UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of the Sterol Sensing Domain in HMG-CoA Reductase Regulation in *Saccharomyces cerevisiae*

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

in

Biology

by

Deeba Pourmand

Committee in charge:

Professor Randy Hampton, Chair
Professor Douglass Forbes
Professor Lorraine Pillus

2011

The Thesis of Deeba Pourmand is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2011

DEDICATION

I would like to dedicate this thesis to everyone who has stood by me and supported me for my Masters degree. Ranging from family, friends, to colleagues in the lab, I thank you all for helping me make this possible.

In recognition of Sam Alsalem, who spent a lot of time and effort formatting this thesis. Thank you – I will remember you for many years to come.

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ACKNOWLEDGEMENTS

I would like to acknowledge the chair of my thesis committee Dr. Randy Y. Hampton, who contributed with positivity and motivation to this research project. His guidance and advice has been valuable and will stay with me.

I would like to acknowledge Chandra Theesfeld for guiding me through this thesis project. She has been generous with the amount of attention, discussion, and time she helped me approach this project. She has contributed to the editing of my thesis.

I would like to acknowledge Tai Davis for making the 30 SSD residue mutations for this study. I would like to acknowledge Richard Gardner for making regional mutations in the N-terminal domain of Hmg2, and some of these mutants were tested in this study.

In Chapter 1, I would like to acknowledge that figures 1-6 were adapted from the following published paper (Hampton R.Y., 2002). Chapter 1, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary

investigator of this material. I would like to acknowledge that Tai Davis contributed on generating the topology diagram of Figure 9.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Chapter 5, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Chapter 6, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material. I would like to acknowledge Richard Gardner for creating the site-directed mutations throughout the N-terminal domain of Hmg2. Some of those mutations were examined in this current study (Richard, R.G., 2000 PhD Thesis).

Chapter 7, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material

ABSTRACT OF THE THESIS

The Role of the Sterol Sensing Domain in HMG-CoA Reductase regulation in *Saccharomyces cerevisiae*

by

Deeba Pourmand

Master of Science in Biology

University of California, San Diego, 2011

Professor Randy Hampton, Chair

Sterol sensing domain (SSD) containing proteins are required for lipid regulation, and are conserved among different organisms. 3-hydroxy-3-methyglutaryl-CoA reductase (HMGR) is a key enzyme for sterol synthesis that contains an SSD. In both mammals and yeast, HMGR undergoes regulated degradation in response to feedback of the mevalonate pathway. The configuration of HMGR changes when regulated by mevalonate

molecules, which targets the protein for regulated degradation in the endoplasmic reticulum. The N-terminus of HMGR is necessary and sufficient for regulated degradation. The SSD of HMGR ranges from 5 out of 8 transmembrane spans of the N-terminus, and the SSD of HMGR is conserved between many organisms. In this work we investigated the role of the SSD in regulation of HMGR in *Saccharomyces cerevisiae*. We made 30 mutations in highly conserved residues of the SSD of budding yeast HMGR, and examined their phenotypes with respect to HMGR regulated degradation. To do this we used flow cytometry to measure HMGR levels in response to pharmacological manipulations of the mevalonate pathway that either increase or decrease degradation signals. We found that the SSD is involved in sensing FPP-derived molecule and oxysterol molecules. The SSD also contributed to the destabilization of HMGR during regulated degradation of the protein.

Chapter 1

Introduction

Regulation of cholesterol levels is critical for many functions in a cell. Cholesterol is an important component of lipoproteins, bile acids, sterol hormones, membrane integrity, and components in cellular membranes, such as lipid rafts that are important for cell signaling and protein sorting (Kuwabara P. E., and Labouesse M. 2002). Deficiencies in cholesterol levels perturb these cellular functions. Overaccumulation of cholesterol in the cell can lead to toxic effects, such as cardiovascular disease and atherosclerosis in mammals. Therefore, it is important to regulate cholesterol levels in the cell. Intracellular cholesterol can be taken up from the bloodstream into cells through low-density lipoprotein particles (LDLs), or through synthesis in the endoplasmic reticulum (ER).

The mevalonate pathway produces isoprene lipids and ultimately cholesterol (Figure 1). Isoprene products have diverse functions in the cell. An isoprene side chain is a component of ubiquinone, which is involved in the electron transport chain, dolichol is used for glycosylation, and isopentyladenine is a component of tRNA. Prenylation of proteins uses isoprene groups, such as farnesyl to anchor proteins into cellular membranes (Goldstein J.L., and Brown M.S. 1990).

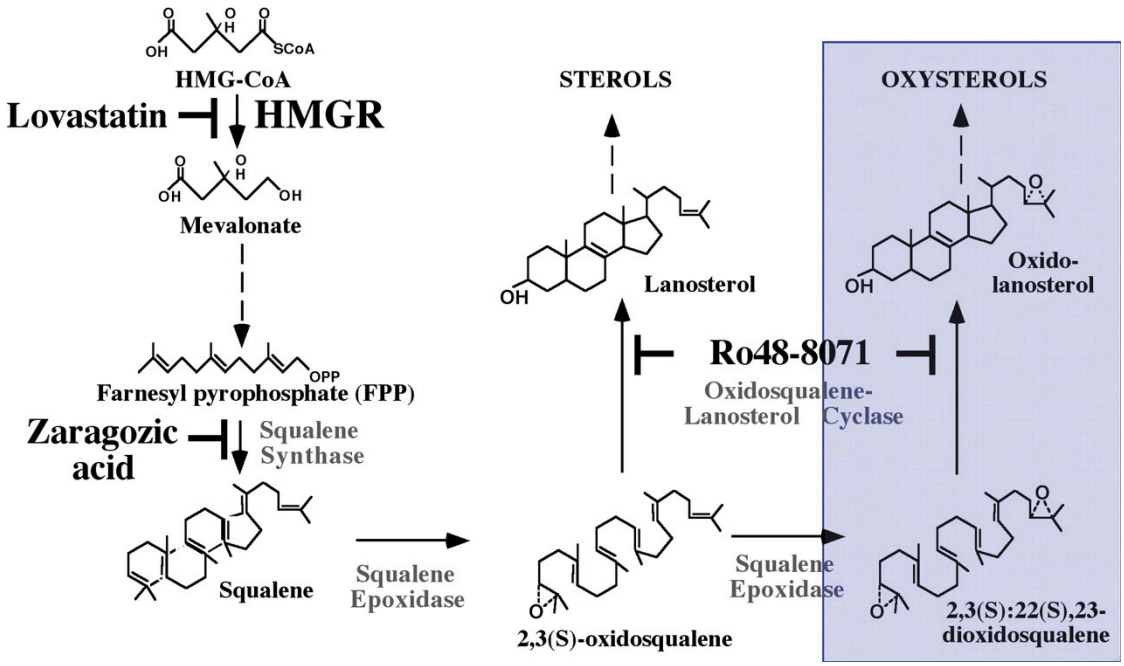

Figure 1: The Mevalonate Pathway. A representation of the mevalonate pathway following the rate-limiting step catalyzed by HMGR. Note that isoprene, sterol, and oxysterol products are synthesized from this pathway. The drugs lovastatin (*Lova*), zaragozic acid (*ZA),* and Ro48-871 (*Ro48*) manipulate the flux of the mevalonate pathway.

Mevalonate products are also components of intercellular messengers, and steroid hormones. These diverse critical processes necessitate careful control of the mevalonate pathway.

One way the mevalonate pathway is controlled is through feedback regulation of HMG-CoA reductase (HMGR). HMGR, an integral membrane protein of the ER, is the rate-limiting enzyme for synthesizing lipids of the mevalonate pathway (Figure 1). This reductase uses NADPH to reduce HMG-CoA to mevalonate. HMGR is involved in maintaining the homeostasis of mevalonate pathway lipids through multivalent feedback regulation. One form of control is the feedback-regulated degradation of HMGR in response to flux through the mevalonate pathway (Figure 2). When there is a high flux through the mevalonate pathway, the pathway products feedback on to HMGR, and induce rapid degradation of HMGR (Hampton R.Y., 2002)(Hampton, R.Y., and Garza, R.M. 2009). When there is a low flux through the pathway, then HMGR degradation is slowed, and HMGR levels are upregulated. HMGR undergoes feedback regulation in both mammals and yeast.

Mammalian and *Saccharomyces cerevisiae* HMGR have structural and functional similarities (Figure 3). *S. cerevisiae* has two isozymes of HMGR located in the ER: Hmg1, and Hmg2. Hmg1 is a stable protein and Hmg2 undergoes rapid regulated degradation depending on the flux of the mevalonate pathway products (Hampton R.Y. 2002).

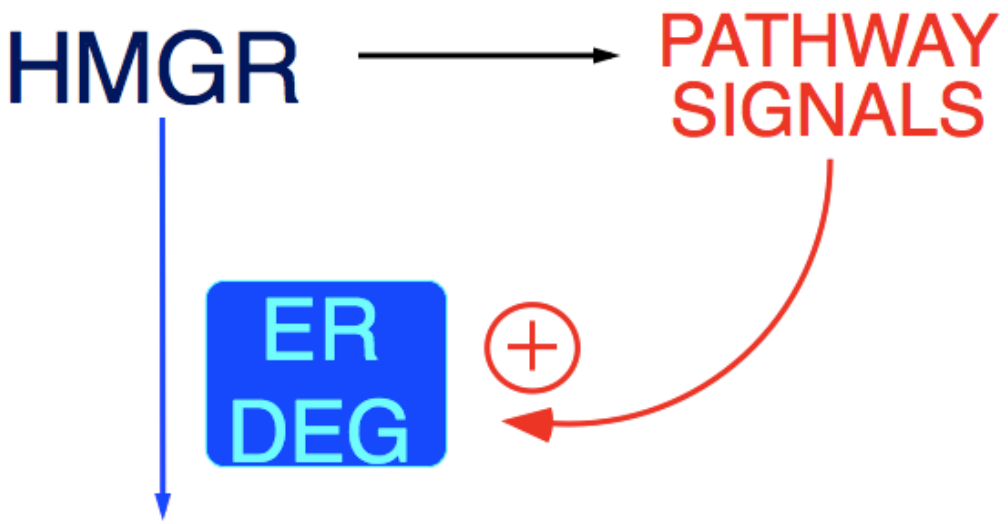

Figure 2: Feedback Regulation of HMGR Stability. Specific mevalonate pathway products feedback onto HMGR and induce regulated degradation of the protein, which is executed at the ER membrane.

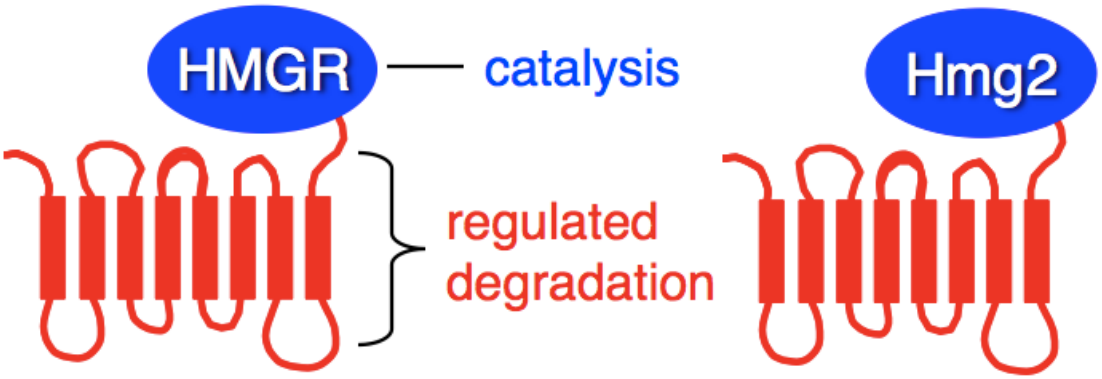

Figure 3: Conservation between mammalian and yeast HMGR.
Mammalian HMGR and yeast HMGR have conserved structure and function of the proteins. There are 8 transmembrane spanning domains at the N-terminus of the proteins (red). The function of the C-terminal catalytic domain is also conserved (blue).

Both mammalian and yeast HMGR have a transmembrane-spanning N-terminal domain that is sufficient and necessary for regulated degradation (Hampton R. Y., and Rine J. 1994)(Hampton R.Y. et al. 1996)(Hampton, R.Y., and Garza, R.M. 2009). Both organisms have a C-terminal catalytic domain that is not necessary for degradation of the protein. The C-terminal catalytic domain is located in the cytosol, which is connected by a linker domain to the N-terminal transmembrane region necessary for ER localization (Figure 3)(Hampton R. Y., and Rine J. 1994). The similarity of the proteins is underscored by the ability of mammalian HMGR to substitute for yeast HMGR *in vivo*, and expression of mammalian HMGR can rescue the functioning of HMGR depleted cells in yeast. Although there are two isozymes of yeast HMGR (Hmg1 and Hmg2), only the Hmg2 enzyme undergoes regulated degradation.

The mevalonate pathway products that cause feedback regulation of HMGR are similar between mammalian and yeast HMGR. Both mammalian and yeast HMGR respond to non-sterol and isoprene molecules. Regulated degradation of mammalian HMGR can be induced by sterols such as lanosterol, 24,25-dihydrolanosterol, and oxysterol 25-hydroxycholesterol (Espenshade P.J., and Hughes A.L. 2007). Hmg2 responds to an oxysterol molecule too, however the specific oxysterol signal has not yet been determined. For both mammalian HMGR and Hmg2, a non-sterol isoprenoid

signal is needed for complete and rapid degradation of HMGR. Through genetic and pharmacological manipulation of the mevalonate pathway, the Hampton lab discovered that 15-carbon farnesyl pyrophosphate (FPP), an FPP derived molecule, is a positive signal for Hmg2 degradation (Gardner R.G., Hampton R.Y. 1999b). When there are high levels of FPP present in the cell, Hmg2 is rapidly degraded, which results with low amounts of Hmg2 protein. When there are low amounts of FPP in the cell, then Hmg2 is more stable and there is slow degradation of the protein (Refer to Figure 1 to see where these molecules are produced in the mevalonate pathway).

Budding yeast Hmg2 and mammalian HMGR undergo regulated degradation by ubiquitin-dependent proteolysis (Ravid T., and Hochstrasser M. 2008)(Hampton, R.Y., and Garza, R.M. 2009). A major site of ubiquitin-dependent proteolysis degradation is at the ER, where secretory and integral membrane proteins of the ER are assembled (Ravid T., and Hochstrasser M. 2008). ER-associated degradation (ERAD) can either be selective or non-specific.

Selective ERAD is a form of regulated degradation, which occurs during specific circumstances in a cell. For instance, cell division coordinates regulated degradation to signal events leading to the division of the cell (Hochstrasser M. 1996). When a regulatory event occurs, an identifiable sequence of the protein becomes exposed, which targets the protein for

selective degradation. The sequences exposed that targets the protein for degradation is known as a “degron” (Ravid T., and Hochstrasser M. 2008). A degron may become exposed during a regulatory event upon covalent modification, removal of an effector protein that otherwise would shield the degron, or altered transcription of receptors that would ordinarily bind to the degron. An example of a degron is the Deg1 sequence of the *MATalpha2* transcription regulator in yeast.

ERAD also employs of quality control, in which misfolded proteins are targeted for degradation. Quality control pathways target proteins for degradation based on the folding state of the protein, rather than sequence. If a protein is misfolded, and chaperones cannot correct the misfolded configuration, the protein is targeted for degradation, such as in the disease cystic fibrosis. In cystic fibrosis, there is a mutation in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which prevents the protein from folding into a functional protein (Hochstrasser M. 1996). The mutated misfolded CFTR protein is constitutively degraded by the 26S proteasome. In this case, the quality control machinery recognizes common structural motifs of a misfolded protein rather than recognizing specific degrons of the protein**.**

Targeting ERAD substrates to the proteasome is accomplished by marking the protein with a polyubiquitin chain. Ubiquitin is a small regulatory

protein that's function is to direct proteins to the proteasome. In order to ubiquitinate a target substrate, a cascade of enzymes is necessary (Figure 4). First, ubiquitin is activated with an E1 ubiquitin-activating enzyme. This charged ubiquitin is transferred from an E1 enzyme to an E2 enzyme (ubiquitin-conjugating enzyme). The E2 then transfers the activated ubiquitin to the protein that is being targeted for degradation with assistance from an E3 ubiquitin-ligase complex. Regulated degradation of Hmg2 is accomplished by the HRD (HMG-CoA Reductase Degradation) pathway, which was demonstrated by the Hampton lab. The HRD pathway is a main pathway that targets quality control substrates for ERAD. Hrd1 is the E3 ubiquitin ligase in yeast cells, and it is rate limiting for tagging proteins with ubiquitin for degradation (Bays, N., et al 2001). Therefore this machinery is utilized primarily for ERAD, and it primarily targets misfolded proteins for degradation.

Hmg2 regulated degradation occurs by the HRD pathway (Hampton R.Y. et al 1996). The HRD machinery primarily recognizes misfolded ERAD substrates; yet somehow Hrd1 is able to execute regulated degradation of Hmg2. There is evidence that upon sensing FPP Hmg2 undergoes a structural transition to a less folded state that is more susceptible to HRD-mediated ERAD (Figure 5). This structural transition was demonstrated through an *in vitro* trypsin proteolysis assay to assess Hmg2 protein folding in microsomes (Shearer A.G., Hampton R.Y. 2005).

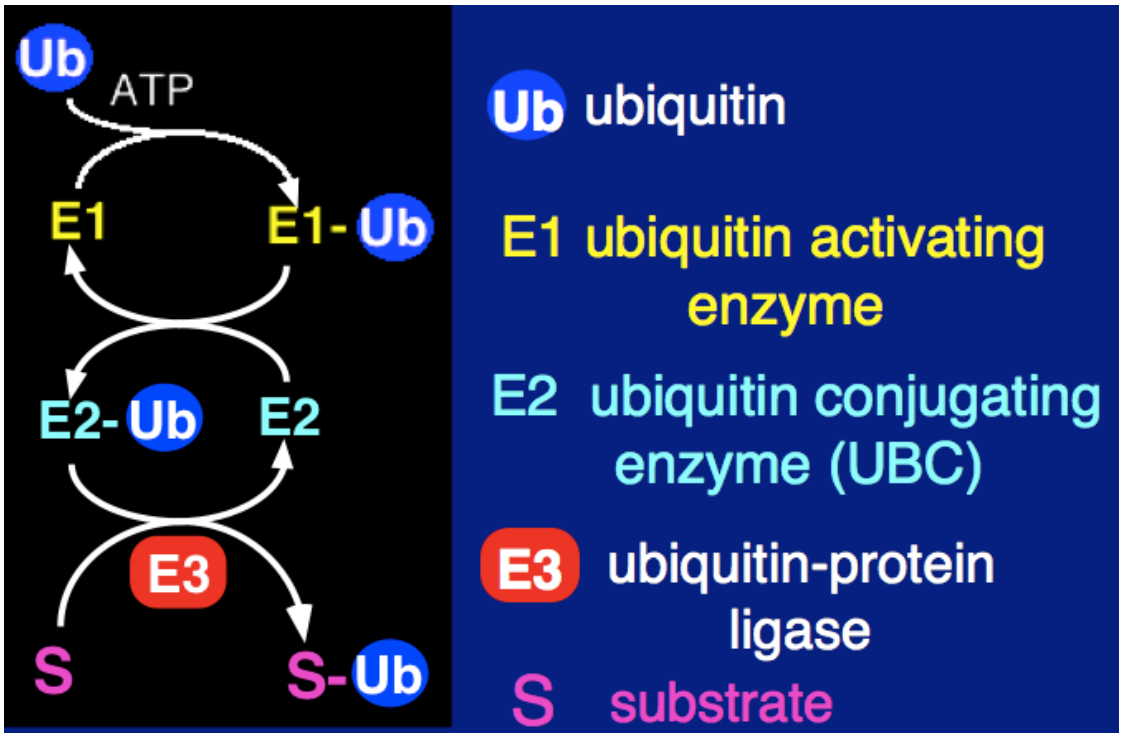

Figure 4: Ubiquitin Cascade. The following enzymes are involved in tagging ubiquitin onto substrates for targeted degradation to the 26S proteasome. E1 activates ubiquitin and transfers ubiquitin to E2, the ubiquitin-conjugating enzyme. The E2 and E3 ubiquitin-ligase directs the ubiquitin onto the targeted substrate. Repetition of this cycle generates multi-ubiquitin chains that target substrates to the proteasome for degradation.

When microsomes were treated with DMSO, trypsin did digest some Hmg2 by 10 min. However, when farnesol (which is an FPP derived molecule) was added to microsomes, trypsin digestion of Hmg2 was amplified, indicating that trypsin was able to more easily access more areas of Hmg2. This finding implies that the protein had changed into a less folded confirmation in a signal dependent manner (Figure 5). This change in folding appears to allow Hmg2 to enter the HRD quality control pathway in a regulated manner. Therefore the currently known model of Hmg2 regulated degradation is that once induced by FPP, Hmg2 undergoes a structural transition into a misfolded substrate, which allows Hrd1 to lead it to degradation (Figure 6).

Regulated entry of Hmg2 into the HRD pathway requires sequences distributed in the N-terminus of Hmg2. A previous study had shown that mutations (ranging from 6 residues or less per region) in the Hmg2 N-terminus yielded mutants that underwent constitutive and unregulated degradation, such as the mutant TFYSA 348-352 ILQAS (Gardner R.G., and Hampton R.Y. 1999a). The TFYSA mutation of Hmg2 did not sense FPP molecule and underwent constitutive degradation. This finding implied that there might be residues in the N-terminus of Hmg2 necessary for sensing regulatory feedback molecules. Another mutation K6R made Hmg2 stable, and showed no degradation of Hmg2 (Gardner R.G., and Hampton R.Y. 1999a).

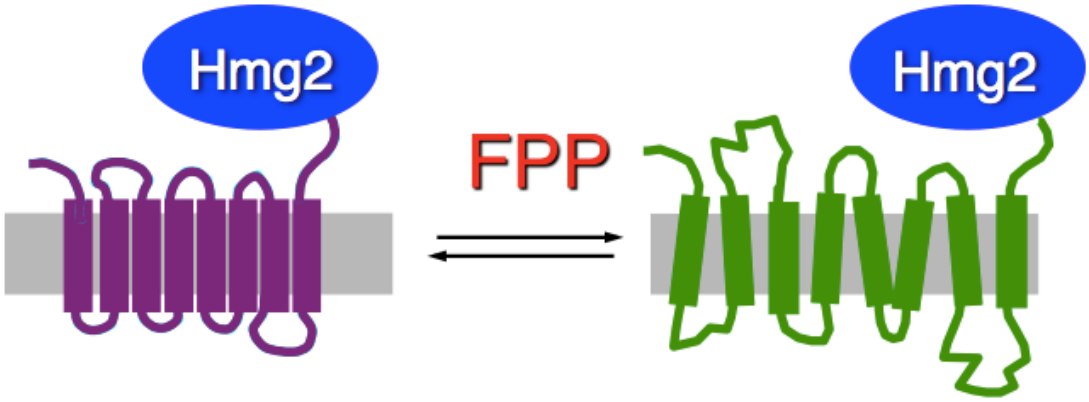

Figure 5: Structural Transition of Hmg2. When FPP derived molecule induces regulated degradation, a structural change in Hmg2 confirmation is initiated.

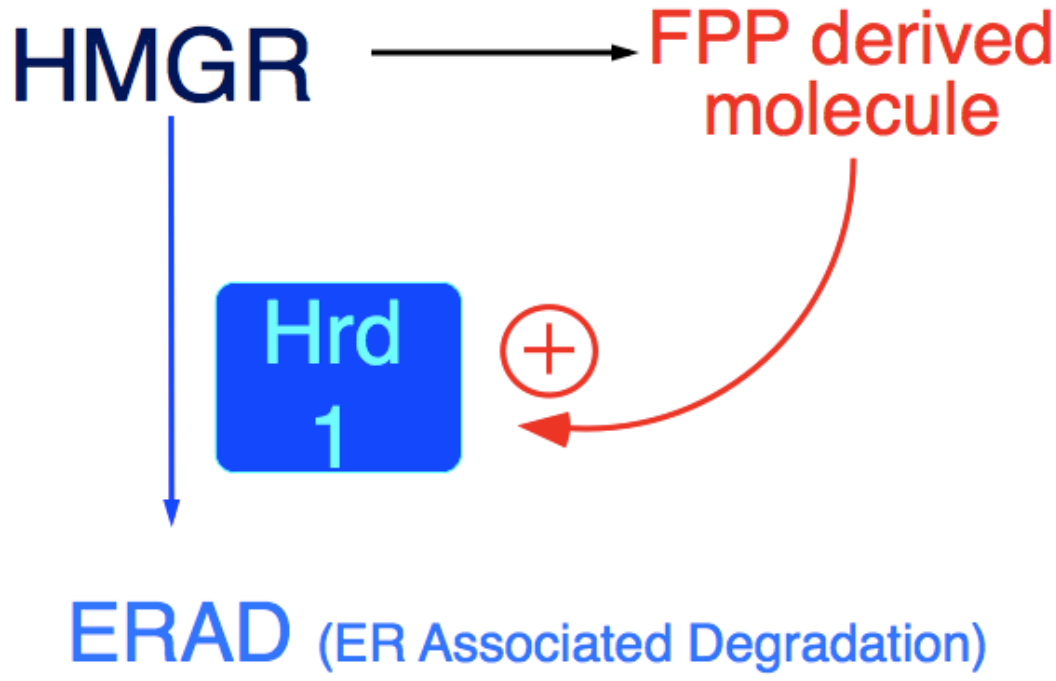

Figure 6: **Specified Feedback Regulation of HMGR.** Specifically FPP derived molecules feedback onto HMGR, and promotes regulated ERAD of this protein. The HRD machinery recognizes HMGR and assists in targeting the protein for degradation.

The K6 residue is thought to be a site for tagging ubiquitin to Hmg2 that's necessary for degradation, and when the K residue was mutated, Hmg2 did not respond to FPP and remained stable. This finding thus demonstrated that there are residues in the N-terminus that are necessary for regulated degradation of Hmg2.

Based on these observations, we hypothesize that residues dispersed throughout the N-terminus are important for regulated degradation of Hmg2. Some of these residues are necessary for sensing the regulatory signals that induce Hmg2 degradation, and some residues are necessary for the destabilization of Hmg2 during regulated degradation. Therefore Hmg2 has aspects of undergoing selective and quality control ERAD. The way that the protein utilizes both types of degradation is currently being investigated.

Within the Hmg2 N-terminal domain is a sterol-sensing domain (SSD). The SSD of Hmg2 spans transmembranes 2-6 of the 8 transmembrane spans. The SSD is a structural motif found in proteins involved in lipid regulation (Hampton, R.Y., and Garza, R.M. 2009). The topology of the SSD is conserved between HMGR, SREBP cleavage activating protein (SCAP), and Niemann-Pick C1 protein (Davies J.P., and Ioannou Y.A., 2000). SSD containing proteins appear to respond to a variety of lipid molecules. For instance, cholesterol regulates SCAP, isoprenes and lanosterol regulates

mammalian HMGR, and an FPP derived molecule primarily regulates Hmg2 (Espenshade P.J., and Hughes A.L. 2007).

SSD containing proteins share conserved sequences, and the SSD of HMGR is conserved among many different organisms ranging from *Homo sapiens*, *Cricetulus griseus,* to *S. cerevisiae* (Figure 7). The SSD (transmembrane spans 2-6) is a conserved motif of residues distributed throughout the transmembrane spans. Even hamster SCAP (*C. griseus)* has sequence homologies to the SSD of HMGR despite their apparent functional differences (Figure 7). Based on this conservation, the SSD appears to be a functionally important domain for regulation of lipids since the residues are conserved throughout different organisms.

Since the N-terminus sequences have demonstrated to be important for regulated degradation of Hmg2, and the SSD spans a significant portion of the N-terminal domain, we proposed that the SSD plays a role in regulated degradation of Hmg2. To test this role, mutations were made in conserved residues of the SSD of Hmg2, and the regulatory behavior of each mutant was examined in a variety of ways (Figure 8).

In this study, highly conserved SSD residues were mutated because we reasoned that conserved residues are important for function. These mutants were assayed for protein stability and regulation by mevalonate pathway molecules to assess the role of these residues in Hmg2 regulated degradation.

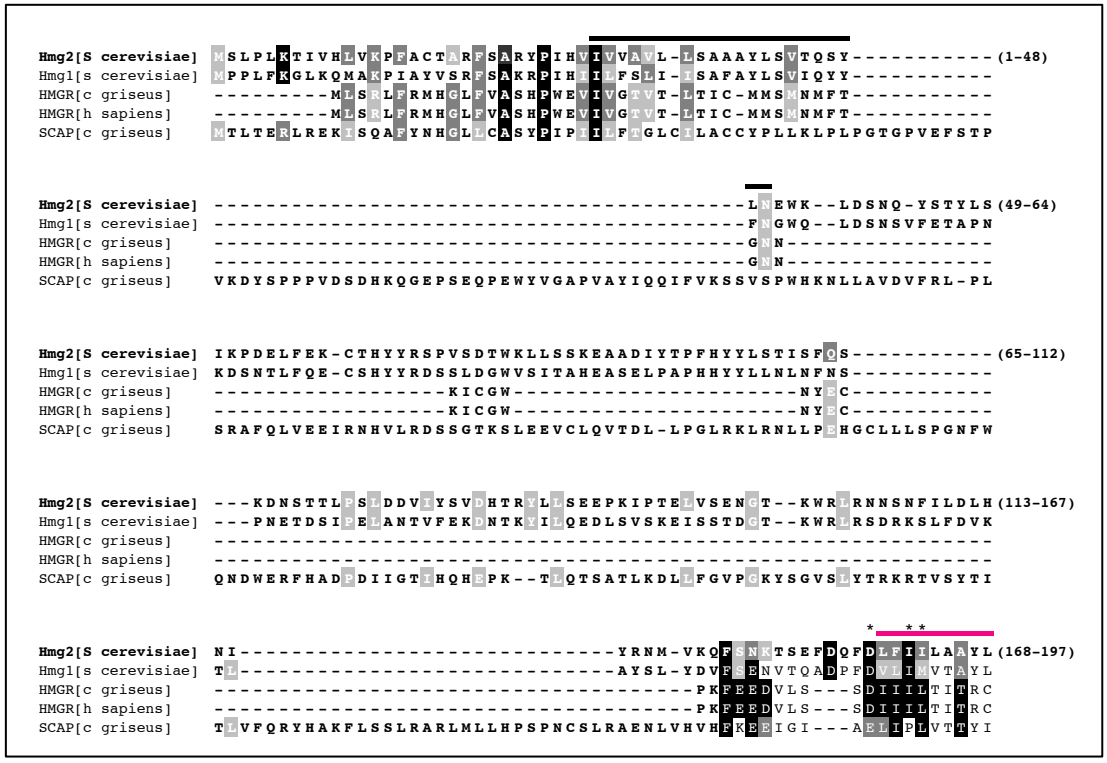

Figure 7: Conserved amino acid residue alignment of SSD-containing proteins. Sequence homology of Hmg2 N-terminal domain to corresponding SSD containing proteins. Sequences of Hmg2 [*S. cerevisiae*] (systematic name YLR450W), and Hmg1 [*S. cerevisiae*] (systematic name YML075C) were obtained from Saccharomyces Genome Database. HMGR [*C. griseus*] (Accession # P00347), HMGR [*H. sapiens*] (Accession # AAG21343.1), and SCAP [*C. griseus*] (Accession # AAB19103.1) sequences were obtained from GenBank. Sequence alignment was assessed with T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk /Tools/msa/tcoffee/). The areas of shaded conservation are consistent with Figure 8, however additional speculation of conservation is shaded. Black regions show identical residues of conservation, and grey regions indicate chemical conservation. Transmembrane domain regions are over-lined, and the SSD region is over-lined in red. Dashes represent breaks in amino acid sequences of the SSD containing proteins, which allows sequence alignment with Hmg2. Asterisks represent the mutations made in Hmg2. The boxed residues are mutated regions (6 or less residues) of Hmg2 based on the study in (Gardner R.G., and Hampton R.Y. 1999a). The corresponding residue numbers of these Hmg2 regions are noted above each region.

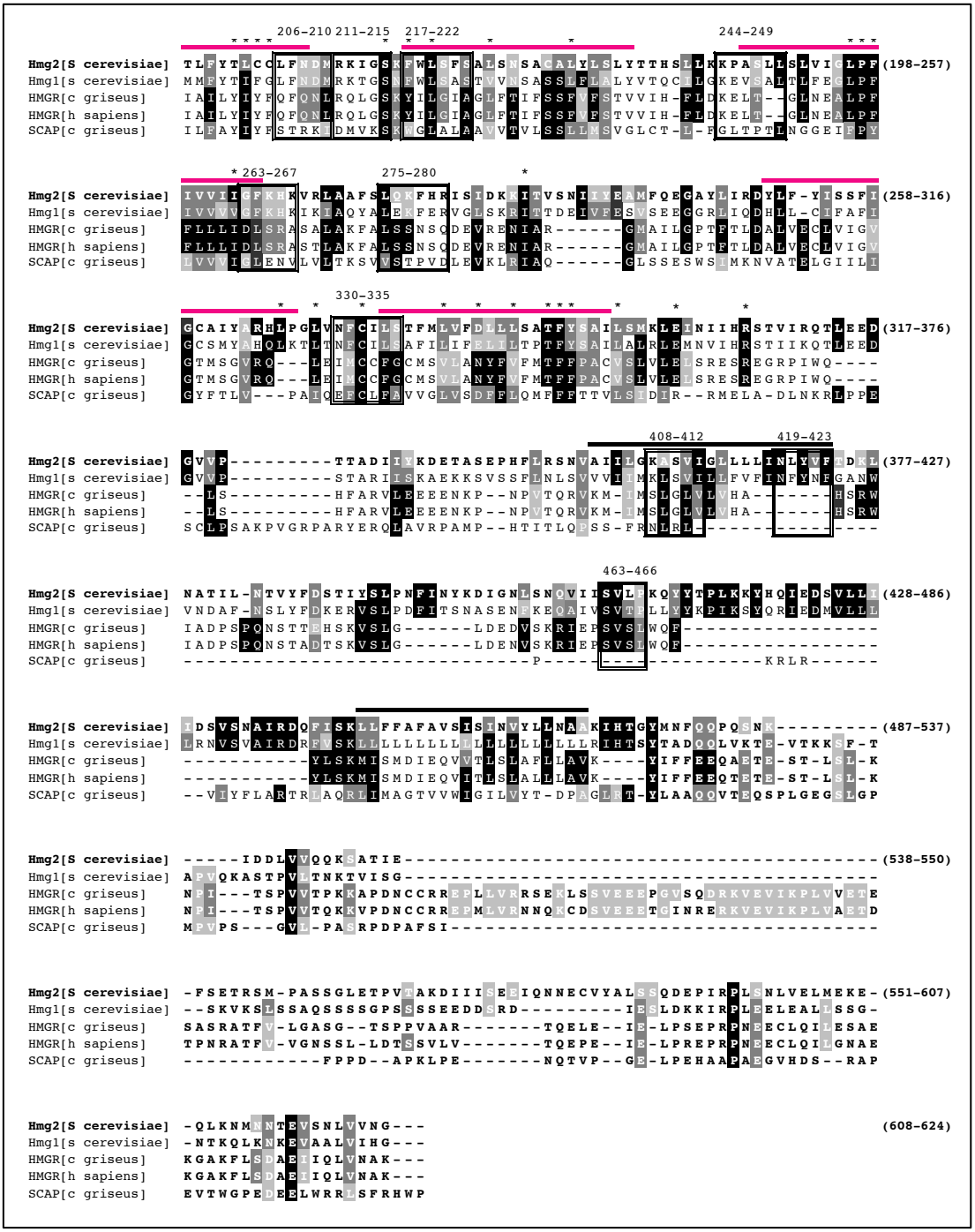

Figure 7: Conserved amino acid residue alignment of SSD-containing proteins, continued.

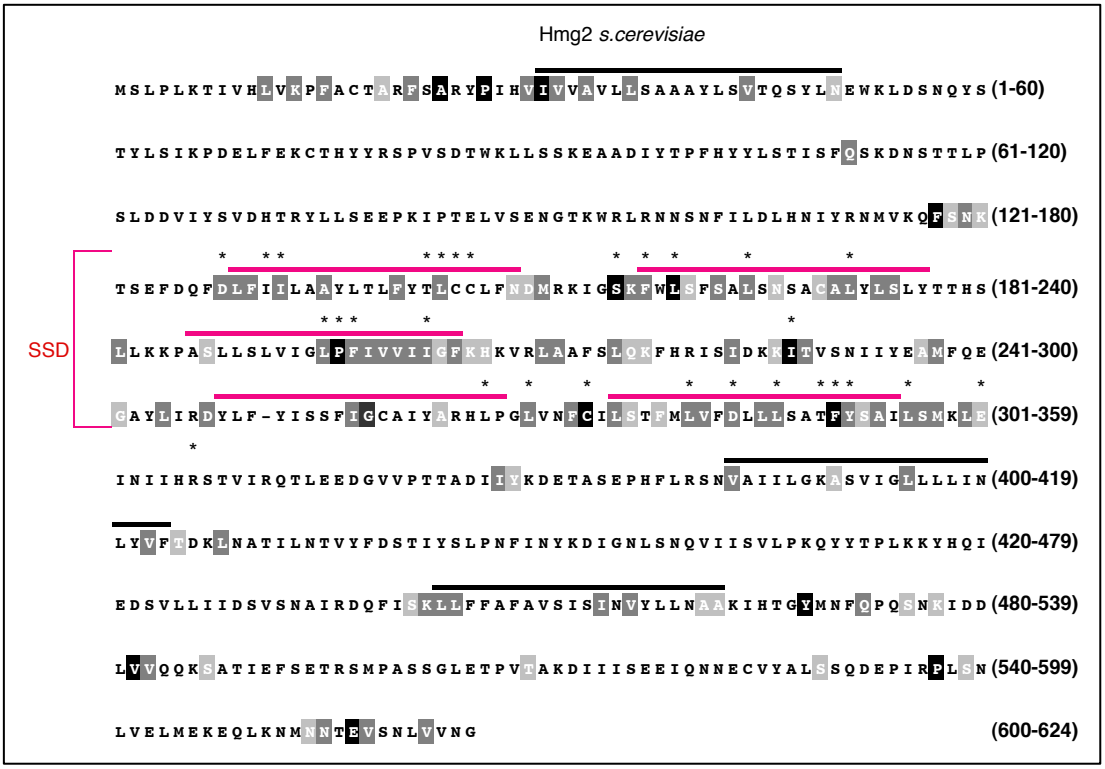

Figure 8: Point mutations in conserved amino acid residues of Hmg2. Sequence analysis of Hmg2 N-terminal domain. Shaded regions denote conservation of residues between Hmg2 and similar SSD-containing proteins (Figure 7). Black residues show identical conservation, dark grey residues show chemical conservation, and light grey residues show semi-conservation. The degrees of conservation were determined by T-Coffee multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/tcoffee/). Transmembrane domains are over-lined, and the SSD region (Transmembrane regions 2-6) is indicated. The asterisks represent mutated residues. There were 30 mutations made total. Some mutants included two or more residues. Hmg2 amino acid sequence was obtained from Saccharomyces Genome Database with systematic name YLR450W.

The results indicate that many conserved residues in the SSD are involved in sensing mevalonate pathway molecules, because these mutated residues show various stunted abilities to sense mevalonate pathway molecules. Surprisingly, all Hmg2 mutations demonstrated variable degrees of increased stabilization, while none showed constitutive degradation indicating misfolding. Therefore, it appears that SSD is involved in destabilizing Hmg2 for regulation. Additional mutations of conserved residues were made outside of the SSD to assess the role of regions outside the SSD for Hmg2 regulated degradation. Results indicated that conserved regions inside and outside the SSD play a role in regulated degradation of Hmg2.

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Chapter 2

Assays for studying Hmg2 SSD mutants

The SSD mutants analyzed in this study are shown in Table 1. 23 out of 30 of these residues were mutated into alanine (A). 5 out of 30 residues were mutated into leucine (L). Mutating these residues into alanine depleted the functional groups normally present in the residues. One residue S215 was mutated into both threonine (T) and alanine (A). The conservation of S215 is observed in SSD containing proteins among different organisms, which is why chemical conservation and chemical depletion of this residue was tested (Figure 7 and 8). One residue L219 was mutated into Phenylalanine (F), which is also a highly conserved residue observed in SSD containing proteins among different organisms (Figure 7 and 8).

Most of the mutations alter a single residue; a few mutations altered a region. For instance, region TLCC 202-205 was mutated into TLCC 202-205 AAAA. T202 (T202A) and L203 (L203A) single residue mutations were also included in this study from this region. This region was examined because it corresponded to the region studied in hamster SCAP. Studies with the mutation Y298C in hamster SCAP (Corresponding to T202A in Hmg2) resulted with an unregulated protein with constitutive activation of sterol regulatory element binding proteins (SREBPs) (Hughes A.L., et al 2008).

Table 1: SSD mutations analyzed in this study. The physical location topology of these residues is depicted in the topology diagram in Figure 9.

Hmg2-GFP derivatives
D188A
I191A
I192A
T202A
L203A
TLCC 202-205 AAAA
S215A
S215T
F217L
L219F
L224A
L231A
L255A
P256A
F257L
I262A
I287A
L325A
L328A
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L339A
D342A
L345A
T348L
F349L
FY349LS
Y350A
L354A
E359A
R365A

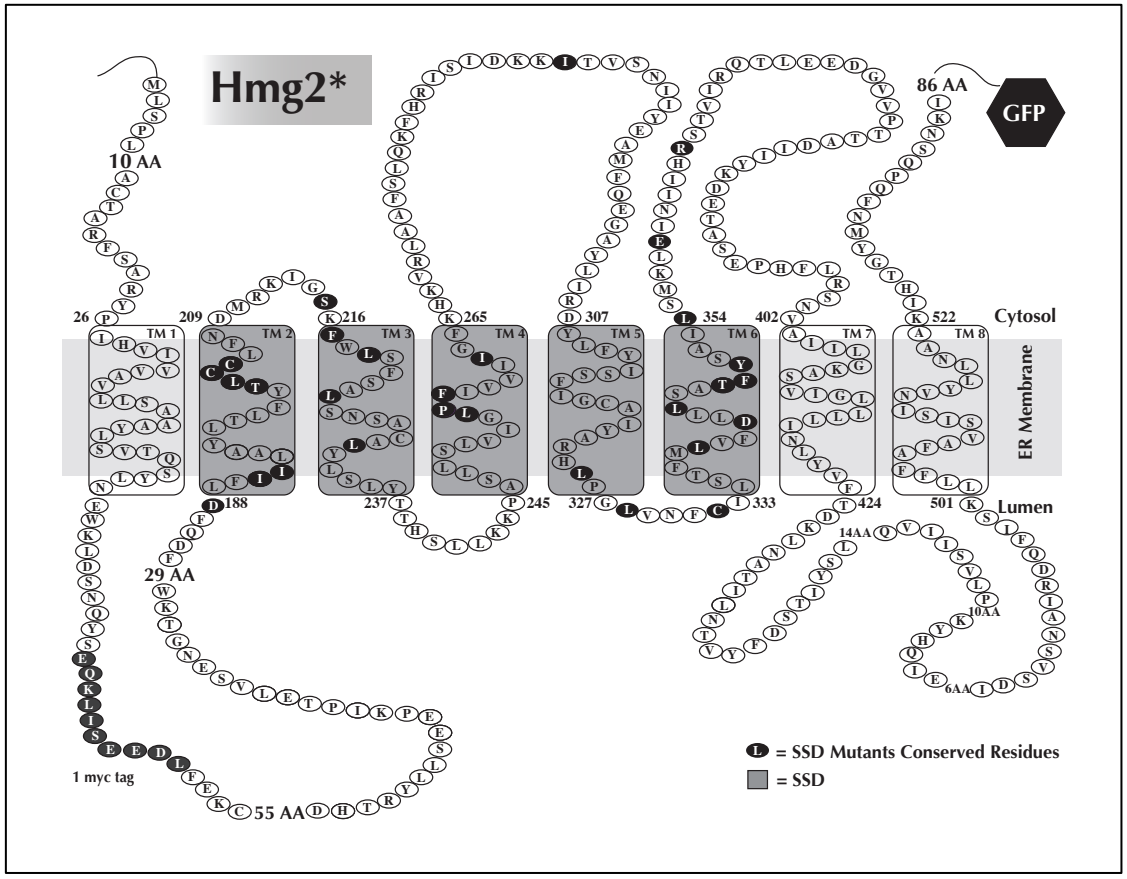

Figure 9: Hmg2 Topology. The topology model of the N-terminal domain of Hmg2 was generated through tests and observations in the Hampton lab at University of California San Diego (UCSD). The SSD is highlighted in dark grey. The mutations analyzed in this study are highlighted in black. Catalytic domain is substituted with a GFP tag (RHY2723), and there is a 1-myc tag between transmembrane domains 1 & 2 in the first luminal loop. This construct is an optical reporter for Hmg2 protein levels and allowed us to perform an assay using flow cytometry.

Therefore, this region was examined in Hmg2 to see if these residues were important for regulated degradation, and to see if there was a correlation in this region to other SSD containing proteins.

The TFYSA mutated region, which caused unregulated degradation of Hmg2, was also further examined in this study, with individual mutations T348L, F349L, Y350A, and the double mutant FY349LS.

A GFP optical reporter was used to qualitatively examine regulated degradation of each mutant. This reporter is a chimeric protein that contains the N-terminal transmembrane domain, and GFP in place of the C-terminal catalytic domain of Hmg2 (Cronin, S.R., and Hampton R.Y., 1999); refer to Table 2 and materials and methods for strain information. The steady-state levels and regulated degradation of these Hmg2-GFP variants was examined optically by flow cytometry.

To test how the SSD plays a role in regulated degradation of Hmg2, FPP levels were manipulated with drug specific enzyme inhibitors. Lovastatin (*Lova*) is a drug that directly binds to HMGR, and halts the production of mevalonate pathway molecules (Figure 1). Lovastatin decreases the pool of FPP in the cell, and decreases Hmg2 degradation, (Figure 10 *Right diagram*) (Hampton R.Y., and Rine J. 1994). Conversely, Zaragozic acid (*ZA*) inhibits the enzyme squalene synthase, which converts FPP to squalene (Figure 1),

causing an accumulation of FPP, and the rapid degradation of Hmg2 (Figure 10 *Right diagram*)(Gardner R.G., and Hampton R.H. 1999b).

The Hmg2 steady state level is established by the rates of synthesis and degradation of the protein. Protein stability was assessed to determine the relationship between loss of regulation and protein stability. To do this, cycloheximide (*CHX*) was added to cells to assess the stability of Hmg2. After addition of CHX, protein levels were assessed overtime to determine how fast the protein was degraded (Figure 10 *Left Panel*).

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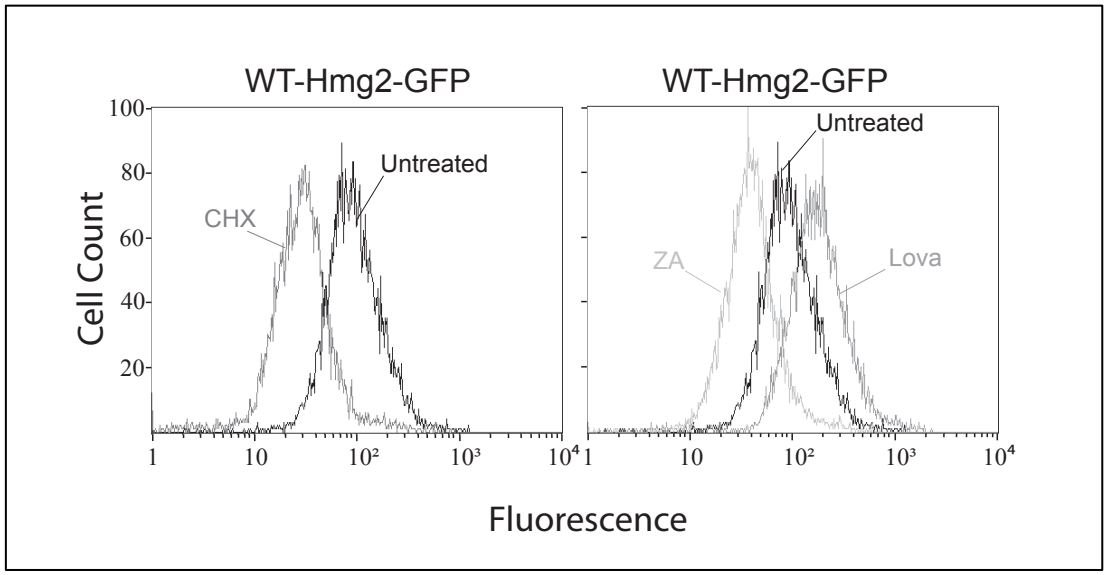

Figure 10: Flow Cytometry of Hmg2-GFP. *Left diagram* CHX chase assay: Hmg2-GFP was grown to log phase at 30°C and incubated for 2 hours with no drug, and 50ug/mL cycloheximide (*CHX*). *Right diagram* Assay for regulated degradation of Hmg2-GFP was grown to log phase at 30°C and incubated for 2 hours with no drug, 25ug/mL lovastatin (*Lova*), and 10ug/mL zaragozic acid (*ZA*). Hmg2-GFP fluorescence was analyzed by flow cytometry. A shift to the right of the histogram indicates higher levels of Hmg2-GFP in the cell. A shift to the left of the histogram indicates Hmg2-GFP levels.

Chapter 3

Materials and Methods

Reagents:

Cycloheximide (*CHX*) was used at 50ug/mL for all experiments and was obtained from Sigma-Aldrich (St. Louis, MO). Lovastatin (*Lova*) was used at 25ug/mL, and Zaragozic acid (*ZA*) was used at 10ug/mL for all the experiments (both donated by Merck). Ro0488071 (*Ro48*) was used at 20ug/mL for all experiments (obtained from Johannes Aebi at F.Hoffman-La Roche Ltd). Mouse monoclonal anti-GFP antibody was obtained from Clontech. Hybridoma supernatant (9E10; obtained from ATTC) was the source of mouse monoclonal anti-myc antibody. Monoclonal anti-ubiquitin antibody was a gift from Richard Gardner from the University of Washington.

Plasmid Construction:

Plasmids were constructed by standard molecular biology techniques as described in Gardner et al. 1998. Hmg2 single residue SSD mutants were synthesized by the splicing by overlap elongation (SOEing) PCR technique, adapted from (Horton et al. 1989), cited in (Sato B.K., and Hampton R. Y. 2006) (Sato B.K. et al 2009).

Strains & Media:

DH5α *Escherichia coli* strains were grown at 37°C in LB medium and 100ug/mL of ampicillin. Yeast strains (Table 2) were grown to log-phase

(A_{600}<0.4 OD/mL) with aeration at 30°C in minimal medium supplemented with the appropriate amino acids and sugars (Hampton R. Y., and Rine J., 1994). The lithium acetate method was used to transform plasmid DNA into yeast, and yeast transformants were selected for uracil prototropy (Ito, H., et al 1983).

Mutants were derived with SOEing using corresponding oligos and parent plasmid pRH1581 (Table 2). pRH1581 derived plasmids have a *TDH3* promoter driving expression of Hmg2-GFP. The Hmg2 catalytic domain is replaced with the GFP tag (Shearer A.G., and Hampton R.Y. 2004). Wildtype RHY2723 was created by integrating pRH1581 at the *ura3-52* locus in parent strain RHY519. The Hmg2-GFP sterol sensing domain (SSD) point mutations were created by integrating the respective mutant plasmid at the *ura3-52* locus, also in RHY519.

Strains overexpressing Hrd1 were made by transforming the Hmg2-GFP variant containing strains with pRH730. This places *TDH3* driving Hrd1 at *TRP1*. Empty vector containing strains were made by integrating pRH311 at *TRP1*. Strains containing 1myc Hmg2 integrated pRH423 at *HMG2*, and were derived from parent strain RHY468.

Degradation Assays:

Cycloheximide chase assays of Hmg2 as well as the preparation of whole cell lysates were performed as previously described, (Garza R. M., et al 2009).

The log phase cell cultures treated with CHX and ZA were incubated at 30°C for 2h before lysis. Hmg2 was detected with anti-myc 9E10 antibody. Goat-anti-mouse conjugated with horseradish peroxidase recognized primary antibody, and chemiluminescence reagents were used for immunodetection.

Ubiquitination:

Ubiquitination of Hmg2-GFP was assayed as in (Gardner R.G., et al 1998) except no $CuSO_4$ was needed. Cell cultures were grown to log phase and were incubated with 10ug/mL of ZA for 10 min. Cells were lysed, and Hmg2-GFP was immunoprecipitated with rabbit polyclonal anti-GFP antibody. Samples were resolved on 8%SDS-PAGE gels, transferred to nitrocellulose, and were immunoblotted with anti-ubiquitin antibody to detect ubiquitination, and monoclonal anti-GFP to detect Hmg2-GFP.

Flow Cytometry analysis:

Flow cytometry analysis was performed as previously described (Gardener R.G., et al 1998) (Cronin S.R., and Hampton, R. Y., 1999) (Garza R. M., et al 2009). 10,000 cells for each sample were analyzed. Each Hmg2-GFP variant was tested at least 3 times in CHX and regulated degradation assays. Final concentrations of 50ug/mL Cycloheximide, 10ug/mL Zaragozic acid, and 25ug/mL of Lovastatin were added separately to cells at mid-log growth phase, and were incubated at 30°C for 2h. A final concentration of 20ug/mL

Ro48 was added to cells at mid-log growth phase, and incubated at 30°C for 4h.

Fluorescence Microscopy:

Fluorescence microscopy with strains expressing Hmg2-GFP was performed on an epifluorescent Nikon Optiphot-2 microscope, using a 63x oil-immersion objective. Fluorescence was observed in live cells at an excitation wavelength of 450-490nm and long-band pass emission through a Nikon B2-A filter. A Cool Snap cf by Photometrics ccd camera was used and images were captured with metacam software. Minimal processing was done.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Table 2: Descriptions of plasmids and strains

RHY 468 derived strains	1mycHmg2 variants
RHY 623	1myc-Hmg2
RHY 628	RHY468 expressing K6R
RHY 1276	RHY468 expressing KASVI 408-412 AAASV
RHY 1277	RHY468 expressing NLYVF 419-423 SWNVV
RHY 1281	RHY468 expressing SVLP 463-466 ALLQ

RHY 7661 derived strains	1mycHmg2-GFP variants
RHY 7937	empty trp vector 1mycHmg2-GFP
RHY 7938	TDH3-driven 3HA-HRD1 1mycHmg2-GFP

RHY 6110 derived strains	1mycHmg2-GFP variants
RHY 7939	empty trp vector TFYSA 348-352 ILQAS
RHY 7940	TDH3-driven 3HA-HRD1 TFYSA 348-352 ILQAS

New Strains	1mycHmg2-GFP variants
RHY 7941	RHY7683 expressing S215A + empty trp vector
RHY 7948	RHY7944 expressing + TDH3-driven 3HA-HRD1
RHY 7935	RHY7684 expressing K6R + empty trp vector
RHY 7936	RHY7935 expressing K6R + TDH3-driven 3HA-HRD1

Plasmid	Expressed protein
pRH467	Hmg2 +catalytic domain (~aa 523 to the end of HMG2 ORF)
pRH1581	Wt-Hmg2-GFP
pRH1692	TFYSA 348-352 ILQAS-Hmg2-GFP (Nr1-Hmg2-GFP)
pRH671	K6R-Hmg2-GFP
pRH2392	D188A-Hmg2-GFP

Table 2: Descriptions of plasmids and strains, continued.

pRH2341	I191A-Hmg2-GFP
pRH2331	I192A-Hmg2-GFP
pRH2336	T202A-Hmg2-GFP
pRH2182	202-205AAAA-Hmg2-GFP
pRH6744	L203A-Hmg2-GFP
pRH2177	S215A-Hmg2-GFP
pRH2184	S215T-Hmg2-GFP
pRH2334	F217L-Hmg2-GFP
pRH2183	L219F-Hmg2-GFP
pRH2337	L224A-Hmg2-GFP
pRH2340	L231A-Hmg2-GFP
pRH2338	L255A-Hmg2-GFP
pRH2176	P256A-Hmg2-GFP
pRH2333	F257L-Hmg2-GFP
pRH2325	I262A-Hmg2-GFP
pRH2247	I287A-Hmg2-GFP
pRH2329	L325A-Hmg2-GFP
pRH2328	L328A-Hmg2-GFP
pRH2245	C332A-Hmg2-GFP
pRH2330	L339A-Hmg2-GFP
pRH2246	D342A-Hmg2-GFP
pRH2326	L345A-Hmg2-GFP
pRH2180	T348A-Hmg2-GFP
pRH2181	F349L-Hmg2-GFP
pRH2185	FY349LS-Hmg2-GFP
pRH2179	Y350A-Hmg2-GFP
pRH2339	L354A-Hmg2-GFP
pRH2327	E359A-Hmg2-GFP
pRH2332	R365A-Hmg2-GFP
pRH423	1myc-Hmg2
pRH909	KASVI 408-412 AAASV-1myc-Hmg2
pRH910	NLYVF 419-423 SWNVV-1myc-Hmg2
pRH914	SVLP 463-466 ALLQ-1myc-Hmg2
pRH582	K6R1myc-Hmg2
pRH311	empty trp vector
pRH730	TDH3-driven 3HA-HRD1

Chapter 4

Results: The Role of the Hmg2 SSD in FPP-stimulated Degradation

We analyzed mutations in conserved residues of Hmg2 (Figure 7 and 8) to test the role of the SSD in regulated degradation. The experiments described in this chapter address whether the SSD plays a role in sensing FPP-derived degradation signal. Lova and ZA were added to cells to manipulate the levels of FPP (Refer to Ch. 3 materials and methods). We expected that a decreased ability to respond to FPP should make the mutants more stable. To examine this, we used a CHX chase assay to examine the protein stability of the mutants. We found that the SSD does play a role in sensing FPP and promoting Hmg2 regulated degradation.

It was possible that the Hmg2 mutants in this study would exhibit phenotypes similar to mutants that had previously been characterized in the lab. First, Some Hmg2 variants could exhibit wild type responses or have altered responsiveness to FPP (Figure 11). Second, some mutants may be constitutively degraded, showing no regulation by FPP (Figure 11)(Hampton R.Y. et al 1996)(Shearer A.G., and Hampton R.Y., 2005). 6myc-Hmg2, Δ40-Hmg2, and Non-responsive mutant (NR1) are all constitutively degraded variants with mutations in the N-terminus of Hmg2. Notably, the NR1-Hmg2 mutation is in the SSD of Hmg2 (region TFYSA 348-352 ILQAS in the sixth transmembrane domain).

One of the residues within TFYSA is highly conserved area of HMGR, suggesting that there is a role in the SSD in sensing FPP, and that loss of FPP-sensing leads to constitutive degradation. In this study, the following mutations were made in this region: T348L, F349L, FY349LS, and Y350A (Table 1) (Figure 9). We expected that some of the SSD mutants might respond in a similar manner as the TFYSA mutant.

A third phenotype observed in previously characterized mutants of the Hmg2 N-terminus are those that are non-responsiveness to FPP and show complete stabilization. In previous studies, residue K6 was discovered to be necessary for degradation of Hmg2. If K6 is mutated to arginine (R), Hmg2 is not responsive for regulated degradation and is stable (Figure 11)(Gardner R.G., and Hampton R.Y. 1999a). Rather than participate in FPP sensing, it is possible that lysine 6 in Hmg2 is an important site for ubiquitination of the protein. None of the mutants analyzed in the current study mutated lysine residues. Nonetheless, it was possible that specific residues in the SSD would be necessary for regulated degradation, and give rise to stable proteins.

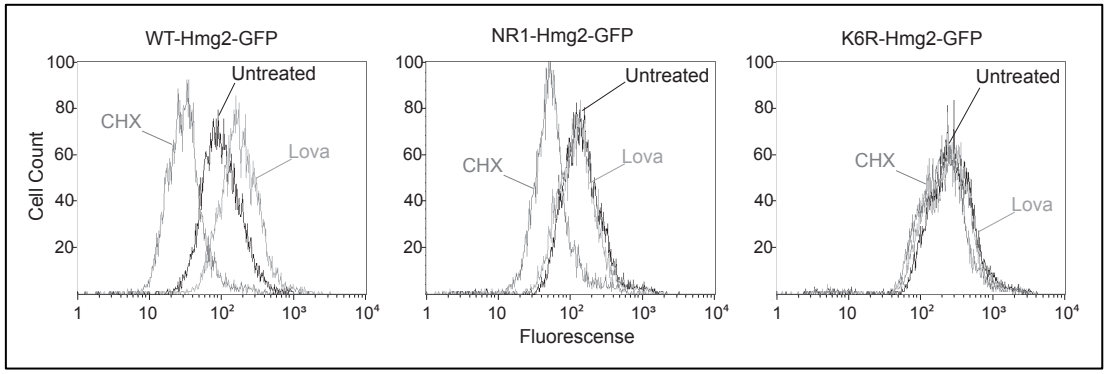

Figure 11: Distinct phenotypes among previously characterized Hmg2 mutants. Strains expressing WT-Hmg2-GFP, NR1-Hmg2-GFP, and K6R-Hmg2-GFP were grown to log phase at 30°C and incubated for 2 hours with no drug, 25ug/mL lovastatin (*Lova*), and 50ug/mL cycloheximide (*CHX*). Hmg2-GFP fluorescence was analyzed by flow cytometry.

Many SSD mutants respond to FPP like wild type Hmg2

To investigate if these SSD residues were important for responding to FPP molecule, we monitored the protein levels in cell cultures treated with Lova to decrease FPP, or ZA to increase FPP. Upon addition of Lova, 17 mutants upregulated the levels of Hmg2 when FPP flux was low, similar to wildtype (Figure 12a represented by examples L224A, L225A, and I287A). Upon addition of ZA, the increased amount of FPP efficiently drove the degradation of these same variants (data not shown). Therefore 17 out of 30 Hmg2 mutants were able to respond to FPP just as well as wild type Hmg2. These mutants also had normal protein stability by CHX chase assay (Figure 12a). This finding indicates that the majority of the conserved SSD residues tested are not critical for FPP sensing. It is possible that these conserved residues are important for other functions, such as sensing other lipids or for FPP-independent functions (see Chapter 5). These 17 mutations are mostly hydrophobic and distributed throughout the SSD (Figure 12b).

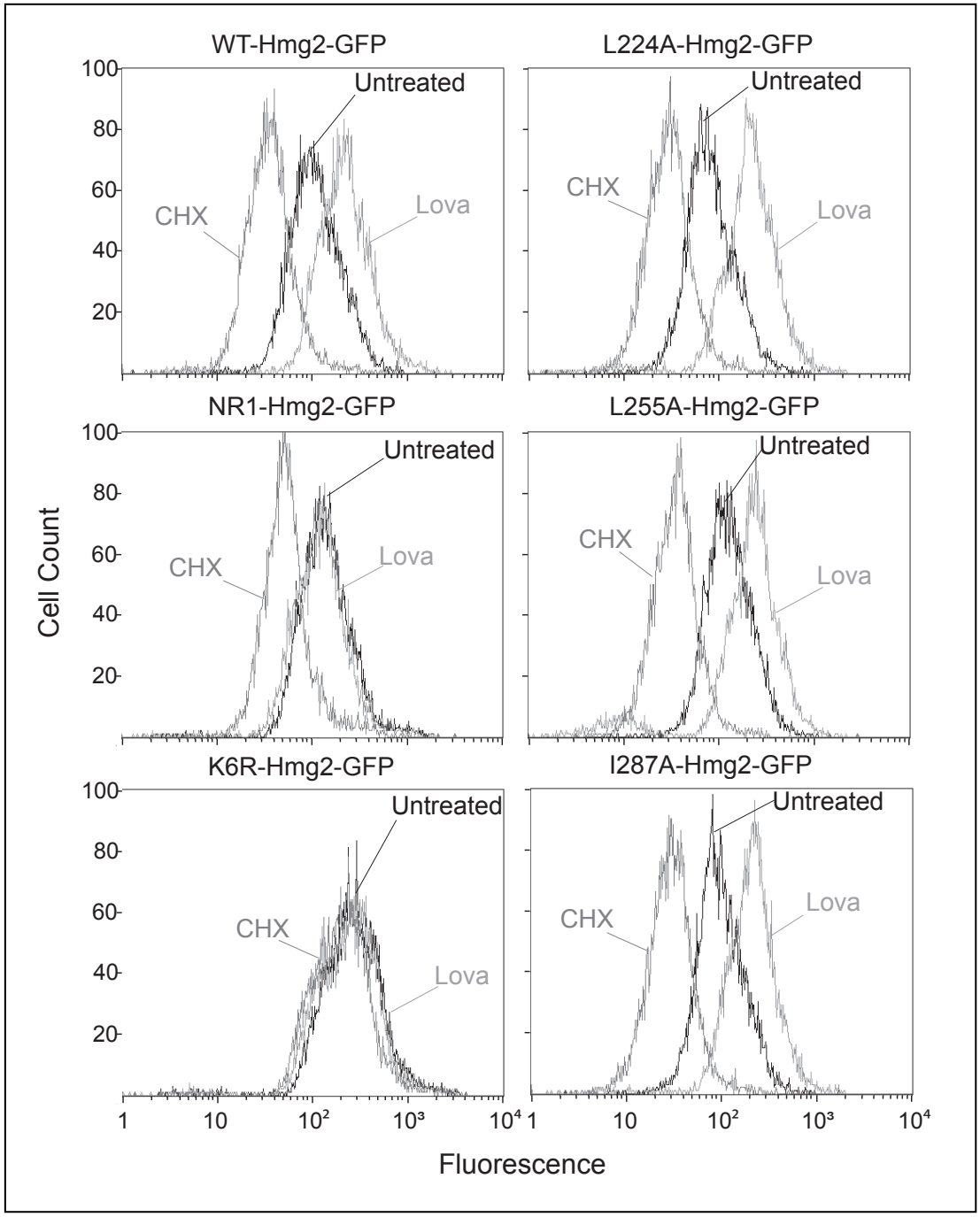

Figure 12: SSD mutants that respond like WT-Hmg2-GFP. a) Strains expressing WT, the controls Nr1 and K6R, and mutant Hmg2-GFP were grown to log-phase at 30°C and incubated for 2 hours with no drug, 25ug/mL Lova, and 50ug/mL CHX. Examples of Hmg2-GFP variants that respond like WT are L224A, L255A, and I287A.

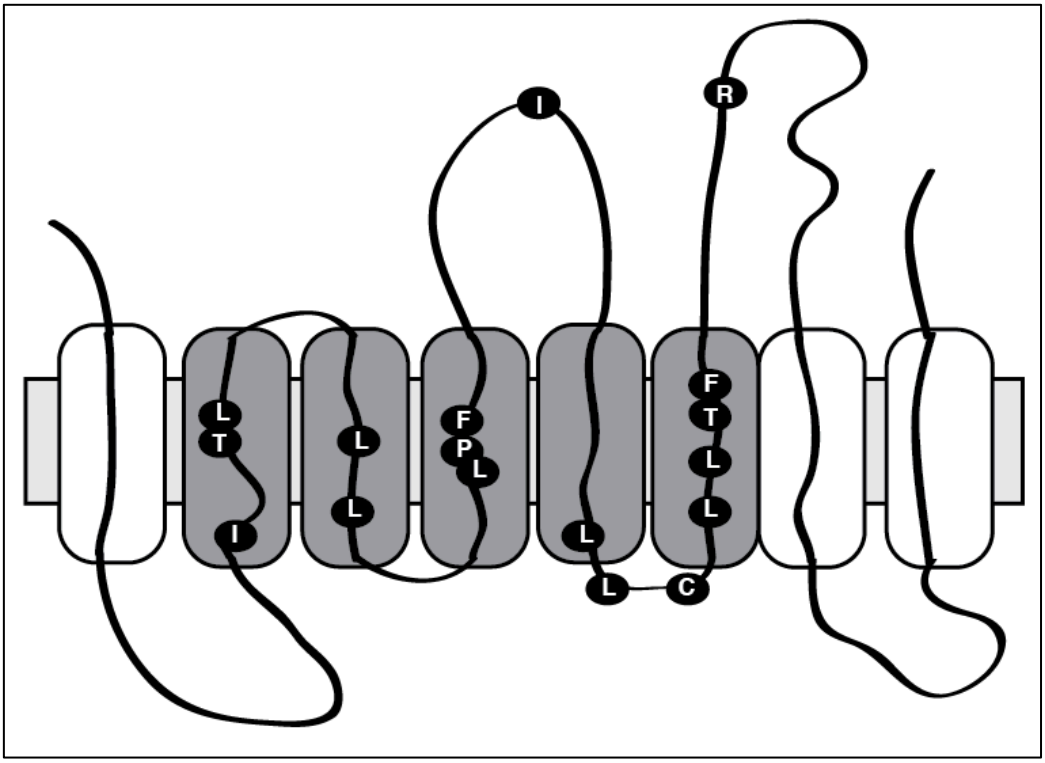

Figure 12: SSD mutants that respond like WT-Hmg2-GFP, continued. **b)** Location of mutants that respond to FPP like wild type. The SSD is represented as the 5 dark grey transmembrane domains. The mutations and residue numbers of wild type like Hmg2 variants are listed below.

Wildtype Response to FPP
I191A
T202A
L203A
L224A
L231A
L255A
P256A
F257L
I287A
L325A
L328A
C332A
L339A
L345A
T348L
F349L
R365A

Hmg2 SSD mutants that partially respond to FPP molecule

Seven mutants have showed decreased responses to FPP, and were accordingly stabilized. Some of these mutants were not as responsive to Lova. I192A, TLCC 202-205 AAAA, and L354A are the examples of these mutants shown in Figure 13. These Hmg2-GFP variants were not significantly upregulated upon treatment with Lova (Figure 13b). A decrease in FPP-derived molecule flux does not up regulate Hmg2 levels as highly as wild type Hmg2, which indicates that there is less ability to regulate Hmg2. To further understand the regulation occurring in these mutants, treatment with ZA was examined to amplify FPP derived molecule to stimulate degradation. These Hmg2 variants were able to respond to FPP when ZA was present (Figure 13b).

We predicted that mutants stunted in response to FPP would be more stable. It appears that these mutants have a higher steady state level then wild type Hmg2 (Refer to Untreated, Figure 13a), indicating that these mutants are slightly stabilized. We believe that the increased stability of these mutants explains why they show a blunted response to Lova. This blunted response to Lova is reflected in increased stability observed in higher steady-state levels of the protein. In these cases, the CHX chase assay was not sensitive enough to detect the slight change in degradation rate (Figure 13a).

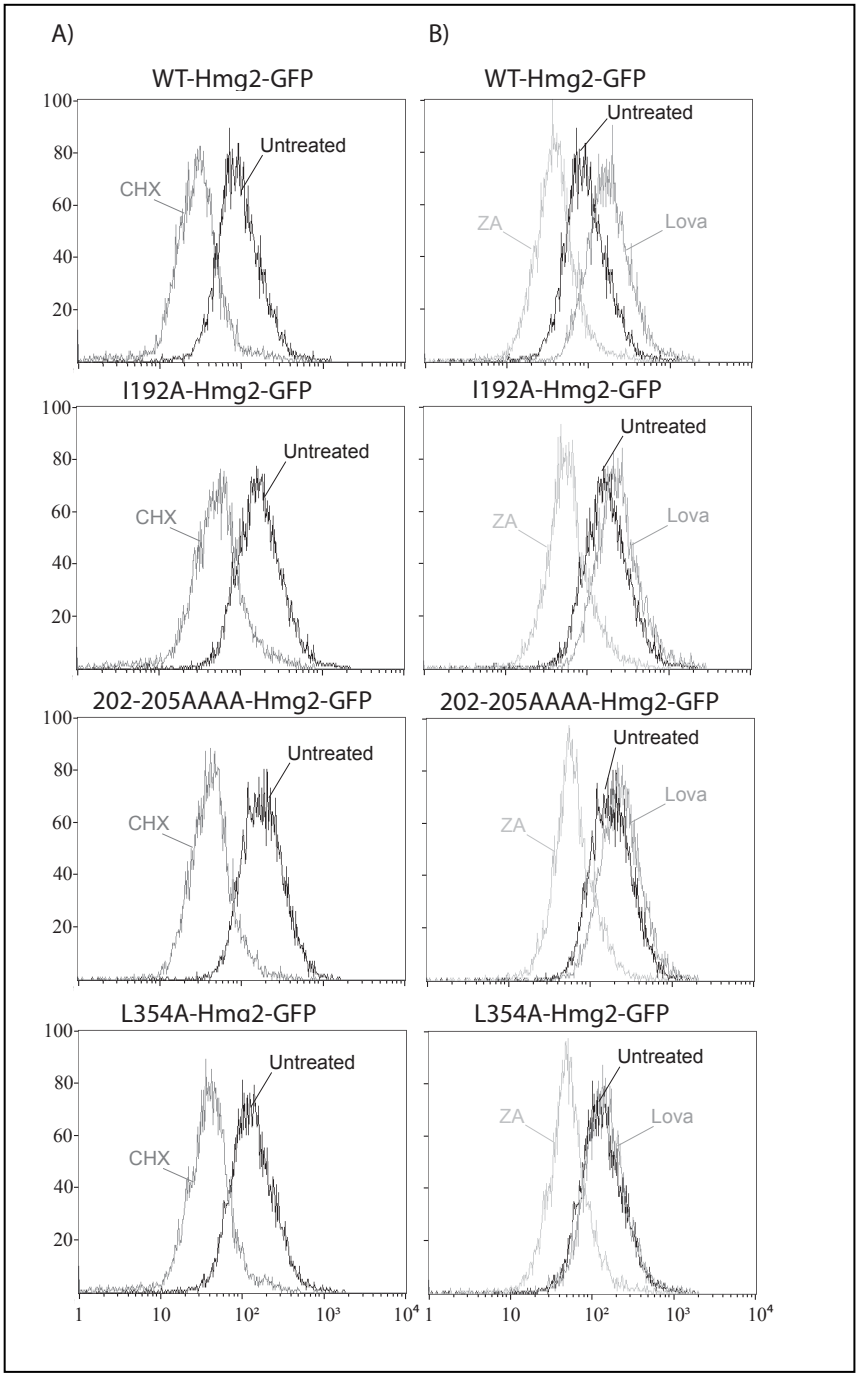

Figure 13: SSD mutants that are partially responsive to FPP. **a)** CHX chase of WT-Hmg2 and mutant Hmg2-GFP strains. Strains were grown to log phase at 30°C and incubated for 2 hours with no drug and 50ug/mL CHX. **b)** Analysis of regulated degradation response to FPP derived signal. WT-Hmg2 and mutant Hmg2-GFP strains were grown to log phase at 30°C and incubated for 2 hours with no drug, 25ug/mL Lova, and 10ug/mL ZA.

However, if FPP is sufficiently increased with ZA treatment, these mutants can be pushed to regulated degradation. Therefore, these mutants partially respond to FPP, and as predicted they were slightly stable.

In summary, there were 7 SSD Hmg2 variants that partially respond to FPP-derived molecule for regulated degradation, and have increased protein stability. These mutants are in SSD transmembrane spans 2,3,4, and 6, and are interspersed in the same regions as those that gave rise to mutants with wild type phenotypes (Figure 13c). These mutants have lost the ability to respond to FPP and simultaneously have become more stable. The results suggest that these residues normally contribute to FPP-sensing and destabilization of the protein. An important avenue for future studies will be to determine if these are in fact recessive loss of function mutations, as we believe them to be.

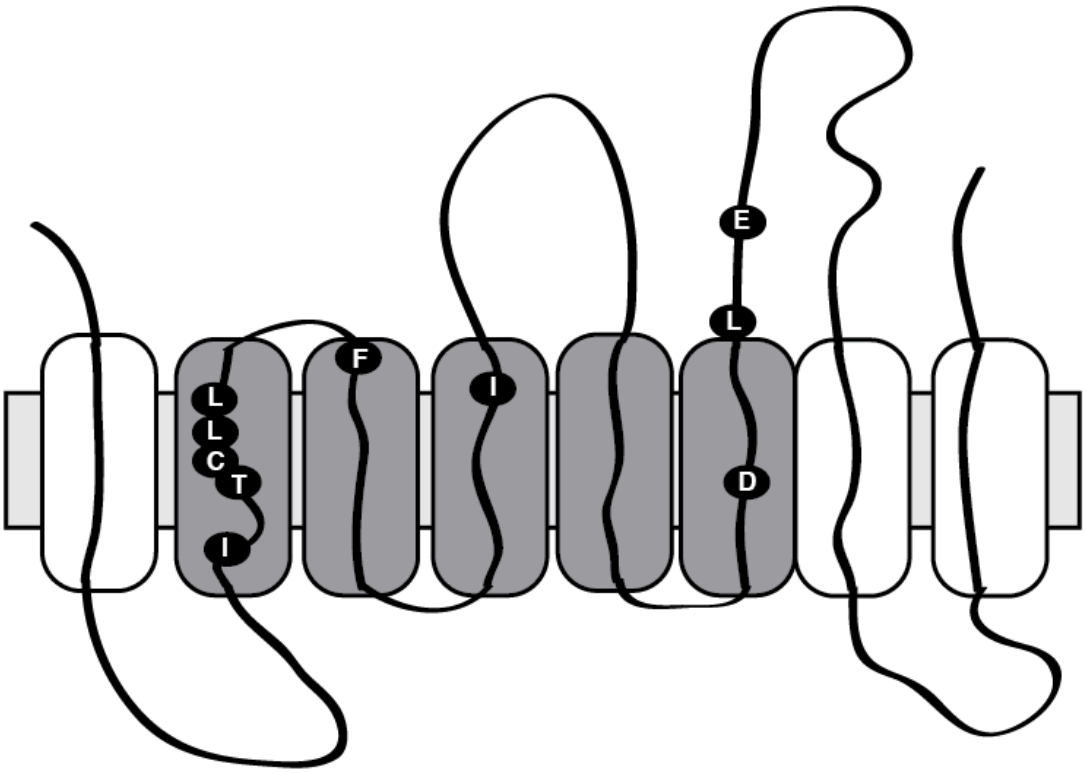

Figure 13: SSD mutants that are partially responsive to FPP, continued. **c)** Location of SSD Hmg2 variants that partially respond to FPP. The SSD is represented as the 5 dark grey transmembrane domains. The mutations and residue numbers of Hmg2 that partially respond to FPP are listed in the following table.

Partial Response to FPP
I192A
TLCC 202-205 AAAA
F217L
I262A
D342A
L354A
E359A

Hmg2 SSD mutants that weakly respond to FPP or do not respond to FPP molecule

Three of the SSD mutants had an even weaker response to FPP than the previous mutants. These mutants are D188A, S215T, and Y350A. To study the regulation, treatments with Lova and ZA were done as previously described. With treatment of Lova, these mutants did not up regulate Hmg2-GFP levels (Figure 14b). S215T and Y350A are shown as examples in Figure 14. When the strains were treated with ZA, these Hmg2 variants weakly responded to FPP. They underwent regulated degradation when exposed to FPP, but at a much slower rate when compared to wild type Hmg2 (Figure 16b).

We expected these mutants to be more stable than wild type. To assess the stability of Hmg2, treatment with CHX resulted with these three partially stable Hmg2 variants (Figure 14a). These mutants are degraded approximately half as fast as wildtype Hmg2, however these variants are not as stable as K6R (Figure 14). These mutants lose the ability to respond to FPP to a greater extent than the other mutants examined thus far. Since these mutants show weaker responses to FPP, these residues may be more important for FPP-sensing.

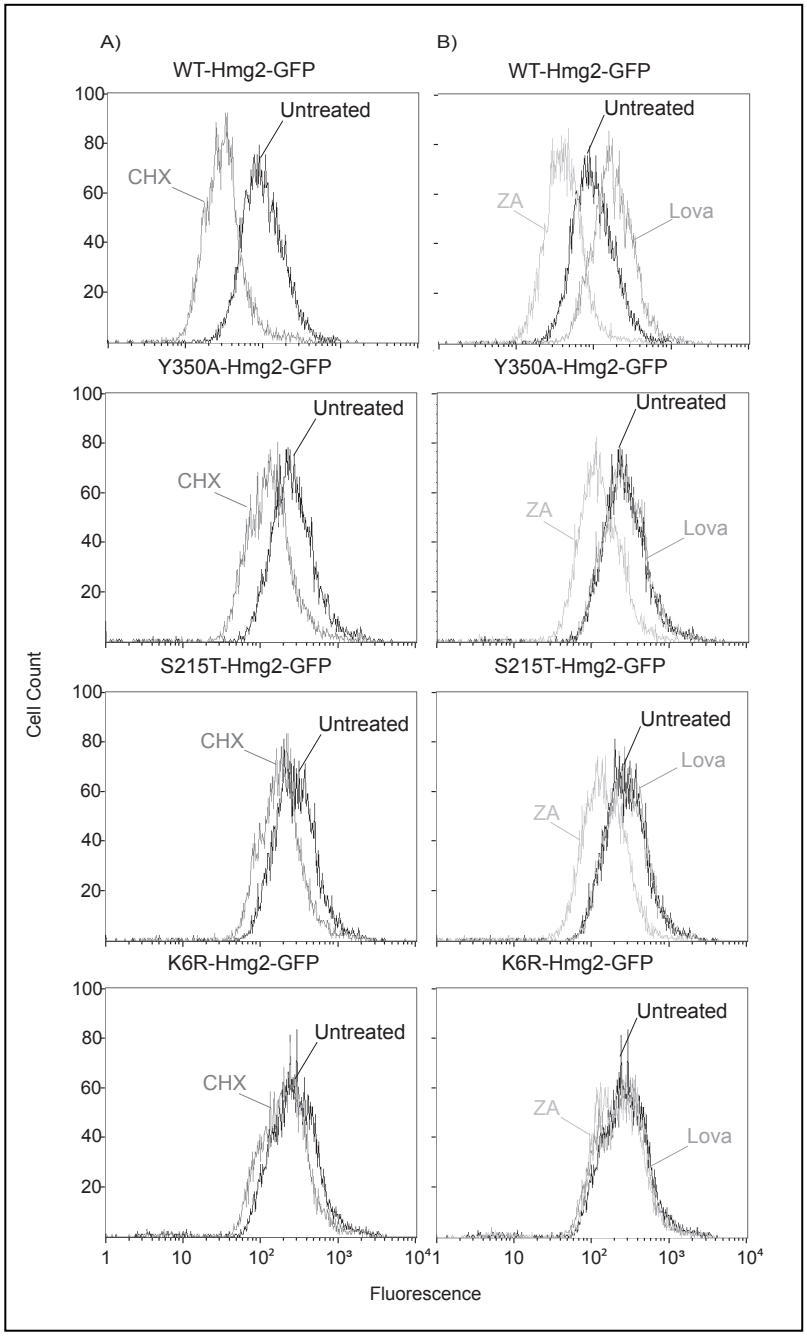

Figure 14: Hmg2 SSD variants that are weak responders to FPP.
a) CHX chase of WT-Hmg2, K6R-Hmg2, and mutant Hmg2-GFP strains. Strains were grown to log phase at 30°C and incubated for 2 hours with no drug and 50ug/mL CHX. **b)** Analysis of regulated degradation response to FPP. WT-Hmg2, K6R-Hmg2, and mutant Hmg2-GFP strains were grown to log phase at 30°C and incubated for 2 hours with no drug, 25ug/mL Lova, and 10ug/mL ZA.

These weakly responding mutants are located in Hmg2 next to mutants with very strong phenotypes (Figure 15b). There were 3 SSD mutants that demonstrated strong non-responsive phenotypes: S215A, L219F, and FY349LS. Treatment of mutants with Lova or ZA showed no responses to FPP (Figure 15a, Lova)(data not shown, ZA). Upon treatment with CHX, these Hmg2-GFP variants were stable, like K6R (the possible non-ubiquitinated mutant)(Figure 15a CHX, compare to K6R in Figure 11). As predicted, these variants were stable because they were not undergoing regulation by FPP.

Thus, these residues in the SSD are necessary for Hmg2 ERAD. These mutants, along with the mutants that weakly responded to FPP, lie in two specific regions of the SSD of Hmg2, and therefore define two critical regions for sensing FPP (Figure 15b).

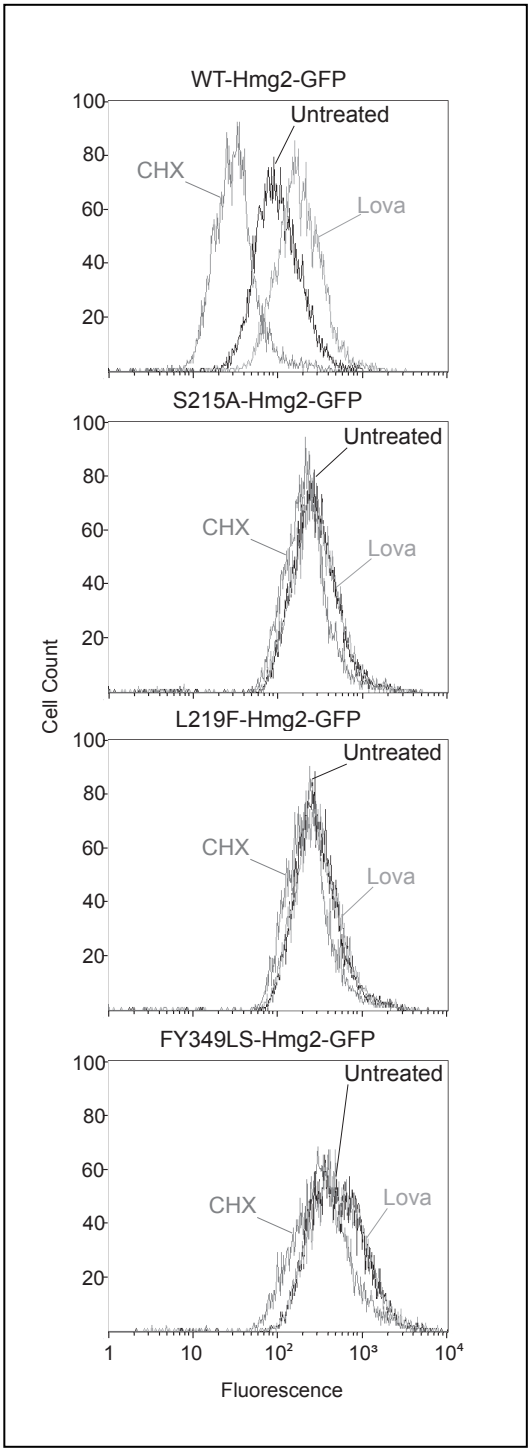

Figure 15: SSD mutants that are stable and not regulated.
a) Strains expressing WT-Hmg2-GFP and mutant Hmg2-GFP were grown to log-phase at 30°C and incubated for 2 hours with no drug, 25ug/mL Lova, and 50ug/mL CHX.

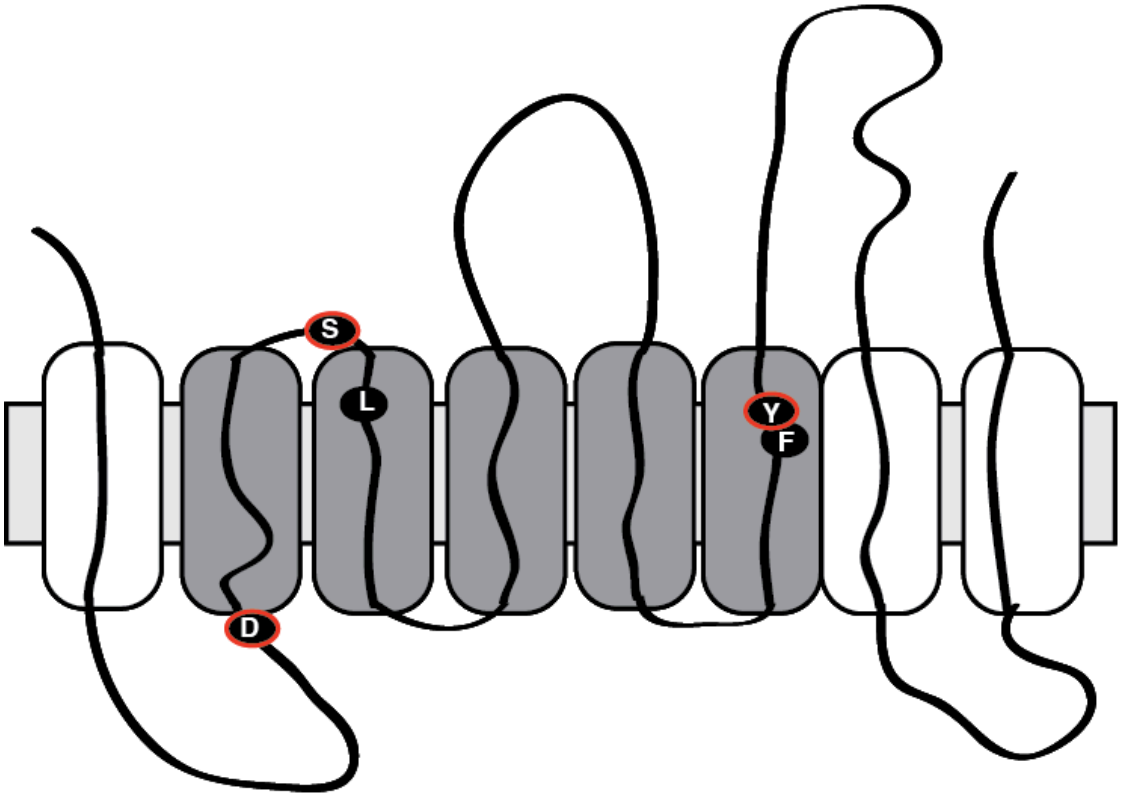

Figure 15: SSD mutants that are stable and not regulated, continued. **b)** Locations of SSD Hmg2 variants that weakly respond and do not respond to FPP. The SSD is represented as the 5 dark grey transmembrane domains. The bordered red residues are mutants that weakly respond to FPP. The solid black residues are the non-responsive mutants. Note that S215T and Y350 weakly respond to FPP, and S215A and FY349LS do not respond to FPP. The mutations and residue numbers of Hmg2 weak responders to FPP (left) and non-responders to FPP (right) are listed in the following tables.

Weak Response to FPP	No Response to FPP
D188A	S215A
S215T	L219F
Y350A	FY349LS

S215A was further examined to investigate why these stable and unregulated mutants showed such phenotypes. These unregulated mutants could have been stable because there was a change in distribution of Hmg2 throughout the cell. The change in localization could prevent these Hmg2 mutants from interacting with the HRD machinery necessary for degradation. The localization was examined optically through fluorescence microscopy (Figure 16a). WT-Hmg2-GFP was dim and the fluorescence was located in the perinuclear and cortex region of the cell where the ER is located in yeast. S215T-Hmg2-GFP was slightly brighter than WT-Hmg2-GFP, and S215A Hmg2-GFP was just about as bright as K6R Hmg2-GFP. All of these Hmg2 variants showed localization of Hmg2 at the perinuclear and cortex regions of the cell. Therefore, the localization of Hmg2 in these mutants was not the reason why they showed stable phenotypes.

Under native levels of Hrd1 expression, these non-responsive mutants were not being degraded. It was possible that these mutants were stable because they weren't being ubiquitinated to undergo degradation, so the level of ubiquitination of S215A-Hmg2-GFP was assessed. Strains were grown to log phase and treated with ZA for 10min to initiate ubiquitination. Results indicated that under native levels of Hrd 1, there is no ubiquitination of S215A similar to K6R (observed through experimentation).

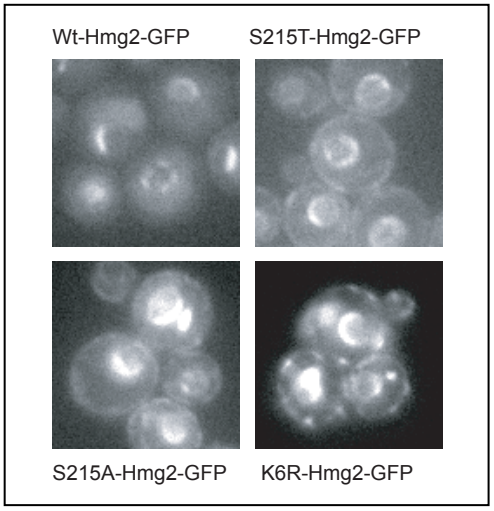

Figure 16a: Localization of S215A-Hmg2-GFP. Fluorescence microscopy of live, log-phase cell cultures of Wt-Hmg2-GFP, S215A, S215T, and K6R-Hmg2-GFP mutants.

It was possible that these mutants were stable and not ubiquitinated because they were not interacting with the HRD machinery. Hrd1 is the rate-limiting enzyme that tags Hmg2 substrate with ubiquitin for degradation. Thus Hrd1 levels were manipulated to examine the abilities of these mutants to be recognized for degradation (Hampton R.Y. 2002). Strains were constructed in the stable mutants so that the *TDH3* promoter overexpressed Hrd1. If the stable mutants were able to interact with Hrd1, then driving overexpression of Hrd1 would push these mutants through ERAD. Overexpression of Hrd1 resulted in degradation of S215A, L219F, and FY349LS (Figure 16b, S215A shown). It is notable that K6R does not undergo degradation even when Hrd1 was overexpressed because the lysine is thought to be necessary for ubiquitination of Hmg2. Therefore the HRD machinery can recognize these mutants.

Since the stable mutants interact with the HRD machinery, it was relevant to test if these mutants can sense FPP when Hrd1 is overexpressed. The results show that S215A-Hmg2-GFP is not regulated even when Hrd1 is overexpressed to drive its degradation (Figure 16c). These results were also observed in L219F and FY349LS (data not shown). Therefore these stable mutants are not capable of being regulated by FPP.

We considered that the serine residue of S215 was important for regulation. We thought that substituting serine with a polar conserved

threonine would be able to recover the function of that residue. However, we found that if a residue were mutated with a conservative replacement, there would be a loss of ability to respond to FPP. S215T weakly responded to FPP, and S215A did not respond to FPP, which indicated that the serine residue was specific for regulation of Hmg2 (Figure 16d). Therefore, conserving the charge of a residue did not recover the functional relevance of the residue, which indicated the specificity of these residues in the Hmg2 SSD.

Overall, the basis for stabilization of S215A, L219F, and FY349LS is because they do not sense FPP. Since they are not regulated they are not tagged with ubiquitin for degradation. They are stable, and only interact with Hrd1 when there is plenty of Hrd1 present in the cell. These residues cannot be replaced by conservative substitution and are highly specific for regulated degradation of Hmg2.

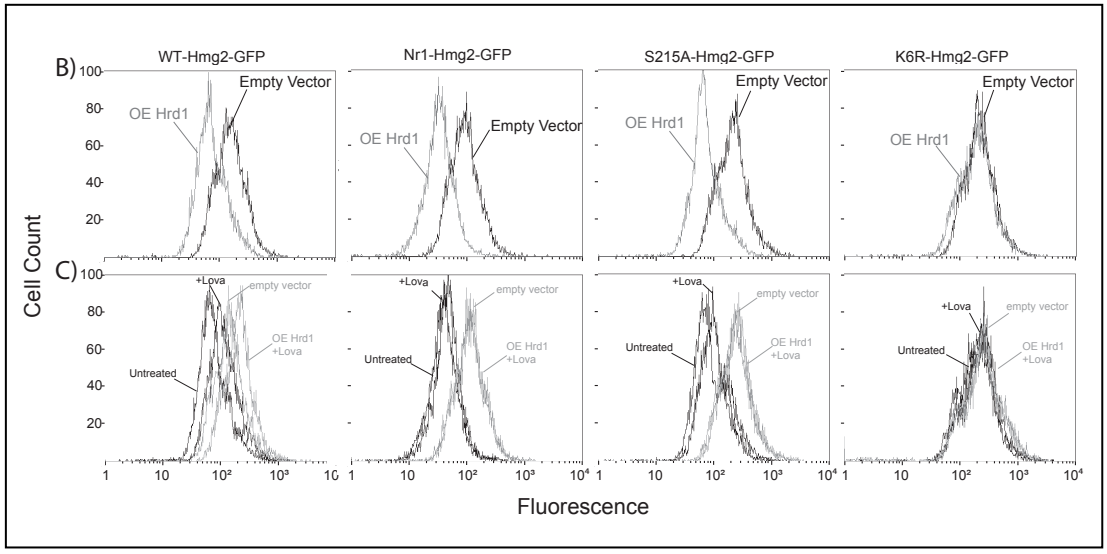

Figure 16b & 16c: Stable SSD mutants are not excluded from ERAD by the HRD pathway. **b)** Over-expressed HRD 1 Hmg2-GFP strains. Cell cultures were grown to log phase, and steady states of Hmg2-GFP was examined by fluorescence flow cytometry. **c)** Regulation of Hmg2-GFP compared to regulation of Hmg2-GFP with over-expressed HRD 1. *In black*: Log phase cell cultures of Wt-Hmg2-GFP and mutant strains were treated with 25ug/mL Lova for 2h, and steady state levels were measured with flow cytometry. *In grey*: Over-expressed Hrd1 Wt-Hmg2-GFP and mutant strains were grown to log phase and were treated with 25ug/mL Lova for 2h.

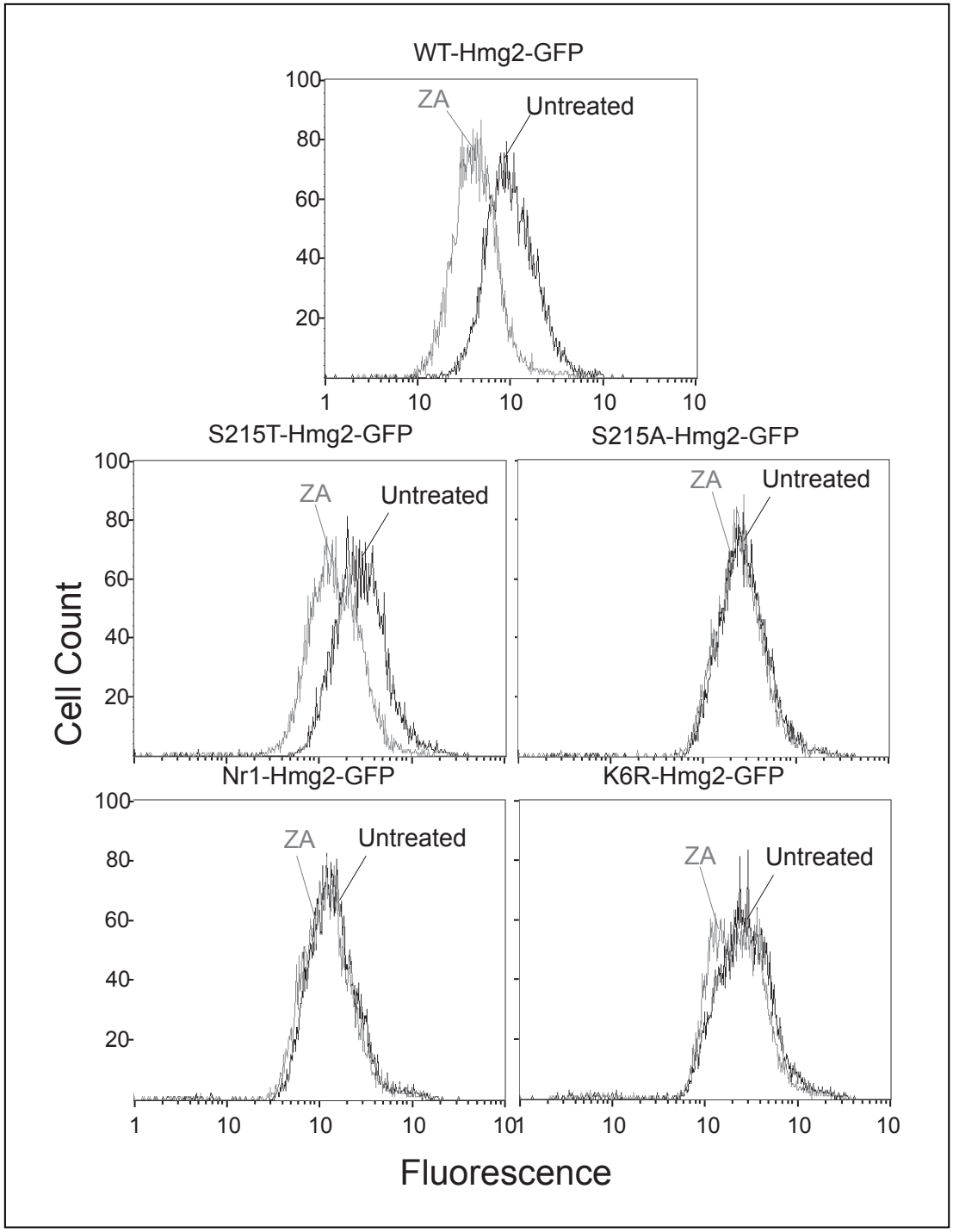

Figure 16d: S215 residue is necessary for sensing FPP derived signal. Steady state levels of Wt-Hmg2-GFP, Nr1 and K6R Hmg2-GFP, and Hmg2-GFP variants were examined. Log phase cell cultures were treated for 2h with 10ug/mL ZA.

Conclusion:

We initiated this study to understand if conserved residues in the SSD are required to respond to FPP, and as a result, we found this to be true. 13 out of 30 mutants responded less well or did not respond to FPP, and were accordingly stabilized. The less able the mutant could respond to FPP, the more stable the mutant was.

Specific conserved residues seemed to be important for sensing FPP. Interestingly, mutations in the most highly conserved residues displayed the strongest phenotypes. Residues S215, L219, and F349 are fully conserved, and D188, and Y350 are chemically conserved between organisms in HMGR and SCAP (Figure 7). Mutations D188A, S215T, S215A, L219F, Y350A, and FY349LS showed strong phenotypes of regulation. These 6 out of 13 interesting mutants were dramatically stunted for sensing FPP and more stable.

These 6 stabilized mutants defined two critical regions for sensing FPP. One of these regions is at the beginning of the SSD (including D188A, S215T, S215A, and L219F), and the other region is towards the end of the SSD (including FY349LS, and Y350A) (Figure 15b). The residues in these SSD regions contain chemically functional side groups that could be important for sensing FPP. For instance, serine and tyrosine residues contain polar side groups that are potential phosphorylation sites, which could be an important

response to senssing FPP. Upon mutation, when the functional group is removed from the residue, such as when serine is removed by an alanine replacement, there is a loss of ability to sense FPP. The polar group of serine is specific, because mutation into conserved polar threonine results with a diminished ability to sense FPP (As shown with S215T and S215A). Therefore, the functional group on a residue is specific, and may be responsible for sensing FPP directly, or modified indirectly in an FPP-dependent manner.

Out of the 13 mutants that demonstrated importance for sensing FPP, there were 7 mutants that partially responded to FPP. These residues also formed critical regions for sensing FPP. Like the six residues that displayed strong phenotypes, mutations in these 7 residues lay in areas towards the beginning and end of the SSD (Figure 13c). Most of these partially responsive mutants are hydrophobic residues. They may be responsible for forming a hydrophobic pocket to directly bind FPP. If this is true, then combining mutations in a single protein may generate a mutant that is more strongly resistant to FPP. Alternatively, these hydrophobic residues could play a role in sensing the lipid composition of the ER membrane. It appears that residues in the SSD (whether containing chemically functional side groups, such as serine, or containing hydrophobic groups) are important for sensing FPP, but may be important for different aspects of the FPP-sensing function.

Mutations in the two critical regions of the SSD weaken the ability of Hmg2 to respond to FPP, and thus make Hmg2 stable. The stability of these Hmg2 variants was further investigated in the 3 mutants that were extremely stable and unregulated. Specifically, S215A was not ubiquitinated or regulated similarly to K6R. Since these mutants are very stable, they may not be capable of undergoing the structural transition to a less-well folded state upon Hmg2 regulation. It would be relevant to test this structural transition with these Hmg2 mutants through an *in vitro* trypsinolysis assay to observe whether the SSD plays a role in the destabilization of Hmg2. It could be that when SSD residues sense FPP, the regulatory signal destabilizes Hmg2 into a less well-folded state that is recognizable for entry into ERAD (Figure 5). Nevertheless, these findings indicate that the SSD must play a role in sensing FPP, as well as stabilizing Hmg2.

Among the mutants in this study, we did not observe any that were constitutively degraded. Nr1-Hmg2 mutant (TFYSA) is a regional mutation in the SSD that is constitutively degraded and unresponsive to FPP. However, when particular residues in TFYSA were mutated, distinct phenotypes were observed. FY349LS and Y350A are quite unresponsive to FPP and stable, indicating that this area is necessary to sense FPP. The TFYSA residues must be important for regulating Hmg2, to the extent that mutating just one residue in this region dramatically altered regulated degradation of Hmg2. Since one

mutation in this region impaired Hmg2 regulation, it's not surprising that 5 mutations in this region altered the protein to be constitutively recognized as a quality control substrate. Therefore, it seems that the SSD residues of Hmg2 synergistically influence the regulation of Hmg2. The TFYSA region by example contains hydrophobic and polar residues that possibly play a role for sensing FPP. Whether the polar residues are modified upon sensing FPP, or the hydrophobic residues respond to altered lipid composition in the membrane, these different types of residues could synergistically influence Hmg2 regulated degradation. It would be relevant to test other mutated regions observed from previous studies that responded like TFYSA, to see which specific residues in the region influence Hmg2 regulation (Gardner, R.G., 2000 PhD Thesis).

The SSD residues that were important for sensing FPP had specific characteristics. The most highly conserved residues, and residues that contained chemically functional groups could provide distinct ways to respond to FPP. Although there were 13 mutations that showed relevance for sensing FPP, there were 17 out of 30 mutations that responded like wildtype, and were not necessary for sensing FPP. These mutations might play a different role in Hmg2 regulated degradation, such as sensing a different lipid molecule that is a positive regulator for Hmg2 ERAD.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Chapter 5

Results: The Role of the Hmg2 SSD in Oxysterol Stimulated Degradation

There are many types of lipids in the cell that provide an array of cellular functions. A type of lipid necessary for cell functions, aside from sterols and isoprene molecules, are oxysterols. Oxysterol molecules partake in apoptosis, protein prenylation, platlet aggregation, and sphingolipid metabolism (Schroepfer G.J., 2000). However, over-accumulation of oxysterol products can be toxic, and contribute to developing stages of atherosclerosis and cardiovascular disease. It is thus important to regulate the levels of oxysterol in the cell. Oxysterols are synthesized downstream of isoprenes in the mevalonate pathway (Figure 1), and these molecules aid in the regulation of cholesterol levels in the cell. Oxysterol molecules have been shown to be positive regulators for mammalian and yeast HMGR ERAD (Espenshade P.J., and Hughes A.L., 2007)(Gardner R.G. et al 2001). Oxysterols are another set of molecules, aside from FPP, that enhances Hmg2 degradation; however, FPP enhances degradation to a greater extent than oxysterols (Gardner R.G. et al 2001). When oxysterol levels are high, Hmg2 undergoes degradation to reduce these accumulating molecules. Since the SSD plays a role in sensing FPP, it is possible that the SSD is also involved in sensing oxysterols.

We tested the ability of the Hmg2-GFP SSD variants to sense oxysterol molecules. Oxysterol molecules were increased in the cell by adding Ro48-

8071 (*Ro48)* to log phase cells *in vivo*. Ro48 is a drug that inhibits oxidosqualene-lanosterol cyclase (OLC) in the mevalonate pathway (Figure 1). Studies have shown that partial inhibition of OLC with Ro48 causes buildup of dioxidosqualene, which is a better substrate for the normally used oxidosqualene, thus causing production of oxysterols upon cyclization of this alternate substrate (Gardner R.G. et al 2001). To test the role of the SSD in response to oxysterols, Ro48 was added to strains expressing the various Hmg2-GFP mutants, and the degradation was examined by flow cytometry.

We found that the response to oxysterol generally mirrored the response to FPP for most of the Hmg2-GFP variants (Figure 17). For example, the Hmg2 variants that responded to FPP like wildtype, responded to oxysterol equally well (Figure 17, see L224A). Mutants that responded partially to FPP molecule had little to no response to oxysterol (Figure 17, see TLCC 202-205 AAAA and D342A). Mutants that responded weakly to FPP molecule made relatively no responses to oxysterol (Figure 17, see S215T). Mutants that did not respond to FPP molecule did not respond to oxysterol (Figure 17, see S215A). These results support the idea that the SSD is involved in sensing lipid molecules to mediate Hmg2 regulated degradation, and suggests that FPP and oxysterol are sensed by a common set of features in the SSD.

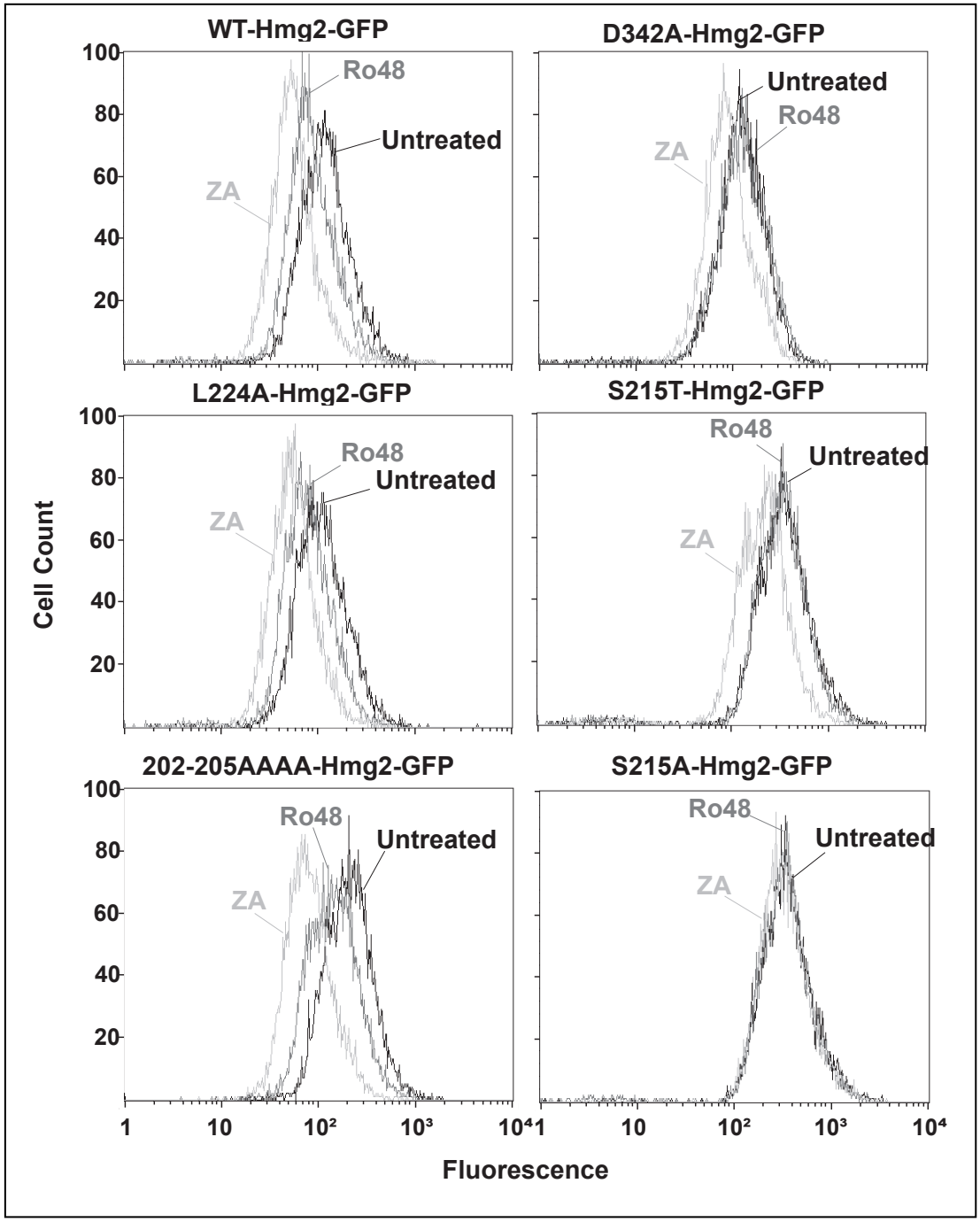

Figure 17: SSD Hmg2 variant oxysterol response mirrors FPP response. Strains expressing WT-Hmg2-GFP and mutant Hmg2-GFP were grown to log-phase at 30°C and incubated for 4 hours with no drug, 20ug/mL Ro48-8071 (*Ro48*), and 10ug/mL ZA. Not that 202-205AAAA is abbreviated for the variant TLCC 202-205 AAAA.

However, not all of the mutants in this study demonstrated mirrored responses to FPP and oxysterol molecule. Six mutants responded distinctly. Mutants I191A, L231A, P256A, F257L, L328A, and L345A could respond to FPP (by ZA treatment), but not to oxysterol (by R048 treatment)(Figure 18a). In the presence of ZA, these mutants responded to FPP just as well as wildtype, resulting in rapid Hmg2 degradation. However, they did not respond to oxysterol molecule upon addition of Ro48 (Figure 18a).

Aside from the observed independent oxysterol and FPP response, when treated with CHX, these mutants were degraded at similar rates to wildtype (data not shown). Generally, the steady-state levels of these mutant proteins are higher than wild type (Figure 18a, see untreated). When Lova was added, these Hmg2 variants slightly upregulated Hmg2 levels (data not shown). The blunted response to Lova was reflected in the increased stability observed in higher steady-state levels of the protein. These mutants were therefore more stable than wild type.

In summary, it is evident that these mutants respond well to FPP molecule, but do not respond to oxysterol molecule. These mutants are located throughout the SSD and do not cluster in the hot spots defined for FPP-sensing (Figure 18b).

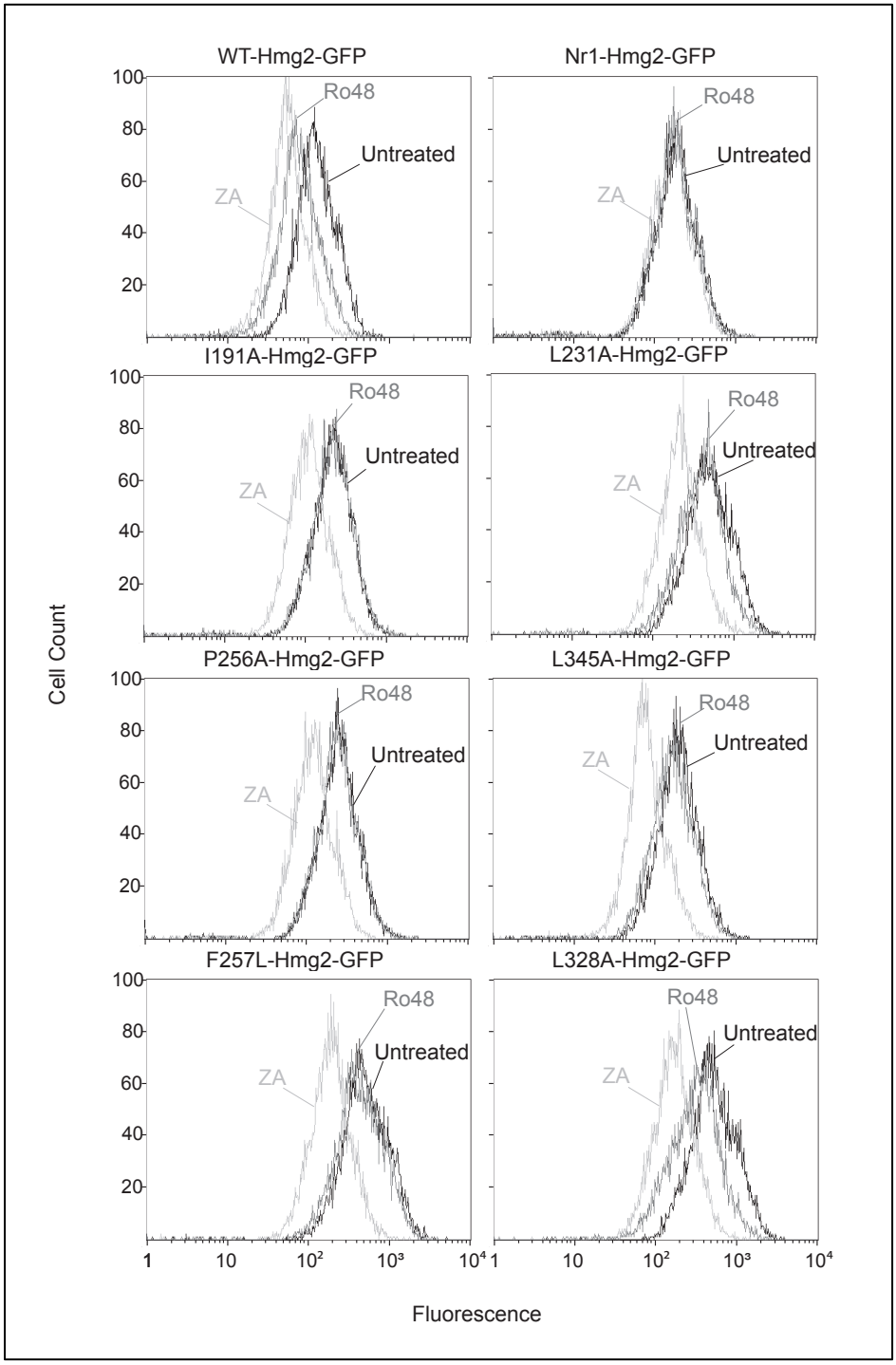

Figure 18: SSD mutants that respond to FPP but not to oxysterol molecule. a) Strains expressing WT and mutant Hmg2-GFP were grown to log-phase at 30°C and incubated for 4 hours with no drug, 20ug/mL Ro48, and 10ug/mL ZA.

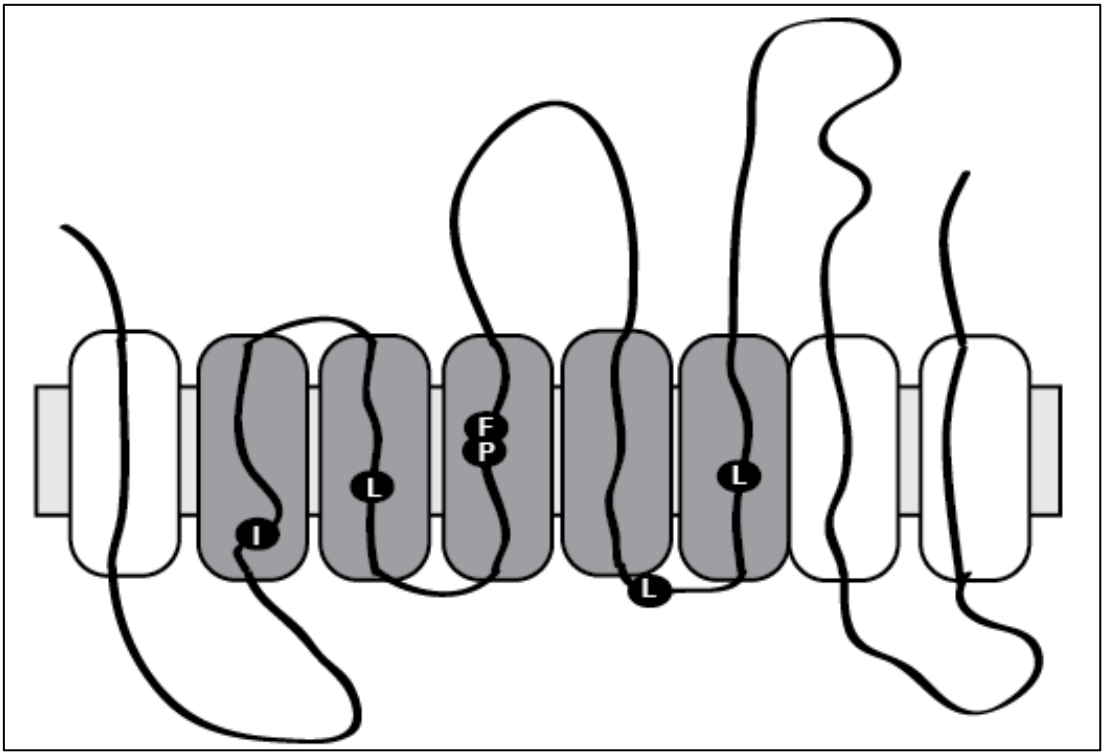

Figure 18: SSD mutants that respond to FPP but not to oxysterol molecule, continued. b) Location of SSD Hmg2 variants that respond only to FPP. The SSD is represented as the 5 dark grey transmembrane domains. The mutations and residue numbers of Hmg2 are listed in the following table.

Independent FPP Oxysterol Response
I191A
L231A
P256A
F257L
L328A
L345A

Conclusion:

The regulation of Hmg2 was tested to see if the SSD plays a role in responding to oxysterol molecule. We found that residues in the SSD are involved in responding to oxysterols, and that some residues appear to be specific for oxysterol response.

There was an overlap in the ability of the SSD to sense oxysterol and FPP molecules, because Hmg2 mutants showed parallel responses to both oxysterol and FPP. The 13 mutants that showed weak or no response to FPP also showed weak or no responses to oxysterol. Therefore, these residues in the SSD play a role in sensing both isoprenes and oxysterols. Recall the two critical regions observed towards the beginning and end of the SSD involved in FPP sensing. These regions must correlate sensing FPP and oxysterol to regulate Hmg2, and are not limited to sensing FPP alone. The different functional groups of residues in these regions, whether chemically active or hydrophobic, must contribute to sensing oxysterol through similar mechanisms. These residues might not be limited to sensing FPP and oxysterols, and could potentially sense other molecules such as sterols.

If Hmg2 uses an overlapping mechanism to sense FPP and oxysterol, then these two molecules could potentially regulate Hmg2 synergistically (Gardner R.G., et al 2001). In our previous work, we observed that oxysterols enhance Hmg2 degradation when FPP is present in the cell. The FPP-derived

molecule was thought to be a primary signal, and oxysterol aided in promoting degradation, especially when there were limited amounts of FPP present in the cell. Based on our results in the present study, we suggest that the molecules are sensed through similar mechanisms, and may influence each other to promote degradation.

Although most mutants showed parallel responses to FPP and oxysterol, there were 6 mutants that responded to FPP, but did not respond to oxysterols. Those findings indicate that there are additional determinants of the SSD employed to sense oxysterols. These 6 residues are hydrophobic, and are distributed across the SSD of Hmg2 (Figure 18b). These residues may constitute a hydrophobic binding pocket specific for oxysterol sensing at these residues. An *in vitro* binding assay can be used to observe such effects.

Thus, there is some overlap in the oxysterol and FPP response of Hmg2, because there were 13 stabilized mutants that showed stunted abilities to respond to both molecules. However, there must be a specific mechanism to sense oxysterol due to the observed 6 hydrophobic residues in the SSD. The proposed hydrophobic pocket could be necessary for sensing oxysterols, and the residues that are important for sensing FPP could influence the sensing of oxysterol. Therefore an overlap between sensing the two molecules can exist, but the 6 hydrophobic residues of the SSD must be necessary to sense oxysterols.

Overall, there were 19 out of 30 SSD residues that showed stunted responses to either FPP or oxysterol molecule. Whether it was a weakened response to one or both of the molecules, these 19 mutants were nonetheless stabilized. Accordingly, these mutations demonstrated altered stability. Hmg2 enters the HRD quality control ERAD pathway by undergoing a structural transition from well folded to misfolded. It would be relevant to test if the altered stabilities of these mutants are due to an altered ability to undergo the structural transition from well folded to misfolded. We can do this using an *in vitro* trypsinolysis assay to see if these mutants are more prone to trypsin digestion. We expect that a mutant incapable of the transition would retain a tight folding that is less prone to digestion, even in the presence of FPP.

There were 11 out of 30 mutants tested that responded to FPP and oxysterol just like wildtype. These residues were not found to be important for sensing these molecules, but they could be responsible for other SSD relevant functions. These residues could be important for sensing other lipids, such as the sterol lanosterol. Lanosterol promotes the stabilization of Hmg2 by promoting the physical interaction with insulin-induced gene proteins (Insigs, but Nsg1 and Nsg2 in yeast)(C.T. unpublished observations).

Mammalian and yeast Nsg proteins are central regulators of cholesterol management because they interact with SCAP and HMGR. Nsg proteins bind to Hmg2 through a lanosterol-induced interaction to hold Hmg2 stable, even in

the presence of high FPP in the cell. It's possible that these other SSD residues are responsible for sensing lanosterol molecule and/or promoting the association with Nsgs. To test this possibility, we could over-express Nsg proteins in these mutant strains to determine if Nsgs stabilize the Hmg2 variants. If a residue were important for binding to Nsgs, then the mutant would be degraded in response to FPP even in the presence of Nsgs. As of now, the SSD demonstrates to respond to FPP, oxysterol, and to alter the folding state of Hmg2 upon regulated degradation. Yet there can be other important functions that the SSD is involved in, and further investigation is needed to understand such functions.

Chapter 5, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material.

Chapter 6

Results: Involvement of regions outside the SSD in response to FPP

The above studies indicate that the highly conserved residues of the SSD are important contributors to regulated degradation of Hmg2 (Figure 7 and 8). Conserved residues are found across the N-terminus of Hmg2, and are not limited to the SSD (Figure 7). The N-terminal domain is necessary and sufficient for regulated degradation of Hmg2, and it is possible that other conserved N-terminus residues are involved in regulating Hmg2.

In fact, we have evidence that regions throughout the N-terminus influence regulated degradation of Hmg2. An independent study was done that created site-directed mutations throughout the N-terminal domain of Hmg2, without regard to the SSD. The mutants had between four to six mutated residues. The mutations were conservative replacements of the residues examined (Gardner R.G., and Hampton R.Y. 1999a). Some of these mutated regions resulted in stable phenotypes. It is noteworthy that there were stable regions found outside of the SSD domain. Those mutated regions that resulted with stable and unregulated phenotypes of Hmg2 are boxed, and labeled by corresponding Hmg2 residue numbers in Figure 7.

We tested regional mutations containing conserved residues outside of the SSD to see if they play a role in regulated degradation of Hmg2. SVLP 463-466 was mutated into ALLQ (Parent strain RHY468). We tested

responsiveness of the mutant to FPP signal by ZA treatment and stability of CHX chase assay. Cells were treated with ZA or CHX for 2 hours and then lysates were prepared for SDS-PAGE and immunoblotting. The protein weakly responded to FPP and was significantly stable (Figure 19). These results indicated that there are regions outside of the SSD that are responsible for Hmg2 regulated degradation. The region lays in the loop located inside the lumen of the ER (Figure 22 residues 463-466). The regions in transmembrane 7 (KASVI 408-412 AAASV and NLYVF 419-423 SWNVV), which have previously been discovered as stable regions were confirmed to be unregulated and stable in this study as well (data not shown). These regions also contain conserved residues (Figure 7), and are localized in Figure 20.

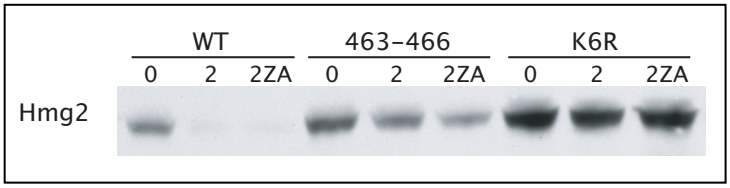

Figure 19: Conserved residues outside of the SSD contribute to Hmg2 regulated degradation. Cycloheximide chase assay of Wt-Hmg2, K6R-Hmg2, and SVLP 463-466 ALLQ. After addition of CHX (50ug/mL), lysates were prepared at 2 hours and were immunoblotted with 9E10 anti-myc antibody. Levels of Hmg2 were also assayed by treatment with ZA (10ug/mL) for 2 hours, and lysates were prepared as in the CHX chase. CHX is represented as 2, and ZA is represented as 2ZA.

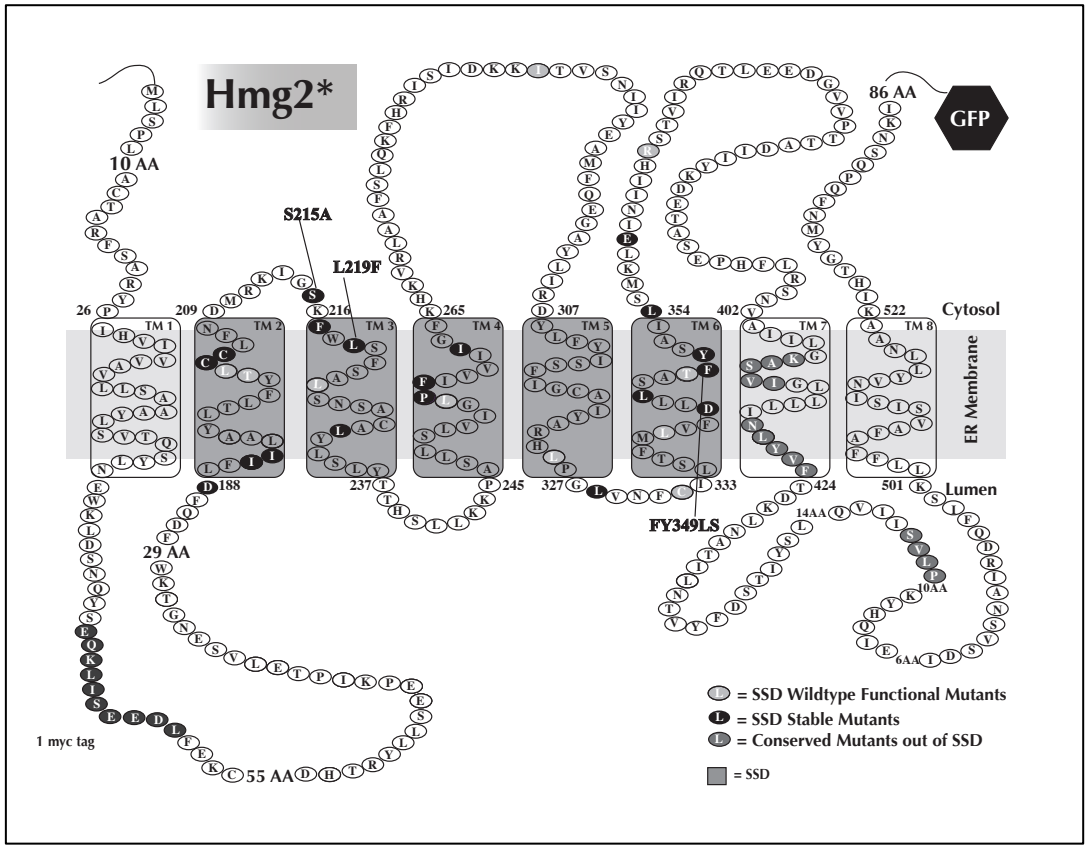

Figure 20: Topology of Hmg2. The following topology diagram indicates the location of mutated residues and the phenotypes associated with the mutation. The SSD is highlighted in medium grey. The mutants that responded to FPP and oxysterol like wildtype Hmg2 are highlighted in light grey. The mutants that showed stunted responses to FPP and oxysterol are highlighted in black. The most stable mutants S215A, L219F, and FY349LS are cited. The three stable regions outside of the SSD are highlighted in medium grey and are mutated as the following: KASVI 408-412 AAASV, NLYVF 419-423 SWNVV, and SVLP 463-466 ALLQ.

Conclusion:

Based on work demonstrated through the previous and present study, it seems that Hmg2 regulated degradation requires residues outside of the SSD. Each of the mutants that indicate blunted regulation alters at least one residue that is conserved between different HMGRs. The identification of residues outside of the SSD are important for lipid sensing, which is a novel finding found in this study of HMGR regulated degradation.

The mutation SVLP 463-466 ALLQ represented a conserved region of Hmg2 outside of the SSD that contributes to regulated degradation (Figure 7) (Figure 20). The serine in SVLP was mutated into S463A in this region, resulting with stable Hmg2 phenotypes, as reflected in S215A. In another conserved region, KASVI 408-412 AAASV, there was also a serine S410A mutated, which resulted with stable phenotypes. It appeared that the serine residues normally contribute to stabilization of these regions, because when mutated to alanine, Hmg2 variant was stable in all three cases. It is possible that serine residues are phosphorylated as a modification for regulation of Hmg2. A pulse-chase analysis can be used to detect if there is phosphorylation occurring at these serine residues. It would be interesting to mutate other conserved serine residues in Hmg2, and see if we observe similar effects.

Overall, creating specific mutations within regions of Hmg2 giving rise to stable phenotypes is necessary to determine which residues are responsible for demonstrating such phenotypes, such as in the mutant TFYSA. There were mutations made in this region that were important for regulation of Hmg2, such as Y350A and FY349LS. Although TFYSA is not a stable protein, it still demonstrates that single residues within that region are necessary for Hmg2 regulation. Therefore pinpointing mutations inside the stable Hmg2 regions would be significant for future studies.

Chapter 6, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material. I would like to acknowledge Richard Gardner for creating the site-directed mutations throughout the N-terminal domain of Hmg2. Some of those mutations were examined in this current study (Richard, R.G., 2000 PhD Thesis).

Chapter 7

Discussion

In this study, the role of the SSD in regulated degradation of Hmg2 was investigated. We showed that the SSD is involved in sensing FPP and oxysterols. The residues in the SSD that contributed to sensing these molecules also contributed to Hmg2 stabilization. The less responsive the mutant was to FPP or oxysterols, the more stable the mutant was. Hence the SSD is involved in the destabilization of Hmg2 during regulation. Regulation of FPP was not limited to the SSD, because conserved residues outside of the SSD also played a role in sensing FPP. Refer to Table 3 to see the phenotypes of the 30 Hmg2 SSD variants, and Figure 20 to view the location of residues and their associated phenotypes.

In addition to HMGR, sterol-sensing domains are found in other proteins that have specific ways of regulating lipids. HMGR undergoes feedback-regulated degradation of mevalonate pathway products, SCAP is necessary for regulating the localization of transcription factors necessary for cholesterol synthesis, and NPC1, a protein affected in Niemann–Pick disease type C1, is necessary for cholesterol trafficking (Kuwabara P. E., et al 2002). Other proteins such as 7-dehydrocholesterol reductase (7DHCR), Patched (Ptc), Dispatched (Disp), and PTR (Ptc-related) all contain an SSD necessary for proper lipid regulation.

Table 3: SSD Mutant Stability and Regulation of Hmg2. The following table shows the phenotypes observed from testing 30 SSD Hmg2-GFP mutants. Mutants are classified into the following phenotype groups. The phenotype observed upon treatment of the following drugs is indicated (Refer to materials and methods for details on drug treatment). (+) Indicates a wildtype phenotype observed upon drug treatment, (+'/-) indicates a partial response to the drug, and (-) indicates stabilization even upon treatment with a drug.

Phenotype	Strain	CHX	ZA	Lova	Ro48
Wildtype Stability/ Regulation	WT #2723	+	+	+	+
	T202A	+	+	+	+
	L203A	+	+	+	+
	L224A	+	+	+	+
	L255A	+	+	+	+
	I287A	+	+	+	+
	L325A	+	+	+	+
	C332A	+	+	+	+
	L339A	+	+	+	+
	T348L	+	+	+	+
	F349L	+	+	+	+
	R365A	+	+	+	+
Partial responders to FPP	I192A	+	+	+'/-	+
	202-205AAAA	+	+	+'/-	+
	F217L	+	+	+'/-	+
	I262A	+	+	+'/-	+
	L354A	+	+	+'/-	+
	D342A	+	+'/-	+	+'/-
	E359A	+	+'/-	+	+'/-
Idependent FPP and oxysterol response	L345A	+	+	+'/-	+'/-
	L231A	+	+	+'/-	+'/-
	L328A	+	+	+'/-	+'/-
	I191A	+	+	+'/-	+'/-
	P256A	+	+	+'/-	+'/-
	F257L	+	+	+'/-	+'/-
Partially stable/ weakly Responsive	D188A	+'/-	+'/-	+'/-	+'/-
	S215T	+'/-	+'/-	-	-
	Y350A	+'/-	+'/-	-	-
Stable/ Non-Responsive	S215A	-	-	-	-
	L219F	-	-	-	-
	FY349LS	-	-	-	-

Since we found that the SSD plays a role in sensing molecules and changing the conformational state of Hmg2, there is insight to the roles of other SSD containing proteins for regulation.

The role of the SSD for regulation of SCAP can be similar to Hmg2, especially since there is similarities found between the two SSD containing proteins. The SSD topological orientation of the proteins is homologous, including an 8 transmembrane spanning N-terminal domain (Davies J.P., et al 2000). The SSD of both proteins ranges transmembranes 2-6, and contains sequences with 25% identity and 55% similarity. The structural conservation of HMGR and SCAP suggests that similar mechanisms could be used for lipid regulation, and the results of this study provide insight to these mechanisms.

It is possible that SCAP and Hmg2 bind directly to lipid molecules for regulation of the proteins. In this study there were hydrophobic residues of Hmg2 involved with sensing FPP and oxysterols. These hydrophobic residues were located in the two critical regions necessary for sensing FPP, and in the 6 residues necessary for sensing oxysterols (Figure 15b)(Figure 18b). These hydrophobic residues could be forming a binding pocket that binds directly to FPP and oxysterol molecules. An *in vitro* binding assay would be needed to assess this possibility, like the *in vitro* binding assay that demonstrated cholesterol to directly bind to the N-terminal domain of SCAP (Radhakrishnan

A. et al 2004). Thus it is possible that both Hmg2 and SCAP regulate lipid levels in the cell by binding directly to lipids to induce regulation.

Since Hmg2 and SCAP might sense lipids through the same mechanism, it's possible that the SSD is involved in lipid sensing for both proteins. The SSD plays a role in both SCAP and Hmg2 regulation, because mutations made in the SSD of both proteins resulted with loss of regulatory function. 3 mutations made in hamster SCAP SSD (D443N, L315F, Y298C) showed unregulated mutants that did not interact with Insig and were constitutively transporting SREBPs to activate cholesterol transcription factors (Yabe D., et al 2002). Hmg2 mutants in this study also revealed 3 mutations in the SSD (S215A, L219F, FY349LS) that were unregulated and stable. Thus SSD residues are important for regulative functions of these proteins.

Aside from the SSD role in sensing lipid molecules, upon regulation of these lipids SCAP and Hmg2 undergo a conformational change. *In vitro* proteolysis demonstrated that there is a structural change occurring in these proteins when induced by specific lipids. Cholesterol was added to crude ER membranes *in vitro*, and a protease protection assay was done to cleave SCAP. It turns out that when adding cholesterol, tripsin digests SCAP into many fragments that differ in size when compared to a tripsin digest with no lipids present (Radhakrishnan A., et al 2004). Hmg2 also undergoes a conformational change when induced by FPP derived molecule (Shearer A.G.,

and Hampton R.Y 2005). *In vitro,* when microsomes were treated with farnesol (which is derived from FPP), tripsin digests Hmg2 at a high rate, which shows that Hmg2 changes configuration. Therefore the regulation of pathway molecules involves a conformational change of both Hmg2 and hamster SCAP.

There are proposed mechanisms for when these conformational changes take place, which depends on the flux of lipids in the cell. When sterol molecules are high in the cell, Insig binds to SCAP in the ER to prevent it from transporting SREBPs. When sterol molecules are depleted in the cell, SCAP undergoes a conformational change that releases binding with Insig. Once SCAP is released, it is able to transport SREBPs (Espenshade P.J., and Hughes A.L. 2007). Hmg2 also goes under a structural transition based on the levels of FPP and oxysterol in the cell. When there are high amounts of these molecules, Hmg2 transitions its conformation into a less-well folded state, which allows HRD machinery to recognize it as an ERAD substrate (Figure 5). Thus these similar SSD containing proteins utilize similar mechanisms during regulatory events.

It is possible that the SSD is responsible for the structural transitions in both SCAP and Hmg2. We found that the SSD is involved in the destabilization of Hmg2 during the conformational change of the protein when induced by FPP, and it turns out that the SSD is involved with the

conformational change of SCAP as well. 3 mutations in hamster SCAP SSD (D443N, L315F, Y298C) blocked the interaction with Insig and resulted in unregulated mutants (Yabe D., et al 2002). The structure of these mutants could not support the interaction with Insig, and the protein could not regulate sterol synthesis as a result of the loss of interaction. Much like the Hmg2 SSD residues examined in this study, the following SCAP SSD residues were needed for the regulated change of conformation in SCAP. Since SCAP and Hmg2 have different functions, the exact types of conformational changes are different. However, they seem to share the commonality that the residues in the SSD do contribute to the structural dynamics of the protein.

The findings in our study for the role of the SSD can expand to other SSD containing proteins. Specifically SCAP protein's SSD is important for regulation, and for inducing a conformational change similarly shown in Hmg2. It would be informative to initiate parallel studies in other SSD containing proteins to understand regulation. Creating mutations in the SSD of other SSD containing proteins would be a step to understand what regions are responsible for regulation of the protein. Through our study, examining conserved residues had shown to be informative areas for regulation.

Mutations that were highly conserved were important for regulation of Hmg2. S215 and L219 are conserved in many HMGR organisms and SCAP (Figure 7). L219 in Hmg2 happens to correspond to the residue L315 in

hamster SCAP (Yabe D., et al 2002). L315 in SCAP is even conserved among many invertebrates and vertebrates that contain SCAP. When mutating L219F in Hmg2 or L315F in SCAP, there is a loss in regulation of these SSD containing proteins. This leucine residue must be a conserved functional motif of SSD containing proteins, and it would be relevant to make the same mutation in other SSD containing proteins. It is relevant to note that S215A and L219F are right next to each other, and compose one of the critical regions for sensing mevalonate pathway products.

The 4-phenylalanine (F) residues in the sixth transmembrane of Hmg2 are also conserved in SCAP and HMGR; however, there are 3F residues and a conserved Y in transmembrane 6 of Hmg2, which corresponds to the mutation FY349LS. The mutational analysis of our study corresponds to an analysis done in mammalian HMGR. Four of the phenylalanine (F) residues in transmembrane 6 were mutated into leucine (L) residues in mammalian HMGR (Figure 7 and Figure 20)(Xu L., and Simoni R.D. 2003). It turns out that a quadruple mutant in HMGR induced an unregulated and stable protein. The hypothesis was that the 4 F residues synergistically acted as a hydrophobic binding pocket that was necessary for the degradation of HMGR. In addition to HMGR, it was found that mutation V439G in SCAP, which exists in transmembrane 6 in hamster SCAP, also shows reduced Insig binding and loss of regulation of SREBP (Hughes A.L., et al (2008). These findings are

consistent with the results of our study because the Hmg2 mutations FY349LS showed stable phenotypes. All of these findings, whether in HMGR or SCAP, suggest that transmembrane 6 residues could be creating a binding pocket that forms a critical region for regulating SSD containing proteins.

Based on the findings in this study it appears that the conserved residues in the SSD are important for the regulation of Hmg2. Functions of regulation are not limited to the SSD; because residues out side of the SSD have shown to contribute to the regulation of Hmg2. Conserved residues in the SSD reveal to be important for the regulation of Hmg2, mammalian HMGR, hamster SCAP, and could extend to other SSD containing proteins. Some conserved residues of the SSD are specific to an organism or protein type, which is the result of evolutionary differences between protein functions and organisms. Nevertheless it appears that the SSD plays similar roles for regulating Hmg2 and SCAP, and it is possible that other SSD containing proteins utilize the same mechanisms as well.

Chapter 7, in part, is currently being prepared for submission for publication of the material. Hampton, Randy Y; Theesfeld, Chandra; Pourmand, Deeba; and Davis, Tai. The thesis author was a primary investigator of this material

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