Mitogen activated protein kinase (MAPK) cascades are predominant among signal transduction pathways and play vital roles in regulating variety of cellular processes. The MAPK module is composed of sequentially organized kinases; the MAPK (also known as ERK, extracellular signal regulated kinase), MAPKK (MEK, extracellular signal regulated kinase kinase) and MAPKKK (MEKK, extracellular signal regulated kinase kinase). Although they vary in numbers and functional complexity, these kinases are conserved from yeast to mammals. Yeast cells are exposed to myriad of stress conditions and they exhibit characteristic response to these stimuli. Among five MAPK pathways present in *S. cerevisiae*, HOG (High Osmolarity Glycerol Response Pathway) is activated in response to high osmolarity stress. The MAPKK, *PBS2* is the central component of this pathway and integrates signals from two different membrane proteins i.e. Sln1p and Sho1p. Sln1p relays its signals through two redundant MAPKKKs, Ssk2p and Ssk22p, whereas Sho1p acts through promiscuous MAPKKK Ste11p. Once activated, the Pbs2p MAPKK facilitates Hog1p phosphorylation on Thr174 and Tyr176, that leads to increased expression of a number of target genes and, as a result, higher accumulation of glycerol inside the cell occurs.

Homologous genes from other species of yeast are potential sources of natural variants of *PBS2*. The characterization of such genes could therefore provide insight into complex physiological role. Recently, an osmosensing MAP kinase pathway has been shown to be present in yeast *Debaryomyces hansenii*. It was isolated from saline environments such as sea water and concentrated brines and is considered to be one of the most halotolerant and osmotolerant species of yeast. This species can grow in the media containing 4 M NaCl, whereas growth of *S. cerevisiae* is limited in the media having more than 1.7 M NaCl. In this study, a *PBS2* homologue from *D. hansenii* was cloned and characterized.

PBS2 homologue was isolated by phenotypic complementation of *S. cerevisiae pbs2* mutant by utilizing a *D. hansenii* genomic library constructed in yeast shuttle vector pRS425. Our screening efforts resulted in two plasmids pDBS14.1 and pDBS16.1. The osmoresistant phenotype was shown to be plasmid linked by retransformation of PB29. Restriction digestions of the plasmids pDBS14.1 and pDBS16.1 revealed that they could possibly be identical, therefore all further studies

were carried out with plasmid pDBS14.1. Plasmid pDBS14.1 was found to contain an insert of 4.0 kb length. Southern hybridization was carried out to confirm that the cloned gene was indeed a *D. hansenii* gene. In order to localize the gene, a restriction map was made. Further sub-cloning proved that the gene was present on a 3.0 kb *Eco*RV fragment and was named *DPBS2*.

To determine that the cloned fragment is a *PBS2* homologue, not an extragenic suppressor, it was subcloned into a yeast centromeric vector YCplac111. The yeast strain PB29 (*pbs2Δ*) transformed with *DPBS2* gene cloned in either centromeric vector (pDP7.1) or multicopy vector (pDP4.1), showed complementation of *pbs2* mutation on plates containing different osmolytes. Thus, the cloned gene is most likely a *PBS2* homologue and not an extragenic suppressor. Interestingly, unlike wild type, PB29 carrying *DPBS2* gene either in multicopy or low copy vector failed to grow in plates containing higher amount of osmolyte. These results indicated that *DPBS2* could complement only partially the *pbs2* mutation in *S. cerevisiae*. It was quite reasonable to assume that the partial complementation could be arising out of the differences in promoter strength of *DPBS2* in heterologous host *S. cerevisiae*. Therefore, to rule out this possibility *DPBS2* was cloned under *S. cerevisiae* promoter. Similar level of complementation was also observed when *DPBS2* was placed under *PBS2* promoter. Pbs2p plays an important but complex role in osmosensing signal transduction pathway. Therefore, it is likely that a *PBS2* homologue was mimicking these complex interactions sub-optimally in a heterologous host and thus showing lower activity.

Northern blot analysis was performed to determine whether expression of the *DPBS2* gene in *D. hansenii* is modulated by changes in osmolarity of the media. Total RNA was isolated from cultures grown at various length of time in the media containing 1 M NaCl and hybridized with *DPBS2* probe. A single transcript of about 2.4 kb was detected in all the samples and the size of transcript correlated well with that of the open reading frame (ORF) identified. When the level of *DPBS2* mRNA expression was normalized with that of actin mRNA level, no significant difference in steady-state level was observed, suggesting that the gene *DPBS2* is constitutively expressed in *D. hansenii*.

From the nucleotide sequence of the clone, an open reading frame of 2049 bp (encoding 683 amino acids) was identified. Comparison of sequences with previously identified *PBS2* homologues from different yeast species using CLUSTAL W program revealed that this sequence is homologous to Pbs2p of *S. cerevisiae,* Wis1p of *S. pombe.* MAPKKs among animals and yeast have highly conserved amino acid residues (Ser-X-X-X-Thr/Ser), located between sub-domains VII and VIII. The phosphorylation of these threonine and serine by MAPKKKs is required for the activation of MAPKKs. Comparison of phosphorylation site showed 100 % homology to that of Pbs2p. Like its *S. cerevisiae* homologue, Dpbs2p also contains a proline rich SH3 binding domain localized at the N-terminus non-kinase region.

In *S. cerevisiae PBS2* is activated by two different, independently acting upstream branches. In order to examine whether Dpbs2p could also be activated by either of these branches, the plasmid pDPS21 was transformed into strain PSY412 (*Δste11, Δpbs2*) and PSY418 (*Δssk2, Δssk22, Δpbs2*). From the result it was evident that Dpbs2 could also be activated by MAPKKKs of either of the upstream branches. However, on salt containing plates, cells with *ste11* genetic background grew marginally better than those with *ssk2, ssk22* genetic background. In the plates, containing more than 0.7 M NaCl the transformants exhibited poor growth compared to control thus confirming that Dpbs2p could only partially complement osmosensitivity of *pbs2* mutation.

Analysis of Dpbs2p sequence revealed that like Pbs2p it also has a long N-terminal and short C-terminal region which are outside the kinase domain. It was observed that these two MAPKKs exhibit considerable sequence divergence in non-catalytic regions although their kinase domains showed strong similarity. It is presumed that these non-catalytic domains may contain functionally important sub-domains and that is why Dpbs2p may not be able to complement fully the *pbs2* mutation in *S. cerevisiae.* To test this, we have made three chimera DPSC1, DPSC2 and DPSC3. In DPSC1 and DPSC3 N-terminal 182 and 322 amino acid residues of Dpbs2p were changed with N-terminal 197 and 337 residues from Pbs2p respectively. In case of DPSC2, amino acid residues 548-683 of Dpbs2p were replaced with residues 567-668 of Pbs2p. Phenotypic analysis showed that the cells harbouring

DPSC2 were significantly more osmotolerant than those having parent *DPBS2*. Phosphorylation of Hog1 is considered as the hallmark of HOG pathway activation. From Western blotting it was evident that level of phosphorylation did not directly correlate to high osmotolerant phenotype of DPSC2. Exposure to high osmolar media causes rapid but transient nuclear localization of Hog1p in *S. cerevisiae*. Therefore, osmostress induced nuclear localization of Hog1p was monitored in cells expressing *DPBS2* or DPSC2. For this purpose we utilized a Hog1-GFP and monitored sub-cellular localization of Hog1-GFP fluorescence upon the osmotic induction. In case of wild type control 89 % of cells showed strong nuclear GFP fluorescence upon exposure to high osmolar conditions. However, a significant difference in number of cells showing nuclear GFP fluorescence was observed for chimera DPSC2 (78 %) and DPBS2 (51 %) under this condition. Thus, it appeared that as compared to *DPBS2*, osmostress induced nuclear translocation of Hog1p was more in cells expressing DPSC2 and consequently these cells could be exhibiting more osmotolerance.

MAPK-docking motif has been identified at the N-termini of different MAPKKs from yeast to human. This motif is comprised of two basic amino acid residues followed by a spacer (2-6 residues) and L/I-X-L/I sequence. Recent studies suggest that *S. pombe* homologue Wis1 has two such motifs. Careful examination of nucleotide derived amino acid sequence of Dpbs2p revealed that sequences closely related to MAPK-docking site consensus were present at residues 178-185 and 241-249. To examine the significance of the sequence mutations were introduced in these motifs. Mutant DPS62 was made by replacing conserved Arg-178 and 179 with Glu. For other docking motif three mutants DPS73, DPS75 and DPS77 were constructed by replacing either conserved Lys-241, 242 with Glu or Leu-247, 249 with Ala or all the four residues simultaneously.

Osmosensitivity of PB29 carrying these mutants was checked. Mutant DPS73 showed significantly reduced growth on plate carrying osmolytes, whereas DPS77 failed to grow on the plates. Moreover, Phenotypic analysis showed that mutation in the second motif completely abolished both Hog1p binding *in vitro* and activation *in vivo*. Therefore, the motif at residues 241-249 could be the primary docking site whereas the other one had minor role. In case of Wis1 also, among the two docking

motifs, one was shown to have a predominant role. Apart from MAPKKs from mammalian sources, docking motifs present in yeast Ste7 and Wis1 have been studied in detail. In all these examples, mutations in docking motif resulted in reduced affinity for the respective MAPKs and such mutations did not show any phenotypic defect. However, in case of Dpbs2p, mutations in the docking motif completely abolished its function. Although importance of docking interaction between MAPKK and MAPK under *in vivo* condition has been an accepted proposition, our result, for the first time, has brought it into experimental perspectives. It is to be noted that the phenotypic analysis was carried out in a heterologous host i.e. *S. cerevisiae*. In such a scenario, it was quite possible that other interactions through active site or due to scaffolding functions were compromised and consequently interaction through docking motif became essential.

Unlike mammalian cells, HOG pathway is the only stress activated MAPK pathway in yeast. Therefore, activation of HOG pathway in *D. hansenii* was monitored under different stress conditions by western analysis. Our results suggested that in *D. hansenii* this pathway was activated by osmostress as well as oxidative stress. However, heat stress did not induce this pathway. In this respect, *D. hansenii* is quite similar to *C. albicans* as HOG pathways in these species are activated by high osmolarity and oxidative stress. In *S. cerevisiae*, high osmolarity conditions mainly induce this pathway. Recent studies suggest that it is also induced under heat stress although at much lower level. On the other hand high osmolarity, oxidative or heat stress seems to activate this pathway in *S. pombe*. Thus, it appears that types of stress conditions that activate HOG pathway differ among yeast species. Considering that these species live in different ecological niche, it is not strange that they have evolved with different strategies to respond to various stress conditions using the same element but in distinct ways.

HOG pathway is the main regulator of yeast adaptation to hyper-osmolarity. However, dynamics of HOG pathway activation in *D. hansenii* has not been studied. Such studies gained more importance in the context of recent observations that kinetics of activation of this pathway in *S. cerevisiae* differs significantly under low and high osmolarity conditions. Thus, activation of HOG pathway in *D. hansenii*

under different osmotic conditions was examined by immunoblotting. Under moderate stress condition (0.7 M NaCl), phosphorylation of Dhog1p reached maximum within 5 min and disappeared between 15-30 min after the shock. Under severe osmolarity condition (2.0 M NaCl) Dhog1p phosphorylation was also observed within 5 min but significant amount of phosphorylated Dhog1p could be detected over a very long time (15 h), after the onset of the osmotic stress. In case of *S. cerevisiae*, severe osmotic stress leads to delayed induction of stress responsive genes. It was shown that Hog1p dephosphorylation coincided with the activation of HOG pathway target genes which was found to precede resumption of cell growth after osmotic stress. By monitoring growth of *D. hansenii*, we could observe that the culture had reached early stationary phase within 15 h after exposure to 2.0 M NaCl and at this point the level of phosphorylated Dhog1p was significantly high. Thus it appeared that complete deactivation of the HOG pathway was not necessary for resumption of growth in *D. hansenii*. Since Dhog1p remained phosphorylated for very long time under severe osmotic stress, it was checked whether this situation could be reversed by exposing the cells to normal medium. A wild type *D. hansenii* culture at logarithmic phase was stressed for 90 min with 2.0 M NaCl and down-shocked to YEPD medium (without salt). It was evident from our result that phosphorylated form of Dhog1p disappeared after 30 min of down-shock.

Translocation of activated Hog1p from cytoplasm to nucleus is an important event during the response to osmotic stress in yeast. Therefore, sub-cellular distribution of Dhog1p was monitored under severe osmotic conditions by indirect immunofluorescence. Nuclear accumulation of Dhog1p around 90 min after application of stress suggested that nuclear translocation of Dhog1p was significantly delayed in *D. hansenii*, although it is highly osmotolerant yeast. At 150 min the fluorescence was observed only in the cytoplasm, demonstrating cytoplasmic re-entry of Dhog1p within this period. Beyond this point, Dhog1p was detected only in the cytoplasm although it is phosphorylated. Thus, our results strongly suggested that under extreme osomotic stress *D. hansenii* cells maintained an activated form of Dhog1p in the cytoplasm.

Present study provides a novel insight into the dynamics of HOG pathway activation in a highly osmotolerant and halotolerant yeast *D. hansenii*. In presence of 2.0 M NaCl, nuclear translocation of Dhog1p was significantly delayed. This observation appears to be paradoxical. As a osmotolerance yeast, *D. hansenii* is expected to respond very rapidly at higher level of stress. Therefore, it is possible that activated Dhog1p plays an important role in cytoplasm as a part of osmoadaptation process before being translocated to the nucleus. In *S. cerevisiae*, hyperosmotic stress inflicts a number of molecular damages that cause shrinkage of cell, disassembly of cytoskeleton, changes in membrane permeability and cell wall remodeling. Precise role of Hog1p in all these processes is not known. The observation that *D. hansenii* maintains activated Dhog1p in the cytoplasm during its growth under high osmolarity, further supports this view. Recently, role of HOG pathway in cell wall remodeling and golgi protein localization has also been implicated. Therefore, the possibility that activated Dhog1p is playing an important role in the cytoplasm cannot be ruled out. Besides causing an initial damage to the cell, severe osmolarity condition also imposes a recurring hazard for its growth in this condition. To overcome such a situation, *D. hansenii* could be maintaining phosphorylated Dhog1p in the cytoplasm during its growth under severe osmotic stress. Although a number of nuclear targets of HOG pathway have been identified recently, only one cytoplasmic target has been discovered so far. Present study indicates that identification of cytoplasmic target/s of this pathway could unravel interesting facets of osmoadaptation process in yeast.