

## Introduction

This proposal has been substantially modified in response to very helpful reviews. We were gratified to find strong overall interest in the questions being asked, and our ability to answer them. The general weaknesses of the grant as submitted were that the proposal was too diffuse, trying to follow too many leads, and that the experiments were correspondingly sketchy in detail. We have revamped the proposal to address all these concerns and to include progress made on the proposed experiments since the first submission.

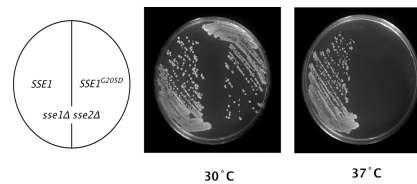
The focus and scope of the proposal has been greatly tightened. First, the previous third aim was to characterize the role of Sse1 in function of the Mediator transcriptional complex. While we are still interested in this novel discovery, pursuit of that line of investigation would require substantial investment of time and money in reagents specific for that project, and detract from our core goal of understanding Sse1 function at a mechanistic level. We have therefore eliminated this aim, reducing the proposal to two overarching Aims with the goal of understanding the interactions and roles of the Hsp110 chaperone specifically with Hsp70 (Aim 1) and Hsp90 (Aim 2). Second, Aim 2 has been supplemented with a new series of experiments that test specific hypotheses about the mechanism of Sse1 interaction with Hsp90 using a model substrate, the glucocorticoid receptor. Substantial additional preliminary data is included both in the Preliminary Studies section, and within the new experimental design sections as appropriate.

Reducing the proposal to two aims allows us to increase the depth of our investigations as detailed below. In addition, a simultaneous discovery by us and a recently published report have identified the MAP kinase Sit2 as the relevant Hsp90 target in the cell integrity pathway (Mills et al., 2005). These results allow us to ask a series of very direct questions about the role of Sse1 and Hsp90 in supporting signal transduction through the pathway, eliminating much of the "fishing" we had planned in the first application.

We specifically address below comments from the Summary Statement. New or substantially altered sections in the proposal are enclosed in brackets {}.

1. The reviewers felt that our analysis of the mechanism by which Sse1 affects Hsp70 activities was too superficial and cursory. In response, we have significantly expanded our experiments to determine if/how Sse1 modulates Ssa1 ATPase activity to enhance Ssa1 in vivo functions, while reducing work with the Ssb chaperones. We have carefully separated out the possible modes by which Sse1 could influence the reaction cycle, specifically addressing hydrolysis and nucleotide exchange rates as components of observed steady-state ATPase activity. In addition, we have incorporated the use of nucleotide binding-defective or ATPase-defective mutants of both Sse1 and Ssa1 that will allow us to parse relative contributions of each chaperone.

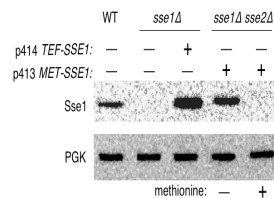
2. Reviewer #1 pointed out that many of our experiments relied on phenotypic effects due to loss of Sse1, while Sse2 remained functional in the cell, perhaps masking potential results due to functional redundancy between the duplicated genes. Although Sse1 appears to account for the vast majority of Hsp110 chaperone function (whatever that may be) in the cell, this is a valid and important criticism. We have therefore generated two strains to address this concern. The first strain contains a temperature sensitive *SSE1* allele fortuitously resulting from substitution of glycine 205 by aspartate, and as



**Figure A. SSE1-G205D is a temperature sensitive allele inactivated at 37°C, resulting in conditional**

shown in Fig. A allows wild type growth at 30°C but is completely non-functional at 37°C in the *sse1Δ sse2Δ* background. Additionally, intermediate growth rates are obtained at semi-permissive temperatures, which will allow us to modulate “total” Sse status as necessary.

The second strain we created expresses *SSE1* under the control of the repressible *MET3* promoter in the *sse1Δ sse2Δ* background. As shown by Western blot in Fig. B, Sse1 protein is expressed at levels comparable to wild type, and lower than the moderate-strength TEF promoter, in the absence of added methionine in the medium. Growth of cells in the presence of methionine results represses Sse1 production to a level virtually undetectable by anti-Sse1 antibody. Correspondingly, these cells grow normally on plates lacking methionine but exhibit a pronounced slow-growth phenotype in the presence of methionine. Together, these strains will allow us to precisely modulate Sse levels in our experiments.



**Figure B. A *MET3-SSE1* promoter fusion abolishes Sse1 production in the presence of added methionine.**

3. All three reviewers were concerned that the links between Hsp90, Sse1 and the cell integrity signaling pathway were not sufficiently solid, notably the lack of a demonstrated client protein. We believe the additional preliminary data we have obtained, and the identification of Slt2 as an Hsp90 target protein, strongly support our case. Moreover, these developments have allowed us to focus our investigations on signaling and interaction between Slt2 and its downstream effectors Rlm1 and SBF. Importantly, these studies will follow analysis of the role of Hsp70 in mediating the Sse1•Hsp90 interaction, both physically and phenotypically. We now realize we had put the cart before the horse, and will now couple investigation of a novel Sse•Hsp90-dependent signaling pathway with analysis of a proven, well-documented substrate.

4. Reviewer #2 expressed concern that our experiments designed to delineate the interaction sites between the chaperones were not extensive enough. We have expanded this section to include new experiments using affinity resins to test accessibility of the nucleotide binding pockets of both chaperones. In addition, we will test the hypothesis that the nucleotide binding pockets must be occupied for stable heterodimerization of Sse1 and Ssa1. Lastly, we will use this information to propose a “structural” model for the heterodimer.

5. Reviewer #2 felt the use of “semi-purified” chaperones from *E. coli* and yeast extracts as the first step in reconstitution was perhaps unwarranted. We have already completed this sub-aim, as demonstrated in Fig. 14, and will be working directly with purified components. We have already purified the Ydj1 co-chaperone, and are working to produce the Sse1, Ssa1 and Ssb1 chaperones.

6. Reviewer #3 felt that we had failed to make clear our ideas of how Sse1 participates in Hsp90-dependent functions. The addition of new experiments in sections D.2.1 and D.2.2 directly address this important question with the hypothesis that Sse1 contributes to Hsp90 functions via its heterodimerization with Ssa1. Along these lines, the reviewer noted that many of the proposed experiments were interesting but descriptive, lacking clearly spelled out and testable hypotheses. We have addressed this important concern by formulating hypotheses (typeset in **bold**) when appropriate and redesigning experiments as necessary to specifically test them.