Summary

Due to conservation of biological pathways and ease of genetic manipulations the budding yeast *S. cerevisiae* has become preferred model organisms for understanding basic aspects of cellular biology. It has provided insight into the basics of eukaryotic processes such as transcription, translation, cellular signaling, protein trafficking and mitochondrial biogenesis. Hsps are known to control wide range of biological functions from prokaryotes to eukaryotes for preventing the aggregation of misfolded proteins to activation of key components of signal transduction pathway. Hsp70 along with its co-chaperones forms the major machinery that promotes folding of proteins but there exists different class of proteins that are transferred from Hsp70 to Hsp90 for proper protein folding. The list of Hsp90 clients includes kinases, steroid hormone receptors and E3 ligases suggesting the possible role in fine tuning of signal transduction pathway.

Eukaryotic cell is composed of various Hsp70 isoforms which are present in different parts of cells and perform distinct function. Hsp70 is important for Hsp90 chaperoning pathway but the function of individual cytosolic Hsp70 in Hsp90 client protein maturation is not known. Using *S. cerevisiae*, v-Src over-expression is less toxic in cells expressing stress inducible Ssa3 or Ssa4 as the only source of Hsp70 than the constitutive ones. Western results show that v-Src expression is 50% less in strains expressing Ssa4 compared to Ssa2. Western blot also revealed that protein tyrosine phosphorylation associated with matured form of v-Src is significantly lower in A4 compared to A2 strain. As immature client proteins of Hsp90 are processed for degradation through ubiquitination proteasomal pathway we found higher degradation rate of v-Src in A4 than A2 cells. The interaction studies using immunoprecipitation of FLAG-v-Src revealed that the client interacts poorly with Hsp90s in A4 than A2 strain. We also showed that v-Src interaction with Ssa4 is relatively weaker than

with Ssa2 which could be the basis of relatively poor interaction observed between v-Src and Hsp90 in A4 strain. Pull down studies revealed that Both Ssa4 and Ssa2 interact similarly with purified Hsp90. Client protein is known to interact either directly to Hsp70 or through Hsp40. The pull down study using purified Ydj1 shows that the co-chaperone interaction with v-Src is similar in both A2 and A4 strains. Further, the pull down study revealed that Ydj1 interaction with Ssa2 is stronger than with Ssa4. In vitro refolding assay revealed that Ssa4 is less efficient in refolding luciferase compared to Ssa4. Similarly we found Ssa4 less efficient in luciferase refolding when experiment was carried in the presence of Sti1 and Hsp90. Hsp70 has three functional domains; NBD (Nucleotide-Binding Domain), SBD (Substrate-Binding Domain), and CTD (C-terminal domain). Using hybrids between NBD and CTD of Ssa2 and Ssa4 we showed that CTD of Ssa4 governs its functional distinction with regard to v-Src mediated toxicity as hybrid H224 reduced toxicity better than H442. We show using western blot that v-Src level is very low in strains expressing H224 as sole source of Hsp70. Our data show that Ssa4 mediated reduction in v-Src toxicity is downstream effect of impairment in Ydj1 interaction with Ssa4.

In another study we performed hydroxylamine mutagenesis to identify sites in Hsp70 Ssa1 isoform which inhibits Hsp90 chaperoning action. T175N and D158N mutants in NBD of Ssa1 reduced v-Src mediated toxicity. Western blot analysis of v-Src revealed that it is expressed similarly in Ssa1, Ssa1-T175N or Ssa1-D158N as sole source of Hsp70 expressing strains. Tyrosine kinase activity associated with active v-Src show that v-Src formed in A1-T175N or A1-D158N are less active compared to A1 strain. Immature form of v-Src formed in yeast cells is known to aggregate and fractionate into pellet fraction of cells. We found that immature v-Src formed in A1-T175N or A1-D158N strains is prone to higher aggregation. We also found no major difference in fractionation of Hsp70 and Hsp90 A1, A1-T175N and A1-D158N strains. Changes in expression level of major chaperones Hsp70, Hsp90, and co-

chaperones Ydj1, Sse1 and Hsp104 are known to effect client protein maturation. We found no major change in level of Ydj1, Hsp70, Sse1 Hsp90, and Hsp104 in A1-T175N and A1-D158N compared to wt-A1 strains. This suggests that the reduction in v-Src mediated toxicity is not due to alteration in chaperones and co-chaperones known to effect v-Src folding in *S. cerevisiae*. Using FLAG tagged v-Src, we found higher interaction with Ssa1-T175N and Ssa1-D158N compared to wt-Ssa1. Contrary to above we found much reduced interaction of FLAG-v-Src with Ssa1-T175N compared to Ssa1-D158N and wt-Ssa1. Collectively our data show an altered interaction of FLAG-v-Src with Hsp70 mutants results in inhibition of Hsp90 chaperoning action with regard to v-Src mediated toxicity.

We have also studied mechanism of Hsp90 chaperoning cycle inhibition by Ssa1 mutants. In this study we found that Hsp70-Hsp90 direct interaction is negatively affected in Ssa1-T175N and Ssa1-D158N mutants. This region in NBD of Ssa1 also serves as binding site for Hsp40s. We show that multiple single knockout study of various Hsp90 co-chaperones in the presence of Ssa1-T175N, Ssa1-D158N or wt-Ssa1 resulted in lethality in *sti1Δ* background. Over expression of Sti1 and Sti1Δlinker complemented for absence of Sti1 in A1-T175N and A1-D158N background. Linker connecting DP1 and TPR2A negatively effects ternary complex formation between Hsp70 and Hsp90 but even smaller fractions of ternary complex formed supports growth in *sti1Δ* background. NBD of Hsp70s serves as binding site for Hsp40s like Ydj1 in *S. cerevisiae*. Pull down using His_6-Ydj1 revealed higher interaction with both Ssa1-T175N and Ssa1-D158N compared to wt-Ssa1. Higher interaction with Ssa1 mutants with Ydj1 was confirmed in vitro using Bio-layer interferometry. Functional assay revealing synergistic action of Ydj1 and Hsp70 using luciferase revealed higher folding activity with both Hsp70 mutants relative to Ssa1. The luciferase refolding is further increased upon addition of Hsp90 and Sti1 to the purified proteins. In contrast to higher luciferase refolding with Ssa1 mutants and Ydj1, we observed lower or similar luciferase refolding activity with

Ssa1-T175N and Ssa1-D158N respectively compared to Ssa1 when luciferase refolding assay was carried out in the presence of Sti1 and Hsp90 with either of the Hsp70 mutants. Our in vivo pull down using His_6 tagged Hsp70s show much reduced interaction between Hsp90 and Ssa1-T175N or Ssa1-D158N compared to wt-Ssa1. Lower interaction between Ssa1 mutants (Ssa1-T175N and Ssa1-D158N) was confirmed using BLI. Thus all the above results show that relative to Ssa1 both Ssa1-T175N and Ssa1-D158N interacts with higher affinity to Ydj1. Both these mutations negatively effects their interaction with Hsp90. Impairment in Hsp70-Hsp90 interaction negatively effects Hsp90 client protein maturation.