THE ROLES OF CELLULAR CHAPERONES IN POSITIVE SENSE RNA VIRUS REPLICATION COMPLEX ASSEMBLY AND FUNCTION

by

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DEDICATION

To Nana, a constant source of support and motivation. Even though you didn't live to see its completion, I know that you are proud. I love you.

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This work would not have been possible without the support and assistance of many people. In particular I'd like to acknowledge the help of my advisor, Dave Miller, and my committee, whose input and high standards drove me to do better science. In addition to the input of my committee, my collaborators were very helpful by supplying reagents and advice on how to use them. Their generosity simplified all of the work that I did.

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ABSTRACT

Positive strand (+) RNA viruses are a significant health threat today, yet the diseases that they cause are difficult to treat due to a lack of antiviral drugs, which is in part due to the tight association of their replication with host cellular functions. In order to design improved therapeutic agents for use against (+) RNA viruses, we must increase our understanding of their replication mechanisms in host cells, and what cellular factors are involved in their life cycle. The early events in (+) RNA virus replication include translation of the genome into the viral replication proteins, followed by the folding, trafficking, and assembly of the viral replication factories on intracellular membranes, which are critical for all (+) RNA viruses, and, therefore viable targets for potential antivirals. To better understand these early events and the cellular proteins that are involved, I used Flock House virus, a model (+) RNA virus, in a *Saccharomyces cerevisiae* model system to take advantage of the facile and well understood genetics of yeast. In these studies, I examined the impact of the major chaperone and cochaperone systems in FHV RNA replication in yeast. I identified a number of chaperones that impacted FHV RNA replication both negatively and positively, including the J-domain family of Hsp40s, the main cellular Hsp70 chaperone system, as well as a number of minor chaperones. In addition, I found that some cellular requirements were membrane-specific, and that there were differences between the requirements seen in *Drosophila* cells and those in yeast. Finally, I began to develop a method for high-throughput analysis of the early events in FHV RNA replication based on the accumulation of the viral polymerase. Therefore, in this body of work, I confirmed that cellular chaperones

play a role in the genome replication of a positive strand RNA virus, and identified a number of proteins in the cell as targets for future study.

CHAPTER I

GENERAL INTRODUCTION

Positive-sense (+) RNA viruses cause a number of human diseases, and are among those pathogens that are emerging as threats to our public health and economy, yet despite this, there are few treatments for (+) RNA virus infections. Though we understand some aspects of viral replication, we are limited in our ability to counter these viruses in part because we lack critical information about the mechanisms involved in the early events in the (+) RNA virus replication cycle. These early events in the (+) RNA virus replication cycle are the translation, folding, targeting and trafficking of the replication machinery to a specific intracellular membrane. Chaperones are good candidates for facilitating these steps in the viral life cycle due to their known roles in the translation, folding, targeting and trafficking of cellular proteins. There is evidence that viruses use chaperones at many steps in their life cycle, and so we hypothesize that (+) RNA viruses use them for the early events in replication as well. In order to gain a better understanding of the connections between (+) RNA viruses and host machinery, our lab uses a model virus in two genetically tractable hosts. This allows us to both identify factors that are responsible for enhancing or regulating steps in the viral life cycle, and also to compare and contrast these factors in a host-dependent way. Using a model

pathogen allows us to make observations and study (+) RNA viruses in a controlled setting, and use this information to make predictions about more clinically relevant pathogens. We are able to do this because of the significant similarities between the life cycles of all (+) RNA viruses.

Positive-sense RNA viruses, their global impact and the challenges of their treatment. Positive sense (+) RNA viruses are responsible for causing a number of significant current and emerging diseases in humans and other animals (69). These include dengue hemorrhagic fever, the respiratory disease caused by SARS coronavirus, and several viral encephalitic diseases, such those caused by West Nile virus and the equine encephalitic viruses. In addition to these newer threats, other more classical viral diseases are reemerging due to resurgence in vector populations, such as yellow fever, as well as dengue hemorrhagic fever. Several factors are responsible for the increase in occurrence and spread of these viruses. Climate change and increasing world temperatures have shifted habitats for many insect species, causing vector populations to move into newer areas or rebound in areas that were previously under control. The modernization of the world has also played its part, allowing for easier travel from previously isolated areas of the world, as well as encroachment into unpopulated areas. In addition to the human health impacts of these viruses, they are also the causes of many animal, plant and insect diseases that have the potential to negatively impact the world's agriculture. Many of the crop disease viruses fall in to the (+) RNA virus group, such as tobacco mosaic virus, tomato bushy stunt virus, cucumber necrosis virus, and the cereal crop pathogens, barley yellow dwarf virus, and cereal yellow dwarf virus. Barley yellow dwarf virus alone can cause up to 50% yield loss in cereal crops (26, 93).

Despite the significance and emergence of (+) RNA viruses, there are relatively few antiviral drugs available for clinical treatments, and agricultural control is largely limited to pesticide use to limit vector populations. Treatment of (+) RNA viruses is complicated by a number of factors, including the extensive use of the host factors to accomplish their life cycle, and the relative lack of understanding of what those host factors are. Compared to the life cycles of viruses with DNA, double stranded RNA, or

negative sense RNA genomes, which all require enzyme steps in order to convert their genome into a translatable form, (+) RNA viruses enter the cell with their genomes already in the coding sense, allowing them to be translated immediately by the host machinery. This intimately ties the viral genome and proteins to the host cellular machinery making virus-specific inhibition difficult. Despite this known dependence on the host cell machinery, what that machinery is and how it is used by the virus are not well understood. Therefore, further understanding into the (+) RNA virus life cycle and its ties to the host machinery would allow us to identify potential drug targets for the development inhibitors that would greatly increase the effectiveness of the treatment of (+) RNA virus infection.

The (+) RNA virus life cycle. The (+) RNA virus life cycle begins when the infectious virus particle binds at the surface of the cell and is either taken up in a vesicle or releases its genome directly into the cytosol (Figure 1.1, step 1). Upon release of the genome, which is in the positive coding sense, it is bound by the cellular ribosomal complex and translated into the viral replication machinery, which then must be folded (Figure 1.1, step 2). Some viral genomes are capped with a 5' 7-methylguanosine, and so bind the ribosome like host mRNAs, while others encode a cap independent internal ribosome entry site that binds the translation machinery without needing a 5' cap (71). There is evidence at this step for host factors playing a role in the specific translation of viral polymerases (13, 52), as well as cell specific tropism based on translation of viral proteins (7, 17, 32).

Next, the viral replication proteins must be targeted and trafficked to a specific intracellular membrane for genome replication (Figure 1.1, step 3). It is at this step that all (+) RNA viruses share a common requirement; in order to be active, the replication machinery needs to be associated with some intracellular membrane (1). The specific

membrane is dependent on the virus, but there is a need for an intracellular membrane (1). There is some debate as to whether the membrane serves as a scaffold for viral replication, or if there is some protein or lipid component of particular membranes that is important for certain viruses, but these are not mutually exclusive possibilities and the membrane likely serves multiple roles for replication. There is specificity of membrane targeting for viral replication complexes: many encephalitic viruses replicate on endosomes (27, 74), flaviviruses replicate on membranes derived from the endoplasmic reticulum (23, 65), whereas many plant and insect pathogens replicate on the mitochondria or chloroplasts (50, 63, 79). In some models the viral polymerase can be retargeted to another membrane without deleterious effects on function (51, 64), which lends credibility to the non-specific argument of the membrane as a scaffold, yet the fact that there is specificity at all among membrane use would argue for a specific effect. Upon membrane association, the viral replication complex causes a rearrangement of the membrane itself and begins to replicate the genome (Figure 1.1, step 4). The first step of genome replication is the transcription of the (+) RNA genome to a negative strand, which is then used as a template for further (+) RNA genome production. Often a subgenome is produced from a secondary site on the genome during active replication, which encodes proteins that are needed for virion production, immune system evasion and other functions (55, 57). There is evidence for the involvement of host factors as replication complex members (6, 27, 88, 91, 97) as well as others that have been shown to be necessary for RNA replication but not to be complex members (2, 15).

Following genome replication and translation of the structural proteins, the virus particle assembles and encapsidates the genomic RNA (Figure 1.1, step 5), and then exits the cell (Figure 1.1, step 6). The encapsidation of replicated genome has been shown to be linked spatially and functionally to active replication complexes (7, 95).

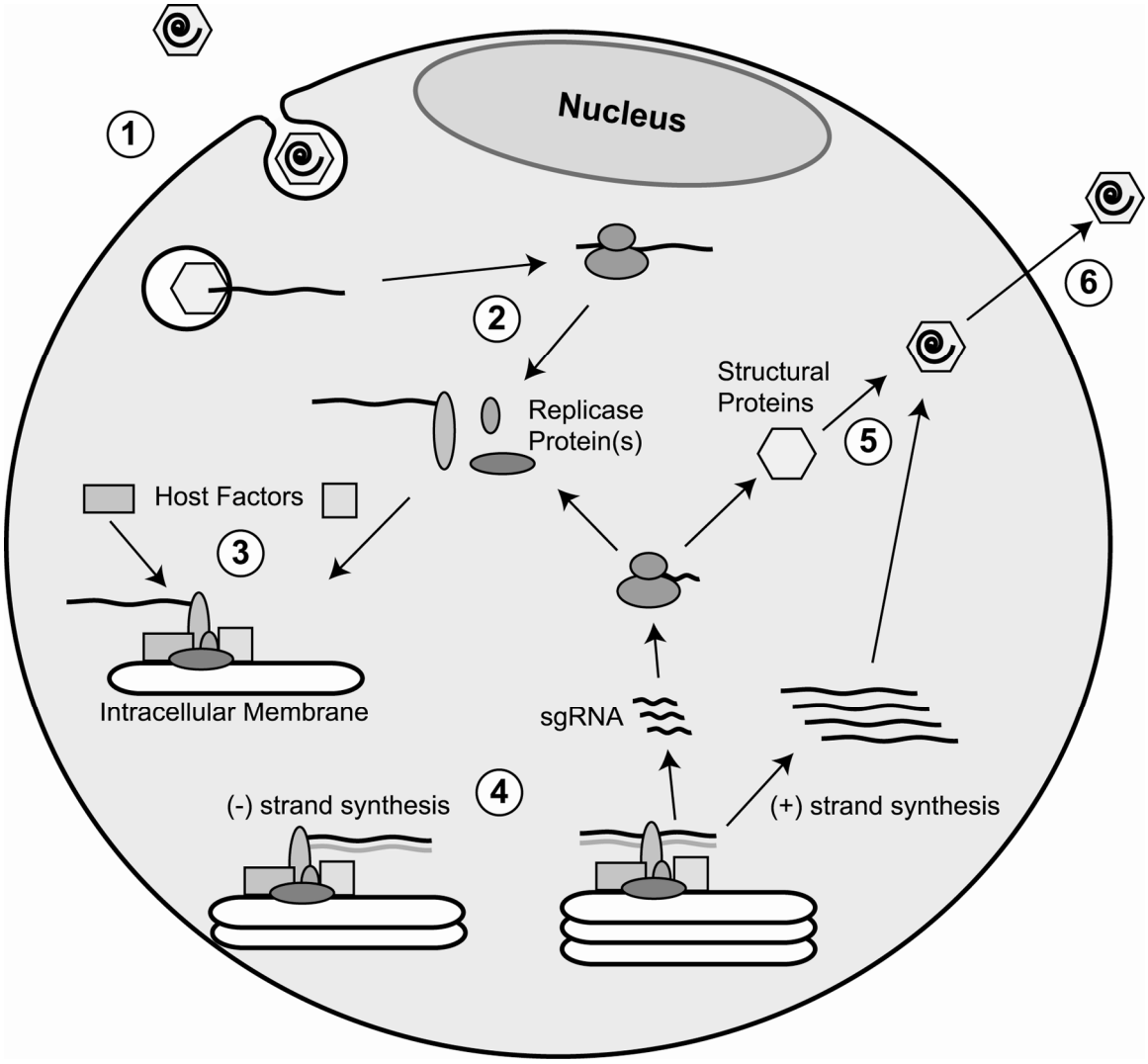


Figure 1.1 - The positive strand RNA virus life cycle. The steps in (+) RNA virus replication: Virion entrance and uncoating (1), Translation of viral genome (2), Assembly of viral replication complex (3), Maturation of replication complex, RNA replication and sgRNA synthesis (4), Virion assembly and encapsidation (5), Virion exit (6). These events are drawn as separate steps out of convenience although there are likely significant links between steps. Abbreviation: sgRNA – subgenomic RNA.

This suggests that the replication cycle progresses continuously and not stepwise as it is often depicted and described.

While some steps are fairly well understood, the critical early steps of the translation, folding, trafficking and membrane association of the replication complex proteins are poorly understood. When thinking of a viral protein as an entity in the cytosol, it faces essentially the same challenges that cellular proteins face. Some viral replicase proteins are translated in the cytosol away from their target membrane, and so must be properly targeted to their site of action. They also must be folded appropriately to function and to hide hydrophobic residues that would cause aggregation, and as membrane associated proteins, many viral replication proteins have hydrophobic domains that must be shielded until they become associated with the target membrane. Further, because replicase proteins function as a part of a large macromolecular complex (27, 70, 88, 97), association with other cellular and viral proteins and complex assembly is a necessary step. When considering cellular proteins, these same challenges are often overcome by association with chaperones.

Cellular chaperones and their role in the cell. Cellular chaperones are a class of cellular proteins involved in the translation, folding, trafficking, and maturation of cellular proteins (21, 42, 104), as well as the assembly of multi-protein complexes (105). The main chaperone complexes in the cell are those of the heat shock protein (Hsp) 90 and Hsp70 (Figure 1.2). Together these two complexes are responsible for the majority of protein folding, maturation, and quality control in the cell (11, 39, 61).

Hsp70 is a ubiquitous chaperone, responsible for the folding and shielding of nearly every protein [(11) and reviewed in (42, 104)]. Hsp70 functions through cycles of binding and hydrolyzing ATP, causing changes that increase or decrease affinity to substrate proteins. These cycles of binding and release continue until the substrate

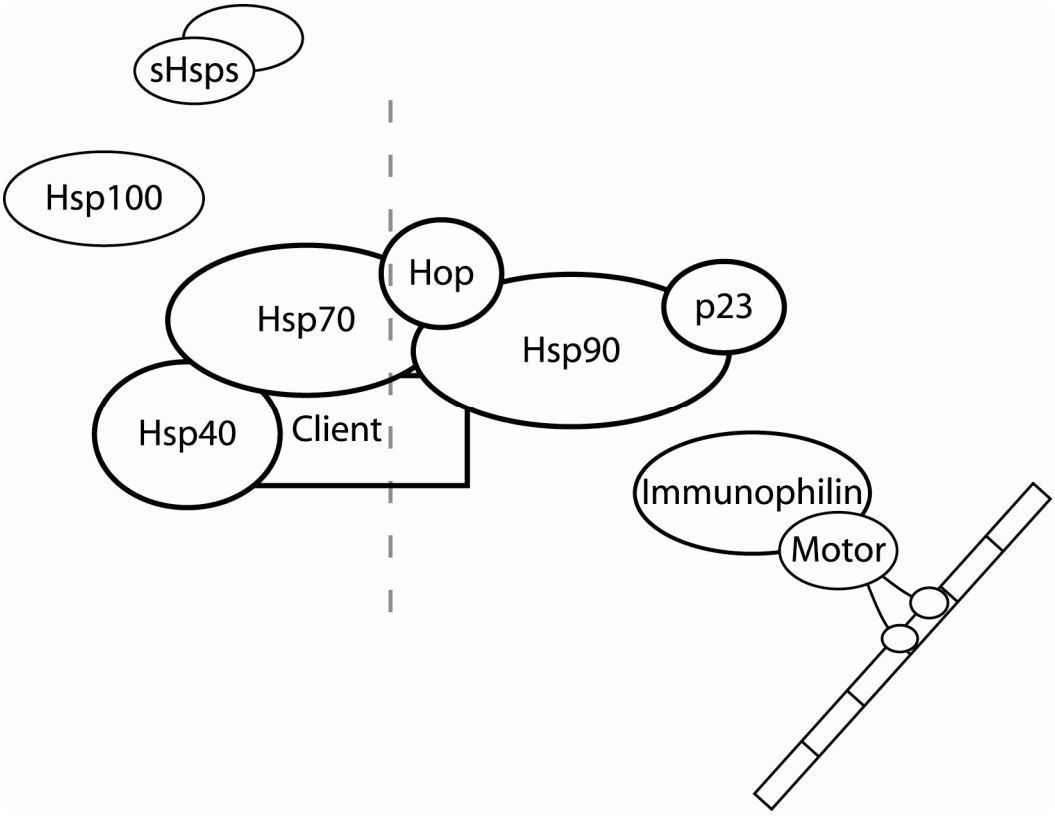


Figure 1.2 – The main chaperone complexes of the cell. The Hsp70 chaperone complex (left) consists of Hsp70 and Hsp40, and can work with Hsp100 for special refolding functions. The Hsp90 chaperone complex (right) includes the Hsp70 complex as well as the Hsp organizing protein (Hop) and p23. Hsp90 can interact with motor proteins through immunophilins and traffic client proteins in the cell. Small heat shock proteins (sHsps) act largely independently of the main chaperone complexes.

protein is in the proper form, and then it is released (11). The ATPase activity of Hsp70s alone is intrinsically weak, and so the chaperone cycle would progress slowly without enhancement by Hsp40 cochaperones (Figure 1.2, left). Hsp40s or J-domain proteins, all contain a J-domain, which binds to the Hsp70, enhancing the weak ATPase activity and increasing the efficiency of the Hsp70s folding function (92). J-domain proteins are so named because they are orthologs of the bacterial DnaJ protein, which regulates the bacterial Hsp70, DnaK. J-domain proteins (also called J-proteins) are grouped based on their homology to DnaJ. DnaJ contains three domains: a J-domain, a glycine/phenylalanine rich domain, and a carboxy terminal domain that contains a zinc finger. The necessity of the glycine/phenylalanine domain has been debated, and its function is not well defined. The carboxy terminal domain and zinc finger are believed to aid the binding of unfolded proteins by Hsp70. The J-domain alone is able to enhance the Hsp70 ATPase activity. J-domain proteins may have all of these domains (type I), be missing the glycine/phenylalanine rich region (type II), or just the J-domain with other domains that are not similar to those of DnaJ (type III). Type I J-domain proteins are thought to function like DnaJ, binding unfolded proteins, and enhancing the ATPase activity of the partner Hsp70. The cellular activities of type II and type III J-proteins are not all defined, and are believed to be more compartmentalized, binding Hsp70s to various sites in the cell, where their activities are more localized [reviewed in (96)].

Hsp90 is a more specialized chaperone, interacting with a subset of specific client proteins (106). Hsp90 is responsible for the maturation of these proteins beyond the usual folding and shielding that Hsp70 does. The Hsp90 complex contains many components, including Hsp70 and a number of cochaperones that are added in turn to bind and mature the client protein (8, 14, 60) (Figure 1.2, right). Perhaps the best studied client proteins of Hsp90 are the steroid receptors. It has been shown that maturation of steroid receptors is dependent on association with the Hsp90 chaperone

complex, and that the receptor is not able to bind ligand until this interaction has taken place (76). Further, the Hsp90 complex holds the steroid receptor in this ligand-ready conformation until binding, and then traffics the complex to the nucleus, where the activated receptor acts as a transcription factor (76). This nuclear trafficking by Hsp90 has been shown to progress through interactions with immunophilins and cellular motor proteins that traffic to the nucleus (29, 30, 75). In addition to its role in trafficking steroid receptors and other transcription factors to the nucleus, Hsp90 has been shown to be important for import of proteins into the mitochondria (105).

Other than the major groups of chaperones in the cell, there are a number of cochaperones and minor chaperones that specialize in other cellular functions. These include the immunophilins, the Hsp100 (Clp) family, and the small Hsps. The immunophilin/cyclophilin family of peptidyl-prolyl isomerases bridge chaperones to motor proteins (29, 30, 60, 75, 76) and are responsible for client protein trafficking. The small Hsps and the Hsp100 chaperones work with the main chaperone complexes and are part of the recovery from heat shock or cellular stress. The Hsp100 (Clp) group are responsible for unfolding and refolding aggregated proteins (39, 73), and the small Hsps refold denatured proteins and prevent the formation of aggregates (43, 44, 81). Much of what we know about chaperones comes from the yeast model, where chaperones have been shown to be key regulators of prion proteins (40, 84, 89), which are related to the aggregate diseases in mammals such as bovine spongiform encephalopathy, Creutzfeldt–Jakob disease, scrapie and chronic wasting disease.

Viruses use cellular chaperones for their replication. Based on their role in the cellular processes of folding, targeting, trafficking, and assembly of protein complexes, chaperones are good candidates for fulfilling these functions for viral proteins. Consistent with this, viral infection has been shown to upregulate cellular chaperones in

host cells (9, 19, 38, 68), suggesting that cellular chaperones are important for viral replication. Infection could nonspecifically upregulate chaperones due to a stress response, but some of these studies show that this has a positive effect on viral replication (9, 38), suggesting that this may be a fortuitous upregulation. Beyond expression studies, there is increasing literature that many types of viruses are using cellular chaperones for their replication and that this is a necessary step. The earliest reference to this was work on the λ bacteriophage. A host genetic screen was done looking for host genes necessary for phage replication, and identified two genes, DnaK and DnaJ, that were required for both host and phage DNA replication (103). DnaK and DnaJ were identified as chaperones, the bacterial Hsp70 and 40, respectively. Along with another Hsp70 cochaperone, GrpE, were later shown to initiate phage DNA replication in vitro (109).

Studies have now identified cellular chaperones as important for the replication of many groups of viruses, and at many steps in the life cycle (Table 1.1). Chaperones have been linked to entry into the cell (37, 80), RT activity of retroviruses (5, 46-48, 72), genomic replication (24, 27, 67, 70, 88, 91, 100, 101, 107, 108), and virion assembly (18, 33, 98). More recently, work on (+) RNA viruses has identified a requirement for chaperones in viral RNA replication (88, 91, 100, 101). Further, purification of some viral replication complexes has revealed that chaperones can be members of viral polymerase complexes (27, 70, 88). Recent work in our lab has identified a link between chaperones and the synthesis of the Flock House virus polymerase in *Drosophila* cells, further implicating host factors into the earliest events in viral replication (13, 52).

Flock House virus is a versatile model for studying virus-host interactions. To study the use of the host machinery by an (+) RNA virus, we use Flock House virus (FHV) as a model pathogen. FHV is the best studied member of the *Nodaviridae* family

Table 1.1 - The role of host chaperones in viral replication

Type	Virus[1]	Chaperone	Role	Ref.
DNA	λ phage	Hsp70/40 (DnaK/J)	Genome replication initiation	(45, 103, 109)
	HSV-1	Hsp40 (hTid-1)	Genome replication	(24)
	Vaccinia	Hsp90	Viral Replication	(49)
	Adenovirus	Hsp40	Viral replication	(38)
	Polyomavirus	Hsc70	Capsid assembly	(18)
	HPV	Hsp40 (hTid-1)	Interacts with viral oncoprotein	(85)
DNA with an RNA intermediate	HBV	Hsp60	RT interaction	(72)
	Duck HBV	Hsp70/40	RT activity	(5)
	HBV	Hsp90	RT activitiy	(46)
	Hepadnavirus	Cdc37	Assembly and replication	(98)
	HBV	Hsp40 (Hdj1)	Negative regulation of replication	(90)
dsRNA	Reovirus	Hsp90	Viral attachment	(37)
(-) RNA	Influenza	Hsp90	Genome replication	(67)
	Measles	Hsp70	Genome replication	(107, 108)
(+) RNA	DENV	Hsp70/90	Viral attachment[2]	(80)
	FHV	Hsp90	Synthesis of polymerase	(13, 52)
	BMV	Hsp40 (Ydj1)	RdRp transport and activity	(91)
	HCV	Hsp90	RdRp maturation	(101)
		Cyclophilin B	RdRp complex activity	(100)
	CuNV	Hsp70 (Ssa)	RdRp complex activity	(88)
	SINV	Hsc70	RdRp complex member	(27)
	ToMV	Hsp70	RdRp complex member	(70)
	Poliovirus	Hsp90	Capsid protein folding	(33)

1 – Abbreviations – HSV-Herpes simplex virus, HPV-Human papillomavirus, HBV-Hepatitis B virus, DENV-Dengue virus, FHV-Flock House virus, BMV-Brome mosaic virus, HCV-Hepatitis C virus, CuNV-Cucumber necrosis virus, SINV-Sinbis virus, ToMV-Tomato mosaic virus.
2 – The functional role of this has been debated (12)

of (+) RNA viruses, which include the two genera alpha and beta. Alphanodaviruses infect insects, while betanodaviruses infect fish. FHV was isolated in 1983 from the grass grub, *Costelytra zealandica* at the field station in Flock House, New Zealand (86). FHV has been used as a model virus for many studies of the viral replication cycle, including those examining capsid assembly and genome packaging (95), the cellular response to a viral infection (55, 58, 99), and RNA complex assembly and function (22, 56, 62, 63, 77, 78, 94, 102). FHV is a very useful model pathogen, for a number of reasons. FHV can replicate its genome in a wide array of cell types, including insect cells (63), *C. elegans* (55), mammalian cells (3), plant cells (87), and the brewer's yeast, *Saccharomyces cerevisiae* (78). The ability to replicate in a number of hosts suggests that FHV requires host factors that are either well conserved or few in number, and allows us to make comparisons of requirements between host cells. FHV also has a well defined RNA replication target membrane. During replication the FHV RNA-dependent RNA polymerase, protein A, is targeted to the outer membrane of the mitochondria (63). This targeting is due to an N-terminal sequence of protein A that resembles the targeting signal of cellular mitochondrial proteins (62). FHV infection of *Drosophila* cells causes a rearrangement of the mitochondria membranes, and forms invaginations of the outer membrane into the intermembrane space between the outer and inner membranes of the mitochondria, called spherules (63), which have recently been modeled using cryogenic-electron microscopy to develop a three dimensional picture of FHV RNA replication complexes (53).

FHV has a small 4.5 kilobase genome, consisting of two capped, non-polyadenylated segments (Figure 1.3), which makes expression possible in many cell types. The larger 3.1 kilobase segment, RNA1, encodes protein A, the FHV RNA-dependent RNA polymerase (RdRp) and the only viral protein required to form a replication complex. The smaller 1.4 kilobase segment, RNA2, encodes the structural

protein precursors, which are dispensable for RNA replication, but are required for virion formation.

Upon entering the cell, RNA1 is translated to protein A. Protein A can then recognize, bind and traffic RNA1 to the mitochondrial outer membrane (94), where it forms a replication complex. During active replication, a third, 0.4 kb subgenomic RNA is produced from the 3' end of RNA1, called RNA3. RNA3 encodes protein B2, which functions as an inhibitor of the RNAi response (55). Protein B2 functions by binding to dsRNA molecules and blocking their recognition by innate defenses (16). Because of this function, protein B2 is necessary for RNA replication in cells with an active RNAi system, such as insects (28, 55, 99) and other invertebrates (58), and plants (55).

Yeast is a useful and genetically tractable model host. In order to explore the roles of host proteins in the replication of a (+) RNA virus, we use the brewer's yeast *Saccharomyces cerevisiae*. We chose to work with yeast as a model host because it is a well studied and genetically tractable system, allowing for simple transition from hypothesis to experimental design. Yeast has been well studied as a eukaryotic cell, and has been used to dissect a number of cellular functions including trafficking, translocation (20, 31, 34, 41) and heat shock (8, 14, 20, 21, 81, 83), allowing our studies to tap into a large amount of published information about these cellular functions. Yeast has been identified as a useful model for some genetic and transmissible human diseases (25, 59, 84) and also as a viable model host for (+) RNA viruses (4, 22, 62, 64, 88, 91, 97) due to the orthology of many yeast genes to those of higher eukaryotes. Chaperone systems in particular have been well studied in yeast cells, including the diversity of the Hsp70 (10) and J-domain protein systems (83, 96). An in depth area of chaperone study is the role of chaperones regulating prions in yeast cells, where it has been shown that prions are highly affected by cellular chaperones through their functions

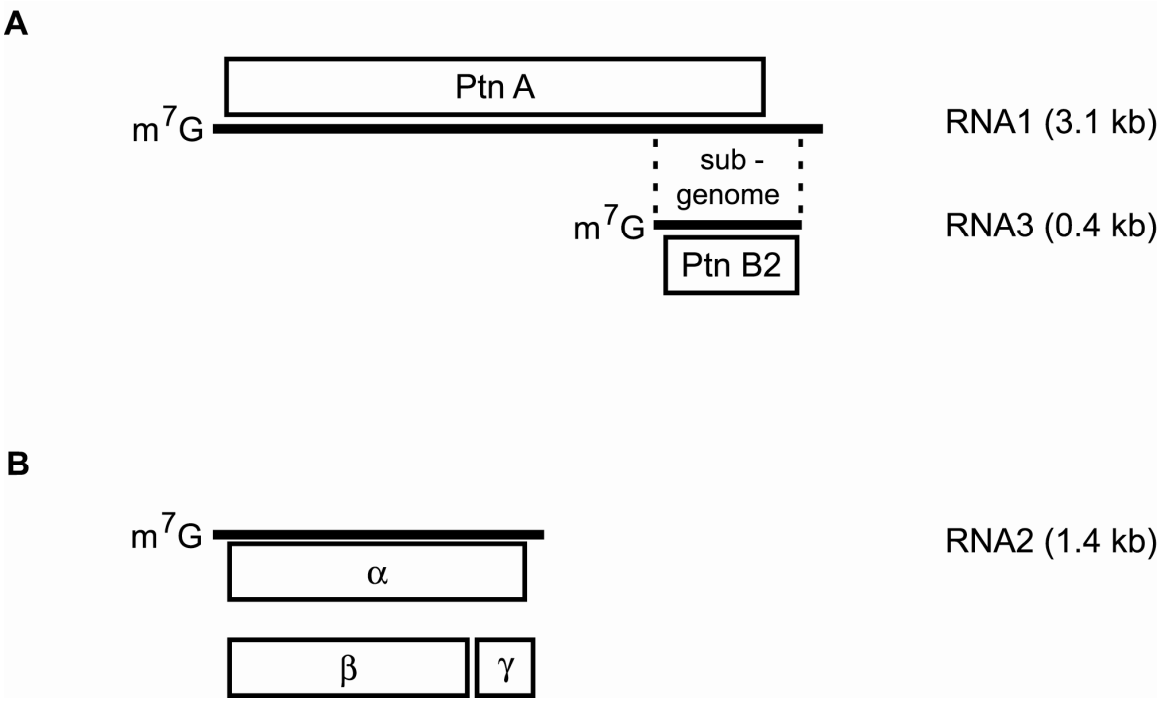


Figure 1.3 - Schematic of the Flock House virus genome. (A) RNA1 encodes the FHV RNA-dependent RNA-polymerase, protein A. During RNA replication, a subgenomic RNA3 is generated from the 3' end of RNA1. RNA3 encodes protein B2, which is an inhibitor of RNA interference. (B) RNA2 encodes the structural protein precursor.

of protecting, unfolding, and refolding prion aggregates (84).

Perhaps the most useful feature of yeast as a host cell for our studies is the availability of genetic libraries including knockout (36), regulated expression (66), and tagged libraries (35, 54, 82) that cover most of the genome. These many options allow for detailed analysis of a gene by selecting a strain that is appropriate for a particular experiment, rather than constructing one. This is useful for both individual studies and also for the design and development of high throughput studies (36).

We study FHV in a yeast model using a replicon system (62). These are cDNA copies of RNA1 that are tightly regulated by a *GAL1* promoter. Upon induction with galactose, these constructs express an authentic viral RNA1 that is then translated and sets up a replication complex on the mitochondria (62). A detailed description of these constructs and their use in our studies can be found in Chapter II (Figure 2.1).

In the following chapters, we have taken advantage of these established model systems in order to study the roles of cellular chaperones in the genome replication of a (+) RNA virus. We will show that host chaperones are required for efficient FHV RNA replication, and that there are host- and membrane-specific differences in that requirement. Further, we used a targeted analysis of cytosolic chaperones in yeast to identify those that affect FHV RNA replication. In that study, we have shown that the Hsp70 system plays contrasting roles in the regulation of viral replication, and also identified a number of other chaperones as important for further study based on their RNA replication phenotypes. Finally, we have begun to develop a method for genome-wide or chemical library based high throughput screening for those host proteins or chemical compounds that impact the synthesis and stability of protein A in yeast cells, allowing for future large scale analysis of host systems in the replication of a (+) RNA virus.

References

1. **Ahlquist, P., A. O. Noueiry, W. M. Lee, D. B. Kushner, and B. T. Dye.** 2003. Host factors in positive-strand RNA virus genome replication. J Virol **77:**8181-6.

2. **Ariumi, Y., M. Kuroki, K. Abe, H. Dansako, M. Ikeda, T. Wakita, and N. Kato.** 2007. DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J Virol **81:**13922-6.

3. **Ball, L. A.** 1992. Cellular expression of a functional nodavirus RNA replicon from vaccinia virus vectors. J Virol **66:**2335-45.

4. **Ball, L. A.** 1995. Requirements for the self-directed replication of flock house virus RNA 1. J Virol **69:**2722.

5. **Beck, J., and M. Nassal.** 2003. Efficient Hsp90-independent in vitro activation by Hsc70 and Hsp40 of duck hepatitis B virus reverse transcriptase, an assumed Hsp90 client protein. J Biol Chem **278:**36128-38.

6. **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.

7. **Bink, H. H., J. Schirawski, A. L. Haenni, and C. W. Pleij.** 2003. The 5'-proximal hairpin of turnip yellow mosaic virus RNA: its role in translation and encapsidation. J Virol **77:**7452-8.

8. **Bohen, S. P.** 1998. Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. Mol. Cell. Biol. **18:**3330-3339.

9. **Bolt, G.** 2001. The measles virus (MV) glycoproteins interact with cellular chaperones in the endoplasmic reticulum and MV infection upregulates chaperone expression. Arch Virol **146:**2055-68.

10. **Boorstein, W. R., T. Ziegelhoffer, and E. A. Craig.** 1994. Molecular evolution of the HSP70 multigene family. J Mol Evol **38:**1-17.

11. **Bukau, B., and A. L. Horwich.** 1998. The Hsp70 and Hsp60 chaperone machines. Cell **92:**351-66.

12. **Cabrera-Hernandez, A., C. Thepparit, L. Suksanpaisan, and D. R. Smith.** 2007. Dengue virus entry into liver (HepG2) cells is independent of hsp90 and hsp70. J Med Virol **79:**386-92.

13. **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:**8412-8420.

14. **Chang, H. C., D. F. Nathan, and S. Lindquist.** 1997. In vivo analysis of the Hsp90 cochaperone Sti1 (p60). Mol Cell Biol **17:**318-25.

15. **Chang, P. C., C. W. Chi, G. Y. Chau, F. Y. Li, Y. H. Tsai, J. C. Wu, and Y. H. Wu Lee.** 2006. DDX3, a DEAD box RNA helicase, is deregulated in hepatitis virus-associated hepatocellular carcinoma and is involved in cell growth control. Oncogene **25:**1991-2003.

16. **Chao, J. A., J. H. Lee, B. R. Chapados, E. W. Debler, A. Schneemann, and J. R. Williamson.** 2005. Dual modes of RNA-silencing suppression by Flock House virus protein B2. Nat Struct Mol Biol **12:**952-7.

17. **Chiu, W. W., R. M. Kinney, and T. W. Dreher.** 2005. Control of translation by the 5'- and 3'-terminal regions of the dengue virus genome. J Virol **79:**8303-15.

18. **Chromy, L. R., J. M. Pipas, and R. L. Garcea.** 2003. Chaperone-mediated in vitro assembly of Polyomavirus capsids. Proc Natl Acad Sci U S A **100:**10477-82.

19. **Collins, P. L., and L. E. Hightower.** 1982. Newcastle disease virus stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins. J Virol **44:**703-7.

20. **Craig, E., P. J. Kang, and W. Boorstein.** 1990. A review of the role of 70 kDa heat shock proteins in protein translocation across membranes. Antonie Van Leeuwenhoek **58:**137-46.

21. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol Rev **57:**402-14.

22. **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.

23. **El-Hage, N., and G. Luo.** 2003. Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. J Gen Virol **84:**2761-9.

24. **Eom, C. Y., and I. R. Lehman.** 2002. The human DnaJ protein, hTid-1, enhances binding of a multimer of the herpes simplex virus type 1 UL9 protein to oris, an origin of viral DNA replication. Proc Natl Acad Sci U S A **99:**1894-8.

25. **Foury, F.** 1997. Human genetic diseases: a cross-talk between man and yeast. Gene **195:**1-10.

26. **Freeman, A.** 2008, posting date. Barley Yello Dwarf Virus (BYDV) and Cereal Yellow Dwarf Virus (CYDV). State of Victoria, Department of Primary Industries. [Online.]

27. **Frolova, E., R. Gorchakov, N. Garmashova, S. Atasheva, L. A. Vergara, and I. Frolov.** 2006. Formation of nsP3-specific protein complexes during Sindbis virus replication. J. Virol. **80:**4122-4134.

28. **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.

29. **Galigniana, M. D., J. M. Harrell, P. J. Murphy, M. Chinkers, C. Radanyi, J. M. Renoir, M. Zhang, and W. B. Pratt.** 2002. Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and in vivo evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. Biochemistry **41:**13602-10.

30. **Galigniana, M. D., J. M. Harrell, H. M. O'Hagen, M. Ljungman, and W. B. Pratt.** 2004. Hsp90-binding immunophilins link p53 to dynein during p53 transport to the nucleus. J Biol Chem **279:**22483-9.

31. **Garcia, P. D., W. Hansen, and P. Walter.** 1991. In vitro protein translocation across microsomal membranes of Saccharomyces cerevisiae. Methods Enzymol **194:**675-82.

32. **Gardner, C. L., C. W. Burke, M. Z. Tesfay, P. J. Glass, W. B. Klimstra, and K. D. Ryman.** 2008. Eastern and Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and macrophages: impact of altered cell tropism on pathogenesis. J Virol **82:**10634-46.

33. **Geller, R., M. Vignuzzi, R. Andino, and J. Frydman.** 2007. Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. Genes Dev **21:**195-205.

34. **George, R., T. Beddoe, K. Landl, and T. Lithgow.** 1998. The yeast nascent polypeptide-associated complex initiates protein targeting to mitochondria in vivo. Proc Natl Acad Sci U S A **95:**2296-301.

35. **Ghaemmaghami, S., W. K. Huh, K. Bower, R. W. Howson, A. Belle, N. Dephoure, E. K. O'Shea, and J. S. Weissman.** 2003. Global analysis of protein expression in yeast. Nature **425:**737-41.

36. **Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis, and M. Johnston.** 2002. Functional profiling of the Saccharomyces cerevisiae genome. Nature **418:**387-91.

37. **Gilmore, R., M. C. Coffey, and P. W. Lee.** 1998. Active participation of Hsp90 in the biogenesis of the trimeric reovirus cell attachment protein sigma1. J Biol Chem **273:**15227-33.

38. **Glotzer, J. B., M. Saltik, S. Chiocca, A. I. Michou, P. Moseley, and M. Cotten.** 2000. Activation of heat-shock response by an adenovirus is essential for virus replication. Nature **407:**207-11.

39. **Glover, J. R., and S. Lindquist.** 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell **94:**73-82.

40. **Gokhale, K. C., G. P. Newnam, M. Y. Sherman, and Y. O. Chernoff.** 2005. Modulation of prion-dependent polyglutamine aggregation and toxicity by chaperone proteins in the yeast model. J Biol Chem **280:**22809-18.

41. **Hansen, W., P. D. Garcia, and P. Walter.** 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-alpha-factor. Cell **45:**397-406.

42. **Hartl, F. U., and M. Hayer-Hartl.** 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science **295:**1852-8.

43. **Haslbeck, M.** 2006. Recombinant expression and in vitro refolding of the yeast small heat shock protein Hsp42. Int J Biol Macromol **38:**107-14.

44. **Haslbeck, M., N. Braun, T. Stromer, B. Richter, N. Model, S. Weinkauf, and J. Buchner.** 2004. Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. Embo J **23:**638-49.

45. **Hoffmann, H. J., S. K. Lyman, C. Lu, M. A. Petit, and H. Echols.** 1992. Activity of the Hsp70 chaperone complex--DnaK, DnaJ, and GrpE--in initiating phage lambda DNA replication by sequestering and releasing lambda P protein. Proc Natl Acad Sci U S A **89:**12108-11.

46. **Hu, J., and C. Seeger.** 1996. Hsp90 is required for the activity of a *Hepatitis B virus* reverse transcriptase. Proc. Natl. Acad. Sci. USA **93:**1060-1064.

47. **Hu, J., D. Toft, D. Anselmo, and X. Wang.** 2002. In vitro reconstitution of functional *hepadnavirus* reverse transcriptase with cellular chaperone proteins. J. Virol. **76:**269-279.

48. **Hu, J., D. O. Toft, and C. Seeger.** 1997. Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. Embo J **16:**59-68.

49. **Hung, J. J., C. S. Chung, and W. Chang.** 2002. Molecular chaperone Hsp90 is important for Vaccinia virus growth in cells. J. Virol. **76:**1379-1390.

50. **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.

51. **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.

52. **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.

53. **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.

54. **Kumar, A., S. Agarwal, J. A. Heyman, S. Matson, M. Heidtman, S. Piccirillo, L. Umansky, A. Drawid, R. Jansen, Y. Liu, K. H. Cheung, P. Miller, M. Gerstein, G. S. Roeder, and M. Snyder.** 2002. Subcellular localization of the yeast proteome. Genes Dev **16:**707-19.

55. **Li, H., W. X. Li, and S. W. Ding.** 2002. Induction and suppression of RNA silencing by an animal virus. Science **296:**1319-21.

56. **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.

57. **Lommel, S. A., T. L. Kendall, Z. Xiong, and R. C. Nutter.** 1991. Identification of the maize chlorotic mottle virus capsid protein cistron and characterization of its subgenomic messenger RNA. Virology **181:**382-5.

58. **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.

59. **Mager, W. H., and J. Winderickx.** 2005. Yeast as a model for medical and medicinal research. Trends Pharmacol Sci **26:**265-73.

60. **Mayr, C., K. Richter, H. Lilie, and J. Buchner.** 2000. Cpr6 and Cpr7, two closely related Hsp90-associated immunophilins from Saccharomyces cerevisiae, differ in their functional properties. J Biol Chem **275:**34140-6.

61. **McClellan, A. J., M. D. Scott, and J. Frydman.** 2005. Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways. Cell **121:**739-48.

62. **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.

63. **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.

64. **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.

65. **Miorin, L., P. Maiuri, V. M. Hoenninger, C. W. Mandl, and A. Marcello.** 2008. Spatial and temporal organization of tick-borne encephalitis flavivirus replicated RNA in living cells. Virology **379:**64-77.

66. **Mnaimneh, S., A. P. Davierwala, J. Haynes, J. Moffat, W. T. Peng, W. Zhang, X. Yang, J. Pootoolal, G. Chua, A. Lopez, M. Trochesset, D. Morse, N. J. Krogan, S. L. Hiley, Z. Li, Q. Morris, J. Grigull, N. Mitsakakis, C. J. Roberts, J. F. Greenblatt, C. Boone, C. A. Kaiser, B. J. Andrews, and T. R. Hughes.** 2004. Exploration of essential gene functions via titratable promoter alleles. Cell **118:**31-44.

67. **Momose, F., T. Naito, K. Yano, S. Sugimoto, Y. Morikawa, and K. Nagata.** 2002. Identification of Hsp90 as a stimulatory host factor involved in Influenza virus RNA synthesis. J. Biol. Chem. **277:**45306-45314.

68. **Morozov, A., J. Subjeck, and P. Raychaudhuri.** 1995. HPV16 E7 oncoprotein induces expression of a 110 kDa heat shock protein. FEBS Lett **371:**214-8.

69. **Morse, S. S.** 1997. The public health threat of emerging viral disease. J Nutr **127:**951S-957S.

70. **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.

71. **Otto, G. A., and J. D. Puglisi.** 2004. The pathway of HCV IRES-mediated translation initiation. Cell **119:**369-80.

72. **Park, S. G., and G. Jung.** 2001. Human hepatitis B virus polymerase interacts with the molecular chaperonin Hsp60. J Virol **75:**6962-8.

73. **Parsell, D. A., A. S. Kowal, M. A. Singer, and S. Lindquist.** 1994. Protein disaggregation mediated by heat-shock protein Hsp104. Nature **372:**475-8.

74. **Peranen, J., and L. Kaariainen.** 1991. Biogenesis of type I cytopathic vacuoles in Semliki Forest virus-infected BHK cells. J Virol **65:**1623-7.

75. **Pratt, W. B., P. Krishna, and L. J. Olsen.** 2001. Hsp90-binding immunophilins in plants: the protein movers. Trends Plant Sci **6:**54-8.

76. **Pratt, W. B., and D. O. Toft.** 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev. **18:**306-360.

77. **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.

78. **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.

79. **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.

80. **Reyes-Del Valle, J., S. Chavez-Salinas, F. Medina, and R. M. Del Angel.** 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol **79:**4557-67.

81. **Rossi, J. M., and S. Lindquist.** 1989. The intracellular location of yeast heat-shock protein 26 varies with metabolism. J Cell Biol **108:**425-39.

82. **Ross-Macdonald, P., P. S. Coelho, T. Roemer, S. Agarwal, A. Kumar, R. Jansen, K. H. Cheung, A. Sheehan, D. Symoniatis, L. Umansky, M. Heidtman, F. K. Nelson, H. Iwasaki, K. Hager, M. Gerstein, P. Miller, G. S. Roeder, and M. Snyder.** 1999. Large-scale analysis of the yeast genome by transposon tagging and gene disruption. Nature **402:**413-8.

83. **Sahi, C., and E. A. Craig.** 2007. Network of general and specialty J protein chaperones of the yeast cytosol. Proc Natl Acad Sci U S A **104:**7163-8.

84. **Sapriel, G.** 2001. Prion model in yeast. Res Microbiol **152:**531-8.

85. **Schilling, B., T. De-Medina, J. Syken, M. Vidal, and K. Munger.** 1998. A novel human DnaJ protein, hTid-1, a homolog of the Drosophila tumor suppressor protein Tid56, can interact with the human papillomavirus type 16 E7 oncoprotein. Virology **247:**74-85.

86. **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:**181-189.

87. **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.

88. **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:**2162-2169.

89. **Shorter, J., and S. Lindquist.** 2008. Hsp104, Hsp70 and Hsp40 interplay regulates formation, growth and elimination of Sup35 prions. Embo J.

90. **Sohn, S. Y., J. H. Kim, K. W. Baek, W. S. Ryu, and B. Y. Ahn.** 2006. Turnover of hepatitis B virus X protein is facilitated by Hdj1, a human Hsp40/DnaJ protein. Biochem Biophys Res Commun **347:**764-8.

91. **Tomita, Y., T. Mizuno, J. Diez, S. Naito, P. Ahlquist, and M. Ishikawa.** 2003. Mutation of host DnaJ homolog inhibits Brome mosaic virus negative- strand RNA synthesis. J. Virol. **77:**2990-2997.

92. **Tsai, J., and M. G. Douglas.** 1996. A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. J Biol Chem **271:**9347-54.

93. **USDA** 2007, posting date. More about Barley Yellow Dwarf Virus. USDA. [Online.]

94. **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.

95. **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.

96. **Walsh, P., D. Bursac, Y. C. Law, D. Cyr, and T. Lithgow.** 2004. The J-protein family: modulating protein assembly, disassembly and translocation. EMBO Rep **5:**567-71.

97. **Wang, R. Y., and P. D. Nagy.** 2008. Tomato bushy stunt virus co-opts the RNA-binding function of a host metabolic enzyme for viral genomic RNA synthesis. Cell Host Microbe **3:**178-87.

98. **Wang, X., N. Grammatikakis, and J. Hu.** 2002. Role of p50/CDC37 in hepadnavirus assembly and replication. J Biol Chem **277:**24361-7.

99. **Wang, X. H., R. Aliyari, W. X. Li, H. W. Li, K. Kim, R. Carthew, P. Atkinson, and S. W. Ding.** 2006. RNA interference directs innate immunity against viruses in adult Drosophila. Science **312:**452-4.

100. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol Cell **19:**111-22.

101. **Waxman, L., M. Whitney, B. A. Pollok, L. C. Kuo, and P. L. Darke.** 2001. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc. Natl. Acad. Sci. USA **98:**13931-13935.

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

103. **Yochem, J., H. Uchida, M. Sunshine, H. Saito, C. P. Georgopoulos, and M. Feiss.** 1978. Genetic analysis of two genes, dnaJ and dnaK, necessary for Escherichia coli and bacteriophage lambda DNA replication. Mol Gen Genet **164:**9-14.

104. **Young, J. C., V. R. Agashe, K. Siegers, and F. U. Hartl.** 2004. Pathways of chaperone-mediated protein folding in the cytosol. Nat Rev Mol Cell Biol **5:**781-91.

105. **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.

106. **Young, J. C., I. Moarefi, and F. U. Hartl.** 2001. Hsp90: a specialized but essential protein-folding tool. J Cell Biol **154:**267-73.

107. **Zhang, X., J. M. Bourhis, S. Longhi, T. Carsillo, M. Buccellato, B. Morin, B. Canard, and M. Oglesbee.** 2005. Hsp72 recognizes a P binding motif in the measles virus N protein C-terminus. Virology **337:**162-74.

108. **Zhang, X., and M. Oglesbee.** 2003. Use of surface plasmon resonance for the measurement of low affinity binding interactions between HSP72 and measles virus nucleocapsid protein. Biol Proced Online **5:**170-181.

109. **Zylicz, M., D. Ang, K. Liberek, and C. Georgopoulos.** 1989. Initiation of lambda DNA replication with purified host- and bacteriophage-encoded proteins: the role of the dnaK, dnaJ and grpE heat shock proteins. Embo J **8:**1601-8.

CHAPTER II

THE HEAT SHOCK PROTEIN 70 CO-CHAPERONE *YDJ1* IS REQUIRED FOR EFFICIENT MEMBRANE-SPECIFIC FLOCK HOUSE VIRUS RNA REPLICATION COMPLEX ASSEMBLY AND FUNCTION IN YEAST

The assembly of RNA replication complexes on intracellular membranes is an essential step in the life cycle of positive-sense RNA viruses. We have previously shown that Hsp90 chaperone complex activity is essential for efficient Flock House virus (FHV) RNA replication in *Drosophila* S2 cells. To further explore the role of cellular chaperones in viral RNA replication, we used both pharmacologic and genetic approaches to examine the role of the Hsp90 and Hsp70 chaperone systems in FHV RNA replication complex assembly and function in *Saccharomyces cerevisiae*. In contrast to results with insect cells, yeast deficient in Hsp90 chaperone complex activity showed no significant decrease in FHV RNA replication. However, yeast with a deletion of the Hsp70 cochaperone *YDJ1* showed a dramatic reduction in FHV RNA replication that was due in part to reduced viral RNA polymerase accumulation. Furthermore, the absence of *YDJ1* did not reduce FHV RNA replication when the viral RNA polymerase and replication complexes were retargeted from the mitochondria to the endoplasmic reticulum. These results identify *YDJ1* as an essential membrane-specific host factor for FHV RNA replication complex assembly and function in *S. cerevisiae*, and are consistent with known differences in the role of distinct chaperone complexes in organelle-specific protein targeting between yeast and higher eukaryotes.

Introduction

Genome replication of positive-sense RNA viruses occurs within membrane-associated macromolecular complexes (5). Although the assembly of these highly active enzymatic complexes in association with intracellular membranes is a critical step in the positive-sense RNA virus life cycle, the mechanisms responsible for viral protein translation, folding, and transport to the appropriate membrane compartment within cells during viral RNA replication complex assembly are poorly understood. The heat shock proteins (Hsps) are a diverse set of molecular chaperones that facilitate cellular protein translation, folding, and trafficking (15). These abundant chaperones also participate in the assembly of membrane-associated protein complexes (47), suggesting that positive-sense RNA viruses may also use cytosolic Hsps as chaperones to assemble viral RNA replication complexes. Consistent with this hypothesis, cellular chaperones have been associated with the replication of numerous positive-sense RNA viruses, including hepatitis C virus (HCV) (42), cucumber necrosis virus (39), brome mosaic virus (BMV) (40), tomato mosaic virus (29), and Sindbis virus (13).

To study the role of cellular chaperones in viral RNA replication complex assembly and function, we use Flock House virus (FHV), a versatile positive-sense RNA virus and well-studied member of the *Nodaviridae* family (2). The utility of FHV as a model pathogen derives in part from its relatively small genome and robust replication in multiple eukaryotic hosts, including *Drosophila melanogaster* (14, 26), *Caenorhabditis elegans* (23) and *Saccharomyces cerevisiae* (22, 25, 27, 32, 33). The FHV genome is bipartite and consists of two capped but non-polyadenylated RNA segments (38). The larger 3.1-kb segment, RNA1, encodes protein A, the FHV RNA-dependent RNA polymerase (RdRp), which is essential for the assembly of functional viral RNA replication complexes (1, 2, 18, 22, 25, 33). The smaller 1.4-kb segment, RNA2, encodes the structural capsid protein precursor, which is dispensable for RNA replication

but necessary for infectious virion production (2). During viral RNA replication, protein A generates a subgenomic 0.4-kb RNA, RNA3, which is colinear with the 3' end of RNA1. RNA3 encodes the RNA interference suppressor protein B2 (21), which is required for FHV RNA replication in insects (21), plants (21), and nematodes (23), but not in yeast (33).

FHV RNA replication complexes assemble on the mitochondrial outer membrane in both insect cells (26) and yeast (25), and protein A is sufficient for their appropriate intracellular localization (25). FHV replication complexes are targeted and anchored to the mitochondrial outer membranes in part by an amino-proximal domain in protein A that resembles the transmembrane stop-transfer sequences present in several cellular mitochondrial outer membrane proteins (25, 27). The protein A mitochondrial targeting signal contains no discernable enzymatic function, as fully functional FHV RNA replication complexes are formed when the mitochondrial targeting signal is replaced with a sequence that contains an endoplasmic reticulum (ER) targeting domain (27). Thus, FHV provides a versatile system to examine the role of both general and membrane-specific host factors in viral RNA replication complex assembly and function.

We have previously shown that the cellular chaperone Hsp90 facilitates the assembly of functional FHV RNA replication complexes in *Drosophila* S2 cells (8, 19), consistent with the demonstrated role of this abundant cytosolic chaperone in the transport of cellular mitochondrial proteins in higher eukaryotes (47). In this report, we further explore the role of cellular chaperones in FHV RNA replication complex assembly and function using *S. cerevisiae* as a eukaryotic host. The facile genetics and ready availability of yeast strains with deletions, mutations, or regulated expression of individual genes makes *S. cerevisiae* a useful model host to examine the impact of unique cellular proteins on viral RNA replication (20, 39, 40). We demonstrate that disruption of Hsp90 chaperone complex activity did not significantly impact FHV RNA

replication in *S. cerevisiae.* In contrast, deletion of *YDJ1,* an Hsp40 family co-chaperone required for Hsp70 chaperone complex activity (7), significantly reduced FHV RNA production when replication complexes were targeted to the mitochondrial membrane, but had only a marginal effect on ER-targeted FHV RNA replication complex function. These results demonstrate both host- and membrane-specific differences in the role of cellular chaperones in viral RNA replication complex assembly and function.

Materials and Methods

Yeast strains, transformation, and culture conditions. The diploid *S. cerevisiae* strain BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0*) was used for all experiments to minimize complications related to second site mutations more common in a haploid strains. Wild type (wt) BY4743 and diploid strains with homozygous deletions of *SBA1*, *STI1*, or *YDJ1* were purchased from the American Type Culture Collection (Manassas, VA). Yeast were cultured and transformed as previously described (25), except that the temperature-sensitive phenotype of *Δydj1* yeast required growth at 25°C for 7 days after transformation for satisfactorily large colonies to develop. Due to the temperature-sensitive nature of FHV RNA replication complexes (unpublished observations), and slow growth or inviability of the mutant strains at higher temperatures, as well as to maintain consistency with *Drosophila* studies, all experiments were performed at 25°C. For induction of *Δsba1* and *Δsti1* yeast, individual clones were transferred to liquid selective minimal media containing 2% glucose and grown to stationary phase, washed with sterile distilled water and resuspended in selective minimal media with 2% galactose at an optical density unit at 600 nm (OD_{600}) of 0.1, which was equivalent to approximately 2.5 x 10^6 cells per ml for both wt and deletion strains. For induction of *Δydj1* yeast, cells were grown in liquid selective minimal media with 2% raffinose, washed in sterile distilled water and resuspended in selective minimal media with 2% raffinose plus 2% galactose at an OD_{600} of 0.1. Unless otherwise stated, experiments were performed with two independently derived clones and results are representative of at least three independent experiments.

Plasmids. FHV expression plasmids pF1, $pF1_{fs}$, and pFA-C/HA have been previously described (22, 25, 27, 33). The retargeted protein A expression plasmid

pFA(HCV)-C/HA was generated by inserting the NheI/HindIII fragment from pFA-C/HA into pFA-HCV (27). FHV RNA1 expression plasmids pF1 and $pF1_{fs}$ encode galactose-inducible cDNA copies of FHV RNA1 with authentic viral 5' and 3' ends that are generated by precise transcription initiation and a hepatitis delta ribozyme, respectively, and thus contain the necessary cis elements to serve as replication templates (Figure 2.1). In addition, the RNA1 transcribed from pF1 can be translated into protein A and initiate RNA replication in cis, which links protein A accumulation to viral RNA replication (Figure 2.1, left). In contrast, the RNA template transcribed from plasmid $pF1_{fs}$ contains an early frameshift-induced stop codon and therefore can not be translated into protein A. Functional protein A is provided from a second plasmid, pFA-C/HA or pFA(HCV)-C/HA to initiate RNA replication in trans, where protein A accumulation is independent of viral RNA replication (Figure 2.1, right). The protein A expression plasmids pFA-C/HA and pFA(HCV)-C/HA encode a galactose-inducible C-terminally hemagglutinin (HA)-tagged protein A open reading frame (ORF) flanked by an upstream *GAL1* leader sequence and a downstream *CYC1* polyadenylation signal sequence. RNAs transcribed from pFA-C/HA and pFA(HCV)-C/HA are efficiently translated into protein A, but do not have the necessary cis elements to serve as replication templates.

The yeast complementation plasmid pYDJ1 was generously provided by Masayuki Ishikawa (Hokkaido University, Sapporo, Japan) (40) and the plasmids pSC-FLGR and pSC-cGR, which encode the steroid dependent full-length or constitutively active C-terminally truncated glucocorticoid receptor (GR), respectively, and the steroid-responsive reporter plasmid pSC-GRE-LacZ, were generously provided by Jorgé Iñiguéz (University of Michigan) (17). The plasmid pGAL-LacZ-HA, a galactose-inducible, HA-tagged β-galactosidase expression construct was generated by inserting a PstI/SalI fragment containing the *LacZ* gene encoding a C-terminally HA-tagged protein into the PstI/SalI sites of pFA (33).

Antibodies and reagents. Rabbit polyclonal antibodies against FHV protein A have been previously described (26). Rabbit polyclonal antibodies against the HA epitope tag were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibodies against porin and 3-phosphoglycerate kinase (PGK) were purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies against yeast Ydj1p were generously provided by Masayuki Ishikawa (Hokkaido University, Sapporo, Japan). All secondary antibodies for immunoblotting were purchased from Jackson Immunoresearch (West Grove, PA). The Hsp90-specific inhibitor geldanamycin was purchased from Sigma (St. Louis, MO) and stored as a stock solution in dimethylsulfoxide at -20^{o}C. Deoxycorticosterone was generously provided by Jorgé Iñiguéz and was also stored as a stock solution at -20^{o}C.

Immunoblot and Northern blot analysis. Total protein was isolated from an equivalent number of yeast as previously described (25) and stored at –20^{o}C until analysis. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as previously described (19). Total RNA was isolated from yeast using hot acidic phenol as previously described (33) and stored at –80^{o}C until analysis. RNA samples were denatured in sample buffer containing 60% formamide and 7% formaldehyde, separated on formaldehyde-1% agarose gels, transferred to ZetaProbe nylon membranes (Bio-Rad, Hercules, CA) by passive capillary transfer overnight, UV-crosslinked at 120 mJ per cm^2 and probed with either digoxygenin-UTP or ^{32}P-UTP labeled strand-specific riboprobes as previously described (26). Protein and RNA bands were quantitated by densitometry using either AlphaEaseFC (Alpha Innotech, San Leandro, CA) or Image Quant TL (Amersham Biosciences, Piscataway, NJ) software.

Glucocorticoid receptor-based Hsp90 activity assay. Yeast transformed with pSC-GRE-LacZ and pSC-FLGR were inoculated into selective media in 96-well plates

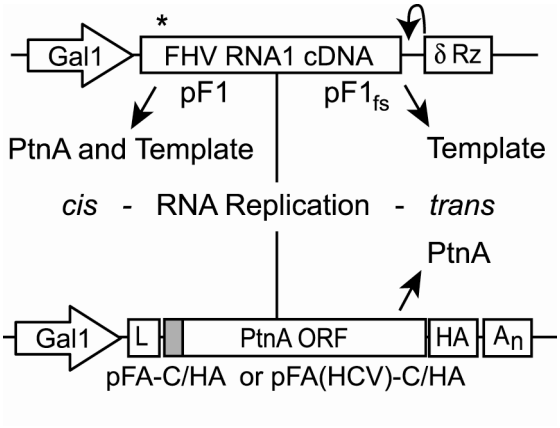


Figure 2.1. Schematics of FHV replicons. RNA replication in cis (left) is initiated when wild type FHV RNA1 is transcribed from plasmid pF1 by host DNA polymerase II. Authentic 5' and 3' ends are generated by precise transcription initiation and a hepatitis delta ribozyme (δ Rz), respectively, and therefore RNA1 from pF1 can both be translated into protein A (PtnA) and function as an RNA replication template. RNA replication in trans (right) requires that the replication template and PtnA are provided by separate plasmids. The RNA1 template is transcribed from plasmid $pF1_{fs}$, which contains an early frame shifting mutation (asterisk) that introduces a premature stop codon. Functional protein A is provided in trans from a second plasmid, either pFA-C/HA or pFA(HCV)-C/HA, which generate mRNAs with modified 5' and 3' untranslated regions that optimize their translation by introduction of the *GAL* leader (L) and *CYC1* polyadenylation signal (A_n) but prevent their utilization as replication templates. The shaded box indicates the location of the membrane targeting signal that is modified in pFA(HCV)-C/HA.

and grown in a moist chamber until saturation, diluted 40-fold into fresh selective test media containing vehicle, 10 μM deoxycorticosterone, or 10 μM deoxycorticosterone and 10 μM geldanamycin, and incubated overnight at 25°C to induce GR-responsive and Hsp90-dependent β-galactosidase expression. For Hsp90- and steroid-independent GR activity, yeast transformed with pSC-GRE-LacZ and pSC-cGR were cultured in a similar manner but without deoxycorticosterone or geldanamycin. To quantitate reporter gene activity, cells were permeabilized with 120 mM sodium phosphate (pH 7.0), 10 mM potassium chloride, 1 mM magnesium sulfate, 2.5% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 20 mM dithiothreitol for 15 min at 37°C and incubated with 0.5 mM chlorophenol red-β-D-galactopyranoside (Roche). Substrate conversion was measured by monitoring the change in OD_{550} over time and β-galactosidase activity was normalized to cell density.

Membrane flotation and carbonate extraction. Thirty OD_{600} units of yeast were harvested by centrifugation, washed once with distilled water, and converted to spheroplasts with 1000 U lyticase in 2 ml spheroplasting (SP) buffer (1 M sorbitol, 0.1 M potassium phosphate [pH 7.6]) containing 0.2% β-mercaptoethanol for 20 min at 25°C. Spheroplasts were washed once in SP buffer, resuspended in 0.5 ml lysis buffer (50 mM HEPES [pH 7.5], 50 mM potassium chloride, 5 mM EDTA, 2 mM magnesium chloride, and a 1:100 dilution of yeast protease inhibitor cocktail [Sigma P8215]), stored on ice for 10 min, lysed with 20 strokes of a 2 ml Dounce homogenizer, and centrifuged at 500 x *g* for 5 min to pellet unlysed cells and nuclei. Clarified lysates were mixed with 50% nycodenz to a final concentration of 37.5% and 1.2 ml were loaded under a 3.4 ml 5-35% discontinuous nycodenz gradient in Beckman 13 x 51 mm ultracentrifuge tubes and centrifuged at 100,000 x *g* in a Beckman MLS 50 rotor for 24 h at 4°C. Equal volume fractions were collected from both top (low density, LD) and bottom (high density, HD)

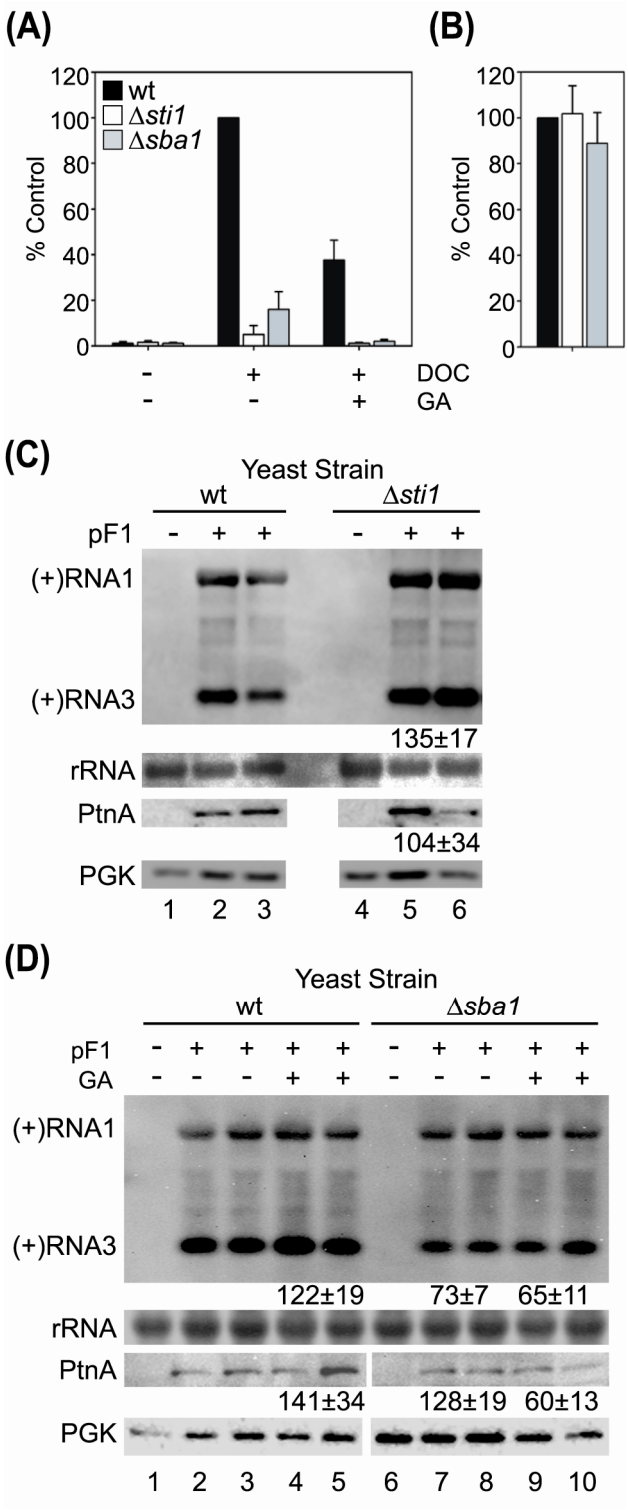
regions of the gradient as previously described (25, 27). For carbonate extraction experiments, LD fractions were pelleted by centrifugation at 17,000 x *g* for 15 min at 4°C, resuspended in either lysis buffer or in 0.1 M sodium carbonate (pH 11), incubated on ice for 30 min, and centrifuged at 17,000 x *g* for 15 min at 4°C to separate samples into carbonate-extracted supernatant (CS) and pellet (CP) fractions. Final samples were analyzed by SDS-PAGE and immunoblotting as described above.

Statistics. A two-tailed Student's *t*-test assuming unequal variances was used for all statistical analyses, and a p-value < 0.05 was considered statistically significant.

Results

FHV RNA replication in *S. cerevisiae* is independent of Hsp90 chaperone complex activity. We have previously shown that inhibition of Hsp90 activity suppresses FHV RNA replication in *Drosophila* S2 cells (19). Since cellular chaperone complexes in yeast and higher eukaryotes are similar in composition but may differ in function (47), we examined the impact of the Hsp90 chaperone complex on FHV RNA replication in *S. cerevisiae* (Figure 2.2). Yeast are less sensitive to Hsp90-specific pharmacologic inhibitors such as geldanamycin due in part to decreased cell permeability (3), and thus we used both pharmacologic and genetic approaches to disrupt *S. cerevisiae* Hsp90 chaperone complex activity. Yeast express two Hsp90s that are thought to be functionally redundant, which are encoded by the constitutive *HSC82* and the inducible *HSP82* genes (4). The deletion of both genes is lethal (28), and therefore we used yeast strains with homozygous deletions of the Hsp90 cochaperones *STI1* and *SBA1*. These genes encode the yeast orthologs of Hsp-organizing protein and p23, respectively, and their deletions disrupt Hsp90 chaperone complex activity without significantly altering cell viability (3, 9). To verify the functional chaperone defect in *Δsti1* and *Δsba1* yeast, we used a glucocorticoid receptor (GR)-based reporter assay (17). The maturation of mammalian steroid receptors into a ligand-ready state is dependent upon a functional Hsp90 chaperone complex (31). Since the *S. cerevisiae* genome does not encode a GR, we transformed yeast with both a mammalian GR expression plasmid and a glucocorticoid response element-driven β-galactosidase reporter plasmid, induced cells with deoxycorticosterone, and measured reporter gene activity by β-galactosidase assay (Figure 2.2A). In wt BY4743 yeast transformed with the Hsp90-dependent reporter system, 10 μM deoxycorticosterone induced the robust production of β-galactosidase. Consistent with the reduced sensitivity of yeast to the inhibitory activity of

Figure 2.2 - FHV RNA replication in *S. cerevisiae* is independent of Hsp90 chaperone complex activity. (A) Inducible Hsp90-dependent GR activity. Wt (black bars), *Δsti1* (white bars), and *Δsba1* (grey bars) yeast transformed with pSC-GRE-LacZ and pSC-FLGR were induced with 10 μM deoxycorticosterone (DOC) in the presence or absence of 10 μM geldanamycin (GA), and β-galactosidase activity was measured by colorimetric assay. Results are expressed as the percent activity relative to DOC-induced β-galactosidase in wt yeast. (B) Constitutive Hsp90-independent GR activity. Wild type (black bars), *Δsti1* (white bars), and *Δsba1* (grey bars) yeast transformed with pSC-GRE-LacZ and pSC-cGR were assayed for β-galactosidase activity, and results are expressed as the percent activity relative to wt yeast. (C) FHV RNA replication in *Δsti1* yeast. Yeast transformed with vector control (lanes 1 and 4) or pF1 (lanes 2-3 and 5-6) were induced with galactose for 24 h, and total protein and RNA were isolated and analyzed by immunoblot and Northern blot, respectively. The position of FHV genomic and subgenomic positive-sense RNA ((+)RNA1 and (+)RNA3, respectively) are shown on the left. Ribosomal RNA (rRNA) and PGK are shown as loading controls. (D) FHV RNA replication in *Δsba1* yeast. Yeast transformed with vector control (lanes 1 and 6) or pF1 (lanes 2-5 and 7-10) were induced with galactose for 24 h in the presence of vehicle (lanes 1-3 and 6-8) or 10 μM GA (lanes 4-5 and 9-10), and total protein and RNA were isolated and analyzed by immunoblot and Northern blot, respectively. Labels and loading controls are as in (C). Numbers represent the percent accumulation of (+)RNA3 and protein A (PtnA) in *Δsti1* or *Δsba1* samples compared to wt control (lanes 2 and 3). There was no difference in growth between wt, *Δsti1* and *Δsba1* yeast after induction with galactose (data not shown). When cropping was necessary, all panels were from the same exposure of the blot, and all contrast adjustments to the initial image were done prior to cropping.


(A)
(B)
wt
Δsti1
Δsba1
% Control
120
100
80
60
40
20
0
- + + DOC
- - + GA
(C)
Yeast Strain
wt
Δsti1
pF1 - + + - + +
(+)RNA1
(+)RNA3
135±17
rRNA
PtnA
104±34
PGK
1 2 3 4 5 6
(D)
Yeast Strain
wt
Δsba1
pF1 - + + + + - + + + +
GA - - - + + - - - + +
(+)RNA1
(+)RNA3
122±19
73±7
65±11
rRNA
PtnA
141±34
128±19
60±13
PGK
1 2 3 4 5 6 7 8 9 10

pharmacologic Hsp90 inhibitors (3), 10 μM geldanamycin reduced β-galactosidase reporter gene activity by 60% in wt yeast (Figure 2.2A), in comparison to the greater than 90% reduction of GR-dependent reporter gene activity in *Drosophila* S2 cells treated with 5 μM geldanamycin (19). In contrast to results with wt yeast, deletion of *STI1* reduced β-galactosidase activity by greater than 90% even in the absence of geldanamycin (Figure 2.2A). Similar results were obtained with *Δsba1* yeast, where in the absence of geldanamycin steroid induced-β-galactosidase activity was 16% of wt, and this residual activity was reduced to background levels with 10 μM geldanamycin (Figure 2.2A). A truncated and constitutively active form of the GR without ligand-binding domains, which is Hsp90- and steroid-independent, induced equivalent β-galactosidase activity in wt, *Δsti1*, and *Δsba1* yeast (Figure 2.2B), indicating that the decrease in deoxycorticosterone-induced reporter activity seen in the absence of *STI1* and *SBA1* was due to a defect in Hsp90 chaperone complex-dependent GR maturation.

To examine the impact of Hsp90 activity on FHV RNA replication in yeast, we transformed wt, *Δsti1*, and *Δsba1* yeast with pF1, induced cells with galactose for 24 h, and analyzed FHV protein A and RNA accumulation by immunoblotting and Northern blotting, respectively (Figures 2.2C and 2.2D). In contrast to the potent inhibitory effect on Hsp90-dependent reporter gene activity (Figure 2.2A), the deletion of *STI1* did not suppress FHV RNA replication, measured by positive strand (+)RNA1, (+)RNA3 or protein A accumulation (Figure 2.2C, lanes 5 and 6). Although there was an approximate 30% reduction in FHV RNA replication in *Δsba1* yeast (Figure 2.2D, lanes 7 and 8), this was potentially due to an Hsp90-independent function of *SBA1* (11, 12), as the addition of geldanamycin did not further suppress FHV RNA replication in *Δsba1* yeast (Figure 2.2D, lanes 9 and 10) despite its inhibitory effect on residual GR-dependent reporter gene activity (Figure 2.2A). Furthermore, we saw no reduction in

FHV RNA replication in wt yeast treated with geldanamycin (Figure 2.2D, lanes 4 and 5), despite a 60% reduction in Hsp90 complex chaperone activity (Figure 2.2A). Taken together, these results indicated that FHV RNA replication in *S. cerevisiae* was not dependent upon a functional Hsp90 chaperone complex.

FHV RNA replication in *S. cerevisiae* is dependent on the Hsp70 co-chaperone *YDJ1.* One previously described difference in the chaperone activity between yeast and higher eukaryotes is the differential role of Hsp70 and Hsp90 in the delivery of preproteins to the mitochondrial import machinery. In higher eukaryotes, mitochondrial protein targeting and import are facilitated by both the Hsp70 and Hsp90 chaperone complexes, whereas in yeast only the Hsp70 complex is required (47). FHV RNA replication complexes assemble on mitochondrial membranes in both *Drosophila* S2 cells and yeast (25, 26). Since our results indicated that FHV RNA replication was not dependent upon functional Hsp90 chaperone complex activity in *S. cerevisiae,* we subsequently examined the role of the Hsp70 chaperone complex. *S. cerevisiae* encodes at least nine cytosolic isoforms of Hsp70, some of which are thought to have partially overlapping functions (43). The Hsp70 SSA subfamily has been specifically implicated in import into and transport across cellular membranes (6, 10), suggesting that this group of yeast cytosolic Hsp70 chaperones may be particularly relevant to FHV RNA replication complex assembly and function. Deletion of all four SSA genes is lethal (43), and therefore to examine the role of the Hsp70 chaperone complex in FHV RNA replication we used a diploid yeast strain with a homozygous deletion of the Hsp70 co-chaperone *YDJ1*. *YDJ1* encodes the yeast ortholog of Hsp40/DnaJ, whose deletion is not lethal but still disrupts Hsp70 chaperone complex activity (7).

Initial experiments indicated that deletion of *YDJ1* in the BY4743 background resulted in a prominent growth defect in selective minimal media with glucose as the carbon source that produced a doubling time approximately two- to three- fold longer

than wt yeast, and induced a growth arrest in selective minimal media with galactose as the sole carbon source (data not shown). Thus, to induce FHV replicon expression from the *GAL1* promoter-driven plasmid pF1, we initially cultured *Δydj1* and wt yeast in selective media with raffinose and transferred to a raffinose-galactose combination to induce replicon expression. To minimize the effects of cell growth on RNA replication, we harvested cells prior to stationary phase for both wt and *Δydj1* yeast. Under these growth and induction conditions, deletion of *YDJ1* greatly reduced FHV RNA replication in yeast, when measured by the accumulation of (+)RNA1, (+)RNA3, or protein A (Figure 2.3A). To ensure that the defect in FHV RNA replication was not due to reduced *GAL1* promoter activity in the absence of *YDJ1*, we transformed yeast with the reporter plasmid pGAL-LacZ-HA, induced for 16 h, and assayed for β-galactosidase activity (Figure 2.3B). There was no difference between wt and *Δydj1* yeast in galactose-induced β-galactosidase activity, indicating that the reduced FHV RNA replication in *Δydj1* yeast was not due to a defect in *GAL1* promoter activity. Since FHV protein A accumulation and RNA replication are linked processes in cells transformed with pF1 (see Figure 2.1), we could not determine from these experiments whether the primary defect in *Δydj1* yeast was reduced viral RNA polymerase accumulation or replication complex function. Nevertheless, these results suggested that in contrast to the Hsp90 co-chaperones *STI1* and *SBA1*, the Hsp70 co-chaperone *YDJ1* was important for efficient FHV RNA replication in *S. cerevisiae*.

We further explored FHV RNA replication in *Δydj1* yeast using the two-plasmid trans replication system depicted in Figure 2.1 to separate viral RNA polymerase accumulation from function, and complemented *Δydj1* mutant yeast with a constitutive exogenous copy of the co-chaperone expressed from plasmid pYDJ1 (Figure 2.4). We transformed wt, *Δydj1*, and pYDJ1-complemented *Δydj1* yeast with pFA-C/HA and $pF1_{fs}$,

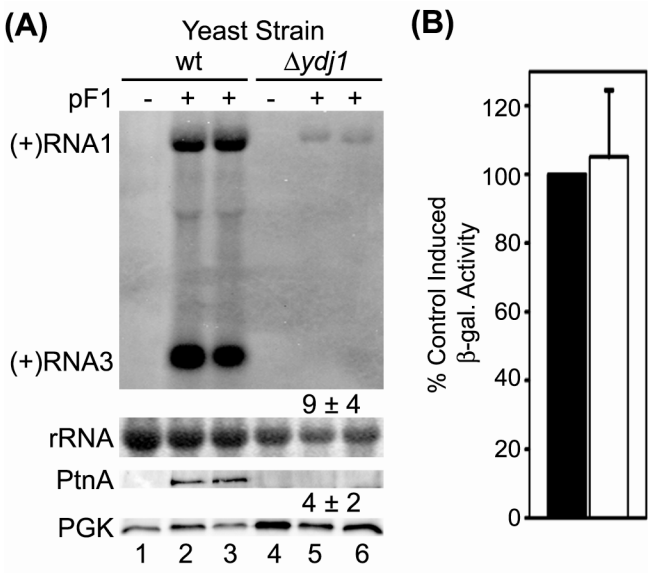
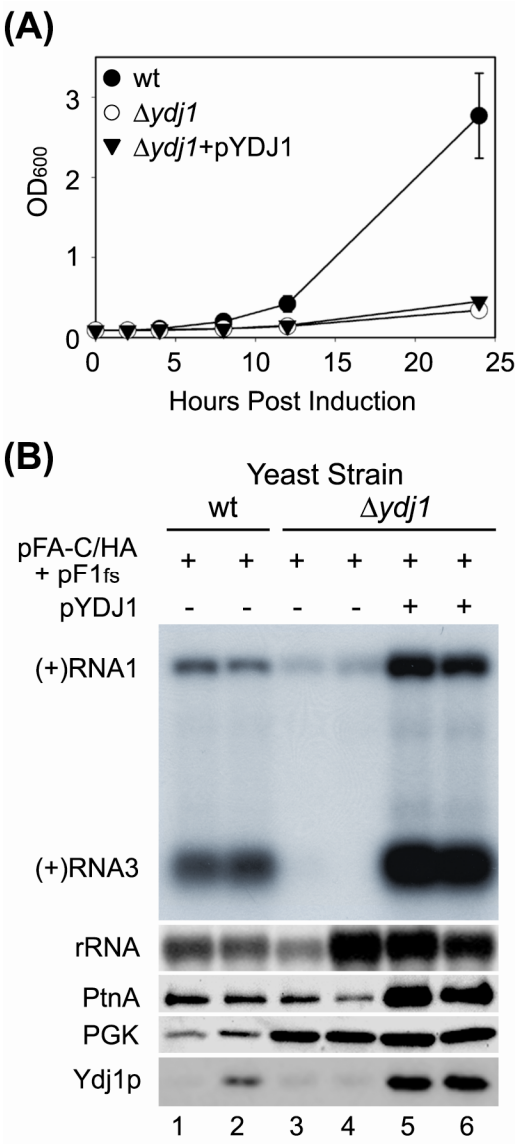


Figure 2.3 - FHV RNA replication is dependent on the Hsp70 co-chaperone *YDJ1*. (A) Wt and *Δydj1* yeast transformed with vector control (lanes 1 and 4) or pF1 (lanes 2-3 and 5-6) were induced with galactose for 24 h, and total protein and RNA were isolated and analyzed by immunoblot and Northern blot, respectively. Results are presented as described in Figure 2. Total protein and RNA recovery in *Δydj1* yeast were increased compared to an equivalent number of wt yeast, and therefore sample loading was adjusted and resulted in underloading for *Δydj1* RNA samples. Numbers represent the percent accumulation of (+)RNA3 and protein A compared to wt control and normalized to total RNA and protein levels (lanes 2 and 3). (B) *GAL1* promoter activity in *Δydj1* yeast. Wt (black bars) and *Δydj1* (white bars) yeast transformed with pGAL-LacZ-HA were induced with galactose for 16 h and β-galactosidase activity was measured by colorimetric assay. Results were adjusted to cell number and are expressed as the percent activity relative to wt induced samples. β-galactosidase activity in uninduced samples was < 1% of wt induced levels.

Figure 2.4 - FHV RNA replication in *trans* in *Δydj1* yeast. (A) Growth of wt, *Δydj1, and* complemented (*Δydj1*+pYDJ1) yeast. Yeast transformed with pFA-C/HA and $pF1_{fs}$ were induced in selective media with 2% raffinose plus 2% galactose and cell density was measured by spectrophotometry (OD_{600}). Note that *Δydj1* and complemented (*Δydj1*+pYDJ1) yeast have the same growth kinetics. Wt yeast saturate at an OD_{600} of 4 to 5 in selective media with 2% raffinose and 2% galactose (data not shown). (B) FHV RNA replication in wt, *Δydj1*, and complemented *Δydj1* yeast. Yeast transformed with pFA-C/HA and $pF1_{fs}$ were induced for 24 h and total RNA and protein were isolated from an equal number of cells and analyzed by Northern blotting and immunoblotting, respectively. Note that total RNA and protein accumulation was increased in the *Δydj1* strain in both the presence and absence of complementation. We confirmed the genetic deletion of the *YDJ1* locus in *Δydj1* yeast by PCR (data not shown), and thus the faint bands in lanes 3 and 4 in the Ydj1p blot represent co-migrating cellular proteins that cross reacted with the Ydj1p antisera. Note that protein in lane 1 is under loaded (PGK and Ydj1p blots) and reflects some variability between replicates, which is the reason two clones are used for each condition.


(A)
wt
Δydj1
Δydj1+pYDJ1
OD600
0
1
2
3
0
5
10
15
20
25
Hours Post Induction
(B)
Yeast Strain
wt
Δydj1
pFA-C/HA
+ pF1fs
pYDJ1
+ + + + + +
- - - - + +
(+)RNA1
(+)RNA3
rRNA
PtnA
PGK
Ydj1p
1 2 3 4 5 6

induced with raffinose-galactose, and initially examined cell growth for 24 h under induction conditions (Figure 2.4A). Proliferation of *Δydj1* yeast was markedly reduced compared to wt yeast with calculated doubling times of 12 h and 5 h, respectively. Surprisingly, this growth defect was not rescued by pYDJ1-mediated complementation despite Ydj1p overexpression (see Figure 2.4B, Ydj1p blot lanes 5 and 6), potentially due to unregulated co-chaperone expression. Nevertheless, the similar growth phenotypes of *Δydj1* yeast in the presence and absence of pYDJ1 complementation allowed us to directly examine the impact of the Hsp70 co-chaperone on FHV RNA replication without potential confounding effects related to cell growth.

We analyzed FHV RNA replication and protein A accumulation in wt, *Δydj1*, and pYDJ1-complemented *Δydj1* yeast transformed with pFA-C/HA and $pF1_{fs}$ at 24 h after galactose induction by quantitative Northern blotting and immunoblotting (Figure 2.4B). FHV RNA replication in trans was significantly reduced in *Δydj1* compared to wt yeast (Figure 2.4B, compare upper blot lanes 1 and 2 to lanes 3 and 4), consistent with cis replication results (Figure 2.3). Furthermore, FHV RNA replication was rescued by complementation with pYDJ1 (Figure 2.4B, lanes 5 and 6) in contrast to the growth phenotype noted above. When we quantified FHV RNA accumulation normalized to cell number, *Δydj1* yeast displayed a greater than 70% reduction in (+)RNA3 levels compared to wt, whereas complementation with pYDJ1 resulted in an increase in (+)RNA3 accumulation to levels greater than in wt yeast (Table 2.1). Similar results were seen with genomic (+)RNA1 accumulation, although the reduction in *Δydj1* yeast was attenuated likely due to the contribution of replication-independent genomic (+)RNA1 production from host RNA polymerase II-directed transcription. The defect in RNA replication was also seen in vitro using extracts from wt, *Δydj1* and complemented yeast (Appendix, Figure 2.8). These results indicated that *YDJ1* was necessary for

Table 2.1 - Quantitative analysis of the effect of *YDJ1* on FHV RNA replication in *S. cerevisiae*

		Mitochondrial targeted protein A			ER targeted protein A		
Normalization		*Δydj1*	*Δydj1*+ pYDJ1	Fold Increase[a]	*Δydj1*	*Δydj1*+ pYDJ1	Fold Increase[a]
Cell Number	(+)RNA1	73 ± 26	371 ± 63*	5.1	143 ± 30	314 ± 13*	2.2
	(+)RNA3	27 ± 19	319 ± 41*	11.8	102 ± 27	177 ± 25	1.7
	Protein A	140 ± 11	1130 ± 445	8.1	245 ± 83	513 ± 69	2.1
Total RNA	(+)RNA1	44 ± 14	220 ± 40*	5.0	112 ± 32	245 ± 16*	2.2
	(+)RNA3	16 ± 11	188 ± 19*	11.8	80 ± 21	138 ± 21	1.7
Total Protein	Protein A	32 ± 8	208 ± 29*	6.5	62 ± 15	160 ± 22*	2.6

Numbers represent percent of wt control and are the mean ± SEM from three independent experiments.
Asterisk indicates a *p-value* <0.05 compared to *Δydj1* yeast.
[a] Fold increase of pYDJ1-complemented yeast over *Δydj1* yeast.

efficient assembly and function of mitochondrial-targeted FHV RNA replication complexes in *S. cerevisiae*.

In contrast to FHV RNA accumulation, the deletion of *YDJ1* had less of an apparent impact on protein A accumulation (Figure 2.4B). When we quantitated immunoblot results and normalized to cell number, there was no significant decrease in protein A accumulation in *Δydj1* yeast, although complementation with pYDJ1 increased protein A recovery eight-fold (Table 2.1). However, it should be noted that the slow growth phenotype of *Δydj1* mutant and complemented yeast (Figure 4A) resulted in a three- to five-fold increase in the per cell accumulation of total protein and RNA compared to wt yeast (Figure 2.4B, compare rRNA gel and PGK blot in lanes 1 and 2 to lanes 3 through 6). When we normalized protein accumulation results to total protein loading controls, there was a 68% reduction in protein A accumulation in *Δydj1* yeast that was fully complemented by pYDJ1 (Table 2.1). These results suggested that the FHV RNA replication defect in *Δydj1* yeast was due in part to decreased viral polymerase accumulation.

***YDJ1* is not essential for efficient ER-targeted FHV RNA replication complex activity.** Although cytosolic Hsps are ubiquitous chaperones that influence a wide range of cellular processes, membrane-specific activities for particular chaperones have been described (6, 10). To examine whether the role of *YDJ1* in FHV RNA replication complex assembly and function was membrane-specific, we used a replicon-based system that retargets FHV RNA replication complexes to the ER (27). The wt amino-proximal mitochondrial targeting signal in protein A can be replaced with the ER targeting signal from the HCV NS5B protein to generate the chimeric protein A expression plasmid pFA(HCV)-C/HA (27). We transformed wt, *Δydj1*, and pYDJ1-complemented *Δydj1* yeast with pFA(HCV)-C/HA and $pF1_{fs}$, induced with raffinose-

galactose for 24 h, and analyzed (+)RNA1, (+)RNA3, and protein A accumulation (Figure 2.5 and Table 2.1). In contrast to results with FHV RNA replication complexes targeted to mitochondria (Figure 2.4B), deletion of *YDJ1* had a minimal impact on ER-targeted FHV RNA replication complex activity (Figure 2.5, lanes 3 and 4). After we normalized results for total protein and RNA levels, ER-targeted FHV protein A accumulation was reduced only 38% compared to 68% for mitochondria-targeted protein A in *Δydj1* yeast, whereas there was no substantial decrease in (+)RNA1 or (+)RNA3 accumulation (Table 2.1). These results were also mirrored in vitro using extracts from wt, *Δydj1* and complemented yeast (data not shown). Complementation with pYDJ1 did increase (+)RNA1, (+)RNA3 and protein A accumulation (Figure 2.5, lanes 3 and 4 compared to lanes 5 and 6), and a quantitative analysis showed an approximate two-fold increase in FHV RNA and protein A accumulation with Ydj1p overexpression (Table 2.1). This is consistent with the finding that overexpression of Ydj1p also increased the accumulation of β-galactosidase expressed from a *GAL1* promoter-driven plasmid (data not shown), suggesting that co-chaperone overexpression stimulated galactose-inducible promoter activity. These results indicated that in contrast to FHV RNA replication complexes targeted to the mitochondria, those targeted to the ER did not require *YDJ1* for efficient assembly and function.

Protein A is membrane associated in *Δydj1* yeast. FHV protein A is tightly associated with intracellular membranes in *S. cerevisiae* (25), and this membrane association is important for complete viral RNA replication complex activity (44, 45). Thus, we examined whether the defect in FHV RNA replication in *Δydj1* yeast was due in part to decreased membrane association of the viral polymerase (Figure 2.6). Protein A has an amino-proximal transmembrane domain that mediates its membrane association and renders it resistant to alkaline extraction (25). We used this biochemical

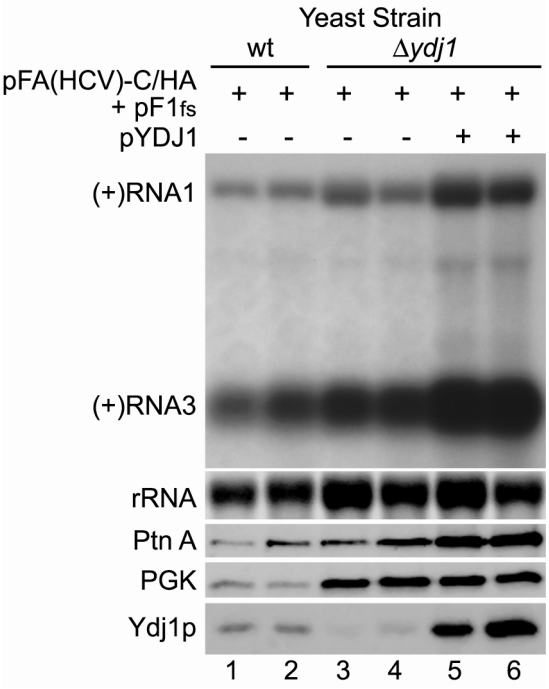


Figure 2.5 - *YDJ1* is not essential for ER-targeted FHV RNA replication complex activity. Wt, *Δydj1*, and complemented *Δydj1* yeast transformed with pFA(HCV)-C/HA and $pF1_{fs}$ were induced for 24 h, and total RNA and protein were isolated and analyzed as described in Figure 4.

characteristic in a density-based flotation technique to examine protein A membrane association in *Δydj1* yeast (Figure 2.6A). We subjected whole cell lysates to equilibration centrifugation in nycodenz gradients and isolated low density (LD) and high density (HD) fractions, which represented membrane-associated and soluble proteins, respectively (25). The LD fraction was further subjected to alkaline extraction and differential centrifugation to separate peripheral from integral membrane proteins. We analyzed fractions by immunoblotting for FHV protein A, the cytosolic enzyme PGK, and the integral membrane protein porin (Figure 2.6B). Consistent with previous observations (25, 26), the majority of protein A was recovered in the LD fraction from wt yeast, and neither the deletion of *YDJ1* nor its complementation altered this distribution (Figure 2.6B, lanes 1 and 2). Furthermore, 84%, 91%, and 81% of membrane-associated protein A was resistant to alkaline extraction in LD fractions from wt, *Δydj1*, and pYDJ1-complemented strains, respectively (Figure 2.6B, lanes 3 and 4). We obtained similar results with ER-retargeted protein A (Figure 2.6C). These results indicated that the functional defect in RNA replication complex activity in *Δydj1* yeast was not due to grossly altered protein A membrane association.

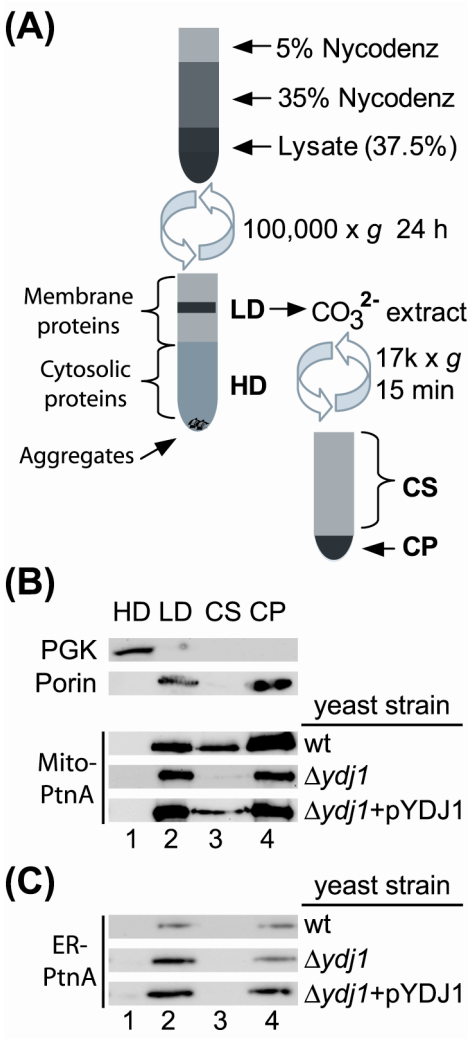


Figure 2.6 - Protein A is tightly membrane associated in *Δydj1* yeast. (A) Schematic of membrane flotation protocol. Whole cell lysates were mixed with nycodenz to 37.5%, loaded under a discontinuous 5-35% nycodenz gradient, and spun to equilibrium at 100,000 x *g*. Equal volume samples were taken from the top low density (LD) fraction, and bottom high density (HD) fraction. LD fractions were further subjected to carbonate extraction at pH 11 for 30 min on ice, and subsequently pelleted and separated into supernatant (CS) and pellet (CP) fractions by differential centrifugation. (B) Membrane flotation and carbonate extraction of membranes from wt, *Δydj1*, and complemented (*Δydj1*+ p*YDJ1*) yeast transformed with pFA-C/HA and $pF1_{fs}$. Yeast 3-phosphoglycerate kinase (PGK) and porin are cytosolic and membrane proteins, respectively. (C) Membrane flotation and carbonate extraction of membranes from wt, *Δydj1*, and complemented (*Δydj1*+ p*YDJ1*) yeast transformed with pFA(HCV)-C/HA and $pF1_{fs}$.

Discussion

In this report we examined the role of cellular chaperone complexes in FHV RNA replication complex assembly and function in *S. cerevisiae*. We demonstrated that deletion of *YDJ1*, an Hsp70 co-chaperone, selectively reduced viral RNA replication when replication complexes were targeted to the mitochondria but not those targeted to the ER. The defect in mitochondrial targeted FHV RNA replication was due in part to decreased viral polymerase accumulation. These results identify *YDJ1* as a cellular host factor essential for mitochondrial targeted FHV RNA replication in yeast. Furthermore, they demonstrate that *S. cerevisiae* and FHV is a versatile host-pathogen model system to investigate the role of membrane-specific host factors in viral RNA replication complex assembly and function.

The observation that FHV RNA replication in yeast was not significantly reduced in the absence of functional Hsp90 chaperone complex activity (Figure 2.2) was in contrast to our results in *Drosophila* S2 cells despite similar temperature conditions, where direct genetic or pharmacologic disruption of Hsp90 function greatly reduced FHV RNA replication (19). One potential explanation for these contradictory results is the previously described differences in chaperone complex activity between yeast and higher eukaryotes with respect to mitochondrial protein targeting (47). The delivery of cellular preproteins to the mitochondrial import machinery is facilitated by both the Hsp70 and Hsp90 chaperone complexes in higher eukaryotes, whereas only the Hsp70 chaperone complex is required in yeast (47). This suggests that FHV uses established cytosolic chaperone pathways to facilitate the synthesis and transport of its RNA polymerase to the appropriate cellular membrane during replication complex assembly. Alternatively, FHV may require Hsp90 activity that is independent of the co-chaperones *STI1* or *SBA1*, similar to the direct role of Hsp90 in the maintenance of the cystic fibrosis transmembrane conductance regulator in yeast (46). However, the selective and direct

Hsp90 inhibitor geldanamycin had no effect on FHV RNA replication in yeast despite its moderate suppression of Hsp90-dependent GR activity (Figure 2.2). Recent work has identified an extensive network of cochaperones used by Hsp70 and Hsp90 in yeast that has raised the possibility of novel chaperone activities (36, 48), and studies are currently underway to examine the contribution of the complete set of known and hypothesized Hsp70 and Hsp90 cochaperones on FHV RNA replication.

The demonstration that the Hsp70 co-chaperone *YDJ1* was essential for efficient FHV RNA replication is consistent with its previous identification as a host factor necessary for BMV RNA replication (40). However, there are significant differences in the role that *YDJ1* may play in FHV and BMV RNA replication complex assembly and function. The most obvious difference is that BMV normally assembles its viral RNA replication complexes on the ER (34, 35), whereas we found no significant defect in *Δydj1* yeast when FHV RNA replication complexes were retargeted to this intracellular membrane compartment (Figure 2.5). The ubiquitous nature of Hsp70 chaperone complex activity on numerous cellular processes made the differential effect of *YDJ1* deletion on FHV RNA replication complexes targeted to mitochondria or the ER an unanticipated result. One potential explanation for the selective defect in mitochondrial targeted replication complexes is the observation that ER-retargeted FHV RNA replication complexes demonstrate increased activity via an unknown mechanism (27), and the deletion of *YDJ1* may have been insufficient to significantly impact maximal or near maximal RNA replication complex function. However, Ydj1p overexpression enhanced ER-retargeted FHV RNA replication (Figure 2.5), suggesting that maximal replication complex activity was not achieved in wt yeast. Interestingly, when we examined ER-retargeted FHV RNA replication in *Δsti1* and *Δsba1* yeast, we found that while these deletions had no effect on expression of protein A, there was a significant

reduction in the accumulation of viral RNA (data not shown). These results along with ongoing studies in our lab suggests that there are distinct chaperone requirements for FHV RNA replication on the ER versus the mitochondria, and that a broader targeted analysis of the chaperone network in yeast would be beneficial to understanding the complex cellular requirements for viral RNA replication, and suggest that membrane-specific activities can be differentiated with the FHV system in *S. cerevisiae*.

An additional difference between the role of *YDJ1* in FHV and BMV RNA replication is that the Hsp70 co-chaperone facilitates assembly of BMV RNA replication complexes by maintenance of viral polymerase cytosolic solubility prior to membrane association, but does not influence its accumulation or recruitment to the ER membrane (40). Although we also found normal membrane association of the FHV RNA polymerase in Δ*ydj1* yeast (Figure 2.6), a detailed quantitative analysis revealed that Ydj1p facilitated protein A accumulation (Table 2.1). Furthermore, we found no evidence for FHV protein A aggregation in Δ*ydj1* yeast (Figure 2.6), whereas the BMV RNA polymerase forms high-density cytosolic aggregates in the absence of functional Ydj1p (40). However, immunofluorescence microscopy demonstrated that a small fraction of wild-type FHV protein A did not co-localize with mitochondrial markers in *Δydj1* yeast (Appendix, Figure 2.7), suggesting that Ydj1p could influence the cytosolic fate of the FHV RNA polymerase but was not necessary to prevent the formation of insoluble aggregates (Figure 2.6). These results indicated that cellular chaperones can have virus-specific effects on positive-strand RNA virus replication, consistent with the limited degree of overlap in host factors identified in genome-wide screens of related positive-strand RNA viruses (20, 30).

There are three general hypotheses to explain to role of cellular chaperones in positive-strand RNA virus replication. The first hypothesis suggests a direct role

whereby cellular chaperones physically yet transiently interact with viral RNA replication complex components to facilitate protein folding and stabilization during translation, intracellular targeting, or membrane association. The presence of hydrophobic viral proteins that are required for the association of RNA replication complexes with intracellular membranes (25, 37, 41) may necessitate the use of cytosolic chaperones to prevent premature or inappropriate protein folding prior to membrane association, similar to the role of these chaperones in the maturation of endogenous hydrophobic cellular proteins (15, 16). The second hypothesis also suggests a direct role whereby cellular chaperones constitute an essential component of the functional membrane-associated viral RNA replication complex, and may function to stabilize viral proteins or RNA. This hypothesis is consistent with the co-purification of Hsp70 chaperones with the membrane-associated replication complexes of cucumber necrosis virus (39), tomato mosaic virus (29), and Sindbis virus (13). Interestingly, Ydj1p has been shown to specifically interact with the SSA family of Hsp70s in yeast (24), and preliminary results suggest that deletion of both *SSA1* and *SSA2* suppresses FHV RNA replication in *S. cerevisiae* (S. Weeks and D. Miller, unpublished results), similar to results with cucumber necrosis virus (39). The third hypothesis suggests an indirect role whereby cellular chaperones promote the maturation of particular cellular proteins that subsequently facilitate viral RNA replication complex assembly or function. We are currently using both targeted genetic and global biochemical approaches within the framework of these three hypotheses to further define the role of cellular chaperones in FHV RNA replication.

The three hypotheses outlined above are not mutually exclusive, and there is a high likelihood that individual cellular chaperones may have virus- and cell-specific mechanisms of action. Nevertheless, there is increasing evidence that the use of cellular chaperones may be a common feature during virus replication. Further studies

on the roles of these ubiquitous cytosolic proteins on the replication of both closely related and highly divergent pathogens will provide important insight into the elaborate methods that viruses use to complete their life cycles, and may identify common targets for the development of novel therapeutics.

References

1. **Ball, L. A.** 1995. Requirements for the self-directed replication of flock house virus RNA 1. J Virol 69:2722.

2. **Ball, L. A., and K. L. Johnson.** 1998. *Nodaviruses* of Insects, p. 225-267. *In* L. K. Miller and L. A. Ball (ed.), The Insect Viruses. Plenum Publishing Corporation, New York, N. Y.

3. **Bohen, S. P. 1998.** Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. Mol. Cell. Biol. 18:3330-3339.

4. **Borkovich, K. A., F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist.** 1989. Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. Mol. Cell. Biol. 9:3919-3930.

5. **Buck, K. W.** 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv. Virus Res. 47:159-251.

6. **Bush, G. L., and D. I. Meyer.** 1996. The refolding activity of the yeast heat shock proteins Ssa1 and Ssa2 defines their role in protein translocation. J. Cell. Biol. 135:1229-1237.

7. **Caplan, A. J., D. M. Cyr, and M. G. Douglas.** 1993. Eukaryotic homologues of *Escherichia coli* dnaJ: a diverse protein family that functions with HSP70 stress proteins. Mol. Biol. Cell. 4:555-563.

8. **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. 81:8412-8420.

9. **Chang, H. C., D. F. Nathan, and S. Lindquist.** 1997. In vivo analysis of the Hsp90 cochaperone Sti1 (p60). Mol Cell Biol 17:318-25.

10. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature 332:800-805.

11. **Freeman, B. C., S. J. Felts, D. O. Toft, and K. R. Yamamoto.** 2000. The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. Genes. Dev. 14:422-434.

12. **Freeman, B. C., and K. R. Yamamoto.** 2002. Disassembly of transcriptional regulatory complexes by molecular chaperones. Science 296:2232-2235.

13. **Frolova, E., R. Gorchakov, N. Garmashova, S. Atasheva, L. A. Vergara, and I. Frolov.** 2006. Formation of nsP3-specific protein complexes during Sindbis virus replication. J. Virol. 80:4122-4134.

14. **Gallagher, T. M., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. 62:3399-3406.

15. **Hartl, F. U., and M. Hayer-Hartl.** 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852-8.

16. **Hoogenraad, N. J., L. A. Ward, and M. T. Ryan.** 2002. Import and assembly of proteins into mitochondria of mammalian cells. Biochim. Biophys. Acta 1592:97-105.

17. **Iniguez-Lluhi, J. A., D. Y. Lou, and K. R. Yamamoto.** 1997. Three amino acid substitutions selectively disrupt the activation but not the repression function of the glucocorticoid receptor N terminus. J. Biol. Chem. 272:4149-4156.

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

19. **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.

20. **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. USA 100:15764-15769.

21. **Li, H., W. X. Li, and S. W. Ding.** 2002. Induction and suppression of RNA silencing by an animal virus. Science 296:1319-21.

22. **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.

23. **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.

24. **Lu, Z., and D. M. Cyr.** 1998. Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. J. Biol. Chem. 273:27824-27830.

25. **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.

26. **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.

27. **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.

28. **Nathan, D. F., M. H. Vos, and S. Lindquist.** 1997. In vivo functions of the *Saccharomyces cerevisiae* Hsp90 chaperone. Proc. Natl. Acad. Sci. USA 94:12949-12956.

29. **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.

30. **Panavas, T., E. Serviene, J. Brasher, and P. D. Nagy.** 2005. Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. Proc. Natl. Acad. Sci. USA 102:7326-7331.

31. **Pratt, W. B., and D. O. Toft.** 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev. 18:306-360.

32. **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.

33. **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.

34. **Restrepo-Hartwig, M., and P. Ahlquist.** 1996. Brome mosaic virus helicase- and polymerase-like proteins colocalize in the endoplasmic reticulum at sites of viral RNA synthesis. J. Virol. 70:8908-8916.

35. **Restropo-Hartwig, M., and P. Ahlquist.** 1999. Brome mosaic virus RNA replication proteins 1a and 2a colocalize and 1a independently localizes on the yeast endoplasmic reticulum. J. Virol. 73:10303-10309.

36. **Sahi, C., and E. A. Craig.** 2007. Network of general and specialty J protein chaperones of the yeast cytosol. Proc Natl Acad Sci U S A 104:7163-8.

37. **Schmidt-Mende, J., E. Bieck, T. Hügle, 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.

38. **Scotti, P. D., S. Dearing, and D. W. Mossop.** 1983. Flock House virus: a nodavirus isolated from *Costelytra zealandica* (White) (*Coleoptera: Scarabaeidae*). Arch. Virol. 75:181-189.

39. **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. 80:2162-2169.

40. **Tomita, Y., T. Mizuno, J. Diez, S. Naito, P. Ahlquist, and M. Ishikawa.** 2003. Mutation of host DnaJ homolog inhibits Brome mosaic virus negative- strand RNA synthesis. J. Virol. 77:2990-2997.

41. **Towner, J. S., T. V. Ho, and B. L. Semler.** 1996. Determinants of membrane association for Poliovirus protein 3AB. J. Biol. Chem. 271:26810-26818.

42. **Waxman, L., M. Whitney, B. A. Pollok, L. C. Kuo, and P. L. Darke.** 2001. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc. Natl. Acad. Sci. USA 98:13931-13935.

43. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 7:2568-2577.

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

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

46. **Youker, R. T., P. Walsh, T. Beilharz, T. Lithgow, and J. L. Brodsky.** 2004. Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. Mol. Biol. Cell. 15:4787-4797.

47. **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.

48. **Zhao, R., M. Davey, Y. C. Hsu, P. Kaplanek, A. Tong, A. B. Parsons, N. Krogan, G. Cagney, D. Mai, J. Greenblatt, C. Boone, A. Emili, and W. A. Houry.** 2005. Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell 120:715-727.

APPENDIX

Materials and Methods

Immunofluorescence Microscopy. We harvested yeast by centrifugation, washed once in sterile water, and fixed cells in 0.1 M potassium phosphate (pH 6.5) with 5.25% formaldehyde for 60 min at 25°C. Fixed cells were washed twice in SP buffer, spheroplasted with lyticase, permeablized in SP buffer with 0.1% Triton X-100 for 10 min at room temperature, and attached to polyethyleneimine-coated microscope slides. Slides were incubated with HA-specific mouse and SSC1-specific rabbit antibodies overnight at room temperature, washed in PBS (50 mM phosphate, 150 mM sodium chloride [pH 7.4]), incubated with separate rhodamine redX, and fluorescein-labeled secondary antibodies, and mounted with ProLong Gold (Molecular Probes, Eugene, OR). We examined slides with a Zeiss LSM510 confocal microscope equipped with a CCD camera, and processed images with Zeiss LSM Image Browser software and Adobe Photoshop.

In vitro RdRp activity. Replication complex activity in vitro was measured using RdRp assays as previously described (2) with several modifications. Briefly, LD membrane fractions were pelleted by centrifugation, washed once and resuspended in lysis buffer without EDTA or protease inhibitors. Total membrane protein concentrations were measured using the DC protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Reaction mixtures containing 2.5 µg of total membrane protein, 50 mM Tris (pH 8.0), 50 mM potassium acetate, 15 mM magnesium acetate, 40 U rRNasin (Promega, Madison, WI), 10 µCi α^{32}P-rUTP (Amersham Biosciences, Piscataway, NJ), 1 mM unlabelled rCTP, rATP, rGTP, and 5 µg actinomycin D per ml in a 25 µl final volume were incubated at 25°C for 3 h, extracted once with phenol-chloroform, desalted with RNAse-free Micro Bio-Spin 30 columns (Bio-Rad), and

separated in 1% non-denaturing agarose gels. After electrophoresis the gels were dried, and [^{32}P]-labeled products were detected by autoradiography and quantified by densitometry as described above.

Results

FHV Protein A intracellular targeting in *Δydj1* yeast. FHV can assemble functional RNA replication complexes on intracellular membranes other than the mitochondrial outer membrane when specifically retargeted (2). However, to determine whether the absence of *YDJ1* altered wt protein A targeting, we examined the intracellular localization of FHV protein A in wt, *Δydj1*, and complemented *Δydj1* yeast by confocal immunofluorescence microscopy with the mitochondrial specific marker SSC1 (3). In the absence of FHV protein A expression, yeast mitochondria display a cage-like morphology with a fairly uniform distribution around the cell periphery (Fig. 2.7, top panel). The expression of protein A in wt yeast altered this distribution such that the mitochondria clustered in discreet regions of the cell, where the majority of protein A colocalized with SSC1 (Fig. 2.7, second panel) (1). In contrast, the protein A signal in *Δydj1* yeast only partially colocalized with SSC1, and the mitochondrial morphology did not completely change to a clustered distribution (Fig. 2.7, third panel). This altered protein A localization was due to the absence of the Hsp70 co-chaperone, as the wt phenotype was restored upon complementation with pYDJ1 (Fig. 2.7, bottom panel). These data indicated that *YDJ1* did affect FHV protein A intracellular localization, and suggested that a portion of the functional defect in viral RNA replication in *Δydj1* yeast (Fig. 2.4 and Table 2.1) may be due to improper protein targeting. It is difficult to determine what this defect represents on a cellular level, as it may be due to an effect on the levels of protein A in the cell.

In vitro RdRp activity in *Δydj1* yeast. The differences in growth rate between *Δydj1* and wt yeast (Fig. 4A) made it difficult to directly compare FHV RNA replication complex activity and differentiate the effects of *YDJ1* on protein A accumulation versus function in vivo. Thus, to further investigate FHV RNA replication complex function in

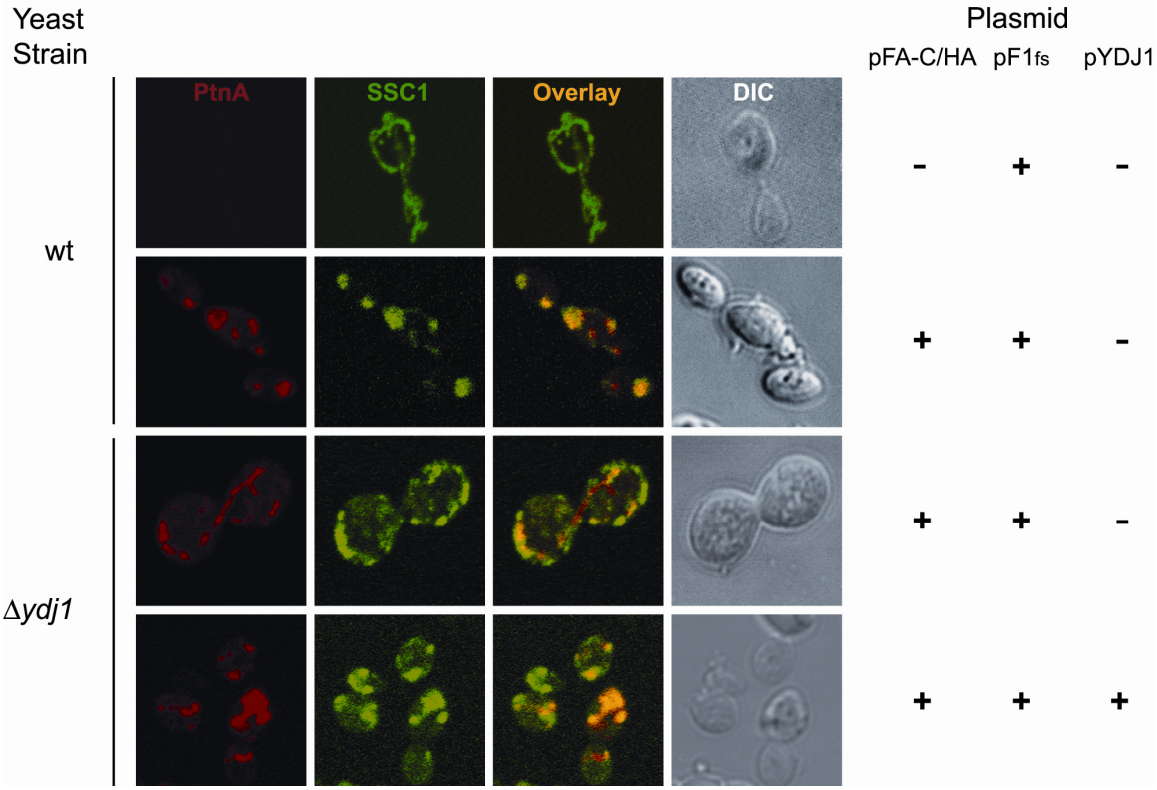


Figure 2.7 - Protein A intracellular localization in *Δydj1* yeast. Wt and *Δydj1* yeast were transformed with pFA-C/HA, $pF1_{fs}$ and pYDJ1 as indicated, induced for 24 hours, fixed, spheroplasted, permeablized and immunolabeled with mouse antibodies specific for HA-tagged protein A, and rabbit antibodies specific for the mitochondrial chaperone *SSC1*. Representative images for protein A (PtnA-red), SSC1 (green), merged signals (overlay), and corresponding differential interference contrast (DIC) are shown. The merged images represent a digital superimposition of red and green signals in which areas of fluorescence co-localization are yellow. Images are representative of three independent experiments.

Δydj1 yeast we used a cell-free system to examine protein A RdRp activity in vitro (Fig. 2.8). We isolated membrane fractions from whole cell lysates by equilibrium centrifugation (see Fig. 2.6A) and performed in vitro RdRp reactions with radiolabeled nucleotides as previously described (2). We added no exogenous RNA to the reactions, and therefore any RdRp activity was due to the presence of endogenous viral template. We normalized samples to total membrane protein, which resulted in approximately equal amounts of protein A measured by immunoblot analysis (Fig. 2.8, top panel). Membrane fractions from *Δydj1* yeast showed a greater than 80% reduction in RdRp activity compared to membrane fractions from wt yeast (Fig. 2.8, compare first two lanes), consistent with the in vivo RNA replication results (Fig. 2.4 and Table 2.1). Furthermore, membrane fractions from complemented *Δydj1* yeast showed a partial restoration of RdRp activity to approximately 50% of wt levels (Fig. 2.8, third lane). The absence of complete restoration of FHV RdRp activity in vitro with pYDJ1 complementation despite in vivo restoration to greater than wt levels (Table 2.1) suggests that Ydj1p also influenced viral RNA accumulation via a replication-independent mechanism, such as RNA stabilization. We also examined the in vitro RdRp activity in membrane fractions from yeast expressing the ER-targeted protein A. Consistent with in vivo results (Fig. 2.5), there was no decrease in ER-targeted in vitro RdRp activity in membrane fractions from *Δydj1* yeast (data not shown). These results suggested that the absence of *YDJ1* resulted in the assembly of dysfunctional mitochondrial-targeted FHV RNA replication complexes. The defect in RdRp activity in extracts from *Δydj1* cells could be explained by a functional problem with the complex itself, or also by a limiting factor for replication, such as (-) RNA1 template. Experiments have not been performed to address the limiting factors in RdRp complex activity, so at present this is only a confirmation of the in vivo phenotype.

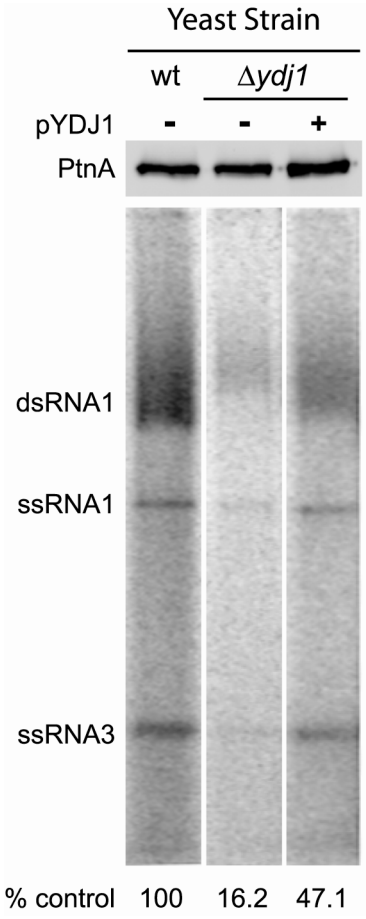


Figure 2.8 - In vitro RdRp activity in membrane fractions from *Δydj1* yeast. Membranes isolated by density gradient equilibrium centrifugation were adjusted for total membrane protein and either immunoblotted for FHV protein A (upper panel) or incubated with [^{32}P]UTP and unlabeled ribonucleotides, and reaction products were separated by nondenaturing agarose gel electrophoresis and visualized by autoradiography (lower panel). The positions of dsRNA1, ssRNA1 and ssRNA3 based on previous studies (2) are shown on the left. All panels for the RdRp results were from the same gel exposure, and all contrast adjustments to the initial image were done prior to cropping. Numbers represent the sum of all radiolabeled products expressed as the percent of wt control.

References

1. **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.

2. **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.

3. **Sakuragi, S., Q. Liu, and E. Craig.** 1999. Interaction between the nucleotide exchange factor Mge1 and the mitochondrial Hsp70 Ssc1. J Biol Chem **274:**11275-82.

CHAPTER III

A TARGETED ANALYSIS OF CELLULAR CHAPERONES IN YEAST REVEALS CONTRASTING ROLES FOR HSP70 IN FLOCK HOUSE VIRUS RNA REPLICATION

Cellular chaperones play a role in the replication of positive strand RNA viruses. We have previously shown that *YDJ1*, a yeast Hsp40, is involved in the RNA replication of Flock House virus, and that its deletion severely reduces RNA replication in yeast. To further understand how chaperones impact RNA replication in the yeast model, we examined a panel of deletion mutants lacking cytosolic proteins that have a known or hypothesized chaperone activity. This analysis revealed that the deletion of several J-domain proteins impact FHV RNA replication, including *YDJ1*, which has a negative effect on RNA replication, and *ZUO1*, which has a positive effect. J-domain proteins, of which *YDJ1* and *ZUO1* are the most highly expressed members, play a critical role by increasing the ATPase activity of their partner Hsp70s. Overexpression of other J-domain proteins in *YDJ1* and *ZUO1* deletion strains revealed that while the effect of *YDJ1* deletion is able to be complemented by different J-domain proteins, the effect of deleting *ZUO1* is only able to be complemented by a similar J-domain protein, consistent with previous reports about the specificity of J-domains with their Hsp70 partners. Finally, a multiple deletion of the corresponding Hsp70 chaperones mimicked the deletion of each J-domain protein. These results identify Hsp70 chaperones as critical regulators of Flock House virus RNA replication.

Introduction

Positive-sense (+) RNA viruses represent the largest group of named human viral pathogens, yet there are relatively few treatments directed against them. One reason for this lack of effective antiviral tools is poor understanding the mechanisms of the early events in the viral replication cycle. The ability to target early events in a viral infection may improve the prognosis of patients that present with a (+) RNA viral infection. One promising early target event is the assembly of genome replication complexes within membrane-associated macromolecular complexes in host cells (5). Although the assembly of these highly active enzymatic complexes in association with intracellular membranes is a critical step in the positive-sense RNA virus life cycle, the mechanisms responsible for viral protein translation, folding, and transport to the appropriate membrane compartment within cells during viral RNA replication complex assembly are poorly understood. Cellular chaperones are a diverse set of proteins that facilitate cellular protein translation, folding, and trafficking (20). These abundant chaperones also participate in the assembly of membrane-associated protein complexes (53), suggesting that positive-sense RNA viruses may also use cytosolic chaperones to assemble viral RNA replication complexes. Consistent with this hypothesis, cellular chaperones have been associated with the replication of numerous positive-sense RNA viruses, including hepatitis C virus (HCV) (49), cucumber necrosis virus (43), brome mosaic virus (BMV) (44), tomato mosaic virus (34), and Sindbis virus (12).

To study the role of cellular chaperones in viral RNA replication complex assembly and function, we use Flock House virus (FHV), a versatile positive-sense RNA virus and well-studied member of the *Nodaviridae* family (2). The utility of FHV as a model pathogen derives in part from its relatively small genome and robust replication in multiple eukaryotic hosts, including *Drosophila melanogaster* (15, 32), *Caenorhabditis elegans* (28) and *Saccharomyces cerevisiae* (27, 31, 33, 37, 38). The FHV genome is

bipartite and consists of two capped but non-polyadenylated RNA segments (42). The larger 3.1-kb segment, RNA1, encodes protein A, the FHV RNA-dependent RNA polymerase (RdRp), which is essential for the assembly of functional viral RNA replication complexes (1, 2, 22, 27, 31, 38, 42). The smaller 1.4-kb segment, RNA2, encodes the structural capsid protein precursor, which is dispensable for RNA replication but necessary for infectious virion production (2). During viral RNA replication, protein A generates a subgenomic 0.4-kb RNA, RNA3, which is colinear with the 3' end of RNA1. RNA3 encodes the RNA interference suppressor protein B2 (26), which is required for FHV RNA replication in insects (26), plants (26), and nematodes (28), but not in yeast (38).

FHV RNA replication complexes assemble on the mitochondrial outer membrane in both insect cells (32) and yeast (31), and protein A is sufficient for their appropriate intracellular localization (31). FHV replication complexes are targeted and anchored to the mitochondrial outer membranes in part by an amino-proximal domain in protein A that resembles the transmembrane stop-transfer sequences present in several cellular mitochondrial outer membrane proteins (31, 33). The protein A mitochondrial targeting signal can be replaced with other targeting signals and used to retarget protein A. Fully functional FHV RNA replication complexes are formed when the mitochondrial targeting signal is replaced with a sequence that contains an endoplasmic reticulum (ER) targeting domain (33). Thus, FHV provides a versatile system to examine the role of both general and membrane-specific host factors in viral RNA replication complex assembly and function.

We use *Saccharomyces cerevisiae* as a model host in part because it is a genetically tractable, easily manipulated, and well understood host cell. Also, the availability of deletion, modified expression, and tagged libraries make it possible to perform larger throughput analyses that would be difficult in other host cells.

We have previously shown that the cellular chaperone Hsp90 facilitates the synthesis of protein A in *Drosophila* S2 cells (6, 23), consistent with the demonstrated role of this abundant cytosolic chaperone in cellular translation (11, 46, 52). We have also shown that there are membrane- and host-specific differences between the role of chaperones in FHV genome replication (50). To further examine the roles that cellular chaperones play in the early events of FHV replication, we analyzed RNA replication in a panel of 31 yeast deletion strains which lack cytosolic proteins of known or hypothesized chaperone activity. In addition to identifying a number of interesting phenotypes associated with this deletion panel, we found that deletion of some of the family of J-domain containing proteins had profound effects on FHV RNA replication. Deletion of the J-domain protein *ZUO1* increased the accumulation of both the viral polymerase protein A and the subgenomic RNA3. Further, we showed that overexpression of other J-domain proteins in the *Δydj1* and *Δzuo1* strains partially to fully compliment the phenotypes, consistent with published reports of complementation among J-domain proteins. Consistent with the known role of Hsp40 as a regulator of an Hsp70, multiple deletions of partner Hsp70s caused a similar phenotype as those of the individual J-domain protein deletions. These results implicated Hsp70s in the regulation of RNA replication in yeast, and suggest that there are divergent roles for Hsp70 in FHV RNA replication.

Materials and Methods

Yeast strains, transformation, and culture conditions. The diploid *S. cerevisiae* strain BY4743 (*MAT*a/*MAT*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0*) was used as the parent for the panel of deletions to minimize complications related to second site mutations more common in a haploid strains. Wild type (wt) BY4743 and diploid strains with homozygous deletions of individual members of the Hsp70 (*SSA1*, *SSA2*, *SSA3*, *SSA4*, *SSB1*, *SSB2*, *SSE1*, *SSE2*, and *SSZ1*), Hsp90 (*HSC82* and *HSP82*), or J-domain/Hsp40 (*APJ1*, *DJP1*, *JJJ1*, *JJJ2*, *JJJ3*, *SWA2*, *XDJ1*, *YDJ1*, and *ZUO1*) families of chaperones, as well as other proteins with known or hypothesized chaperone activity (*EGD1*, *EGD2*, *BTT1*, *HSP26*, *HSP42*, *HSP104*, *CPR1*, *CPR6*, *CPR7, GIM4*, and *GIM5*) were purchased from the American Type Culture Collection (Manassas, VA), and were confirmed by PCR. An overview of these chaperones can be found in Table 3.1. Wildtype strain DS10 (*MAT* α *his3 leu2 lys2 Δtrp2 ura3*) and *SSA1/ SSA2* double deletion strain in that background were generously provided by Peter Nagy (University of Kentucky, Lexington, KY) and have been described previously (43, 51). Wildtype strain MH272-3f a/α (*ura3/ura3 leu2/leu2 his3/his3 trp1/trp1 ade2/ade2 HML*a/*HML*a) and haploid *SSZ1* (*MAT* a) deletion strain, and SS*B1/SSB2* (*MAT* α) double deletion strain in that background were provided by Sabine Rospert (Universität Freiburg, Freiburg, Germany) and have been described previously (39). Yeast were transformed as previously described (31), and incubated on agar plates at 25^{o}C until colonies formed. Due to the temperature-sensitive nature of FHV RNA replication complexes (data not shown), and slow growth or inviability of some mutant strains at higher temperatures, as well as to maintain consistency with *Drosophila* studies, all experiments were performed at 25^{o}C. For analysis of the mutant panel, individual clones were transferred to liquid selective minimal media containing 2%

Table 3.1 – Yeast cytosolic chaperones and their functions in the cell.

Family	Chaperone	Partners	Cellular Function[1]
Hsp70	SSA	YDJ1, JJJ1, SWA2	Protein folding/sorting
	SSB	ZUO1, SSZ1	Translation and folding
	SSE	Hsp90/70	Nucleotide exchange factor
	SSZ1	ZUO1, SSB	Translation and folding
Hsp90	Hsc82/Hsp82	Hsp70, STI1, SBA1, SSE, CPR6, CPR7	Specialized folding
Hsp40	APJ1	Unknown	Protein folding
	XDJ1	Unknown	Protein folding
	YDJ1	SSA	Protein folding/sorting
	DJP1	SSA	Peroxisome biogenesis
	SIS1	SSA	Translation initiation
	JJJ1	SSA	Ribosome biogenesis
	JJJ2	Unknown	Unknown
	JJJ3	Unknown	Unknown
	SWA2	SSA	Clathrin uncoating
	ZUO1	SSZ1, SSB	Translation and folding
Others	EGD/BTT	YDJ1, SSA1	Protein folding and sorting
	STI1	Hsp90	Hsp90 complex activity
	SBA1	Hsp90	Hsp90 complex activity
	CPR1	Unknown	Protein folding
	CPR6	Hsp90	Protein folding and transport
	CPR7	Hsp90	Protein folding and transport
	GIM4	Chaperonin	Protein folding
	GIM5	Chaperonin	Protein folding
	Hsp26	unknown	Unfolded protein binding/folding
	Hsp42	unknown	Unfolded protein binding/folding
	Hsp104	YDJ1, SSA	Refolding aggregates

[1] Cellular functions are known or hypothesized, and are collected from the S*accharomyces* Genome Database website www.yeastgenome.org

glucose and grown to stationary phase, washed with sterile distilled water and resuspended in selective minimal media with 2% galactose at an optical density unit at 600 nm (OD_{600}) of 0.2, which was equivalent to approximately 5 x 10^6 cells per ml. For induction of *Δydj1* yeast, cells were grown in liquid selective minimal media with 2% raffinose, washed in sterile distilled water and resuspended in selective minimal media with 2% raffinose plus 2% galactose at an OD_{600} of 0.2. For dilution plating, yeast were grown as above, then adjusted to 1.0 OD_{600}/ml and serial diluted five times at 1:10 in a 96 well plate. 10 µl of each dilution was spotted onto agar plates containing the indicated supplements and incubated at 30°C for three days until colonies formed. Unless otherwise stated, experiments were performed with two independently derived clones and results are representative of at least two independent experiments.

Plasmids. FHV expression plasmids pF1, $pF1_{fs}$, pFA-C/HA, and pFA(HCV)-C/HA have been previously described (27, 31, 33, 38, 50). FHV RNA1 expression plasmids pF1 and $pF1_{fs}$ encode galactose-inducible cDNA copies of FHV RNA1 with authentic viral 5' and 3' ends that are generated by precise transcription initiation and a hepatitis delta ribozyme, respectively, and thus contain the necessary cis elements to serve as replication templates (Fig. 2.1). In addition, the RNA1 transcribed from pF1 can be translated into protein A and initiate RNA replication in cis, which links protein A accumulation to viral RNA replication (Fig. 2.1, left). In contrast, the RNA template transcribed from plasmid $pF1_{fs}$ contains an early frameshift-induced stop codon and therefore can not be translated into protein A. Functional protein A is provided from a second plasmid, pFA-C/HA or pFA(HCV)-C/HA to initiate RNA replication in trans, where protein A accumulation is independent of viral RNA replication (Fig. 2.1, right). The protein A expression plasmids pFA-C/HA and pFA(HCV)-C/HA encode a galactose-inducible C-terminally hemagglutinin (HA)-tagged protein A open reading frame (ORF) flanked by an upstream *GAL1* leader sequence and a downstream *CYC1*

polyadenylation signal sequence. RNAs transcribed from pFA-C/HA and pFA(HCV)-C/HA are efficiently translated into protein A, but do not have the necessary cis elements to serve as replication templates.

J-domain yeast complementation plasmids were described previously (41), and were moved into a pRS416 background to be compatible with our system.

Antibodies and reagents. Rabbit polyclonal antibodies against FHV protein A have been previously described (32). Rabbit polyclonal antibodies against the HA epitope tag were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibodies against porin and 3-phosphoglycerate kinase (PGK) were purchased from Molecular Probes (Eugene, OR). Rabbit polyclonal antibodies against yeast Ydj1p were generously provided by Masayuki Ishikawa (Hokkaido University, Sapporo, Japan). Polyclonal antibodies against yeast Ssb1p, Ssz1p and Zuo1p were generously provided by Sabine Rospert (University of Freiburg, Freiburgh, Germany). All secondary antibodies for immunoblotting were purchased from Jackson Immunoresearch (West Grove, PA).

Immunoblot and Northern blot analysis. Total protein was isolated from an equivalent number of yeast using post alkaline extraction (25) and stored at –20^{o}C until analysis. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as previously described (23). Total RNA was isolated from an equivalent number yeast using hot acidic phenol as previously described (38) and stored at –80^{o}C until analysis. RNA samples were denatured in sample buffer containing 60% formamide and 7% formaldehyde, separated on formaldehyde-1% agarose gels, transferred to ZetaProbe nylon membranes (Bio-Rad, Hercules, CA) by passive capillary transfer overnight, UV-crosslinked at 120 mJ per cm^2 and probed with ^{32}P-UTP labeled strand-specific riboprobes as previously described (32). Protein and RNA bands were quantitated by densitometry using either

AlphaEaseFC (Alpha Innotech, San Leandro, CA) or Image Quant TL (Amersham Biosciences, Piscataway, NJ) software.

Statistics. A two-tailed Student's *t*-test assuming unequal variances was used for all statistical analyses, and a p-value < 0.05 was considered statistically significant.

Results

A targeted analysis of cellular chaperones and their impact on FHV RNA replication. To further explore the roles of cellular chaperones in FHV RNA replication, we examined RNA replication in a panel of 31 deletion strains lacking cytosolic proteins with known or hypothesized chaperone function. Strains were grown and induced in groups with a corresponding positive and negative control, and equal numbers of cells were harvested from each strain for protein and RNA analysis. Levels of all quantifications were normalized to loading controls and are expressed in Table 3.2 compared to wildtype levels. We examined three types of FHV RNA replication: replication in cis, replication in trans, and retargeted replication in trans (Figure 2.1). We chose to examine replication in these three ways to gain a further level of insight into replication. Replication in cis (Table 3.2, column 1) is a more authentic representation of infection, with one template being the source of all viral products in the cell. Replication in trans (Table 3.2, columns 2 and 3) allows us to examine the accumulation of the viral polymerase, protein A, independent of the activity of FHV RNA replication complexes, as its message comes solely from cellular transcription rather than cellular transcription and viral RNA replication. Retargeted replication in trans (Table 3.2, columns 4 and 5) allows us the additional condition of examining FHV protein and RNA levels when replication complexes are retargeted to the ER rather than to the mitochondria, which can be used to distinguish between membrane specific effects and general cellular effects.

Hsp70. The Hsp70 family is the largest family of chaperones in yeast, comprised of nine members. There is considerable amount of redundancy built into the Hsp70 system (51), and so a single deletion of one family member may not show a phenotype in this method of analysis. Therefore a lack of effect in a single deletion does not exclude the possibility that Hsp70 family members are involved in FHV RNA replication.

Deletion of *SSA1* caused a minor defect in the accumulation of protein A, but caused a moderate increase rather than decrease in the accumulation of RNA3, which was elevated above wild type levels. The deletion of *SSE1* caused an increase in replication in cis, and a drastic increase in the accumulation of ER-targeted protein A. One Hsp70 member that had a substantial phenotype was *SSZ1*, a ribosome associated atypical Hsp70 that has a role in translational fidelity and early protein folding, and does not have a functionally redundant homolog (16, 17). The deletion of *SSZ1* caused a 10-fold increase in the accumulation of mitochondrial-targeted protein A while having no effect on the accumulation of ER-targeted protein A. RNA replication in this mutant was also dramatically increased, between 30-50 fold, regardless of targeting.

Hsp90. Like the Hsp70 family, the Hsp90 family of chaperones has redundancy due to two copies of functionally similar proteins. Deletion in either of the two Hsp90 members did not have a significant phenotype, which is consistent with our previous study where we found that in yeast, the deletion of Hsp90 cochaperones did not cause a defect in FHV RNA replication (50), but did cause a complete loss of Hsp90-dependent reporter activity, consistent with published reports of their function as Hsp90 cochaperones (3, 7).

Hsp40/J-domain proteins. In contrast to the results from the main chaperones, deletion mutants in the J-domain protein family of Hsp40 family had a number of interesting phenotypes, consistent with our previous identification of *YDJ1*, the most abundant cytosolic J-domain protein (41), as a necessary host protein for FHV RNA replication previously (50). Deletion of *APJ1*, which shares homology with *YDJ1*, had a negative effect on the accumulation of RNA3, but not on that of protein A. Further, this effect was not specific to the mitochondria, decreasing RNA3 accumulation in the ER-retargeted system as well. Another J-domain protein deletion mutant that had a significant phenotype was *ZUO1*, dramatically increasing the accumulation of protein A

Table 3.2 – An analysis of chaperones and their impact on FHV RNA replication.

		Cis	Trans - Mitochondria		Trans – ER	
		(+)3	PtnA	(+)3	PtnA	(+)3
Hsp70	SSA1	0	-	+	0	0
	SSA2	0	0	0	0	0
	SSA3	0	0	0	0	0
	SSA4	0	0	0	0	0
	SSB1	0	0	0	0	0
	SSB2	0	0	ND	-	ND
	SSE1	+	-	ND	+++	ND
	SSE2	0	0	0	0	0
	SSZ1	+++	+++	+++	0	+++
Hsp90	Hsc82	0	-	0	0	0
	Hsp82	0	0	0	0	0
J-Dom.	YDJ1	- -	- -	- -	0	0
	XDJ1	0	0	0	0	0
	APJ1	-	0	-	0	-
	DJP1	0	0	0	0	0
	SWA2	0	+	+	0	0
	ZUO1	+++	++	+++	0	+++
	JJJ1	++	+	++	0	++
	JJJ2	+	+	+	0	+
	JJJ3	0	0	0	0	0
NAC	EGD1	0	0	0	0	-
	EGD2	0	0	0	0	0
	BTT1	0	0	0	0	0
Others	HSP26	0	0	-	0	0
	HSP42	0	0	-	0	0
	HSP104	0	-	-	0	0
	CPR1	0	0	+	0	0
	CPR6	0	-	- -	0	0
	CPR7	++	0	0	0	++
	GIM4	0	0	-	+	0
	GIM5	++	+	+	++	++

Symbols: +++: greater than 1000%, ++: 500-1000%, +: 300-500%,
0: 60- 300%, -:40-60%, - -: less than 40%
Abbreviations: ND – incomplete results

and RNA3 of mitochondria targeted replication, but only the RNA3 of ER-targeted replication. This was the same phenotype as that caused by deletion of the Hsp70 chaperone, *SSZ1*. *ZUO1* and *SSZ1* are the regulatory members of the ribosome associated complex, a chaperone complex involved in translational fidelity and early nascent protein folding. Interestingly, deletion of another ribosome associated chaperone, *JJJ1*, caused the same increases as those of the ribosome associated complex, suggesting a link between translation and replication, consistent with previous reports (6, 48). A similar uncharacterized J-domain protein, *JJJ2*, also caused an increase in the accumulation of protein A and RNA3.

The nascent polypeptide associated complex. In contrast to the results of the deletion of the ribosome associated chaperones above (ZUO1, SSZ1, JJJ1), the deletion of members of another ribosome associated complex, the nascent polypeptide associated complex (*EGD1, EGD2, BTT1*) had very little effect on FHV RNA replication.

Stress response chaperones. Next we examined a number of other known and hypothesized chaperones. Deletion of a number of proteins involved in folding and refolding of nascent and stressed proteins had consequences on FHV RNA replication. Deletion of two small chaperones, *HSP26* and *HSP42*, which are both involved in preventing aggregation of unfolded proteins under stress (21, 40), caused a decrease in RNA3 accumulation in trans, yet had no effect on replication in cis. In addition, deletion of *HSP104* caused a decrease in protein A and RNA3 accumulation. Hsp104p is involved in refolding proteins under stress, and works in cooperation with Ydj1p and the Ssa complex (19, 35).

Immunophillins. *CPR1*, *CPR6*, and *CPR7* are all immunophilins, a class of chaperones involved in protein folding (29) and trafficking (13, 14, 36). Deletion of *CPR1* caused an increase in RNA3 in trans replication targeted to the mitochondria, while the deletion of *CPR6* caused a decrease in protein A and RNA3 accumulation in

the same system. The deletion of *CPR7* caused an increase in replication in cis and in retargeted trans. These proteins have been hypothesized to have different roles in cells (29), and so are intriguing candidates for further study.

Chaperonin complex members. Finally, a deletion of *GIM4* caused a decrease in RNA3 in trans, but an increase in protein A in retargeted trans. Deletion of *GIM5* caused an increase in protein A and RNA3 in all replicon systems. Gim4p and Gim5p are members of the chaperonin complex and have been implicated in microtubule biogenesis (18, 47). Mitochondria are associated with microtubules in cells, and so this may indicate that the cytoskeleton is involved in FHV RNA replication. This would not be suprising, given the dramatic cell morphology changes seen with FHV RNA replication (24, 31-33).

Deletion of ribosome associated chaperones causes a 2-fold change in *GAL1* promoter activity. We next tested the *GAL1* promoter activity in the J-domain protein and deletion strains as well as the Hsp70 chaperone *SSZ1* deletion strain to ensure that the results seen above were specific to FHV RNA replication as opposed to a non-specific effect on the promoter activity of the replicon. Yeast were transformed with a *GAL1* promoter-driven β-galactosidase expression construct and induced as in the FHV experiments (Figure 3.1). Most of the J-domain protein deletion strains had no change in their *GAL1* promoter activity (Figure 3.1, lane 1 vs lanes 4-8 and 10). The deletion of *JJJ2* or *ZUO1* however, increased the accumulation of β-galactosidase by two-fold (Figure 3.1, lane 1 vs 9 and 11). Similar to the results with the *Δzuo1* strain, the accumulation of β-galactosidase was increased in the *Δssz1* strain as well (Figure 3.1, lane 1 vs 3 and 11). The *JJJ1* deletion mutant did not have an increase in promoter activity (Figure 3.1, lane 1 vs 8), and therefore the results above are specific to FHV RNA replication. The moderate 1.5 to 2-fold increase in promoter activity does not fully

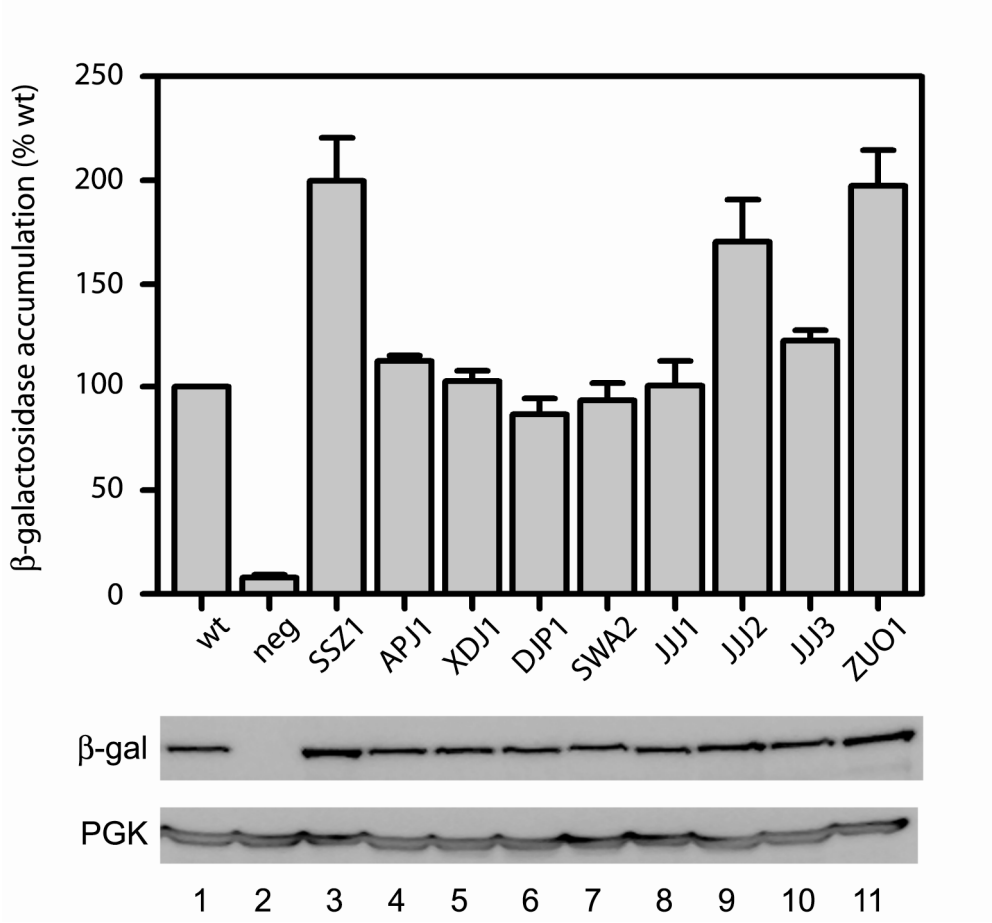


Figure 3.1 – *GAL1* promoter activity in J-domain protein deletion strains.
Yeast expressing a *GAL1* promoter-driven β-galactosidase construct were induced for 24h in galactose containing media. Bars represent quantification of immunoblots normalized to loading controls and are the given as an average of three trials ± the SEM.

account for the dramatic increases in the accumulation of protein A or RNA3 (at least 5-10 fold), and therefore there are specific effects on FHV RNA replication in these strains as well.

The *YDJ1* deletion phenotype can be complemented with unrelated J-domain proteins. The deletion of *YDJ1* caused a drastic reduction in RNA replication and a defect in accumulation of protein A (50). This strain also had a growth defect and heat sensitivity that was consistent with previous studies (41, 44). When we transformed the *Δydj1* strain with constructs expressing various J-domain proteins, we noticed that several complemented strains grew colonies faster than the uncomplimented *Δydj1* strain (data not shown), consistent with previous work on J-domain proteins (41). The other phenotypes that were complemented in that study, such as heat sensitivity (41), were not complemented in our hands (Figure 3.2). This inconsistency could be due to a different parent strain and also our use of centromeric, stable low copy number expression vectors rather than 2-micron high copy vectors

As for the FHV RNA replication phenotypes, the *Δydj1* strain showed a 75% reduction in (+) RNA3 levels, and this reduction was fully complemented by expression of *YDJ1*, consistent with our previous work (Figure 3.3A, lanes 1-2 vs. 4 and 12). In addition to Ydj1p, overexpression of Jjj2p and Sis1p was able to partially complement the defect in (+) RNA3 levels (Figure 3.3A, lanes 7 and 9 vs. 1-2 and 4). This complementation of a *Δydj1* strain with other J-domain proteins is consistent with the previous work examining the growth and heat sensitivity of the *Δydj1* strain (41). Sis1p is an essential chaperone that has been shown to regulate the SSA family of chaperones, similar to Ydj1p, while Jjj2p's function has not been identified. There are no common features that known or characterized for these J-domain proteins other than the

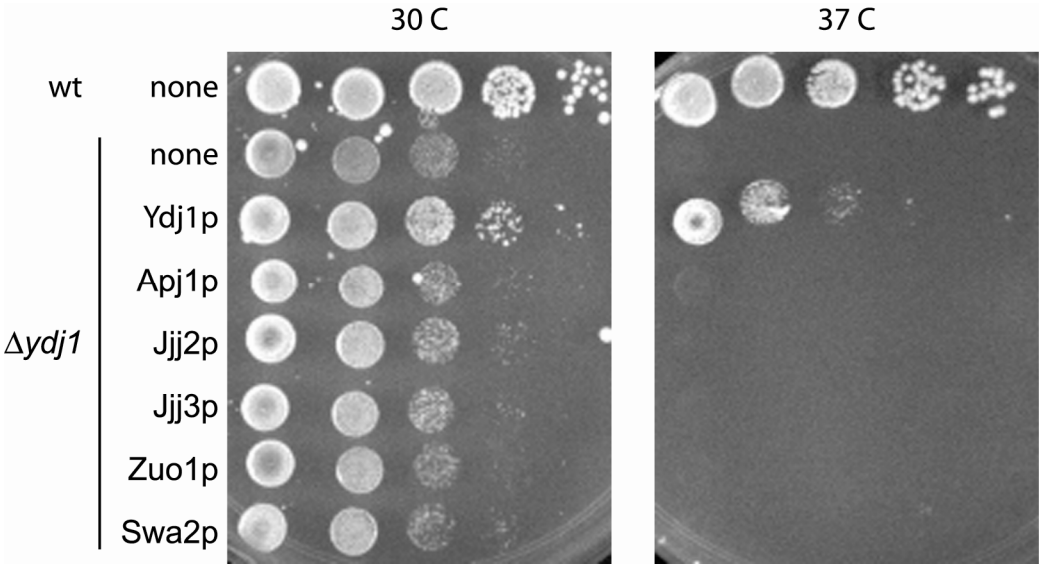


Figure 3.2 – Growth and heat sensitivity of Δydj1 strains complemented with other J-domain proteins. Yeast containing vector controls or the indicated J-domain protein expression vectors were serially diluted 1:10 and spotted on plates. Plates were incubated at the indicated temperature for 2 days and imaged. Data are representative of three experiments.

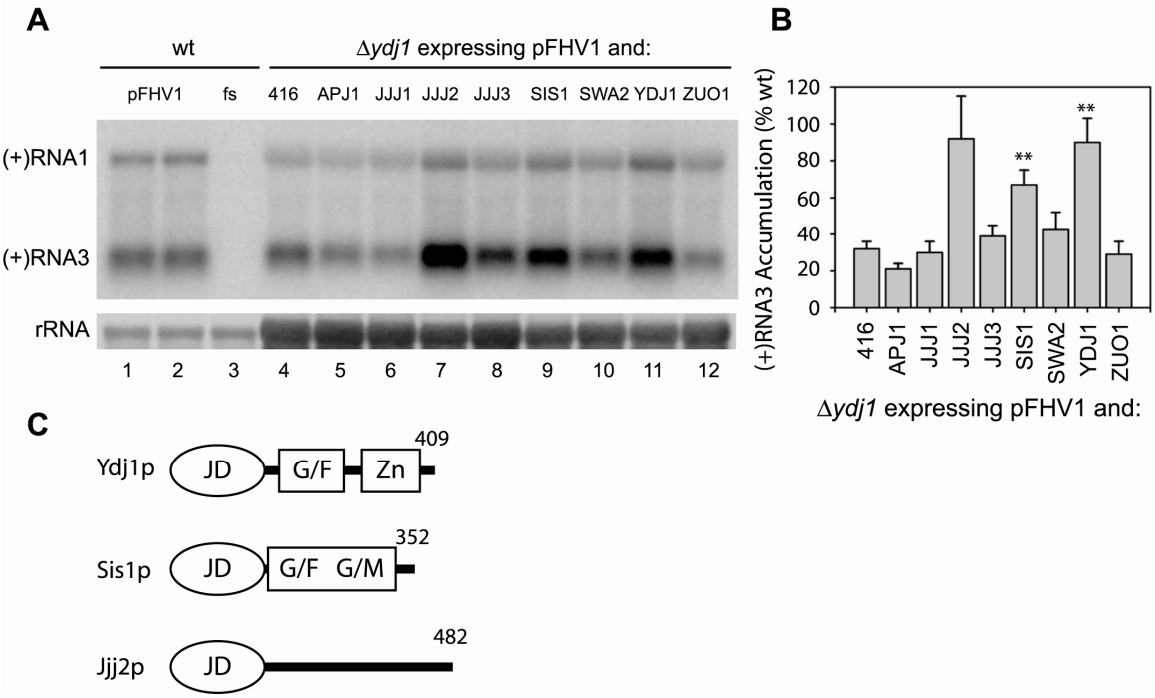


Figure 3.3 - Cross complementation of the *Δydj1* strain. (A) Wt yeast expressing pFHV1 or pFHV1fs, or *Δydj1* yeast expressing pFHV1 and the indictated plasmid were grown and induced in raffinose/galactose media. RNA was extracted as before and analyzed by Northern blot. (B) Quantification of A, lanes 4-12. Bars represent the accumulation of RNA3 compared to wt and are the result of three independent experiments ± SEM. A double asterisk indicates a *p*-value < 0.05 by two-tailed Student's *t*-test assuming unequal variances compared to vector control. (C) Schematics of J-domain proteins: Jjj2p, Sis1p, and Ydj1p are from (41). Numbers indicate length in amino acids of full length proteins. Domain abbreviations: JD - J-domain, G/F or G/M - Glycine/Phenylalanine or Glycine/ Methionine rich region, Zn - Zinc Finger.

J-domain (Figure 3.3C) (41). Therefore, it is possible that the source of the RNA replication phenotype in the *Δydj1* strain is due to a lack of regulation of the partner SSA Hsp70 complex.

Deletions in the major *SSA* family of Hsp70 chaperones decrease FHV RNA replication. Due to the ability of unrelated J-domain proteins to partially rescue the defect in FHV RNA replication in the *Δydj1* strain, we hypothesized that the cause of the defect was actually due to a decrease in activity of the partner Hsp70 for Ydj1p. Ydj1p has been shown to interact with and regulate the ATPase activity of the SSA family of yeast Hsp70s (9, 10). The *SSA* family has four members, all of which are greater than 70% homologous to each other and have been shown to be somewhat redundant in function (4). Therefore, in order to study the role of these chaperones in FHV RNA replication, we must use strains with multiple deletions. In previous studies on cucumber necrosis virus, researchers copurified the *SSA* complex with the CuNV RNA replication complex from yeast (43). Further, the authors were able to show a defect in replication using a double deletion strain of *SSA1/2* compared to wildtype. We obtained the double deletion and its parent wildtype strain and examined FHV RNA replication in cis using the strains (Figure 3.4A). The double deletion strain showed a reduction in (+) RNA3 levels similar to that of the *Δydj1* strain (Figure 3.4A). Further, there was a defect in accumulation of the polymerase in the double deletion strain (Figure 3.4B). This is consistent our hypothesis that the phenotype seen in the *Δydj1* strain is due to a defect in the regulation of the partner Hsp70, and provides more evidence that FHV relies on the main chaperone systems in the host in order to replicate its genome.

The *ZUO1* deletion phenotype can be complemented with the ribosome associated JDP, *JJJ1*. The deletion of *ZUO1* and also that of *SSZ1* yielded the same result, a

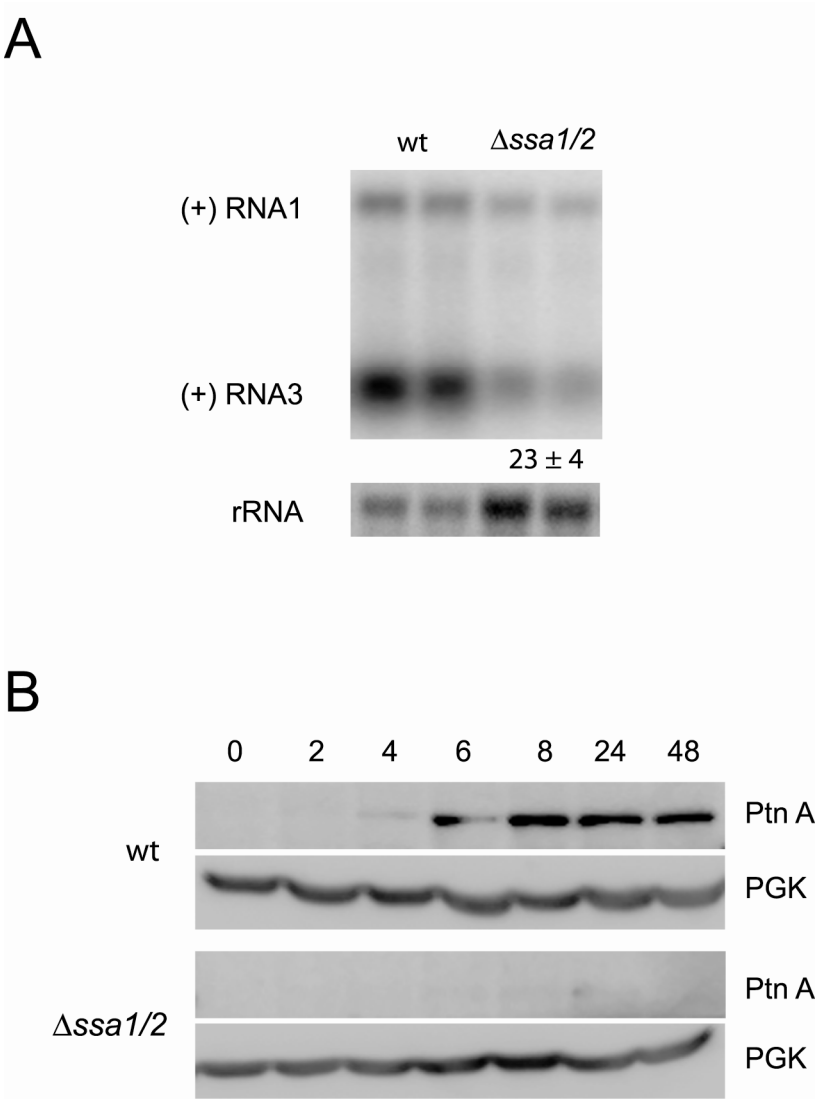


Figure 3.4 - Double deletion of SSA1/2 causes the same phenotype in FHV RNA replication as deletion of *YDJ1*. (A) FHV replication in cis in wt and *Δssa1/2*. Number represents accumulation of (+)RNA3 compared to wild type, and the result of two experiments ± standard deviation. (B) Yeast expressing HA-tagged protein A were induced in raffinose/galactose and harvested at the indicated times and analyzed by immunoblot.

dramatic increase in the levels of protein A and (+) RNA3 (Table 3.2). Zuo1p and Ssz1p function in cells as the ribosome associated complex, and have been reported to be involved in the efficiency and fidelity of translation in yeast (8, 17, 39). They do this by regulating the *SSB* family of Hsp70s, which are also ribosome associated in fungal cells (17). The dramatic increase in FHV RNA replication was the strongest such phenotype in our analysis, and we next sought to complement this phenotype with other J-domain proteins. Consistent with our results from the targeted analysis, deletion of *ZUO1* caused an increase of RNA3 above wildtype levels (Figure 3.5A, lanes 1-2 vs. 4). Complementation of this phenotype in *Δzuo1* yeast was only possible through expression of *ZUO1* or *JJJ1* (Figure 3.5A, lanes 6 and 12).

There may have been some partial complementation in the *APJ1*, *JJJ2*, *SIS1*, and *YDJ1* (Figure 3.5A, lane 4 vs lanes 5, 7, 9, and 11) which were reduced from 400% to about 300%, but the only two J-domain proteins that were able to complement the phenotype to near wildtype levels were Jjj1p and Zuo1p (Figure 3.5B). In addition to the J-domain, both of these proteins contain a zuotin domain, which has been shown to bind Z-DNA in vitro (Figure 3.5C) (54), and a zinc finger domain, as well as a charged domain, which have been hypothesized to bind target proteins.

Deletions in the *SSB* family of Hsp70 chaperones increase FHV RNA replication. Because the deletion of *SSZ1* and *ZUO1* caused the same phenotype, and both are regulators of the *SSB* family of Hsp70s, we next hypothesized that the *SSB* family of Hsp70s was the source of the phenotype in the *Δzuo1* strain. In fungal cells, the *SSB* family of Hsp70s is ribosome associated, and is involved in the regulation and fidelity of translation. Ssb1p and Ssb2p are 99% identical to each other and are functionally redundant, and are 63% identical to the *SSA* family (4). A double deletion is viable, and has been reported to be similar in phenotype to both the *Δzuo1* and *Δssz1* strains

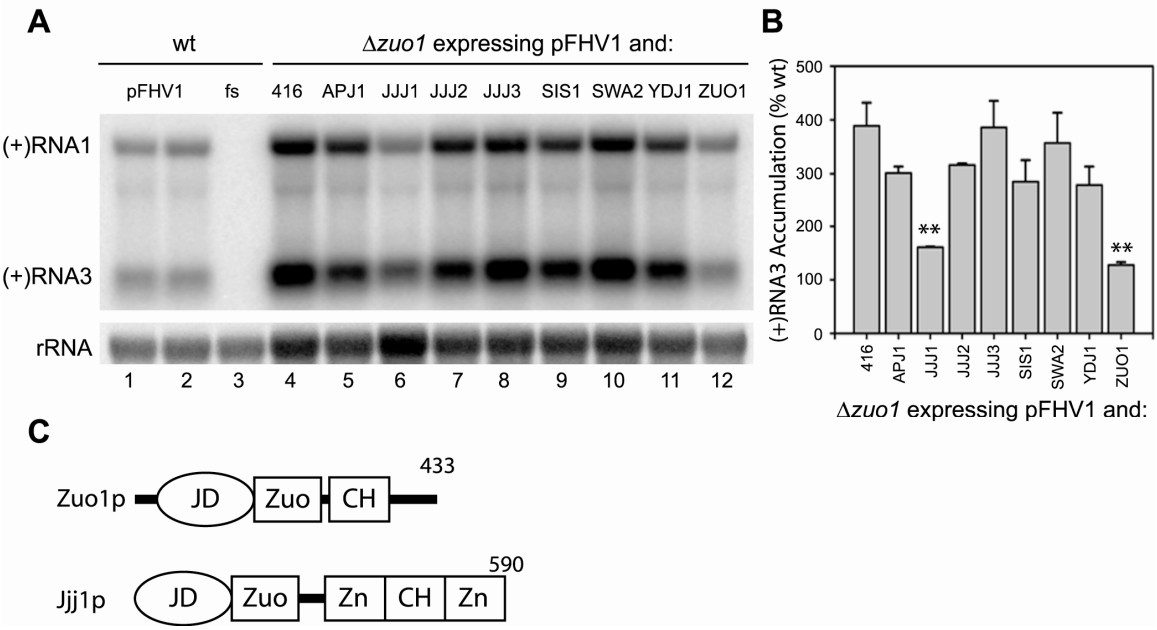


Figure 3.5 - Cross complementation of the *Δzuo1* strain. (A) Wt yeast expressing pFHV1 or pFHV1fs, or *Δzuo1* yeast expressing pFHV1 and the indictated plasmid were grown and induced in raffinose/galactose media. RNA was extracted as before and analyzed by Northern blot. (B) Quantification of A, lanes 4-12. Bars represent the accumulation of RNA3 compared to wt and are the result of three independent experiments ± SEM. A double asterisk indicates a *p*-value < 0.05 by two-tailed Student's *t*-test assuming unequal variances compared to vector control. (C) Schematics of J-domain proteins: Zuo1p and Jjj1p are from (41). Numbers indicate length in amino acids of full length proteins. Domain abbreviations: JD - J-domain, Zuo - zuotin domain, Zn - Zinc Finger, CH - charged domain

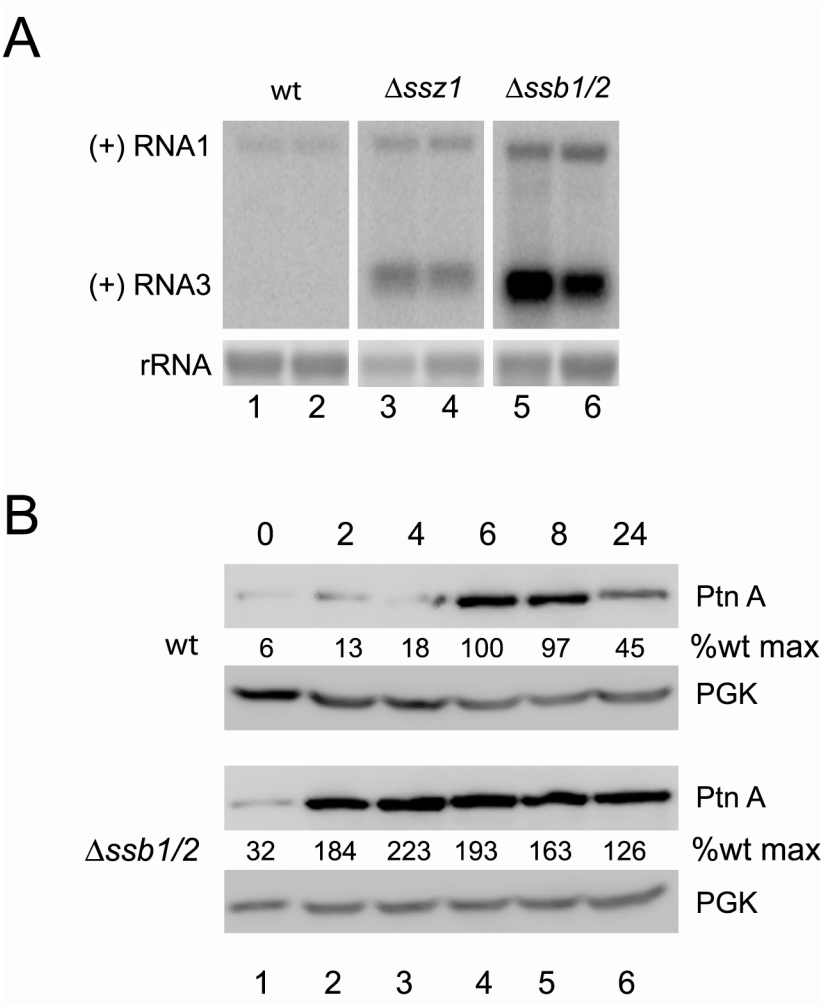


Figure 3.6 - Double deletion of *SSB1/2* causes the same phenotype in FHV RNA replication as deletion of RAC members. (A) FHV replication in cis in wt, *Δssz1*, or *Δssb1/2*. (B) Yeast expressing HA-tagged protein A were induced in galactose and harvested at the indicated times and analyzed by immunoblot.

(16, 17). We obtained this *Δssb1/2* strain as well as a *Δssz1* strain in the same MH272-3f background, and examined FHV replication in cis in all strains (Figure 3.6A). FHV replication in the wild type strain was not as robust as in other strains (Figure 3.6A, lanes 1-2), consistent with previous results from our lab (unpublished data).

The deletion of *SSZ1* in the MH272-3f background caused an increase in the accumulation of RNA3 (Figure 3.6A, lanes 3-4 vs. 1-2), similar to in the results in the BY4743 background. Further, the double deletion of *SSB1* and *SSB2* caused a more exaggerated phenotype, increasing RNA replication two-fold above the increase in the *SSZ1* deletion strain (Figure 3.6A, lanes 5-6 vs. 3-4). Further, the accumulation of protein A expressed from a *GAL1* promoter-driven vector was faster and saturated at a higher level in the *Δssb1/2* strain than in the MH272-3f (Figure 3.6B). These results are consistent with the hypothesis that the role of Zuo1p and Ssz1p in FHV RNA replication is to regulate the activity of Ssb1/2p on the ribosome, and so we hypothesize that the phenotype seen in the *Δssz1* and *Δzuo1* strains is due to a deregulation of the Ssb1/2 chaperone on the ribosome. This hypothesis is based on the known cellular role of Zuo1p and Ssz1p as regulators of the Ssb1/2 chaperone complex, though we can not rule out that Ssz1p, Zuo1p, and Ssb1/2p have separate cellular effects that yield the same phenotype from these data.

Discussion

Cellular chaperones have effects on FHV RNA replication. In this study, we used a panel of deletion strains to examine the roles of cellular chaperones on FHV RNA replication. We used three types of replicons to determine effects on replication in cis, as well as both protein and RNA effects on replication in trans targeted to the mitochondria and retargeted to the endoplasmic reticulum. Analysis of these chaperones revealed several interesting phenotypes. The major chaperone groups of Hsp70/90 are functionally redundant, and therefore we didn't see many phenotypes by deleting individual members. The impact of the major chaperones on FHV RNA replication were instead revealed by deletions of their respective cochaperones, which had much more consistent and significant phenotypes. One family that was particularly interesting was the J-domain containing family of Hsp40 proteins. These proteins serve to regulate the ATPase activity of their partner Hsp70s, and by using what is known about these pairings, we were able to predict which particular Hsp70s were involved. The minor and specialized chaperones were also an interesting group, and all suggest potential cellular functions that are important for FHV RNA replication.

The goal of this targeted analysis was identify chaperones that had a functional role in FHV RNA replication, and also to build a testable model for the early events of FHV RNA replication. Using these primary data, as well as information in the literature about the cellular roles of these chaperones, we can make hypotheses about individual steps of the translation, folding and trafficking of protein A, as well as the formation and activity of FHV RNA replication complexes. This model will be described in the final chapter of this thesis (Chapter V), and will serve as the basis of future experiments to dissect how chaperones influence FHV RNA replication.

The specificity of *Δydj1* and *Δzuo1* phenotypes links the J-domain proteins to their partner Hsp70s. The two strongest phenotypes in our targeted analysis were those caused by the deletion of *YDJ1* and *ZUO1*, and so we next examined these phenotypes in more detail. The deletion of the Hsp40/JDP *YDJ1* caused a drastic decrease in FHV RNA replication, while the deletion of *ZUO1* caused a drastic increase. By over expressing individual J-domain proteins in the *Δydj1* and *Δzuo1* backgrounds, we examined the ability of other J-domain proteins to complement the defects.

The ability of unrelated J-domain proteins to partially complement the defective RNA replication phenotype of the *Δydj1* strain is consistent with what is known about the cellular function of Ydj1p as a regulator of the *SSA* family of Hsp70s (9, 10, 41, 45). We examined this possibility by using a strain with a deletion of two *SSA* members, and found that deletion of *SSA1* and *SSA2* replicated the *Δydj1* phenotype, decreasing RNA3 levels by 75%. This is consistent with the hypothesis that the effect of deleting *YDJ1* is due to a deregulation of the *SSA* chaperone complex.

We performed a similar complementation experiment with the *Δzuo1* strain. This experiment revealed that only Zuo1p or Jjj1p were able to complement the *Δzuo1* phenotype. This result was consistent with previous studies of Zuo1p and, as a part of the ribosome associated complex, its role in the regulation of the ribosome associated *SSB* family of Hsp70 chaperones (41). The fact that Jjj1p could complement the phenotype of the *Δzuo1* strain can also be explained by its cellular role. Jjj1p is also ribosome associated and regulates the *SSA* complex. Jjj1p's cellular role is to recruit Ssa1/2p to the ribosome, where the chaperone is involved in ribosome biogenesis (30, 41). Therefore it has been suggested that overexpression of Jjj1p can recruit Ssa1/2p to the ribosome, where the chaperone complex can complement the defects associated with deletion of the ribosome associated Ssz1p/Zuo1p/Ssb1/2p complex.

Due to Zuo1p and Ssz1p's role of regulating Ssb1/2, we next examined the effect of a double deletion of *SSB1* and *SSB2* on FHV RNA replication. We used a different wild type strain for this experiment, and it appeared restricted for FHV RNA replication. As in the targeted analysis, the deletion of *SSZ1* relieved that restriction, increasing replication to detectable levels. Furthermore, the double deletion of *SSB1* and *SSB2* caused an additional increase in FHV RNA replication. These results are consistent with the hypothesis that the defect in the *Δzuo1* and *Δssz1* strains was caused by a defect in the regulation of the Ssb1/2p chaperone complex. The restriction of FHV RNA replication in the MH272-3f wild type strain is consistent with observations in our lab that there are differences in the robustness of FHV RNA replication between wild type strains, though this is the first time that we have seen a mutation that relieved the restriction. This presents the possibility for further study of restriction factors that negatively regulate FHV RNA replication in yeast.

Two competing hypotheses. Because these studies do not examine the physical interaction of protein A with any of these chaperones, there are two possibilities that could explain our results. The phenotypes that we saw could be due to a direct or indirect on FHV RNA replication. Because chaperones are known to act through transient interactions with their client proteins, it is attractive to hypothesize that these chaperones are acting directly on protein A, or on FHV RNA replication complexes. However, it is equally possible that these chaperones are acting indirectly, through their role as chaperones on other cellular factors, which are then directly influencing protein A or FHV RNA replication. Further studies are necessary to distinguish between these possibilities, yet they are not mutually exclusive, and likely there are aspects of direct and indirect influence involved.

References

1. **Ball, L. A.** 1995. Requirements for the self-directed replication of flock house virus RNA 1. J Virol **69:**2722.

2. **Ball, L. A., and K. L. Johnson.** 1998. *Nodaviruses* of Insects, p. 225-267. *In* L. K. Miller and L. A. Ball (ed.), The Insect Viruses. Plenum Publishing Corporation, New York, N. Y.

3. **Bohen, S. P.** 1998. Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. Mol. Cell. Biol. **18:**3330-3339.

4. **Boorstein, W. R., T. Ziegelhoffer, and E. A. Craig.** 1994. Molecular evolution of the HSP70 multigene family. J Mol Evol **38:**1-17.

5. **Buck, K. W.** 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv. Virus Res. **47:**159-251.

6. **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:**8412-8420.

7. **Chang, H. C., D. F. Nathan, and S. Lindquist.** 1997. In vivo analysis of the Hsp90 cochaperone Sti1 (p60). Mol Cell Biol **17:**318-25.

8. **Conz, C., H. Otto, K. Peisker, M. Gautschi, T. Wolfle, M. P. Mayer, and S. Rospert.** 2007. Functional characterization of the atypical Hsp70 subunit of yeast ribosome-associated complex. J Biol Chem **282:**33977-84.

9. **Cyr, D. M., and M. G. Douglas.** 1994. Differential regulation of Hsp70 subfamilies by the eukaryotic DnaJ homologue YDJ1. J Biol Chem **269:**9798-804.

10. **Cyr, D. M., X. Lu, and M. G. Douglas.** 1992. Regulation of Hsp70 function by a eukaryotic DnaJ homolog. J Biol Chem **267:**20927-31.

11. **Donze, O., and D. Picard.** 1999. Hsp90 binds and regulates Gcn2, the ligand-inducible kinase of the alpha subunit of eukaryotic translation initiation factor 2 [corrected]. Mol Cell Biol **19:**8422-32.

12. **Frolova, E., R. Gorchakov, N. Garmashova, S. Atasheva, L. A. Vergara, and I. Frolov.** 2006. Formation of nsP3-specific protein complexes during Sindbis virus replication. J. Virol. **80:**4122-4134.

13. **Galigniana, M. D., J. M. Harrell, P. J. Murphy, M. Chinkers, C. Radanyi, J. M. Renoir, M. Zhang, and W. B. Pratt.** 2002. Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and in vivo evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. Biochemistry **41:**13602-10.

14. **Galigniana, M. D., J. M. Harrell, H. M. O'Hagen, M. Ljungman, and W. B. Pratt.** 2004. Hsp90-binding immunophilins link p53 to dynein during p53 transport to the nucleus. J Biol Chem **279:**22483-9.

15. **Gallagher, T. M., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. **62:**3399-3406.

16. **Gautschi, M., H. Lilie, U. Funfschilling, A. Mun, S. Ross, T. Lithgow, P. Rucknagel, and S. Rospert.** 2001. RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin. Proc Natl Acad Sci U S A **98:**3762-7.

17. **Gautschi, M., A. Mun, S. Ross, and S. Rospert.** 2002. A functional chaperone triad on the yeast ribosome. Proc Natl Acad Sci U S A **99:**4209-14.

18. **Geissler, S., K. Siegers, and E. Schiebel.** 1998. A novel protein complex promoting formation of functional alpha- and gamma-tubulin. Embo J **17:**952-66.

19. **Glover, J. R., and S. Lindquist.** 1998. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell **94:**73-82.

20. **Hartl, F. U., and M. Hayer-Hartl.** 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science **295:**1852-8.

21. **Haslbeck, M., N. Braun, T. Stromer, B. Richter, N. Model, S. Weinkauf, and J. Buchner.** 2004. Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. Embo J **23:**638-49.

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

23. **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.

24. **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.

25. **Kushnirov, V. V.** 2000. Rapid and reliable protein extraction from yeast. Yeast **16:**857-60.

26. **Li, H., W. X. Li, and S. W. Ding.** 2002. Induction and suppression of RNA silencing by an animal virus. Science **296:**1319-21.

27. **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.

28. **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.

29. **Mayr, C., K. Richter, H. Lilie, and J. Buchner.** 2000. Cpr6 and Cpr7, two closely related Hsp90-associated immunophilins from Saccharomyces cerevisiae, differ in their functional properties. J Biol Chem **275:**34140-6.

30. **Meyer, A. E., N. J. Hung, P. Yang, A. W. Johnson, and E. A. Craig.** 2007. The specialized cytosolic J-protein, Jjj1, functions in 60S ribosomal subunit biogenesis. Proc Natl Acad Sci U S A **104:**1558-63.

31. **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.

32. **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.

33. **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.

34. **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.

35. **Parsell, D. A., A. S. Kowal, M. A. Singer, and S. Lindquist.** 1994. Protein disaggregation mediated by heat-shock protein Hsp104. Nature **372:**475-8.

36. **Pratt, W. B., P. Krishna, and L. J. Olsen.** 2001. Hsp90-binding immunophilins in plants: the protein movers. Trends Plant Sci **6:**54-8.

37. **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.

38. **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.

39. **Rakwalska, M., and S. Rospert.** 2004. The ribosome-bound chaperones RAC and Ssb1/2p are required for accurate translation in Saccharomyces cerevisiae. Mol Cell Biol **24:**9186-97.

40. **Rossi, J. M., and S. Lindquist.** 1989. The intracellular location of yeast heat-shock protein 26 varies with metabolism. J Cell Biol **108:**425-39.

41. **Sahi, C., and E. A. Craig.** 2007. Network of general and specialty J protein chaperones of the yeast cytosol. Proc Natl Acad Sci U S A **104:**7163-8.

42. **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:**181-189.

43. **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:**2162-2169.

44. **Tomita, Y., T. Mizuno, J. Diez, S. Naito, P. Ahlquist, and M. Ishikawa.** 2003. Mutation of host DnaJ homolog inhibits Brome mosaic virus negative- strand RNA synthesis. J. Virol. **77:**2990-2997.

45. **Tsai, J., and M. G. Douglas.** 1996. A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. J Biol Chem **271:**9347-54.

46. **Uma, S., S. D. Hartson, J. J. Chen, and R. L. Matts.** 1997. Hsp90 is obligatory for the heme-regulated eIF-2alpha kinase to acquire and maintain an activable conformation. J Biol Chem **272:**11648-56.

47. **Vainberg, I. E., S. A. Lewis, H. Rommelaere, C. Ampe, J. Vandekerckhove, H. L. Klein, and N. J. Cowan.** 1998. Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. Cell **93:**863-73.

48. **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.

49. **Waxman, L., M. Whitney, B. A. Pollok, L. C. Kuo, and P. L. Darke.** 2001. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc. Natl. Acad. Sci. USA **98:**13931-13935.

50. **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.

51. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2568-2577.

52. **Yang, J., J. M. Yang, M. Iannone, W. J. Shih, Y. Lin, and W. N. Hait.** 2001. Disruption of the EF-2 kinase/Hsp90 protein complex: a possible mechanism to inhibit glioblastoma by geldanamycin. Cancer Res **61:**4010-6.

53. **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.

54. **Zhang, S., C. Lockshin, A. Herbert, E. Winter, and A. Rich.** 1992. Zuotin, a putative Z-DNA binding protein in Saccharomyces cerevisiae. Embo J **11:**3787-96.

APPENDIX

Primary data for targeted analysis of chaperones. Data from Table 3.2 are shown as representative blots.

Figure 3.7 – Targeted analysis of chaperones: replication in cis. Total RNA extracted from an equivalent number of yeast was separated by electrophoresis and analyzed by Northern blot as in the materials and methods. (A) Group 1. (B) Group 2. (C) Group 3. Abbreviations: wt – wild type yeast expressing pFHV1, neg – wild type yeast containing an empty vector, rRNA – ribosomal RNA.

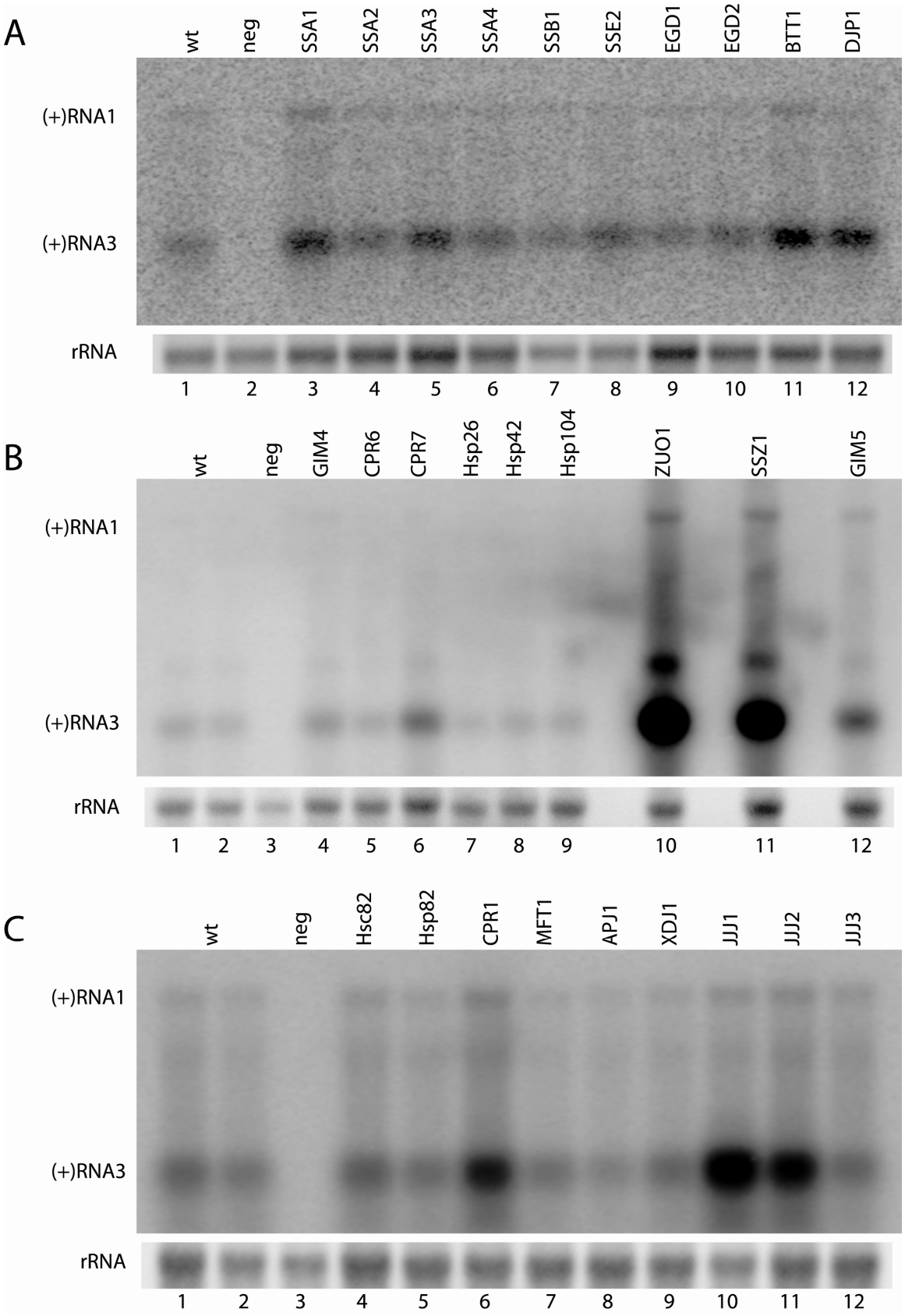
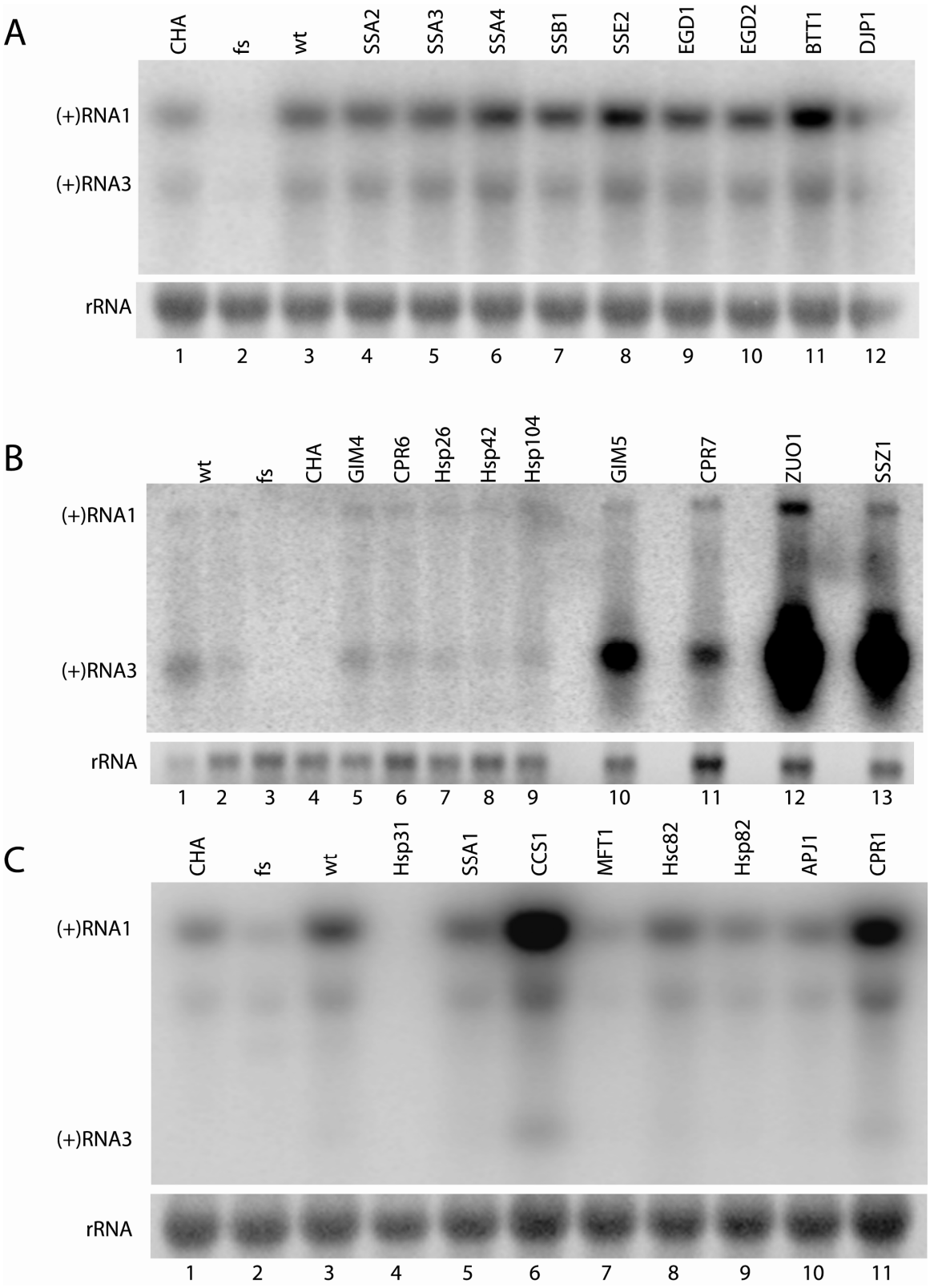

A
wt
neg
SSA1
SSA2
SSA3
SSA4
SSB1
SSE2
EGD1
EGD2
BTT1
DJP1
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12
B
wt
neg
GIM4
CPR6
CPR7
Hsp26
Hsp42
Hsp104
ZUO1
SSZ1
GIM5
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12
C
wt
neg
Hsc82
Hsp82
CPR1
MFT1
APJ1
XDJ1
JJJ1
JJJ2
JJJ3
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.8 – Targeted analysis of chaperones: replication in trans RNA samples. Total RNA extracted from an equivalent number of yeast was separated by electrophoresis and analyzed by Northern blot as in the materials and methods. (A) Group 1. (B) Group 2. (C) Group 3. Abbreviations: CHA – wild type yeast expressing protein A only, fs – wild type yeast expressing replication template only, wt – wild type yeast expressing both protein A and a replication template, rRNA – ribosomal RNA.


A
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SSA2
SSA3
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SSB1
SSE2
EGD1
EGD2
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DJP1
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12
B
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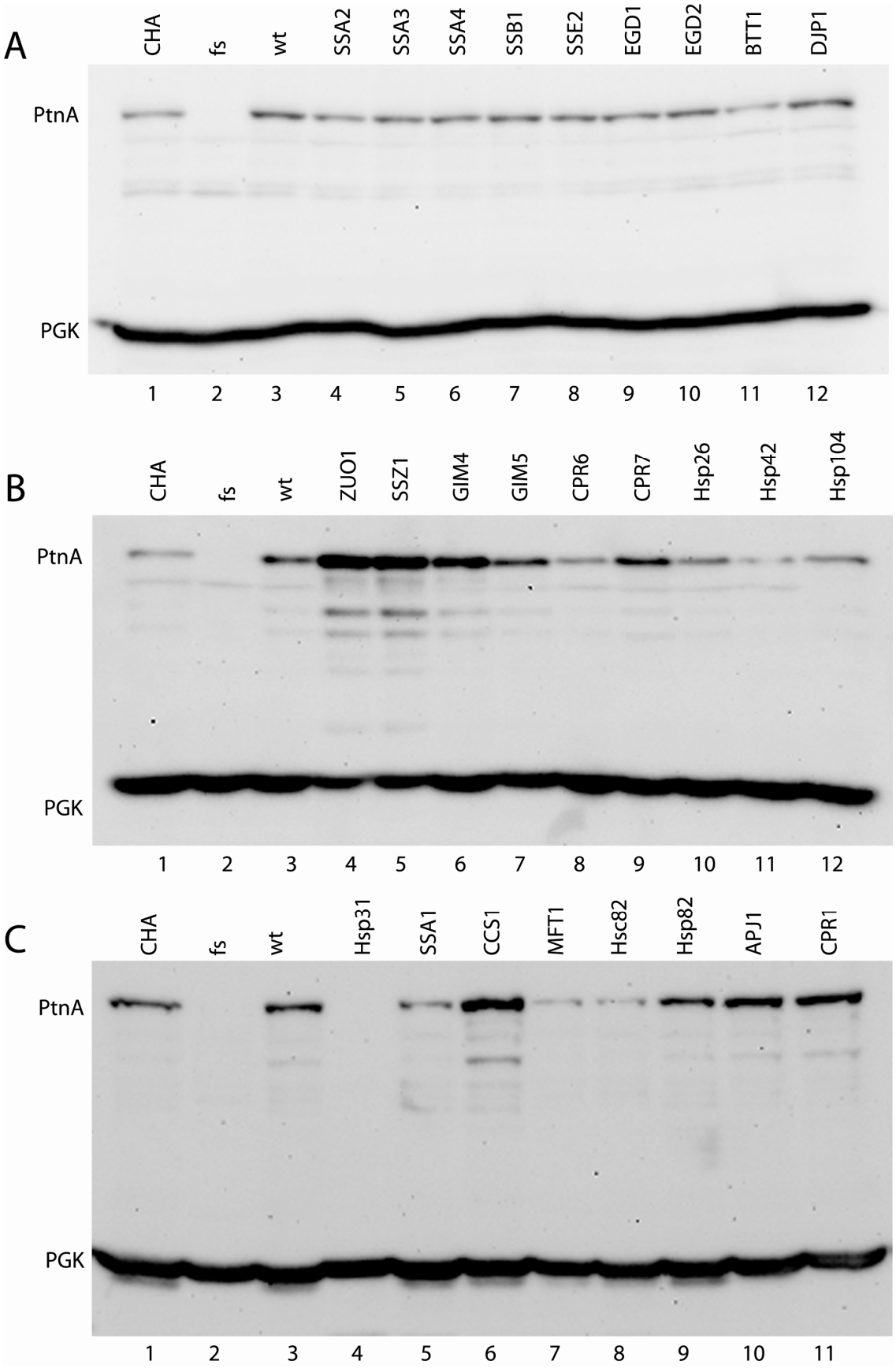
CHA
GIM4
CPR6
Hsp26
Hsp42
Hsp104
GIM5
CPR7
ZUO1
SSZ1
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12 13
C
CHA
fs
wt
Hsp31
SSA1
CCS1
MFT1
Hsc82
Hsp82
APJ1
CPR1
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11

Figure 3.9 – Targeted analysis of chaperones: replication in trans protein samples. Total protein extracted from an equivalent number of yeast was separated by electrophoresis and analyzed by immunoblot as in the materials and methods. (A) Group 1. (B) Group 2. (C) Group 3. Abbreviations: CHA – wild type yeast expressing protein A only, fs – wild type yeast expressing replication template only, wt – wild type yeast expressing both protein A and a replication template, rRNA – ribosomal RNA.


A
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SSA2
SSA3
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SSB1
SSE2
EGD1
EGD2
BTT1
DJP1
PtnA
PGK
1 2 3 4 5 6 7 8 9 10 11 12
B
CHA
fs
wt
ZUO1
SSZ1
GIM4
GIM5
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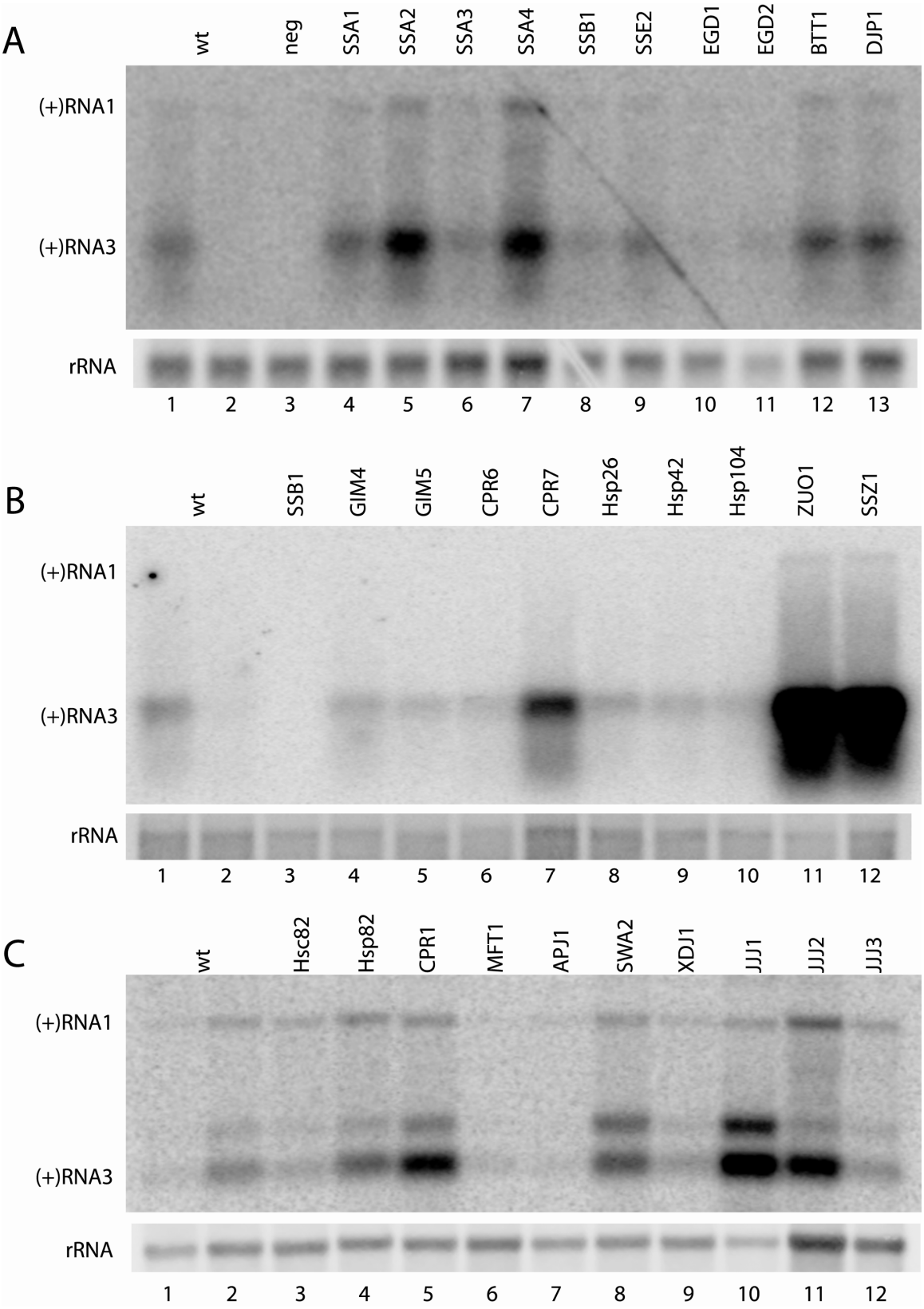
Hsp42
Hsp104
PtnA
PGK
1 2 3 4 5 6 7 8 9 10 11 12
C
CHA
fs
wt
Hsp31
SSA1
CCS1
MFT1
Hsc82
Hsp82
APJ1
CPR1
PtnA
PGK
1 2 3 4 5 6 7 8 9 10 11

Figure 3.10 – Targeted analysis of chaperones: ER-retargeted replication in trans RNA samples. Total RNA extracted from an equivalent number of yeast was separated by electrophoresis and analyzed by Northern blot as in the materials and methods. (A) Group 1. (B) Group 2. (C) Group 3. Abbreviations: wt – wild type yeast expressing both ER-retargeted protein A and a replication template, neg – wild type yeast expressing replication template only, rRNA – ribosomal RNA.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wt
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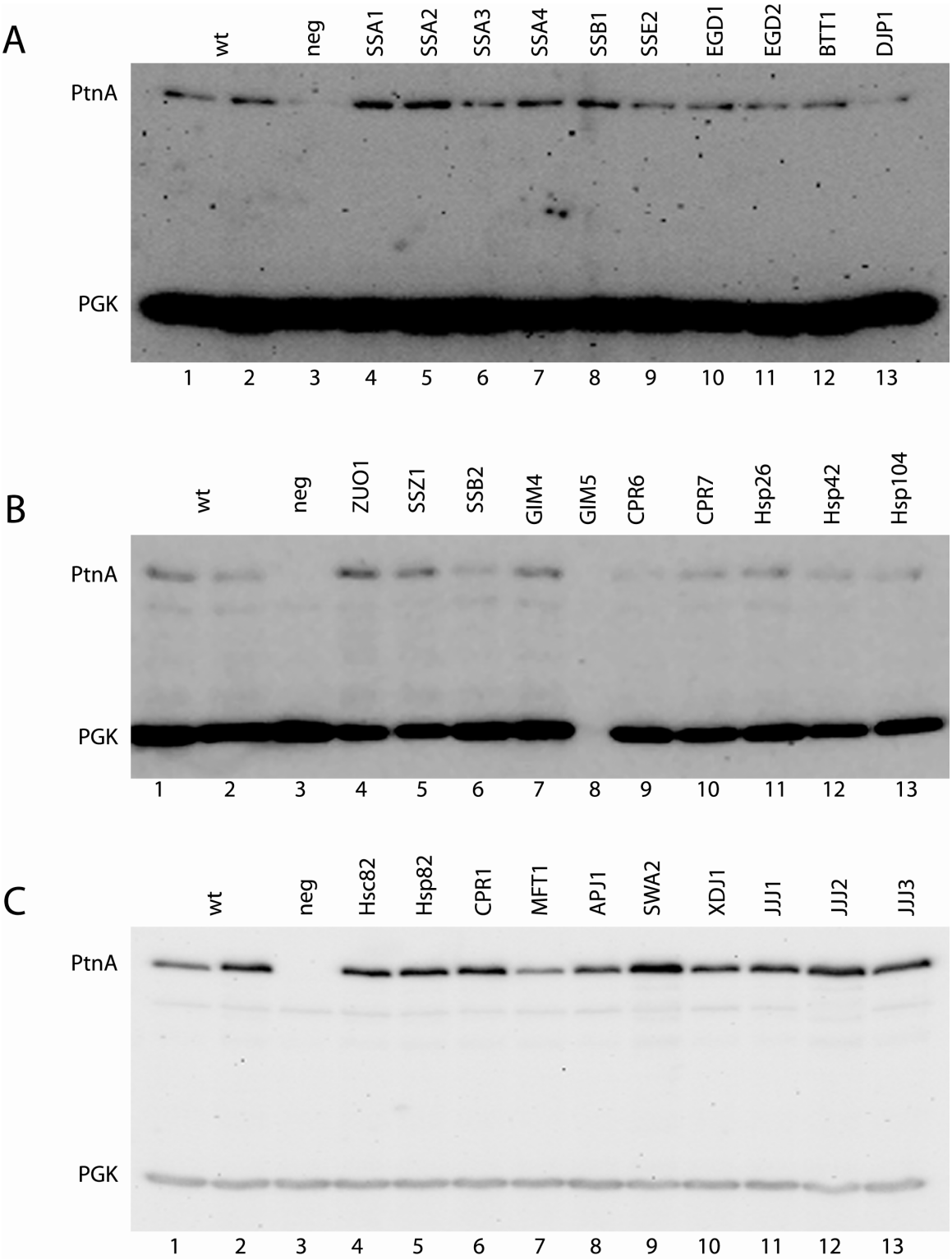
SSA2
SSA3
SSA4
SSB1
SSE2
EGD1
EGD2
BTT1
DJP1
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12 13
B
wt
SSB1
GIM4
GIM5
CPR6
CPR7
Hsp26
Hsp42
Hsp104
ZUO1
SSZ1
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12
C
wt
Hsc82
Hsp82
CPR1
MFT1
APJ1
SWA2
XDJ1
JJJ1
JJJ2
JJJ3
(+)RNA1
(+)RNA3
rRNA
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.11 – Targeted analysis of chaperones: ER-retargeted replication in trans protein samples. Total protein extracted from an equivalent number of yeast was separated by electrophoresis and analyzed by immunoblot as in the materials and methods. (A) Group 1. (B) Group 2. (C) Group 3. Abbreviations: wt – wild type yeast expressing both ER-retargeted protein A and a replication template, neg – wild type yeast expressing replication template only, rRNA – ribosomal RNA.


A
wt
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SSA1
SSA2
SSA3
SSA4
SSB1
SSE2
EGD1
EGD2
BTT1
DJP1
PtnA
PGK
1 2 3 4 5 6 7 8 9 10 11 12 13
B
wt
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ZUO1
SSZ1
SSB2
GIM4
GIM5
CPR6
CPR7
Hsp26
Hsp42
Hsp104
PtnA
PGK
1 2 3 4 5 6 7 8 9 10 11 12 13
C
wt
neg
Hsc82
Hsp82
CPR1
MFT1
APJ1
SWA2
XDJ1
JJJ1
JJJ2
JJJ3
PtnA
PGK
1 2 3 4 5 6 7 8 9 10 11 12 13

CHAPTER IV

DESIGNING A METHOD OF HIGH THROUGHPUT SCREENING FOR CHANGES IN PROTEIN A SYNTHESIS AND STABILITY IN YEAST

Identification of host factors or chemical compounds that modulate the synthesis and stability of viral proteins would be beneficial for designing research tools as well as potential therapeutic targets. Due to the number of chemical and genetic libraries available to researchers today, designing a method to test these libraries in a high throughput way is a necessary step in beginning any kind of comprehensive study into their effects on a given system. Yeast make a useful model system for high throughput screening due to its genetic tractability and ease of use in the laboratory, as well as the number of yeast genes that have orthologs in higher eukaryotes. Therefore we used the Flock House virus model system to develop a method for high throughput screening of genetic and chemical libraries to examine their effects on the synthesis and stability of protein A, the Flock House virus RNA-dependent RNA polymerase. These preliminary studies have identified a number of complicating factors, but have identified two approaches that may be useful in this task.

Introduction

Positive strand (+) RNA viruses are significant health threats today, yet there are relatively few clinical treatments available for those infected with them. Because (+) RNA viruses replication is intimately tied to the host cellular machinery, it is difficult to separate viral processes from those of the cell. Therefore, better understanding of how (+) RNA viruses replicate in host cells is an important first step in the design and development of therapeutic agents to combat viral infections. We use Flock House virus (FHV) as a model (+) RNA viral pathogen because of its robust genomic replication in many eukaryotic cell types including *Drosophila melanogaster* (6, 15), *Caenorhabditis elegans* (12), and *Saccharomyces cerevisiae* (11, 14, 16, 19, 20). This implies that the host factors required for efficient FHV RNA replication are either well conserved or few in number. In order to efficiently identify the specific role of cellular factors in viral replication, we must first identify those factors that impact replication, and then further test their effect. A good way to begin a study like this is through the use of genetic and chemical libraries in a high throughput manner. The use of a model host often can be beneficial because of the genetic tractability and availability of libraries. In our lab we use the brewer's yeast *Saccharomyces cerevisiae* as a model host.

Studies in our lab and others have shown that yeast is a viable model host organism for (+) RNA virus replication studies (11, 14, 16, 19, 20, 22). Yeast is as easy to culture and transform as bacteria, yet is a eukaryotic cell, with many genes that are orthologous to those of higher eukaryotes (5, 13). The true benefits of yeast, however, lie in the availability of numerous genetic libraries that have been constructed, including deletion (8), promoter controlled expression (17), as well as the possibility of using yeast to screen chemical libraries (23). In order to screen the large number of libraries efficiently, it is necessary to design a method of screening them in a high throughput way. Therefore, assay design is critical before scaling up experiments, and thorough

testing of conditions must be done to ensure that sample to sample variability is minimized. In this study, we began the development of a high throughput assay based on host gene deletions that impact the synthesis and stability of protein A, the Flock House virus RNA-dependent RNA polymerase.

Our first goal was to develop an assay based on a selectable tag with a survival-based readout. Survival-based designs allow for screening strains in pools, biologically eliminating negatives and leaving a higher yield of positive strains. When the homologous diploid deletion library that we used was constructed, a molecular barcode was included in the flanking region around the inserted KanMX gene (8). This barcode is unique for each strain and can be amplified by PCR from any strain using the same primer set. This PCR product can be used as a probe for a microchip containing each code in a known location, allowing pools of strains to be grown together and each strain's growth to be tracked by barcode (8). For our first design we chose to tag protein A with the *URA3* gene in order to take advantage of the positive and negative selection that is possible with such a construct (1, 2). This method has been used in screens before (24), and the *URA3* tag has been shown to be active on intracellular membranes (24). Ura3p normally processes orotidine 5-phosphate into uracil, but when grown in media containing the chemical mimic 5-fluoroorotic acid (5-FOA), the enzyme converts it into 5-fluorouracil, which is toxic (2, 25).

Our second goal was to design the screen in a more traditional way, based on the firefly luciferase gene, which has been shown to be active and easy to read in yeast (10). Both the *URA3* and luciferase tags were functional as protein A fusion constructs, and we therefore tested their utility as methods of high-throughput screening.

Materials and Methods

Yeast strains, transformation, and culture conditions. The diploid *S. cerevisiae* strain BY4743 (*MATa/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0*) was used for all experiments to minimize complications related to second site mutations more common in haploid strains. Wild type (wt) BY4743 and diploid strains with homozygous deletions of *MFT1*, *APJ1*, *GIM4*, and *SSZ1* were purchased from the American Type Culture Collection (Manassas, VA). Yeast were cultured and transformed as previously described (14), except that due to the temperature-sensitive nature of FHV RNA replication complexes (unpublished observations), and slow growth or inviability of some mutant strains at higher temperatures, as well as to maintain consistency with *Drosophila* studies, all experiments were performed at 25^{o}C unless otherwise stated. For yeast induction, individual clones were transferred to liquid selective minimal media containing 2% glucose and grown to stationary phase, washed with sterile distilled water and resuspended in selective minimal media with 2% galactose and indicated selection agents at an optical density unit at 600 nm (OD_{600}) of 0.1, which was equivalent to approximately 2.5 x 10^6 cells per ml for both wt and deletion strains. For constitutive expression constructs, individual clones were transferred into liquid selective media containing 2% glucose and grown to stationary phase, washed, and diluted into the same media with indicated selection agents at an optical density unit at 600 nm (OD_{600}) of 0.1. For calculation of the number of doublings, the following equation was used: Log_2 (OD_2/OD_1), where OD_2 and OD_1 are the final and initial OD_{600} readings, respectively. Percent doublings was calculated using the following equation: 100 x ($D_{5\text{-}FOA}/D_{vehicle}$), where $D_{5\text{-}FOA}$ and $D_{vehicle}$ are the number of doublings in the 5-FOA treated and untreated samples, respectively. Unless otherwise stated, experiments were

performed with two independently derived clones and results are representative of at least three independent experiments. For dilution plating, yeast were grown as above, then adjusted to 1.0 OD_{600}/ml and serial diluted five times at 1:10 in a 96 well plate. 10 µl of each dilution was spotted onto agar plates containing the indicated supplements and incubated at 30°C for three days until colonies formed.

Plasmids. FHV expression plasmids pFA-C/HA has been previously described (11, 14, 16, 20). The constitutive expression constructs pRS425-GPD, pRS415-ADH were generously provided by Anuj Kumar (University of Michigan, Ann Arbor, MI). The protein A-URA3 fusion construct pFA-C/URA3 was generated by PCR using primers for the *URA3* gene that change the first amino acid from a methionine to a glycine, and incorporate restriction sites *BspE1* and *AatII* upstream and downstream of the *URA3* coding sequence, respectively (See Table 4.1). Following PCR amplification from pRS416 (ATCC, Manassas, VA) and restriction digestion with the above enzymes, the PCR products were separated by agarose gel electrophoresis and the correctly sized *URA3* fragment was isolated using the PCR and Gel clean up kit per manufacturer's instructions (Promega, Madison, WI). Isolated products were ligated into pFA-C/HA plasmid digested with the above enzymes, generating a URA3 fusion beginning in the linker of the C/HA tag and effectively replacing the tag. Constitutive expression

Table 4.1 - Primers for construction of protein A-C/Ura3

Primer	Sequence[a,b]	Res. Site
Sense	5′-CAACTGTCCGGA**GGT**TCGAAAGCTACATATAAGGAACGTG-3′	*BspEI*
Antisense	5′-GTCGATGACGTCAGTTTTTTAGTTTTGCTGGCCGCA-3′	*AatII*

a – restriction site sequences are underlined
b – First amino acid Met → Gly mutation is shaded

constructs were generated by moving the protein A-C/HA or protein A-C/URA3 open

reading frames from pFA-C/HA or pFA-C/URA3, respectively into pRS425-GPD. Further generation of other weaker constitutive expression constructs was done by moving the protein A-C/URA3 open reading frame into pRS415-ADH or by moving the GPD-protein A-C/URA3 cassette into pRS415 (ATCC, Manasas, VA). Protein A-C/URA3 constructs are summarized in Table 4.1. The yeast protein A-C/fLUC vector was generated in two steps. Firstly, a *Drosophila* based expression vector was generated by PCR amplifying a region encoding fLUC from pTRE2/hyg-LUC, incorporating *AgeI* and *XhoI* sites , digesting the PCR product, and ligating it into the *BspEI*/*XhoI* sites of pS2FA-GalL (15). Next, a *BlpI/HindIII* fragment from the *Drosophila* vector was inserted into the same sites of the yeast vector pFA-C/HA (14).

Table 4.2 – Constitutive protein A-C/URA3 vectors used in this study.

Construct Name	Copy Number	Promoter Activity	Expression Level
p425-GPD-C/URA3	High	High	High
p415-GPD-C/URA3	Low	High	Medium
p415-ADH-C/URA3	Low	Low	Low

Antibodies and reagents. Rabbit polyclonal antibodies against FHV protein A have been previously described (15). Rabbit polyclonal antibodies against the HA epitope tag were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibodies 3-phosphoglycerate kinase (PGK) were purchased from Molecular Probes (Eugene, OR). All secondary antibodies for immunoblotting were purchased from Jackson Immunoresearch (West Grove, PA).

Immunoblot analysis. Total protein was isolated from an equivalent number of yeast via post alkaline extraction (9) and stored at –20^{o}C until analysis. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as previously described (26). Protein bands were

quantitated by densitometry using AlphaEaseFC (Alpha Innotech, San Leandro, CA) software.

In vivo yeast firefly luciferase assay. The determination of firefly luciferase activity in intact yeast was adapted from Leskinen et al. (10) with some modifications. Essentially, yeast expressing fLUC tagged protein A were grown in 2% raffinose media in 96 well plates until saturated (usually 2 days), diluted 1:40 into 2% raffinose/2% galactose induction media, and induced overnight. Plates were then read at 600nm (OD_{600}), and 50 µl was transferred to an opaque plate and used for the luciferase assay. The luciferase assay was performed in a BMG plate reader with dual injectors (BMG labtech, Offenburg, Germany). Plates were shaken at max speed for 30s prior to assay to resuspend cells, and 200 µl of 1.1 mM D(-)-Luciferin (Sigma #L9504, St. Louis, MO) in Na Citrate buffer (pH 3.0) was injected in each well, then plates were shaken for 2s and light emission was detected at max gain for 10 seconds. Finally, light output was normalized to cell density (RLU/OD_{600}), giving a per cell RLU value. Calculations were done with BMG Mars software (BMG labtech, Offenburg, Germany).

Statistics. A two-tailed Student's *t*-test assuming unequal variances was used for all statistical analyses, and a p-value < 0.05 was considered statistically significant.

Results

Our goal is to design an easy to interpret method for high throughput screening of mutations or chemical compounds that decrease the accumulation of the FHV RNA-dependent RNA polymerase, protein A in *Saccharomyces cerevisiae*. To do this, we looked at several potential systems that had been used previously for high throughput screening.

Protein A with a C-terminal Ura3p tag is functional and selectable. In order to perform a viability based screen, we generated a protein A-Ura3 fusion protein and tested the functionality of this construct by selection and counter selection on minus uracil dropout media and 5-FOA media, respectively (Figure 4.1). Wild type yeast containing either C-terminally HA- or Ura3-tagged protein A were serially diluted and spotted on selective galactose plates without uracil or with 0.05% 5-FOA. Yeast expressing either construct were able to grow on media selecting for the plasmid (Figure 4.1, left panel), indicating that there was no difference in toxicity between HA- and Ura3-tagged protein A. Yeast expressing Ura3-tagged protein A were able to grow on uracil dropout media, whereas those expressing HA-tagged protein A were not (Figure 4.1, center panel). Conversely, media containing 5-FOA inhibited the growth of yeast expressing Ura3-, but not HA-tagged protein A (Figure 4.1, right panel). Further experiments were done on the same media at different temperatures, but there was no change in the results (data not shown). Therefore, the protein A-Ura3 fusion construct was functional in our hands, and could be used for selection or counter selection in yeast.

Protein A is toxic to yeast when constitutively expressed. In order to avoid picking up false positives that inhibit expression from an inducible *GAL1* promoter, we next moved the Ura3-tagged protein A ORF into a high copy number vector, with constitutively active *GPD* promoter (Table 4.2). When we transformed this construct,

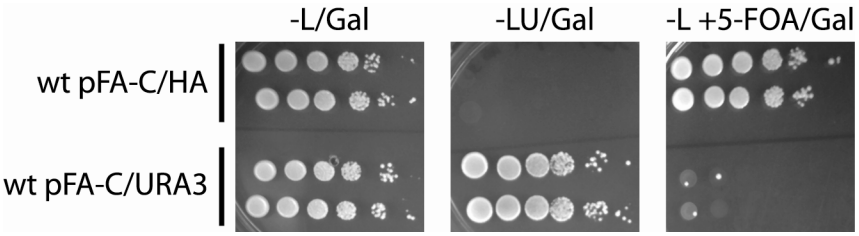

Figure 4.1 - A Ura3 tag is functional on protein A. Wild type yeast containing HA- of Ura3- tagged protein A constructs were serially diluted 1:10 and spotted onto selective media containing galactose. Plates were grown at 30 degrees celsius for three days. Abbreviations: L: leucine, U: uracil, 5-FOA: 0.05% 5-fluoroorotic acid, Gal: 2% galactose.

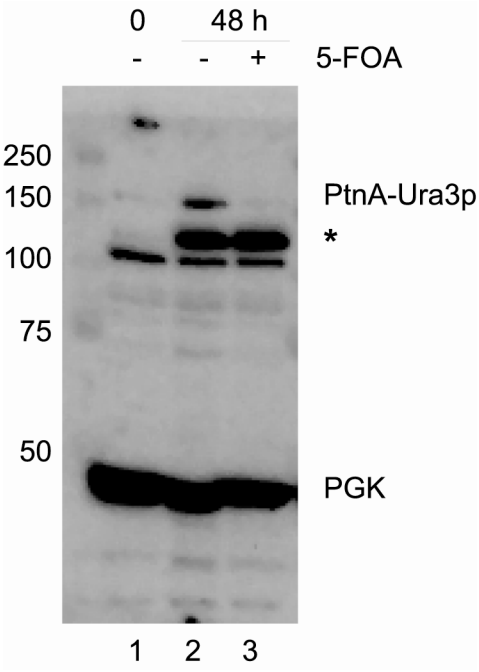

Figure 4.2 - Ura3-tagged protein A is unstable at high expression levels. Wildtype yeast expressing p425-GPD-FA-C/Ura3 were grown for 48h in the presence or absence of 5-FOA. Immunoblot analysis with a polyclonal protein A antibody. The low expression in lane 1 is due to the cells being in stationary phase. Numbers on the left are protein standards in kDa. The asterisk indicates a band the size of untagged protein A. PGK is 3-phosphoglycerate kinase, a loading control.

p425-GPD-C/URA3, into yeast, we noticed a drastic increase in the amount of time to form colonies on plates. Further, the colonies that did form were irregularly shaped (data not shown). This suggested that protein A was toxic when expressed at high levels in yeast, something that we had not observed using *GAL1* inducible constructs, which are also highly expressed. To examine the stability of the Ura3-tagged protein A in wt cells, we grew yeast containing p425-GPD-C/URA3 in the presence or absence of 5-FOA. Immunoblot analysis of total protein extracts from these cultures revealed that protein A-Ura3 was expressed in cells, but at a low level (Figure 4.2, lane 2). In addition to the band at the right size for protein A-Ura3, there was a large band recognized by the polyclonal protein A antibody that was the approximate size of untagged protein A (Figure 4.2, asterisk in lanes 2-3). This suggested that the protein A-Ura3 construct is not stable in cells. Furthermore, the growth of wild type yeast containing p425-GPD-C/URA3 was slow, as was that of the same vector expressing protein A-HA (data not shown), suggesting that protein A itself was toxic to cells at very high levels after an extended period. In order to fully test the value of a Ura3-tagged protein A, we decided to move the ORF into a lower level constitutive expression vector.

Lower expression constructs yield a more stable level of protein A-Ura3. We moved the protein A-Ura3 cassette to a centromeric vector under the same promoter, generating p415-GPD-C/URA3, which decreased the expression levels in yeast by lowering copy number (Table 4.2). We also decided to put the protein A-Ura3 cassette under the control of another weaker constitutive constitutive promoter (18), generating p415-ADH-C/URA3 (Table 4.2). When wild type yeast were transformed with the lower constitutive expression constructs, it was apparent that the constructs were less toxic to cells. Cells transformed with p425-GPD-C/URA3 took 7 days to generate reasonably sized colonies on plates, whereas cells transformed with p415-GPD-C/URA3 or p415-

ADH-C/URA3 took only 4 days (data not shown). Immunoblot analysis of lysates from these strains showed that there was no detectable degradation of protein A-Ura3 in the p415-GPD-C/URA3 or p415-ADH-C/URA3 containing strains, compared to the strain containing p425-GPD-C/URA3 (Figure 4.3, Lanes 2-5 vs. lane 1). The construct p415-GPD-C/URA3 yielded a higher level of full length protein A-Ura3, than in the high copy p425-GPD-C/URA3 vector (Figure 4.3 lanes 4-5 vs. lane 1). Further, the p415-ADH-C/URA3 construct yielded a low level of expression that was a useful control for further studies (Figure 4.3, lanes 2-3 compared to 4-5), allowing a comparison between wild type yeast expressing high or low levels of protein A-Ura3.

Protein A-Ura3 expression was not stable after treatment with 5-FOA, and different expression levels did not correlate with growth differences. In order for these constructs to be useful for a high throughput analysis, two conditions must be met. First, expression of the constructs needs to be stable through treatment, because the cellular levels of protein A-Ura3 are the basis for selection. Second, a difference in cellular levels of protein A-Ura3 would have to correlate to a difference in viability, because survival under counter-selection due to a loss of protein A is the basis for our detection. To test these two conditions, we transformed wild type yeast with one of three plasmids, for no protein A-Ura3 expresison (empty vector), for low protein A-Ura3 expression (p415-ADH-C/URA3), or for high protein A-Ura3 expression (p415-GPD-C/URA3). These three strains were grown in the presence or absence of 5-FOA for 24 hours, and equivalent numbers of cells were harvested from each culture and analyzed by immunoblot (Figure 4.4A). To measure growth, cell density was measured at 5h and 24h post treatment, and the number of doublings was used to calculate a percentage of doublings, as described in the materials and methods (Figure 4.4B). This method of calculating cell growth was chosen because the method of detection in the high-

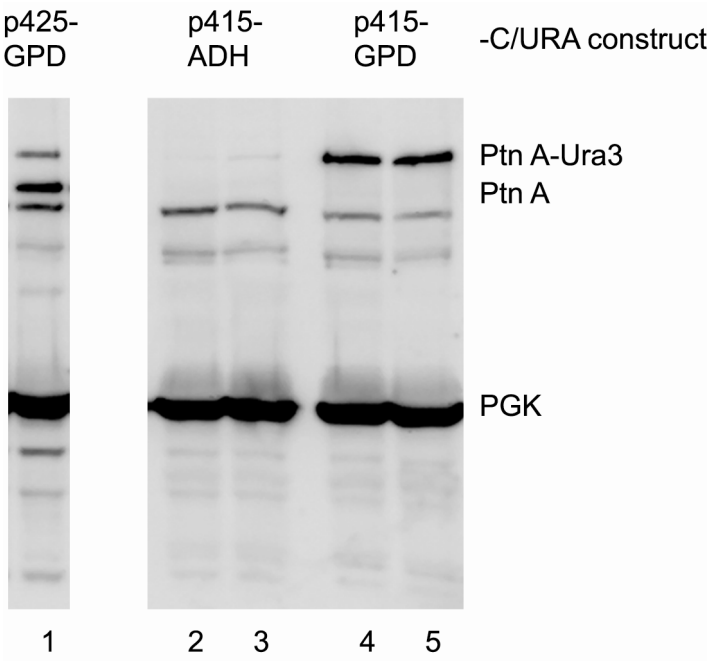

Figure 4.3 - Different expression constructs of protein A. Wild type yeast expressing protein A-Ura3 from different copy number plasmids and promoters. GPD - glyceraldehyde-3-phosphate dehydrogenase promoter, ADH - Alcohol dehydrogenase 1 promoter. PGK is 3-phosphoglycerate kinase, a loading control. Lanes are from the same blot, and all contrast adjustments were done prior to cropping.

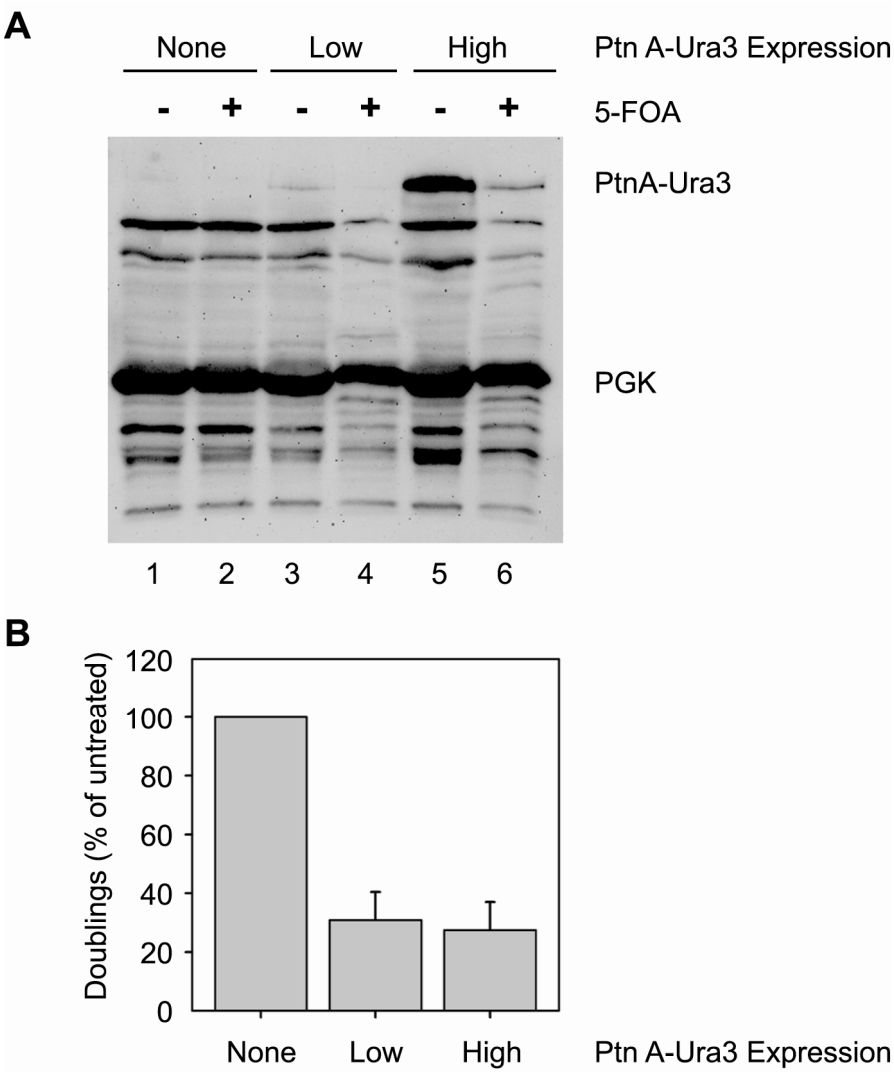

Figure 4.4 - Protein A-Ura3 is not a stable or titratable construct. (A) Protein A-Ura3 is not maintained with counter selection. Total protein samples from wt yeast expressing different levels of protein A-Ura3 after treatment with vehicle only, or treatment with 5-FOA. (B) There is no difference between growth of yeast expressing high or low levels of protein A-Ura3. After 24h of growth, the number of doublings was calculated for each sample. Numbers in B represent the percent of doublings in 5-FOA treated samples compared to untreated samples for wt yeast expressing different levels of protein A-Ura3. Labels: None = empty vector, Low = p415-ADH-C/URA3, High = p415-GPD-C/URA3. All labels between A and B are matched.

throughput screen will be based on an increase in barcode signal over initial reading, which is roughly equivalent to number of doublings of cell density.

In the absence of 5-FOA the low and high expression constructs yielded a low and high level of protein A-Ura3, respectively (Figure 4.4A, lanes 3 and 5). In the presence of 5-FOA, the levels of protein A-Ura3 dropped in both the low and high expressing strains (Figure 4.4A, lanes 4 and 6), indicating that the expression of protein A-Ura3 was not stable through treatment. Further, there was no difference in growth between the high and low expression vectors (Figure 4.4B, high and low bars). This indicates that despite a large difference in the cellular levels of protein A-Ura3 between the high and low expressing constructs, there was no measurable difference in the effect on growth.

Taken together, these data suggested that the Ura3 tag was too active to be useful in our high throughput screen, because our expected results are that in a library of deletions or chemical treatments there will a range of effects on the stability of protein A, and so detection of moderate phenotypes will be necessary. Therefore, we conclude that the protein A-Ura3 construct is not useful for a high throughput analysis, though it may serve some purpose in a different experiment, or if its activity can be reduced.

A firefly luciferase tagged protein A is a good candidate for a high throughput screen. Because the Ura3 tag was too active to be useful in a viability-based assay, we next decided to examine the utility of a non-selectable tag. This would require more hands on time but could be used to identify compounds or deletions that increase the levels of protein A above wild type in addition to those that decrease the levels. Therefore, we generated a firefly luciferase (fLUC) tagged protein A construct (pFA-C/fLUC) as well as an ER retargeted fLUC-tagged protein A (pFA(HCV)-C/fLUC). Previous studies using fLUC have validated that fLUC works in yeast (10), and so we were confident that this approach would work for protein A-fLUC in yeast as well. We

sought to validate these constructs in wild type cells as well as in certain members of the targeted analysis from chapter 3 to test if trends by immunoblot were consistent for a luciferase assay.

We chose to test the protein-fLUC constructs in strains with deletions of *MFT1, APJ1,* GIM4 and *SSZ1*. *MFT1* was initially included in the targeted analysis because it was listed as a mitochondrial targeting gene (7), but further literature searching revealed that it really has a defect in transcription (3) and so it was removed from the data in Chapter III. While it was not useful in our chaperone analysis, it was useful for testing constructs driven from a *GAL1* promoter, such as this validation. By immunoblot, *MFT1* accumulated about 60% less protein A than did wt (data not shown). *APJ1*, *GIM4*, and *SSZ1* were chosen because or their phenotypes were the normal accumulation, slightly increased accumulation, and largely increased accumulation of protein A, respectively (Chapter III, Table 3.2). Yeast were grown in 96 well plates, and induced overnight before luciferase activity was detected by addition of luciferin.

Table 4.3 – Performance of fLUC construct compared to immunoblot.

	wt	*MFT1*	APJ1	GIM4	SSZ1
RLU/cell	100 ±19	4 ±1*	81 ±9	86 ±9	192 ±18*
WB (HA)	100 ±0	41 ±19	125 ±45	227 ±97	1121 ±754

Numbers represent the average ±SEM
Asterisk indicates a *p*-valule of < 0.05 from a Student's *t*-test.

The light signal from each mutant strain was compared to that of the wild type strain sample by a Student's *t*-test (Table 4.3). Yeast expressing HA-tagged protein A showed no light emission (data not shown), while wild type yeast expressing fLUC-tagged protein A showed a robust signal above background (Table 4.3). The *MFT1* deletion strain had a significantly lower signal than wt, following the same trend as its

immunoblot numbers. The *APJ1* and *GIM4* deletion strains both had slightly decreased signals from wt, but not significantly so. Finally, the *SSZ1* deletion strain had a significantly increased signal. The magnitude of these changes was not the same as their immunoblot numbers (Table 4.3), but the trend of changes was consistent, and indicated that all of these strains would have performed similarly in either method of analysis. These data validated that a fLUC tag would be useful for a high-throughput analysis of the synthesis and stability of protein A. Further, due to its sensitivity and dynamic range, it would allow for additional analysis, such as one where conditions caused an increase in the accumulation of protein A.

Discussion

In this study we began the development of a method of high throughput screening for changes in protein A synthesis and stability in yeast. In doing so, we identified several challenges associated with using a toxic or selectable tag in yeast when the anticipated results are an intermediate phenotype. Further, we have explored the utility of a firefly luciferase tag in the same high throughput screen. While this method would be more time consuming, it is highly sensitive and also has the added potential of being able to detect an increase in protein A levels in the cell.

The Ura3 tag was active on protein A, and was too sensitive for the detection of a moderate phenotype. In this study, we showed that the Ura3 gene was active and functional when used as a tag for protein A. In fact it was too active for the detection of a moderate phenotype, which is the predicted and demonstrated phenotype for this type of analysis (Chapter 3). A literature review of the *URA3* gene and its product, orotidine-5'-phosphate decarboxylase, revealed that the activity of this enzyme is quite high; it is able to reduce the reaction half-time from 78 million years to 18 milliseconds (a 10^{17} change) (21). Therefore, in order to be useful, we would need to decrease the activity and increase the titratability of the enzyme by random mutagenesis and screening for less proficient clones. Another alternative would be to follow the model of the membrane based yeast two hybrid analysis, which uses an unstable Ura3 tag that is released upon interaction of two tagged proteins and is degraded, making cells resistant to 5-FOA, or a similar method using a released transcription factor to express a selectable marker (24). We could adapt this to our model using positive selection, where cells in which proteins don't interact would retain the Ura3 tag, and so would grow on uracil dropout media. This method is limited only by our knowledge of the interacting partners of protein A, other than protein A itself (4). As our understanding of the FHV RNA replication complex increases, this will become a more viable screening method.

Firefly luciferase is an active protein A tag in yeast, and is a potentially useful tag for our high throughput screen. Tagging protein A with fLUC yielded an active fusion protein, which gave a reproducible light signal upon treatment with substrate. Further, when tested in strains that were identified in our targeted analysis as having lower than, equal to, or higher than wild type levels, the fLUC tagged protein A performed equally well, with signals significantly lower than, no different than, or significantly higher than wild type signals, respectively. This suggests that strains identified as interesting by immunoblot analysis would have also been identified as hits in a luciferase based high-throughput screen. When the fLUC-tagged protein A was used in replication in trans assays, the tagged protein A was expressed at levels comparable to a C/HA tagged construct, and it was able to replicate, but at a reduced level compared to the C/HA tagged protein A (S. Weeks, A. Leorps, and D. Miller, unpublished observations). This indicated that the large cFLUC tag might interfere with RNA replication, and while this is important to note, it does not impact the utility of the construct in a synthesis and stability based screen. This method of screening for mutations or compounds that alter the per cell levels of protein A requires more hands on time than a live-die assay that could be run in pools and identified by genetic tag amplification, but is highly sensitive, and is currently a more viable option. Additional options for the use of the pool based screen include selection of cells by means other than viability, such as fluorescence. For example, a fluorescently-tagged protein A could be integrated into a wild type strain, and used in a classical mutagenesis screen, and pools of mutants could be sorted using fluorescence-activated cell sorting, isolating those cells that have more or less fluorescent signal than the cutoffs for wild type cells. Protein A has been used in a flow cytometry experiment in yeast before (4), and so this is a possibility for our studies too.

In this study, we have identified certain challenges and limitations to performing a high throughput assay based on the accumulation of protein A in yeast cells. Despite the limitations in the systems that we tested, the benefits of using a genetically tractable

organism like yeast as a model system to perform such a test outweigh the drawbacks and make this a good candidate for future development.

References

1. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet **197:**345-6.

2. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol **154:**164-75.

3. **Chavez, S., T. Beilharz, A. G. Rondon, H. Erdjument-Bromage, P. Tempst, J. Q. Svejstrup, T. Lithgow, and A. Aguilera.** 2000. A protein complex containing Tho2, Hpr1, Mft1 and a novel protein, Thp2, connects transcription elongation with mitotic recombination in Saccharomyces cerevisiae. Embo J **19:**5824-34.

4. **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.

5. **Foury, F.** 1997. Human genetic diseases: a cross-talk between man and yeast. Gene **195:**1-10.

6. **Gallagher, T. M., and R. R. Rueckert.** 1988. Assembly-dependent maturation cleavage in provirions of a small icosahedral insect ribovirus. J. Virol. **62:**3399-3406.

7. **Garrett, J. M., K. K. Singh, R. A. Vonder Haar, and S. D. Emr.** 1991. Mitochondrial protein import: isolation and characterization of the Saccharomyces cerevisiae MFT1 gene. Mol Gen Genet **225:**483-91.

8. **Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis, and M. Johnston.** 2002. Functional profiling of the Saccharomyces cerevisiae genome. Nature **418:**387-91.

9. **Kushnirov, V. V.** 2000. Rapid and reliable protein extraction from yeast. Yeast **16:**857-60.

10. **Leskinen, P., M. Virta, and M. Karp.** 2003. One-step measurement of firefly luciferase activity in yeast. Yeast **20:**1109-13.

11. **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.

12. **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.

13. **Mager, W. H., and J. Winderickx.** 2005. Yeast as a model for medical and medicinal research. Trends Pharmacol Sci **26:**265-73.

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:**9856-9867.

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:**11664-11676.

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:**12193-12202.

17. **Mnaimneh, S., A. P. Davierwala, J. Haynes, J. Moffat, W. T. Peng, W. Zhang, X. Yang, J. Pootoolal, G. Chua, A. Lopez, M. Trochesset, D. Morse, N. J. Krogan, S. L. Hiley, Z. Li, Q. Morris, J. Grigull, N. Mitsakakis, C. J. Roberts, J. F. Greenblatt, C. Boone, C. A. Kaiser, B. J. Andrews, and T. R. Hughes.** 2004. Exploration of essential gene functions via titratable promoter alleles. Cell **118:**31-44.

18. **Mumberg, D., R. Muller, and M. Funk.** 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156:**119-22.

19. **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.

20. **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.

21. **Radzicka, A., and R. Wolfenden.** 1995. A proficient enzyme. Science **267:**90-3.

22. **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:**2162-2169.

23. **Shah, N., and R. D. Klausner.** 1993. Brefeldin A reversibly inhibits secretion in Saccharomyces cerevisiae. J Biol Chem **268:**5345-8.

24. **Thaminy, S. a. S., I.** 2002. The Membrane-based Yeast Two-hybrid System, p. 395-405. *In* E. Golemis (ed.), Protein-Protein Interactions: A Molecular Cloning Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

25. **Umezu, K., T. Amaya, A. Yoshimoto, and K. Tomita.** 1971. Purification and properties of orotidine-5'-phosphate pyrophosphorylase and orotidine-5'-phosphate decarboxylase from baker's yeast. J Biochem **70:**249-62.

26. **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.

CHAPTER V

GENERAL DISCUSSION

In these studies, we have explored the role of chaperones in the replication of a positive strand RNA virus using two approaches. First, we examined the major chaperone complexes in the cell specifically. Second, we used a targeted analysis to explore a larger group of cytosolic chaperones. These methods have allowed us to form a testable model of the early events in FHV RNA replication using a combination of the data from our own studies and also information from the yeast community at large. In addition to these two methods, we have begun to develop a method for a high throughput genomic or chemical library screening to identify yeast genes or chemical compounds that affect FHV RNA polymerase synthesis and stability, allowing us to approach the question of host factors from a more unbiased perspective in the future. These diverse approaches will allow us to generate a large data set that will help to address a central question in our lab: What host factors do positive strand RNA viruses use to accomplish their replication cycle?

Host chaperone requirements for FHV RNA replication are host- and membrane-specific. In our first study we sought to expand upon our data from *Drosophila* cells, where our lab has shown that Hsp90 is necessary for synthesis of protein A, the FHV RNA-dependent RNA polymerase (6, 18). We showed that in contrast to the results in *Drosophila* cells, FHV RNA replication in yeast was Hsp90 independent. Instead, FHV RNA replication was dependent on the Hsp70 chaperone system. Because of redundancy and viability issues with knocking out the main cellular chaperones, we approached this experimentally using yeast strains with a deletion of cochaperones for each complex. To impact the Hsp90 system, we used strains with a deletion of *STI1* or *SBA1*, two cochaperones that are involved in early and late complex activity, respectively. To impact the Hsp70 system, we used a strain with a deletion of *YDJ1*, an Hsp40 that required for efficient Hsp70 activity. This was an indirect approach, but due to previous studies (5, 7, 8, 22, 38) and our own controls (Figure 2.2) we believe that these cochaperone deletions were specific to the corresponding chaperone complex. It is possible however that there was some indirect effect on another system unrelated to Hsp90 or 70. Deletion of *SBA1* for example, did have a small effect on RNA replication, whereas deletion on *STI1* had no effect compared to the drastic reduction seen in *Drosophila* cells. Both cochaperone deletions reduced Hsp90 reporter assay levels to background, and treatment with Hsp90 specific inhibitors had no effect on FHV RNA replication despite causing a 60% reduction in reporter levels. This suggested that the *SBA1* deletion may have had other effects not involving Hsp90 activity. Literature has suggested a role for *SBA1* in the nucleus (37), and so this chaperone may have alternative functions in the cell.

Rather than FHV RNA replication being dependent on the Hsp90 chaperone complex, we found that it was dependent on the Hsp70 complex. The deletion of the Hsp70 cochaperone *YDJ1* caused a reduction in RNA replication, and we suspected that

this defect was two-fold. Both protein A and (+) RNA3 accumulation were down in *Δydj1* cells. Protein A levels were decreased compared to loading controls in *YDJ1* yeast, yet some protein A did accumulate in the cells but there was no replication indicating that the protein A that accumulated was defective. We next showed that this defective protein A was tightly membrane associated, and that a portion of it was accumulating on the mitochondria, indicating that there may be a problem with the complex activity. Finally, we showed that the defect in the *YDJ1* deletion strain was membrane specific. When protein A was retargeted to the endoplasmic reticulum membrane rather than the mitochondria, there was little or no defect in accumulation of protein A or (+) RNA3 (Figure 2.5). This is consistent with the hypothesis that Ydj1p functions at the level of replication complex activity, and that there is likely another component present at the ER that is able to compensate for the absence of Ydj1p. Another possibility is that the ER-targeted FHV replication complexes do not require chaperones.

This difference in FHV host requirements between the *Drosophila* and yeast models is one that we must reconcile in our future studies. Initially we hypothesized that the differences were due to a known difference in the mitochondrial trafficking pathways between yeast and higher eukaryotes (44). Mitochondrial targeting in higher eukaryotes (including *Drosophila*) requires the Hsp70 and 90 chaperone systems, while in yeast, only Hsp70 is required. This hypothesis was consistent with our data in the *Δydj1* strain in chapter II, where there we saw a defect in the FHV RNA replication on the mitochondria, but no defect in the ER-retargeted replication. However, our lab later found that the Hsp90 dependence that we saw in *Drosophila* was at the step of protein A synthesis, and that there was no defect in targeting or stability (6). Further, the data in Chapter 3 from the *Δssa1/2* double mutant strain is consistent with a protein A accumulation defect, consistent with a problem with protein A synthesis rather than trafficking. It is possible, however, that a trafficking defect may cause an decrease in the

stability of protein A, which we can not rule out at this time. More experiments are needed to further distinguish between these two possibilities. If there is a difference in the chaperone requirements for the synthesis of protein A between yeast and higher eukaryotes, this would be an interesting and novel finding.

A targeted analysis of chaperones revealed a contrasting role for Hsp70 in FHV RNA replication. In our second study, we explored a group of 31 cytosolic proteins with known or hypothesized chaperone activity in an attempt to get a broader view of chaperones and their effect on FHV RNA replication. Individual knockouts of Hsp70 or Hsp90 did not have consistent effects, with the exception of the deletion of *SSZ1*, a unique and atypical Hsp70. This is consistent with the known redundancy in the main chaperone systems of yeast cells (41). One group that was very telling was the J-domain family of Hsp40 proteins. These proteins are believed to work as partners of the Hsp70 machinery, and therefore were good indicators that the Hsp70 machinery was involved in FHV RNA replication. Two particular J-domain proteins that were interesting were *YDJ1* and *ZUO1*. In our first study, we found that the deletion of *YDJ1* had a drastic negative effect on FHV RNA replication, and our results in the second study were consistent. In contrast to *YDJ1*, the deletion of *ZUO1* caused a drastic increase in FHV RNA replication. This increase was due to an increase in the accumulation of both protein A and RNA3.

We next explored the ability of other J-domain proteins to complement the RNA replication phenotypes that we saw in our targeted analysis shared the same complementation characteristics as those of growth and temperature sensitivity (32). We found that the decrease in FHV RNA replication caused by deletion of *YDJ1* was able to be complemented by other J-domain proteins, including those that are structurally unrelated to Ydj1p, suggesting the defect in FHV RNA replication was due to

regulation of Hsp70 by a J-domain. In contrast, the drastic increase in FHV RNA replication that we saw in the *ZUO1* deletion strain was complemented by Zuo1p itself or by the closely related and ribosome associated J-domain protein, Jjj1p. Both of these results led us to the same conclusion; the defect seen in the J-domain deletion strains was likely due to the deregulation of the partner Hsp70.

The chaperone partners for Ydj1p are the *SSA* family of Hsp70s, though Ydj1p is only one of many J-domain proteins that partner with that family. This promiscuity is consistent with our ability to complement deletion of *YDJ1* with other J-domain proteins, and led us to examine FHV RNA replication in a strain with a double deletion of *SSA1* and *SSA2*. This strain has been used in other (+) RNA virus studies. Ssa1/2p were purified with the cucumber necrosis virus RNA replication complex, and a double deletion *SSA1/2* strain decreased replication complex activity (34). Like our studies with the *YDJ1* deletion strain, FHV RNA replication in the *Δssa1/2* strain was defective, consistent with the hypothesis that the defect in *Δydj1* cells was due to a deregulation of Ssa1/2p.

The chaperone partners for Zuo1p are the *SSB* family of Hsp70. Zuo1p regulates Ssb1/2 along with its partner, Ssz1p. Ssz1p is an atypical Hsp70, and along with Zuo1p, makes up the ribosome associated complex, which regulates Ssb1/2p (15). Together, this chaperone complex functions on the ribosome and binds nacent polypeptides, ensuring efficiency and fidelity of the translation machinery. No other J-domain proteins have been shown to bind and regulate Ssb1/2p, and so the complementation results of the *ZUO1* deletion phenotype led us to hypothesize that this was due to a deregulation of Ssb1/2p (32). This led us to examine FHV RNA replication in a *SSB1/2* deletion strain. The deletion of both *SSB1* and *SSB2* caused a more drastic increase in FHV RNA replication than did the deletion of either *ZUO1* or *SSZ1*, consistent with our hypothesis that the J-domain protein defect was caused by a

deregulation of the Hsp70. This experiment used a different parent wild type strain than the large scale analysis, and there was no detectable replication in that wild type strain, only in the *SSZ1* and *SSB1/2* deletion strains. This further emphasized the drastic increase in replication caused by the deletion of these chaperones.

The FHV RNA replication phenotypes of the other J-domain deletion strains were interesting as well, and require further study. There was a correlation between type of J-domain protein and impact on FHV RNA replication. Deletion of two of three type I J-domain proteins tested (*APJ1* and *YDJ1*) had negative effects on FHV RNA replication, the only type II J-domain protein (*DJP1*) had no effect, and all but one of the type III J-domain proteins tested had a positive effect when deleted. This is an intriguing area for future study, and may help to determine what the cellular roles of some hypothesized J-domain proteins are.

For some of the strains that we tested in the targeted analysis, deletion caused an increase in protein A or RNA3 above wild type levels. This is intriguing, as there seems to be a saturation point in wild type yeast for both protein A and RNA3 accumulation (Weeks and Miller, unpublished observations). In addition to our targeted analysis, the wild type strain used in the *SSB1/2* experiments appeared to be non-permissive for FHV RNA replication (Figure 3.4), and the deletion of *SSZ1* or *SSB1/2* made the strain permissive. The fact that the deletion of a gene causes an increase in that accumulation above the wild type saturation point suggests that there is a system of negative regulation of FHV replication in yeast. There are viruses that specifically infect fungi (9, 11), and RNA silencing antiviral systems have been described in some fungi (26, 33), but none have been described for *Saccharomyces cerevisiae*. This is a particularly intriguing area for a classical mutagenesis screen, as it implies that one or more cellular factors may be acting to restrict viral RNA replication in *S. cerevisiae*. Possibility for this effect on the accumulation of protein A, it that a defect in the *SSB*

chaperone complex is causing a problem with translational fidelity during the synthesis of protein A. Control of translational fidelity is a known cellular role for the *SSB* chaperone complex, and a mis-translation of RNA1 could cause protein A to become more active. We did not sequence the protein A that accumulated in either ribosome associated complex deletion strains or in the *Δssb1/2* double deletion strains, and therefore we can not rule out this possibility, but this is not the most likely cause for our results. A more likely cause is that the *SSB* chaperone complex is playing a role in protein turnover in cells, and that a defect in this activity is causing an increased accumulation of protein A in mutant cells. This is consistent with other chaperone systems and their role of quality control and protein turnover (24), but this has not been described for the *SSB* chaperone complex. Interestingly, work in a collaborator's lab has suggested that other proteins may be more active in the absence of the *SSB* chaperone complex, and that the chaperone complex may be involved in the degradatory pathway (Sabine Rospert, personal communication with S. Weeks). Our observations in the FHV system are consistent with both of these possibilities.

In addition to the direct effects on protein A, another possibility is that a defect in the *SSB* chaperone complex activity is having an effect on other cellular factors, which are then having an effect on FHV RNA replication. One possibility for this is an effect on the lipid synthesis pathways in the cell. Protein A has been shown to be dependent on certain phospholipids in order to be active (43), and a change in the normal balance of phospholipids on the mitochondria could cause an increase in the activity of protein A. This would be similar to the effect that we see when we retarget protein A to the ER, and it becomes more active (25). We can not rule out a shift in phospholipids levels in *SSB* deficient cells, nor can we rule out mistargeting of protein A in these cells, and therefore these are both possibilities for the phenotypes that we see in ribosome associated complex or *SSB* deletion mutant strains.

Building a testable model for the early events in FHV replication. The first step towards a complete picture of how host factors are co-opted for (+) RNA virus replication is to identify those host proteins that affect viral RNA replication. The results of our targeted analysis gave us an overview of the impact of chaperones on FHV protein and RNA levels. Those data, combined with the known or hypothesized functions of chaperones from literature searches allowed us to form hypotheses about the roles of these proteins in FHV replication. Therefore, we built a model based on the early events in FHV RNA replication, and included the chaperones that we tested at the steps that were consistent with cellular roles and impact on viral products (Figure 5.1). Thus far, experiments in our lab have not been able to show interaction of protein A with any cellular factor, and so we could not distinguish between direct and indirect effects in this model, and so the proximity of the chaperones to protein A or replication complexes is speculative.

Due to their role in ribosome formation and function, the first group of chaperones that we were interested in were the ribosome associated complex (Ssz1p, Zuo1p, and Ssb1/2p), or are known to be ribosome associated (Jjj1p). Deletion of all of these genes resulted in an increase in protein A and (+) RNA3 accumulation, therefore, we hypothesized that they were involved in a negative regulation of protein A synthesis or activity (Figure 3.5, step 1) as discussed above. Jjj2p has no known function, but had a similar phenotype as these chaperones, and therefore it was included at this step as well. Alternatively, this group of chaperones may have played a role in the translation of another cellular factor (X) that negatively regulated FHV replication complex activity.

The next group of chaperones were those that impacted the accumulation of protein A, but are not directly involved with the translation machinery, as they may represent the folding and processing of protein A. We included Ydj1p and Ssa1p in this group, as well as Hsp104p, which is known to interact with the Ssa complex for

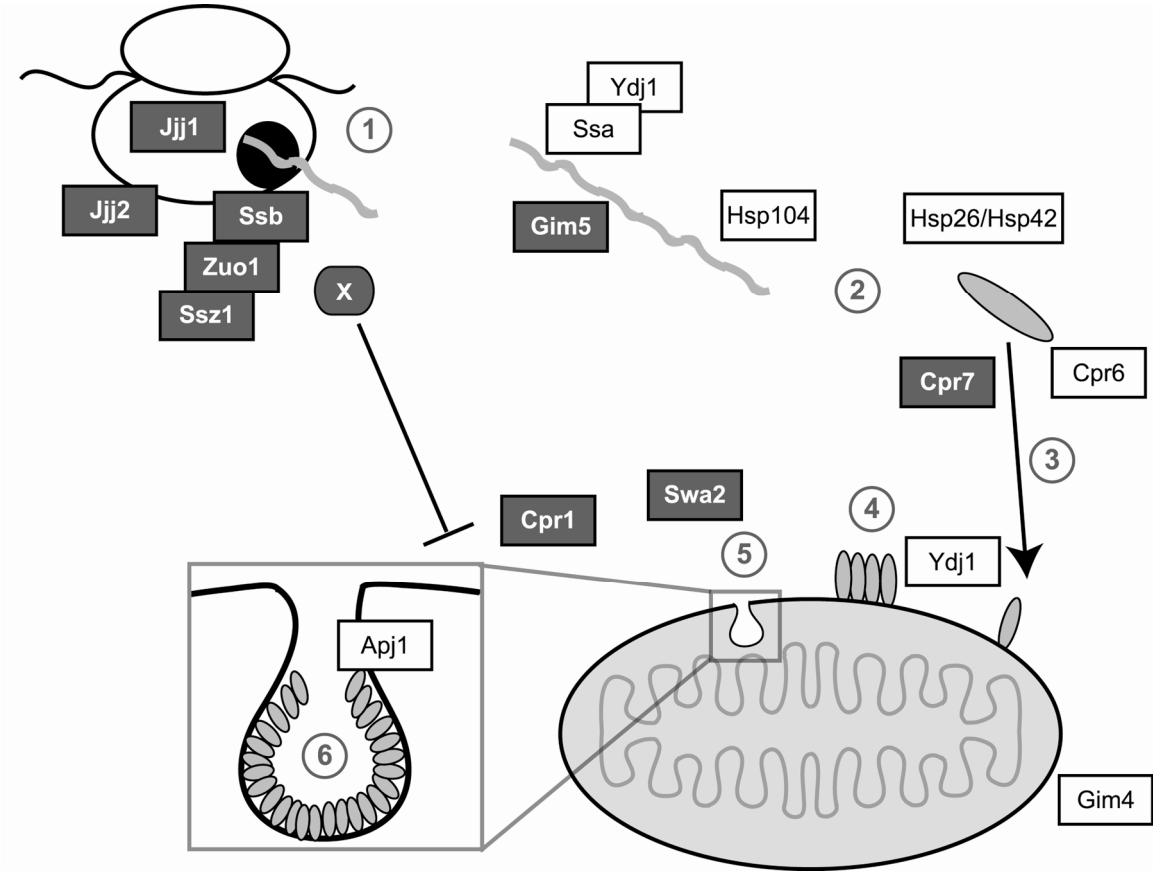

Figure 5.1 - Model for the role of chaperones in the formation and function of FHV RNA replication complexes. The steps in FHV RNA replication: Protein A is translated on the ribosome (1), protein A is folded (2), protein A is trafficked to the mitochondrial outer membrane (3), protein A self associates (4), spherules are formed (5), and RNA replication (6). Proteins boxed in gray are hypothesized to play a negative role in FHV RNA replication, as their deletion resulted in an increase of protein A or (+) RNA 3 accumulation. Proteins boxed in white are hypothesized to play a positive role in FHV RNA replication, as their deletion resulted in a decrease of protein A or (+) RNA3 accumulation. Proteins are placed in the protein A cycle according to their impact on the replicons used in this study as well as their known or hypothesized role in the cell.

specialized refolding. Deletion of these chaperones all had a negative impact on the accumulation of protein A, and so we hypothesized that these facilitate the folding and processing of protein A (Figure 3.5, step 2). The deletion of *GIM5* caused an increase in the accumulation of protein A. Because Gim5p is involved in early protein folding (39), we also include it at this step, though it has also been implicated in microtubule biogenesis (16). It is difficult to examine proper folding in vivo, but this may be able to be tested using an in vitro method such as circular dichroism in the presence or absence of certain cellular chaperones.

Deletion of the small heat shock proteins *HSP26* and *HSP42* had negative effects on the accumulation of (+) RNA3, but not on protein A. These chaperones are known to refold denatured proteins in stressed cells (17, 31), suggesting that there may be a refolding event that is necessary when protein A is expressed at high levels, and so we included them between the steps of folding and trafficking. We also included Cpr6p and Cpr7p, two immunophilins that caused a decrease in the accumulation of protein A, or an increase in RNA replication, respectively. We included the immunophilins at the step of protein A trafficking (Figure 3.5, step 3) because of their cellular role (12, 13, 23, 28), but it remains to be seen whether they play a role in folding or trafficking events. An analysis of the membrane association of protein A in these mutants will aid in determining this.

Once protein A is on the mitochondrial outer membrane, several things have to happen; protein A must associate with itself and possibly other proteins, form invaginations of the membrane called spherules, and begin to replicate the genomic RNA. Therefore, chaperones that impact (+) RNA3 accumulation were included in these steps. Deletion of *YDJ1* likely had more than one effect, because the protein A that did accumulate in trans was not active, and so we included Ydj1p in steps on the mitochondria as well as in the cytosol (Figure 3.5, step 4). Through work that we did not

show, protein A that accumulated in yeast cells was more sensitive to proteases than that from wt cells, indicating that protein A may have been unfolded, not interacting with itself and other proteins, or not forming spherules that would protect the replication complex from proteases or nucleases. Therefore, we included Ydj1p at the step of protein A self-association. Gim4p is a chaperonin loading protein in cells, and has also been shown to effect microtubule biogenesis (16, 39). Because protein A accumulates on the mitochondrial outer membrane and the mitochondria are in association with the microtubules in yeast (36), we included Gim4p at the level of mitochondrial biogenesis. Deletion of *SWA2* caused an increase in the accumulation of protein A and (+) RNA3, and so due to its role in membrane trafficking and vesicle formation (14), it was included at the step of spherule formation (Figure 3.5, step 5). The deletion of *CPR1* caused an increase in (+) RNA3 accumulation, but not in protein A accumulation, and so it was included between membrane association and RNA replication. Finally, Apj1p has been shown to be both cytosolic, and mitochondrial associated (30), and the deletion of *APJ1* caused a decrease in (+) RNA3 accumulation levels in replication targeted to the mitochondria and to the ER, but had no effect on protein A levels. Therefore, we included Apj1 as a potential cofactor in the RNA replication complex, though more experiments need to be done to confirm this.

Because these studies have not examined physical interactions between protein A and host factors, there are two possibilities to explain our results. The first is a direct interaction between protein A and cellular chaperones. This is an attractive possibility, because of the known action of chaperones functioning through a transient interaction with client proteins. There is another possibility that these chaperones are involved in the stability, maturation, or trafficking of other cellular factors that affect protein A accumulation or RNA replication complex activity. We have attempted to test the physical interactions of protein A with cellular factors, both in the cytosolic and

membrane-associated forms, but this data was negative (S. Weeks, K. Stapleford, and D. Miller, unpublished data). Without knowing what factors protein A interacts with, it is difficult to distinguish between the two possibilities. These are not mutually exclusive hypotheses however, and there is likely an overlap between the two, with some chaperones interacting with protein A, and others interacting with cofactors of protein A. Therefore, additional studies are necessary in order to determine whether the phenotypes that we saw in our studies are due to a direct or indirect effect on protein A.

One possibility for an indirect effect is a cellular pathway that is dependent on chaperones, which then impacts protein A accumulation or function. Based on other studies in our lab, we hypothesized that lipid synthesis pathways play a significant role in FHV RNA replication. Enzymes for membrane biosynthesis have been found to be associated with the mitochondria (1, 19, 40, 45), and so this pathway is a good candidate for future study in relation to the chaperone phenotypes that we saw for FHV RNA replication. This model is a starting point for the study of chaperones in FHV RNA replication. We have identified several chaperones that have roles, but not what those roles were. Much work remains to be done to test this model and likely the study of membrane association and cellular localization will be beneficial to narrowing down specific roles for these chaperones.

High-throughput screening based on protein A levels is a viable strategy to identify those genes or chemical compounds that impact (+) RNA virus polymerase synthesis and stability. A useful compliment to our targeted studies would be a high-throughput screen for effects on FHV RNA replication in yeast. This has been done with other (+) RNA virus models (21, 27) using reporter replicons. To accomplish this, we have begun to develop a method to screen at the whole genome or chemical library level. The goal of this screen would be to identify those host proteins or

chemical compounds that impact the synthesis and stability of protein A in yeast cells. The benefits of this type of analysis on our current studies are two fold. First, a high throughput screen would allow us to examine whole libraries in an unbiased approach rather than studying individual genes or compounds. Second, this would allow us to form a more powerful model of FHV RNA replication, based on the large amount of information that is available about the interconnectedness of pathways in the cell, and their cross talk.

Our first approach to develop a high throughput screening method was to generate a construct that could give a readout based on viability. Viability-based screens have the benefit of requiring less hands-on processing time due to a biological elimination of negatives, leaving a high yield of positive hits. Also, due to the molecular barcode design used in the construction of the many yeast genetic libraries, it is possible to screen pools of strains rather than individual strains separately. The method that we decided to use was to tag protein A with the Ura3 enzyme. The Ura3 tag can be selected for or against based on media supplements (3, 4), allowing us to enrich for those strains that contain an altered level of protein A. This tag was functional on protein A and on the mitochondria, but ultimately was not useful in its current form. The high activity and low titratability of the protein A-Ura3 construct made it impossible to distinguish between protein levels by viability, as low or high levels of expression both caused an equal growth defect. Therefore, in order to make this construct more useful, we would need to make the Ura3 tag less active, and therefore be able to more precisely control the viability of cells by titration of the selection agent. Ultimately, we would need to be able to adjust the activity of the construct so that intermediate expression levels would have detectable growth differences, but in the current state the construct is too active. The best approach to decrease the activity of the enzyme would be to use random mutagenesis looking for less active clones.

Our second approach for a high throughput assay design was a more traditional method. We used a firefly luciferase tagged protein A construct in yeast to allow us to use light production as a surrogate for protein A levels. The protein A-fLUC construct was functional, and was able to be read simply and quickly with a luminescence-based plate reader. Further, when this method was compared to data from our targeted analysis, the luciferase based assay was able to reproducibly identify increases and decreases in protein A levels by light production. The dynamic range of luciferase assays is also wider than that of immunoblot quantification, and so allows us to detect smaller changes in protein A levels by statistical means.

Future Directions. The future of this project could go in many directions. The first is the further characterization of the chaperone deletion strains from the targeted analysis. These strains had FHV RNA replication phenotypes, based on the accumulation of viral products after a 24 hour induction, but beyond that we do not know what caused the phenotypes. The cause for these phenotypes could be two fold. Either the effect was on protein A, or the effect was on replication complex activity. To address the protein A effect, we need to examine the membrane association and cellular localization of protein A in each of these strains. To address the question of an effect on replication complex activity, we can use an in vitro RdRp activity assay. Determining if protein A is membrane and mitochondria associated, and also if there is an in vitro defect in FHV RNA replication complexes are two very powerful pieces of data. These data would allow us to strengthen our model by assigning more solid functions to those chaperones that have FHV RNA replication phenotypes.

The future directions for the high-throughput design would include a random mutagenesis of the *URA3* gene and a more detailed comparison of luciferase activity compared to immunoblot data. To make a more useful *URA3* tag, we would need to

make the gene less active, and in so doing make it more titratable. The main problem currently is that the Ura3p is so active any amount of expression is enough to cause a growth phenotype. A less active form would allow for a range of phenotypes based on different levels of expression. A low fidelity PCR amplification followed by screening for less functional forms of the *URA3* gene would be a relatively quick step towards generating a more useful construct. The luciferase-based approach is functional in its current state, but requires more testing in order to better predict the variability of light values, and also to assign a wild type range to use as a cutoff during the screen. This is relatively simple to achieve, and would involve repeating the preliminary experiments done in these studies.

There are other possibilities for high throughput screening that we have not tested. One promising method would be to utilize a fluorescently tagged protein A (10). This construct could be integrated into the genome of wild type yeast, and then used as a starting point for a classical mutagenesis. Groups of mutants could be separated by fluorescence-activated cell sorting, isolating those that had higher or lower fluorescent signals than the parent strain and plating them for future study. This method would have the benefit of the pooled screening approach and isolation of live strains on plates, but would require the identification of individual mutations by genetic methods. Reading fluorescence in live yeast is complicated by the relatively high auto fluorescence associated with the cell wall and other cellular components (2). This method would require some optimization to improve the signal to noise ratio, but due to the potential for high yield would be worth the effort to optimize.

The possibility of a negative regulator of viral replication in yeast is very exciting. There are fungal and yeast viruses (9, 11, 33, 35) and it seems unlikely that there would be no host defense against them. Therefore, to explore this, there are some preliminary results that need to be done to determine if a selectable replicon and mutagenesis

screen would work in yeast. First, the careful choice of the replicon and its selectable marker is essential. A selectable replicon has been used for FHV studies before (29), and it was based on a URA3 gene inserted into a replication template for FHV RNA2. When the RNA is replicated, the URA3 gene is expressed, and the cells are then able to grow on uracil dropout media. We must choose markers that are stable and responsive in the strain background that we choose, as well as not conflict with any selectable markers that we would use in the rest of the screen. Second, the non-permissive wild type strain must restrict the replication of the construct to the point that there is essentially no background expression of the selectable marker. Third, the replicon needs to be expressed in the wild type strain that is non-permissive as well as the *Δssz1* and *Δssb1/2* deletion strains that we know are permissive for FHV replication. These would be the experimental controls for no replication, low replication, and high replication, respectively, and so would need to perform differently in order for the screen to be useful.

The final design of the experiment may vary, but I believe that an integrated cassette containing a promoter-driven selectable replicon would be the best option, as it would ensure consistency in the library construction, and a synchronized 'launch' of the replicon in all cells. As for the mutation of the genome, a transposon based yeast genome library has successfully been generated using bacteria as a carrier (20), and so this is a viable option. Other alternatives include chemical or UV mutagenesis, though these are more difficult to identify candidate genes post screening. Transposon mutagenesis has the benefit of incorporating sequencing primers in the transposon for easy identification of insertion site. Other alternative approaches for identification of potential negative regulators or restriction factors in yeast would include a subtractive hybridization based approach, or potentially a cross mating to a permissive strain to test for dominance. These are not as versatile as a mutagenesis screen, but would be useful

as backup approaches should the primary assay design fail, or as preliminary experiments to confirm that there are restriction factors in yeast.

An interesting model host to consider for the FHV RNA replication experiments is fission yeast, such as *Schizosaccharomyces pombe*. These yeast are similar to the budding yeast used in these studies, yet have some differences that may enrich our studies as well. Fission yeast divide more similarly to higher eukaryotic cells, and may allow some studies into the vertical transmission of FHV infection in culture, as well as the division of mitochondria from mother to daughter cells. Also, fission yeast have been shown to possess an RNA-interference system similar to that of invertebrates, with orthologs to Argonaut and Dicer proteins (42). This could be an interesting model to dissect the role of RNA-interference in yeast and the role of protein B2 as an inhibitor of this pathway. Differences in the chaperone systems between budding and fission yeast have not been well described, but this may provide an additional level of study of this critical host system for FHV RNA replication.

Closing statement. These studies have identified cellular chaperones as being important for the assembly and function of (+) RNA virus replication complexes. We have used a model pathogen and host to take advantage of established cellular studies and well understood genetically tractable systems. These studies represented the first steps towards a more complete understanding of cellular factors that viruses use in their hosts, and will hopefully lead to the development of novel molecular tools or antiviral treatments for laboratory and clinical use. There remains much to be done in these systems, but these early results have suggested that this is a fruitful area of study that will lead to important discoveries using a relatively simple system. Beyond the specific work done in this thesis, these studies have helped to verify that yeast is a useful and potentially high yield model system to study the replication of (+) RNA animal viruses.

References

1. **Ardail, D., F. Gasnier, F. Lerme, C. Simonot, P. Louisot, and O. Gateau-Roesch.** 1993. Involvement of mitochondrial contact sites in the subcellular compartmentalization of phospholipid biosynthetic enzymes. J Biol Chem **268:**25985-92.

2. **Billinton, N., and A. W. Knight.** 2001. Seeing the wood through the trees: a review of techniques for distinguishing green fluorescent protein from endogenous autofluorescence. Anal Biochem **291:**175-97.

3. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol Gen Genet **197:**345-6.

4. **Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol **154:**164-75.

5. **Bohen, S. P.** 1998. Genetic and biochemical analysis of p23 and ansamycin antibiotics in the function of Hsp90-dependent signaling proteins. Mol. Cell. Biol. **18:**3330-3339.

6. **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:**8412-8420.

7. **Chang, H. C., D. F. Nathan, and S. Lindquist.** 1997. In vivo analysis of the Hsp90 cochaperone Sti1 (p60). Mol Cell Biol **17:**318-25.

8. **Cyr, D. M., and M. G. Douglas.** 1994. Differential regulation of Hsp70 subfamilies by the eukaryotic DnaJ homologue YDJ1. J Biol Chem **269:**9798-804.

9. **Dawe, A. L., and D. L. Nuss.** 2001. Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. Annu Rev Genet **35:**1-29.

10. **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.

11. **Esteban, R., and T. Fujimura.** 2003. Launching the yeast 23S RNA Narnavirus shows 5' and 3' cis-acting signals for replication. Proc Natl Acad Sci U S A **100:**2568-73.

12. **Galigniana, M. D., J. M. Harrell, P. J. Murphy, M. Chinkers, C. Radanyi, J. M. Renoir, M. Zhang, and W. B. Pratt.** 2002. Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and in vivo evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. Biochemistry **41:**13602-10.

13. **Galigniana, M. D., J. M. Harrell, H. M. O'Hagen, M. Ljungman, and W. B. Pratt.** 2004. Hsp90-binding immunophilins link p53 to dynein during p53 transport to the nucleus. J Biol Chem **279:**22483-9.

14. **Gall, W. E., M. A. Higginbotham, C. Chen, M. F. Ingram, D. M. Cyr, and T. R. Graham.** 2000. The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. Curr Biol **10:**1349-58.

15. **Gautschi, M., A. Mun, S. Ross, and S. Rospert.** 2002. A functional chaperone triad on the yeast ribosome. Proc Natl Acad Sci U S A **99:**4209-14.

16. **Geissler, S., K. Siegers, and E. Schiebel.** 1998. A novel protein complex promoting formation of functional alpha- and gamma-tubulin. Embo J **17:**952-66.

17. **Haslbeck, M., N. Braun, T. Stromer, B. Richter, N. Model, S. Weinkauf, and J. Buchner.** 2004. Hsp42 is the general small heat shock protein in the cytosol of Saccharomyces cerevisiae. Embo J **23:**638-49.

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. **Kuchler, K., G. Daum, and F. Paltauf.** 1986. Subcellular and submitochondrial localization of phospholipid-synthesizing enzymes in Saccharomyces cerevisiae. J Bacteriol **165:**901-10.

20. **Kumar, A., M. Seringhaus, M. C. Biery, R. J. Sarnovsky, L. Umansky, S. Piccirillo, M. Heidtman, K. H. Cheung, C. J. Dobry, M. B. Gerstein, N. L. Craig, and M. Snyder.** 2004. Large-scale mutagenesis of the yeast genome using a Tn7-derived multipurpose transposon. Genome Res **14:**1975-86.

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. USA **100:**15764-15769.

22. **Lu, Z., and D. M. Cyr.** 1998. Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. J. Biol. Chem. **273:**27824-27830.

23. **Mayr, C., K. Richter, H. Lilie, and J. Buchner.** 2000. Cpr6 and Cpr7, two closely related Hsp90-associated immunophilins from Saccharomyces cerevisiae, differ in their functional properties. J Biol Chem **275:**34140-6.

24. **McClellan, A. J., M. D. Scott, and J. Frydman.** 2005. Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways. Cell **121:**739-48.

25. **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.

26. **Nakayashiki, H.** 2005. RNA silencing in fungi: mechanisms and applications. FEBS Lett **579:**5950-7.

27. **Panavas, T., E. Serviene, J. Brasher, and P. D. Nagy.** 2005. Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. Proc. Natl. Acad. Sci. USA **102:**7326-7331.

28. **Pratt, W. B., P. Krishna, and L. J. Olsen.** 2001. Hsp90-binding immunophilins in plants: the protein movers. Trends Plant Sci **6:**54-8.

29. **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.

30. **Reinders, J., R. P. Zahedi, N. Pfanner, C. Meisinger, and A. Sickmann.** 2006. Toward the complete yeast mitochondrial proteome: multidimensional separation techniques for mitochondrial proteomics. J Proteome Res **5:**1543-54.

31. **Rossi, J. M., and S. Lindquist.** 1989. The intracellular location of yeast heat-shock protein 26 varies with metabolism. J Cell Biol **108:**425-39.

32. **Sahi, C., and E. A. Craig.** 2007. Network of general and specialty J protein chaperones of the yeast cytosol. Proc Natl Acad Sci U S A **104:**7163-8.

33. **Segers, G. C., X. Zhang, F. Deng, Q. Sun, and D. L. Nuss.** 2007. Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. Proc Natl Acad Sci U S A **104:**12902-6.

34. **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:**2162-2169.

35. **Solorzano, A., N. Rodriguez-Cousino, R. Esteban, and T. Fujimura.** 2000. Persistent yeast single-stranded RNA viruses exist in vivo as genomic RNA.RNA polymerase complexes in 1:1 stoichiometry. J Biol Chem **275:**26428-35.

36. **Svoboda, A., and I. Slaninova.** 1997. Colocalization of microtubules and mitochondria in the yeast Schizosaccharomyces japonicus var. versatilis. Can J Microbiol **43:**945-53.

37. **Toogun, O. A., W. Zeiger, and B. C. Freeman.** 2007. The p23 molecular chaperone promotes functional telomerase complexes through DNA dissociation. Proc Natl Acad Sci U S A **104:**5765-70.

38. **Tsai, J., and M. G. Douglas.** 1996. A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. J Biol Chem **271:**9347-54.

39. **Vainberg, I. E., S. A. Lewis, H. Rommelaere, C. Ampe, J. Vandekerckhove, H. L. Klein, and N. J. Cowan.** 1998. Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. Cell **93:**863-73.

40. **Vance, J. E.** 1990. Phospholipid synthesis in a membrane fraction associated with mitochondria. J Biol Chem **265:**7248-56.

41. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2568-2577.

42. **Wood, V., R. Gwilliam, M. A. Rajandream, M. Lyne, R. Lyne, A. Stewart, J. Sgouros, N. Peat, J. Hayles, S. Baker, D. Basham, S. Bowman, K. Brooks, D. Brown, S. Brown, T. Chillingworth, C. Churcher, M. Collins, R. Connor, A. Cronin, P. Davis, T. Feltwell, A. Fraser, S. Gentles, A. Goble, N. Hamlin, D. Harris, J. Hidalgo, G. Hodgson, S. Holroyd, T. Hornsby, S. Howarth, E. J. Huckle, S. Hunt, K. Jagels, K. James, L. Jones, M. Jones, S. Leather, S. McDonald, J. McLean, P. Mooney, S. Moule, K. Mungall, L. Murphy, D. Niblett, C. Odell, K. Oliver, S. O'Neil, D. Pearson, M. A. Quail, E. Rabbinowitsch, K. Rutherford, S. Rutter, D. Saunders, K. Seeger, S. Sharp, J. Skelton, M. Simmonds, R. Squares, S. Squares, K. Stevens, K. Taylor, R. G. Taylor, A. Tivey, S. Walsh, T. Warren, S. Whitehead, J. Woodward, G. Volckaert, R. Aert, J. Robben, B. Grymonprez, I. Weltjens, E. Vanstreels, M. Rieger, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, A. Dusterhoft, C. Fritzc, E. Holzer, D. Moestl, H. Hilbert, K. Borzym, I. Langer, A. Beck, H. Lehrach, R. Reinhardt, T. M. Pohl, P. Eger, W. Zimmermann, H. Wedler, R. Wambutt, B. Purnelle, A. Goffeau, E. Cadieu, S. Dreano, S. Gloux, et al.**

2002. The genome sequence of Schizosaccharomyces pombe. Nature **415:**871-80.

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

44. **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.

45. **Zinser, E., C. D. Sperka-Gottlieb, E. V. Fasch, S. D. Kohlwein, F. Paltauf, and G. Daum.** 1991. Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. J Bacteriol **173:**2026-34.