Protein Phosphorylation, a well known post translational modification of proteins is a primary means of mediating signal transduction events that control numerous cellular events through highly regulated and dynamic interplay of **kinases** and **phosphtases**. These enzymes from extremophiles have become a focus of increasing scientific concern these days with emanating novel discoveries regarding the mechanism of stress tolerance. Since stress tolerance mechanism is not very well understood from halotolerant yeasts, therefore the host selected for the present study was *D. hansenii*. The present study focuses on the Ser/Thr phosphatase class of enzymes, specifically the **P**hosp**h**oprotein **p**hosphatase (PPP) class from *D. hansenii*. For this PPP orthologs were looked in the genome of *D. hansenii* with the help of SMART (Simple Modular Architecture Research Tool) database (Schultz *et al.*, 2000). Nine protein sequences were identified and named as *DhGLC7*, *DhPPG1*, *DhPPQ1*, *DhPPH3*, *DhPPH21*, *DhCMP2*, *DhPPT1*, *DhPPZ1*, and *DhSIT4* respectively. The study describes the detailed molecular genetic characterization of *DhSIT4* in its native host *D. hansenii*. The DhSit4p showed 81.52% identity and 93.63% similarity with Sit4p from *S. cerevisiae*. With *Candida albicans* CaSit4p, it showed 94.58% identity and 99% similarity. For characterizing *DhSit4* gene further in its native host, *D. hansenii* mutant strain *Dhsit4Δ* carrying a deletion in *DhSIt4* gene was generated using homologous recombination. *Dhsit4Δ* mutant in *D. hansenii* resulted in slow growth phenotype. The number of cells at G1 phase of cell cycle was increased significantly in the absence of DhSit4p Sit4 plays an important role in cell cycle regulation in *S. cerevisiae* (Sutton *et al.*, 1991). This function of Sit4p is conserved in *D. hansenii* resulting in the slow growth phenotype of *Dhsit4Δ* mutant. Next, growth of DBH93 and *Dhsit4Δ* mutant on different carbon sources was exercised on agar plate and liquid media. The control strain DBH93, as well as the mutant *Dhsit4Δ*, grew well on

plates containing mono or oligo saccharides e.g. cellobiose, galactose, maltose, and raffinose. However, *Dhsit4Δ* growth on sucrose was slower both in liquid media and plate and this was due to lesser extracellular invertase activity of the *Dhsit4Δ* mutant. The mutant *Dhsit4Δ* also failed to grow on agar plates containing 2% ethanol, 3% glycerol and 1% succinate.

D. hansenii is one of the most halotolerant species of yeast. High amount of salt in the growth medium imposes not only the toxic effects of salt but also other types of stress. Therefore, the role of DhSit4p in tolerance towards toxic cations, and agents causing oxidative or cell wall stress was studied. Although *Dhsit4Δ* was observed to be highly sensitive to toxic cations it grew better on hygromycin B. In this regard, the behavior of *Dhsit4Δ* was quite distinct from *S. cerevisiae* mutant (Manlandro *et al.*, 2005). *Dhsit4Δ* strain was also sensitive to agents known to cause cell wall stress e.g. Calcofluor white and Congo red. From electron microcopy and fluorescent microscopy it was clear that the cell wall in the *Dhsit4Δ* mutant was highly amorphous and thin. These results indicated the role of DhSit4 in maintaining the cell wall integrity in *D. hansenii*. *Dhsit4Δ* was resistant to caffeine. Although caffeine is also a cell wall damaging agent, it acts through TORC1 kinase. In *S. cerevisiae*, Sit4p is a known modulator of TORC1 which is appeared to be conserved in *D. hansenii*. This was further corroborated by the lesser rapamycin sensitivity exhibited by *Dhsit4Δ*.

Like other yeasts, *D.hansenii* possesses a conserved signal transduction pathway comprising MAP kinase Slt2/Mpk1 plays a pivotal role by presenting the first line of defense under different physiological conditions (Minhas *et al.*, 2012). Therefore, the genetic interaction between CWI pathways and DhSit4p was

determined. Constitutive expression of DhSit4p under *DhTEF* promoter suppressed the sensitivity of *Dhmpk1Δ* cells towards toxic cations, congo red and caffeine, whereas the cells expressing DhSit4p from the native promoter could weakly and partially suppress the phenotypic defects of *Dhmpk1Δ* cells. Dosage dependence of this phenotypic suppression was further confirmed by observing the expression of DhSit4p-RFP fusion protein. The previous study from our laboratory showed that the over-expression of DhPpz1p could also suppress the phenotypic defect of *Dhmpk1*Δ cells (Minhas *et al.*, 2012). In this regard, the action of DhSit4p and DhPpz1p were observed to be very similar at the phenotype level. However, the underlying mechanism is not clear at present.

Earlier studies in *S. cerevisiae* suggested opposite roles for Sit4p and Ppz1p in growth and cell cycle regulation (Clotet *et al.*, 1999). Further, to understand the genetic interaction of DhSit4p with DhPpz1p. A double deletion mutant strain DBH938 (*Dhhis4 Dharg1 Dhppz1::DhHIS4 Dhsit4::DhARG1*) was generated. The double deletion clearly indicated that additional deletion in *DhPPZ1* abrogated the salt sensitivity as well as CR and CFW sensitivity of *Dhsit4* mutant. The effect of deletion of *DhPPZ1* gene on the growth defect of *Dhsit4*Δ mutant was also checked by flow cytometry. From this it was concluded that additional deletion of *DhPPZ1* abrogated the growth defect, hypersensitivity to the salt and cell wall destabilizing agents of *Dhsit4Δ* mutant and hence they act opposite to each other in maintaining cell cycle, cell wall biogenesis and salt stress. Similar results were observed from the expression of DhHal3p under a strong *DhTEF* promoter in *Dhsit4*Δ cells, which was equivalent to the deletion of *Dhppz1Δ*. These observations were good in agreement with a previous study from *S.cerevisiae* that shows similar results (Clotet *et al.*,

1999).From this study DhSit4p appears to be crucial for proper orchestration of cell cycle, cell wall integrity, anaerobic metabolism and a cross talk with major regulatory pathways such DhMpk1p/Slt2p in *D.hansenii*. All of these functions are being affected by DhSit4p in one or another way. However, to understand the role of DhSit4p in salt homeostasis a future investigation will be needed to establish effect of *Dhsit4Δ* with ion channels at transcript level including DhNHA1p, DhTrk1p and DhEna1p.

A previous study from the lab established that DhPpz1p and DhHal3p interact with each other and the only known regulator of DhPpz1p in *D. hansenii*. The study also identified a short Ser/Arg rich motif in the N-terminus non-catalytic region of DhPpz1p (Minhas *et al.*, 2012) however, the significance of this motif was still ambiguous. This motif appeared to be important for its role in salt tolerance but not in cell wall integrity pathway. Therefore, the second half of the present study identified the binding domain(s) between DhPpz1p and its regulator(s). Following this, the present study also identified the important structural determinants of interaction between DhPpz1p /DhHal3p and concluded the study by finding another novel binding partner of DhPpz1p in *D. hansenii*.

The present study first probed the recognition domains between DhPpz1p /DhHal3p .The methodology employed to decipher this was yeast two hybrid analysis and β-galactosidase assay which assessed the binding strength between interacting proteins. DhPpz1p is closely related to PPP1 type of phosphatases. Firstly, the structures of the DhPpz1p and DhHal3p were modeled utilizing templates **PDB ID: 1JK7** and **PDB ID: 1E20** respectively. The generated models did not correspond to full length proteins due to lack of any structural similarity by unique N-terminal

domain of DhPpz1p and highly acidic tail of DhHal3p. The models were generated from **SWISS-MODELLER** (Guex *et al.*, 1997; Comeau *et al.*, 2004; Kozakov *et al.*, 2013and Biasini *et al.*, 2014).Further, the reliability was evaluated using online software **PROCHECK.**

The most distinguished feature of DhHal3p is a highly acidic aspartic acid rich tail of this protein. Analysis from amino acid sequence of DhHal3p revealed that DhHal3p also possessed a mimic of "RVxF", the signature sequence motif **"KLHVL"**. This recognition motif is universally present in PP1c binding regulators and was also recognized in DhHal3p.Analysis from DhPpz1p amino acid sequence highlighted a serine, arginine and asparagine rich unusual non-catalytic N-terminus. The docking studies between the homology models of these two proteins from **CLUSPRO 2.0** (Comeau *et al.*, 2004; Kozakov *et al.*, 2013) marked a region spanning from **417-457** residues on DhPpz1p sequence, which represented a small helical domain in DhPpz1p. Further, various truncations of catalytic terminus of DhPpz1p was generated based upon **CLUSPRO 2.0** analysis and were tested with two hybrid analysis. The study revealed a **"hot spot of interaction"** between DhPpz1p and DhHal3p. This hot spot spanned from amino acid region **417-572** residues on DhPpz1p. Similarly, from DhHal3p, it was observed that region spanning from 1-462 are sufficient to recognize DhPpz1p. Furthermore, by site directed mutagenesis of this region, for the first time the study determined the essential role of **"hot spot of interaction"** between DhPpz1p and DhHal3p. Site directed mutagenesis revealed a significant loss in binding between mutant DhPpz1p***A**/DhHal3p. Interestingly, the mutations at positions D443A and D446A in DhPpz1p resulted in stronger binding capability of mutant DhPpz1p***B** as compared to the wild type

DhPpz1p with full DhHal3p (Where "*A" corresponds to the mutations at Asparagine 378 and Arginine 381 positions converted to alanine residues. In "*B", the Aspartic acid-443 and Aspartic acid-446 were mutagenized to alanine.). However, mutation in **hot spot** on DhPPZ1_ (417-572), the fragment lost its binding capability with DhHal3p. This analysis provided a clear evidence for the importance of D443 and D446 residues in DhPpz1p. On the other hand, mutating the C-terminal DhPpz1p constructs, a marginally weaker interaction between mutant DhPPZ1_ (279-572)*A with DhHal3p was observed. Site directed mutagenesis of DhHal3p revealed the importance of Arginine-338, Histidine-344, Arginine-348 and Arginine-349 residues on DhHal3p with DhPpz1p interaction. Together, these results illustrated the region of binding between these two proteins and essential structural determinants of interaction.

The DhPpz1p is the member of type 1 PPP class in *D.hansenii* and DhHal3p is the only known negative regulator of DhPpz1p. The DhHal3p recognizes and binds to catalytic terminus of DhPpz1p to mediate the salt tolerance in *D. hansenii*. However, no information about other physiological substrates was available in the literature. Therefore, to fill this gap we looked for probable conserved orthologs in *D. hansenii* genome similar to Ppz1p/Ppz2p regulators observed in *S. cerevisiae*. Venturi *et al.*, 2000 proposed that ScGlc7p, ScPPZ1p and ScPPZ2p share common interacting partner(s) such as ScGlc8p.Following this, Gene Bank accession number **XP_462343.1** was identified and named as *DhGlc8*. The amino acid sequence of DhGlc8p revealed a **"KGILK"** motif and a **"RKXHY"** motif, present in the amino acid sequences of DhGlc8p. This analysis concluded that DhGlc8p could be a possible DhPpz1p binding target in *D. hansenii*. Yeast two hybrid analysis revealed

that DhGlc8p could only recognize catalytic terminus of DhPpz1p and recognizes independent structural determinants other than Asparagine 378, Arginine 381, Aspartic acid 443 and Aspartic acid 446 on DhPpz1p which is still plausible. These results indicated that DhHal3p recognition, binding and docking motif(s) are non-overlapping with DhGlc8p in *D. hansenii*.

Next to identify the secondary binding sites at N-terminus of DhPpz1p, the N-terminal was further dissected. This analysis was based upon previous lab study which showed that the N-terminus of DhPpz1p in *D. hansenii*, contained few serine arginine rich amino acid stretches. These identified regions of low complexity contained six short sequence motifs. These motifs were deciphered as insignificant for cellular growth and integrity. Further we understood the significance of these serine arginine rich stretches in binding interactions with DhHal3p and DhGlc8p.The yeast two hybrid analysis of N-terminal deletion mutants named DhPpz1_NΔ1, NΔ2, NΔ3, NΔ4, NΔ5 and NΔ6 revealed that deleting NΔ3, NΔ4 and NΔ6 region corresponding to residues spanning 81-104, 106-120, and 241-254 strengthen the binding with DhHal3p. Furthermore, the N-terminal truncations devoid of catalytic domain such as DhPPZ1_(1-72) and DhPPZ1_(81-278) could recognize DhPpz1p_(279-572) whereas, DhHal3p could only recognize DhPPZ1_(81-278) construct of DhPpz1p in two hybrid analysis. In comparison NΔ2, NΔ5 and NΔ6 bind strongly with DhGlc8p. The contrasting binding associations between negative regulator DhHal3p and positive regulator DhGlc8p established the contribution of catalytic domain and regulatory N-terminus of DhPpz1p. The results from the present study for the first time demonstrate the secondary sites of binding over N-terminus of DhPpz1p, which was always speculated in other Ppz1p orthologs. The extensive study proposes a

nested hypothesis about the self-regulatory mechanism existing in DhPpz1p molecule based on homology modeling and protein-protein interaction studies. The study hypothesizes that DhPpz1p falls amidst of PPP1 and PP2A class of proteins structurally. It seems to be evolved from PP2A class of molecule where the protein had a scaffolding domain and an auto regulatory domain within the same molecule to dock with the regulatory proteins to form a heteromeric complex. The DhPpz1p could probably exist in a closed "flap like state" mediated by its N-terminus over catalytic terminus under absence of any stimulus. This closed form facilitates the binding with the regulators upon recognizing them. This associating brings specific conformational changes in DhPpz1p depending upon the target site of action or modulation.