rao.** 2006. Packaging of brome mosaic virus subgenomic RNA is functionally coupled to replication-dependent transcription and translation of coat protein. J Virol **80:**10096-108.

3. **Becker, T., S. Pfannschmidt, B. Guiard, D. Stojanovski, D. Milenkovic, S. Kutik, N. Pfanner, C. Meisinger, and N. Wiedemann.** 2008. Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. J Biol Chem **283:**120-7.

4. **Carman, G. M., and G. M. Zeimetz.** 1996. Regulation of phospholipid biosynthesis in the yeast Saccharomyces cerevisiae. J Biol Chem **271:**13293-6.

5. **Castorena, K. M., S. A. Weeks, K. A. Stapleford, A. M. Cadwallader, and D. J. Miller.** 2007. A functional heat shock protein 90 chaperone is essential for efficient flock house virus RNA polymerase synthesis in Drosophila cells. J Virol **81:**8412-20.

6. **Coonrod, E. M., M. A. Karren, and J. M. Shaw.** 2007. Ugo1p is a multipass transmembrane protein with a single carrier domain required for mitochondrial fusion. Traffic **8:**500-11.

7. **Dye, B. T., D. J. Miller, and P. Ahlquist.** 2005. In vivo self-interaction of nodavirus RNA replicase protein a revealed by fluorescence resonance energy transfer. J Virol **79:**8909-19.

8. **Fritz, S., D. Rapaport, E. Klanner, W. Neupert, and B. Westermann.** 2001. Connection of the mitochondrial outer and inner membranes by Fzo1 is critical for organellar fusion. J Cell Biol **152:**683-92.

9. **Henderson, N. S., L. G. Nijtmans, J. G. Lindsay, E. Lamantea, M. Zeviani, and I. J. Holt.** 2000. Separation of intact pyruvate dehydrogenase complex using blue native agarose gel electrophoresis. Electrophoresis **21:**2925-31.

10. **Hulett, J. M., F. Lueder, N. C. Chan, A. J. Perry, P. Wolynec, V. A. Likic, P. R. Gooley, and T. Lithgow.** 2008. The transmembrane segment of Tom20 is recognized by Mim1 for docking to the mitochondrial TOM complex. J Mol Biol **376:**694-704.

11. **Kemper, C., S. J. Habib, G. Engl, P. Heckmeyer, K. S. Dimmer, and D. Rapaport.** 2008. Integration of tail-anchored proteins into the mitochondrial outer membrane does not require any known import components. J Cell Sci **121:**1990-8.

12. **Koehler, C. M.** 2004. The small Tim proteins and the twin Cx3C motif. Trends Biochem Sci **29:**1-4.

13. **Krimmer, T., D. Rapaport, M. T. Ryan, C. Meisinger, C. K. Kassenbrock, E. Blachly-Dyson, M. Forte, M. G. Douglas, W. Neupert, F. E. Nargang, and N. Pfanner.** 2001. Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. J Cell Biol **152:**289-300.

14. **Kunji, E. R., and A. J. Robinson.** 2006. The conserved substrate binding site of mitochondrial carriers. Biochim Biophys Acta **1757:**1237-48.

15. **Lee, C. S., R. Das, and R. Reed.** 2003. Agarose gel separation/isolation of RNA-protein complexes. Curr Protoc Mol Biol **Chapter 27:**Unit 27 1.

16. **McDonough, V. M., R. J. Buxeda, M. E. Bruno, O. Ozier-Kalogeropoulos, M. T. Adeline, C. R. McMaster, R. M. Bell, and G. M. Carman.** 1995. Regulation of phospholipid biosynthesis in Saccharomyces cerevisiae by CTP. J Biol Chem **270:**18774-80.

17. **Meineke, B., G. Engl, C. Kemper, A. Vasiljev-Neumeyer, H. Paulitschke, and D. Rapaport.** 2008. The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. FEBS Lett **582:**855-60.

18. **Miller, D. J., and P. Ahlquist.** 2002. Flock house virus RNA polymerase is a transmembrane protein with amino-terminal sequences sufficient for mitochondrial localization and membrane insertion. J Virol **76:**9856-67.

19. **Miller, D. J., M. D. Schwartz, and P. Ahlquist.** 2001. Flock house virus RNA replicates on outer mitochondrial membranes in Drosophila cells. J Virol **75:**11664-76.

20. **Mozdy, A. D., J. M. McCaffery, and J. M. Shaw.** 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J Cell Biol **151:**367-80.

21. **Nelson, D. R., C. M. Felix, and J. M. Swanson.** 1998. Highly conserved charge-pair networks in the mitochondrial carrier family. J Mol Biol **277:**285-308.

22. **Ostrander, D. B., D. J. O'Brien, J. A. Gorman, and G. M. Carman.** 1998. Effect of CTP synthetase regulation by CTP on phospholipid synthesis in Saccharomyces cerevisiae. J Biol Chem **273:**18992-9001.

23. **Poch, O., I. Sauvaget, M. Delarue, and N. Tordo.** 1989. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J **8:**3867-74.

24. **Popov-Celeketic, J., T. Waizenegger, and D. Rapaport.** 2008. Mim1 functions in an oligomeric form to facilitate the integration of Tom20 into the mitochondrial outer membrane. J Mol Biol **376:**671-80.

25. **Reichert, A. S., and W. Neupert.** 2002. Contact sites between the outer and inner membrane of mitochondria-role in protein transport. Biochim Biophys Acta **1592:**41-9.

26. **Robinson, A. J., C. Overy, and E. R. Kunji.** 2008. The mechanism of transport by mitochondrial carriers based on analysis of symmetry. Proc Natl Acad Sci U S A **105:**17766-71.

27. **Rodriguez, M., T. A. Good, M. E. Wales, J. P. Hua, and J. R. Wild.** 2005. Modeling allosteric regulation of de novo pyrimidine biosynthesis in Escherichia coli. J Theor Biol **234:**299-310.

28. **Russ, W. P., and D. M. Engelman.** 2000. The GxxxG motif: a framework for transmembrane helix-helix association. J Mol Biol **296:**911-9.

29. **Saksela, K., G. Cheng, and D. Baltimore.** 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J **14:**484-91.

30. **Scorrano, L.** 2008. Caspase-8 goes cardiolipin: a new platform to provide mitochondria with microdomains of apoptotic signals? J Cell Biol **183:**579-81.

31. **Senes, A., D. E. Engel, and W. F. DeGrado.** 2004. Folding of helical membrane proteins: the role of polar, GxxxG-like and proline motifs. Curr Opin Struct Biol **14:**465-79.

32. **Settles, E. W., and P. D. Friesen.** 2008. Flock house virus induces apoptosis by depletion of Drosophila inhibitor-of-apoptosis protein DIAP1. J Virol **82:**1378-88.

33. **Stuart, R. A., and C. M. Koehler.** 2007. In vitro analysis of yeast mitochondrial protein import. Curr Protoc Cell Biol **Chapter 11:**Unit 11 19.

34. **Vachova, L., and Z. Palkova.** 2007. Caspases in yeast apoptosis-like death: facts and artefacts. FEMS Yeast Res **7:**12-21.

35. **Venter, P. A., and A. Schneemann.** 2007. Assembly of two independent populations of flock house virus particles with distinct RNA packaging characteristics in the same cell. J Virol **81:**613-9.

36. **Waizenegger, T., and D. Rapaport.** 2007. Analyzing import intermediates of mitochondrial proteins by blue native gel electrophoresis. Methods Mol Biol **372:**287-95.

37. **Waizenegger, T., S. Schmitt, J. Zivkovic, W. Neupert, and D. Rapaport.** 2005. Mim1, a protein required for the assembly of the TOM complex of mitochondria. EMBO Rep **6:**57-62.

38. **Wittig, I., and H. Schagger.** 2008. Features and applications of blue-native and clear-native electrophoresis. Proteomics **8:**3974-90.

39. **Zaid, H., S. Abu-Hamad, A. Israelson, I. Nathan, and V. Shoshan-Barmatz.** 2005. The voltage-dependent anion channel-1 modulates apoptotic cell death. Cell Death Differ **12:**751-60.

40. **Zara, V., A. Ferramosca, L. Capobianco, K. M. Baltz, O. Randel, J. Rassow, F. Palmieri, and P. Papatheodorou.** 2007. Biogenesis of yeast dicarboxylate carrier: the carrier signature facilitates translocation across the mitochondrial outer membrane. J Cell Sci **120:**4099-106.

41. **Zheng, Y., Y. Shi, C. Tian, C. Jiang, H. Jin, J. Chen, A. Almasan, H. Tang, and Q. Chen.** 2004. Essential role of the voltage-dependent anion channel (VDAC) in mitochondrial permeability transition pore opening and cytochrome c release induced by arsenic trioxide. Oncogene **23:**1239-47.