SUMMARY

All living organisms, whether they are uni- or multicellular, actively interact with their surrounding environment and modulate their physiological status to maintain cellular homeostasis. This adaptation process is highly coordinated via diverse signaling pathways, that are regulated by a series of signaling components, including sensors/receptors, kinases, and transcription factors. Among fungi, a number of two component -like sensor histidine kinases are linked to various downstream signaling pathways to regulate various aspects of cellular physiology e.g. osmo-adaptation, hyphal development, virulence, sporulation (Yamada-Okabe *et al.*, 1999; Santos and Shiozaki, 2001; Nemecek *et al.*, 2006; Viaud *et al.*, 2006; Bahn *et al.*, 2007). Nearly all the fungal two component histidine kinases are hybrid histidine kinases (HHK) because the sensor histidine kinase and the receiver domains are present in a single polypeptide. Available genome sequence data suggest that fungi, in general, have a large repertoire of two component histidine kinases that are classified into eleven groups based on their domain architectures. The prime focus of the present study was the fungal histidine kinases that belong to group III. The group III HHK are ubiquitous in fungi except *S. cerevisiae* and *S. pombe* (Catlett *et al.*, 2003). Nik1p of *Neurospora crassa* (also known as os-1) (Alex *et al.*, 1996; Schumacher *et al.*, 1997), Daf1p of *Botryotinia fuckeliana (Botrytis cinerea)* (Cui *et al.*, 2002), Hik1p of *Magnaporthe grisea* (Motoyama *et al.*, 2005), CaNik1p of *Candida albicans* (Alex *et al.*, 1998) are few well known examples of this group. Group III HHK represented by Nik1p and its orthologs govern "high osmolarity stress response" in fungi, a function that they share with Group VI HHK represented by Sln1p. Group III HHKs are also shown to be involved in the hyphal development and virulence in few fungi. Moreover, they appear to be the target of different fungicidal agents (e.g. phenylpyroles, dicarboximides and aromatic hydrocarbons) as the cells harbouring mutations in these kinases exhibit resistance to these fungicides (Catlett *et al.*, 2003; Oshima *et al.*, 2006; Viaud *et al.*, 2006; Dongo *et al.*, 2009). Thus, Nik1 orthologs are a very important class of HHK in fungi. Group III orthologs contains 5-7 HAMP domain repeats at their N-terminus. Our current understanding about the role of HAMP domain derives from the single HAMP domains of membrane-bound prokaryotic sensor HK that connects transmembrane domain and cytoplasmic kinase output domain. In these transmembrane sensor kinases, signals from input extra-

cellular sensor modules induce conformational changes in the HAMP domain that either activate or inactivate the kinase activity of the output domain. Recent structural insight into the HAMP domain structure implied either helix rotation and/or alterations in their packing stability as the signaling mechanism across the membrane (Hulko *et al.*, 2006, Zhou *et al.*, 2009). However, HAMP repeat organization of group III HHK is quite different from HAMP domain of prokaryotic sensor kinases studied so far. Group III HHK are cytosolic proteins and lack transmembrane domains. Instead, they have a unique amino-terminus consisting of multiple HAMP domains in a single molecule. Various osmosensitive and fungicide resistant mutants implicate the involvement of HAMP domains as mediators of these responses in group III HHK. All these findings clearly suggested that the HAMP domain repeats are very important for the functionality of group III HHK. However, no systematic structure-function studies have been pursued among multiple HAMP containing proteins that could conclusively delineate the functional contribution of each HAMP domain in signaling. Therefore, we undertook the genetic approach to characterize the role of multiple HAMP domains in the regulation of two group III HHK members, DhNik1p from halotolerant yeast *Debaryomyces hansenii* and Hik1p from phytopathogenic filamentous fungus *Magnaporthe grisea*.

Previous genetic study on DhNik1p, a group III HHK from *D. hansenii* had established that each of the five HAMP domain repeats in DhNik1p were functionally distinct (Meena, 2009). Studies on single HAMP domain containing prokaryotic proteins have established that it exists as a dimer where the HAMP monomers interact to form a four helix bundle. Therefore, in the present study we examined whether the distinct role of the HAMP domains in DhNik1p originated due to any structural differences among these domains. To address this issue, yeast two hybrid assay was performed to study the interdomain interactions between the HAMP domain repeats. It was evident from the results that HAMP domains 1, 4 and 5 were capable of forming a dimeric four helix bundle structure whereas HAMP domains 2 and 3 did not exhibit interdomain interactions suggesting that they could be structurally different from the well established dimeric HAMP structure. In the previous study from our laboratory, it was demonstrated that HAMP4 domain was epistatic to HAMP5 domain in a DhNik1p mutant carrying deletion in the first three HAMP domains whereas in the wild type DhNik1, this effect was revoked due to the presence

of other HAMP domains. Therefore, as a part of this study, we also determined the intradomain interactions of HAMP domains 4 and 5 with each of the five HAMP domains individually to address whether the HAMP domain repeats also exhibit intramolecular interactions for regulating DhNik1p activity. The results indicated that HAMP4 domain was capable of interacting strongly with HAMP5 and HAMP2 domains whereas its interaction with HAMP domains 1 and 3 was relatively weak. In contrast, HAMP5 domain showed no interaction with HAMP1 and HAMP3, but strong interaction with HAMP4 and HAMP5. With HAMP2, it showed moderate interaction due to high sequence homology between HAMP2 and HAMP4. Taking these findings into consideration, it is plausible that in the wild type DhNik1, alternative interactions might occur among HAMP domain repeats intra-molecularly to regulate its activity *in vivo*. Further proof in this regard shall be obtained by solving the structure of DhNik1p in future.

Histidine kinases in general are known to function as homodimers. However, this has not been experimentally validated till date for any of the group III HHK. Genetic analysis done in an earlier study demonstrated that autophosphorylation of conserved histidine residues in DhNik1p occurred *in trans* indicating that it was also functional as a homodimer (Meena, 2009). However, conclusive evidence in this regard was lacking. Moreover, the region/s involved in dimerization of DhNik1p also remained undefined. To fill in this lacuna, we investigated whether DhNik1p exists as a homodimer *in-vivo* by using Co-immunoprecipitation assay (Co-IP). Co-IP studies with wild type DhNik1p established that it existed as a homodimer *in-vivo*. Next, we attempted to define the region essential for dimerization of DhNik1p. Bioinformatics analysis predicted HisKA (DHp) domain in DhNik1p as the putative homodimerization domain. However, deletion of the amino acid residues 480-555 encompassing the HisKA (DHp) domain in DhNik1p did not abrogate the dimerization of Δ480-555 mutant suggesting the involvement of additional regions in DhNik1p dimerization. As described in the review section, like HAMP domain, the DHp domain also has four-helix bundle architecture (Tanaka *et al.*, 1998; Marina *et al.*, 2005). The HAMP domains in histidine kinases appear to be linked to DHp domains through a continuous α-helix that comprises the AS2 α-helix of the HAMP domain and the AS1 α-helix of the DHp domain. Both the DHp and the HAMP domains are involved in the homo-dimerization of *E. coli* osmosensor EnvZ (Hidaka

et al., 1997). Also, a recent crystal structure of a HAMP-DHp fusion protein showed that the HAMP and DHp domains made up the cytoplasmic dimerization interface [Protein Data Bank (PDB) ID: 3zrx] (Ferris *et al.*, 2012). Therefore, we utilized a functional DhNik1p mutant ΔH1-4 (Δ53-383) lacking the first four HAMP domains (i.e. having only HAMP5 domain) to generate progressive truncations upto the HisKA domain as represented by the mutants Δ1-439, Δ1-455, Δ1-475, Δ1-500 and Δ1-555 that were subsequently studied for their dimerization ability. The results of the Co-IP assay indicated that like DhNik1p, the mutants' ΔH1-4, Δ1-439, Δ1-455, Δ1-475 and Δ1-500 also existed as homo-dimers *in-vivo*. However, dimerization was abrogated in the mutant Δ1-555 lacking both HAMP5 and HisKA domains, indicating that both these domains most likely comprised the dimerization interface in DhNik1p. However, from these results, the possibility of involvement of other HAMP domains in homo-dimerization of DhNik1p could not be excluded. Inability of the Δ1-455, Δ1-475 and Δ1-500 mutants to demonstrate phenotypic complementation in *S. cerevisiae* strain NM1 on 5-FOA led to another important insight that the connector and AS2 α-helix segments of HAMP5 domain were crucial for the stability of DhNik1p homodimer.

Another aspect of this study focussed on understanding the role of HAMP-like linkers in the functionality of DhNik1p. As mentioned in the results section, the HAMP domain repeats are flanked by linkers regions that exhibit HAMP-like sequence features. DhNik1p has five HAMP domains and four HAMP-like linkers within its N-terminus. So, we investigated the functional contribution of each of the HAMP-like linker regions by deleting or replacing specific linkers with flexible glycine rich peptides of length five (-GGGGS) or fifteen $(GGGGS)_3$ and gauging their effects on the DhNik1p kinase function in *S. cerevisiae* strains NM1 and AMY1000. In the absence of any stress (hyperosmolarity or fungicides), the DhNik1p exists in kinase-on default state in *S. cerevisiae*. Functional analysis of the mutants by phenotypic complementation assay indicated that irrespective of deletion or replacement of HAMP1b, HAMP2b or HAMP3b linkers, DhNik1p was still a functional kinase whereas deletion of HAMP4b linker led to the kinase-off state. This assessment was substantiated by the ability of the linker mutants E-AΔ1b and L5Δ1b to negatively regulate the HOG pathway in *S. cerevisiae* in the absence of *SLN1*. In contrast, cells expressing E-AΔ2b, E-AΔ3b or L5Δ2b, L5Δ3b, L15Δ1b, L15Δ2b or

L15Δ3b mutants exhibited slightly elevated basal phosphorylated Hog1p levels suggesting that the histidine kinase and phosphotransfer activities could be partially affected in these mutants whereas, strong induction in phosphorylated Hog1p levels in E-AΔ4b, L5Δ4b and L15Δ4b mutants confirmed their kinase-off status.

The heterologous expression of group III HHK confers sensitivity to fludioxonil on *S. cerevisiae* cells presumably through the over-activation of HOG pathway. Our earlier work showed that like a typical member of group III HHK, fludioxonil inhibits DhNik1p resulting in a kinase-off state. When the linker mutants were analysed for their fludioxonil sensitivity in AMY1000 strain, only E-AΔ1b and L5Δ1b mutants showed sensitivity to fludioxonil whereas E-AΔ2b, E-AΔ3b, L5Δ2b, and L5Δ3b mutants exhibited fludioxonil resistance growth phenotype. Moreover, these fludioxonil resistant mutants maintained slightly elevated basal phosphorylated Hog1p levels irrespective of fludioxonil treatment. Therefore, a *lacZ* reporter system was employed to assess the *in-vivo* kinase activity of HAMP-like linker deletion and replacement mutants (E-AΔ1b, E-AΔ2b, E-AΔ3b, L5Δ1b, L5Δ2b, and L5Δ3b) and how their activity was modulated by fludioxonil treatment. It was interesting to find out that deletion or replacement of linker HAMP1b marginally increased the kinase activity of E-AΔ1b and L5Δ1b mutants compared to DhNik1p. HK activity of both the mutants was inhibited by fludioxonil treatment, although to a lower extent in L5Δ1b mutant. On the other hand, HAMP2b linker mutants E-AΔ2b and L5Δ2b possessed only partial kinase activity in comparison to DhNik1p. However, unlike DhNik1p, kinase activity of both E-AΔ2b and L5Δ2b mutants remained unaffected upon fludioxonil treatment. Similarly, the mutants E-AΔ3b or L5Δ3b had compromised kinase activity, which was not diminished by fludioxonil treatment. All these results indicate that HAMP-like linkers HAMP2b and HAMP3b play a pivotal role in regulation of DhNik1p kinase activity whereas HAMP-like linker HAMP1b seems dispensable. On the other hand, as indicated by functional analysis, deletion of HAMP-like linker 4b mutant imposed a restrain on the signaling by locking DhNik1p in a kinase-off state.

Both HAMP and alternating HAMP-like linkers exhibit heptad repeat patterns. It is intriguing that there is a break of +4 (or -3) residues, referred as stutters between the repeating HAMP-like linkers and the adjacent HAMP units. Presence of stutter elements is a conserved feature in group III HHK. DhNik1p contains four stutter

elements, one following each 90 residue repeat unit designated as 1a, 2a, 3a and 4a. These stutters/ helical mismatches are proposed to be essential for signal transduction by HAMP domains to control output domain activity in single HAMP and poly-HAMP containing bacterial chemoreceptors and sensor HK (Zhou *et al.*, 2009; Stewart and Chen, 2010; Airola *et al.*, 2010). According to the dynamic bundle hypothesis for HAMP domain mediated signal propagation, a helical mismatch could generate an inverse relationship between packing of HAMP and the kinase-control output module. That is, tight *x-da* packing in HAMP might loosen *a-d* packing in the kinase-control module to inhibit the downstream histidine kinase activity and *vice-versa* (Zhou *et al.*, 2009). Recently, a stutter discontinuity in the heptad repeat pattern was found to be essential for response to signal in *E.coli* nitrate sensor NarX (Stewart and Chen, 2010). Therefore, we determined whether these helical mismatches were similarly involved in mediating signal propagation through the HAMP repeats in DhNik1p, as has been proposed for prokaryotic HAMP-containing proteins. To this end, we constructed four-residue deletions, designated as **Δ1a** (149-152aa; LTNQ), **Δ2a** (241-244aa; LTTQ), **Δ3a** (333-336aa; LTNQ) and **Δ4a** (425-428aa; LTSQ) in DhNik1p. One deletion was made per molecule to remove the associated stutter, thus resulting in a continuous heptad repeat pattern from the HAMP-like AS2 helix to the AS1 helix of the following HAMP domain. Deletion of either 1a, 2a or 3a stutter elements in DhNik1p did not affect its ability to complement *sln1* function in both NM1 and AMY1000 strains indicating that the mutants were functional; however, Δ4b mutant was non-functional as it could not grow on the selection plates. Fludioxonil sensitivity was also determined for the functional stutter mutants after heterologous expression in *S. cerevisiae* strain AMY1000. Δ2a and Δ3a mutants exhibited fludioxonil resistant phenotype in contrast to the Δ1a mutant that retained the fludioxonil sensitive phenotype of DhNik1p. The functional mutants were also analysed for their ability to regulate the HOG pathway both in the absence and presence of fludioxonil. Compared to untreated cells, phosphorylated Hog1p appeared in fludioxonil treated cells expressing DhNik1p or Δ1a mutant, indicating that these kinases were inhibited by fludioxonil. However, phosphorylated Hog1p was not observed in the fludioxonil treated cells expressing Δ2a or Δ3a mutants reconfirming that these kinases were not sensitive to fludioxonil. When the *in-vivo* kinase activity of stutter mutants (Δ1a, Δ2a, Δ3a and Δ4a) was determined by β-galactosidase assay,

it revealed that Δ1a mutant was an overactive kinase but could be inhibited by fludioxonil. However, Δ2a and Δ3a mutants had only ~25% kinase activity in comparison to DhNik1p and were not affected by fludioxonil. On the other hand, Δ4a was a kinase-off mutant. Overall, the analysis showed that only the heptad discontinuity lining the HAMP-like linker 4b and HAMP5 domain in DhNik1p was essential for its function whereas deletion of other heptad discontinuities did not reverse the signaling state. This indicates that the stutter mediated alternating packing states between the successive HAMP and HAMP-like linker domains for delivering opposing conformational signals to the kinase domain may not be a feasible mode of signal transduction in DhNik1p. Another crucial insight gained from the analysis of HAMP-like linker and stutter deletion mutants points out that fludioxonil mediated inhibition of DhNik1p kinase activity is centred on HAMP-like linker domains 2b and 3b.

In the second part of the study, we carried out functional characterization of Hik1p, a six HAMP containing group III HHK from filamentous ascomycete *Magnaporthe grisea*. Previously, heterologous expression of Group III HHK DhNik1p was shown to rescue the lethality of *sln1* deletion in *S. cerevisiae*. So, we investigated whether Hik1p was also proficient in complementing *sln1* function in *S. cerevisiae*. We found that *HIK1* could complement *sln1* function in *S. cerevisiae* strains NM2 and AMY1000 carrying different *sln1* mutations. *SLN1* functions as an osmosensor in the HOG pathway in *S. cerevisiae*, therefore, we determined whether Hik1p also behaved as an osmosensor to regulate the HOG pathway in *sln1* background. For this, we checked the phosphorylated Hog1p levels in AMY1000 cells expressing Hik1p in the absence and presence of 0.5M NaCl shock. Phosphorylated Hog1p was not observed in the absence of osmotic shock, compared to the appearance of a strong phosphorylated Hog1p band within 5 min of osmotic shock that gradually returned to near basal levels indicating adaptation to osmostress. These findings indicated that like Sln1p, Hik1p was also an osmosensor that negatively regulated the HOG pathway in *S. cerevisiae*.

The presence of HAMP domain repeats is a characteristic feature of the group III HHK. Group III HHK from yeasts possesses five HAMP domain repeats whereas filamentous ascomycetes and basidiomycetes harbour six and seven HAMP domain repeats respectively. The role of these domains was not studied in any of the six

HAMP containing group III HHK. Therefore, the role of HAMP domain repeats in the functionality of Hik1p was studied. Hik1p amino terminus contains six HAMP domains that are interlinked via adjacent HAMP-like linker domains. To determine the influence of individual HAMP domains on Hik1p regulation, we constructed a series of mutants in which the HAMP domains were either individually or progressively deleted from the amino terminus. These individual HAMP domain deletion mutants ΔH1, ΔH2, ΔH3, ΔH4, ΔH5 and ΔH6 as well as sequential deletion mutants ΔH1-2, ΔH1-3, ΔH1-4, ΔH1-5 and ΔH1-6 were analyzed for phenotypic complementation in *S. cerevisiae* strains NM2 and AMY1000 as well as checked for their ability to regulate the HOG pathway in AMY1000 strain. Mutants with individual HAMP domain deletions indicated that HAMP domains 1 and 6 were indispensable for Hik1p function as these mutants were non-functional. Surprisingly, deleting HAMP domain 2, 3, 4 or 5 still resulted in functional mutants as they could complement *sln1* function and prevented the activation of the HOG pathway as phosphorylated Hog1p was not detectable in these mutants. Interestingly, the mutants carrying deletion in two or four HAMP domains were non-functional whereas deletion of three or five HAMP domains resulted in functional alleles. To our surprise, ΔH1-6 mutant lacking all six HAMP domains also exhibited phenotypic complementation. Further removal of five additional residues in the ΔH1-6 mutant (ΔH1-6') resulted in a non-functional allele highlighting the importance of this coiled-coil extension in regulating Hik1p activity. Overall, the results revealed that besides HAMP domain 1 or 6, the deletion of HAMP domains 1 and 2 (ΔH1-2) or HAMP domains 1 to 4 (ΔH1-4) also adversely affects Hik1p activity and therefore are crucial for Hik1p activity whereas HAMP domain 3 (ΔH1-3) and 5 (ΔH1-5) seem to be override this effect suggesting their antagonistic role.

As already mentioned, Hik1p contains alternating HAMP and HAMP-like linker domains within its amino terminus and each HAMP plus HAMP-like linker pair comprises a 92-residue repeat unit. Therefore, Hik1p contains five such tandem 90-residue repeat units at its amino terminus. We also investigated the functional contribution of each HAMP-like linker and 92-residue repeat unit by deleting them one at a time and checking their phenotypic complementation in *S. cerevisiae* strains NM2 and AMY1000 as well as HOG pathway regulation in AMY1000 cells. HAMP-like linker mutants ΔH2a, ΔH3a or ΔH4a complemented *sln1* function whereas ΔH1a

or ΔH5a failed to do so. Absence of detectable phosphorylated Hog1p in ΔH2a, ΔH3a or ΔH4a mutants reconfirmed their functional status. However, ΔH1a or ΔH5a mutants were unable to prevent HOG pathway activation as they were kinase-off mutants. 92-residue repeat unit mutants' Δ1+1a, Δ2+2a, Δ3+3a, Δ4+4a and Δ5+5a were similarly analyzed. It was astounding to observe that the mutants Δ1+1a, Δ2+2a, Δ3+3a and Δ4+4a were non-functional whereas the mutant Δ5+5a retained the wild type Hik1p function. Collectively, these results established that irrespective of the variation in the number of HAMP domain repeats in DhNik1p or Hik1p, the penultimate HAMP and associated HAMP-like coiled coil extension negatively regulate their activity.

Finally, Hik1p mutants were analyzed for their fludioxonil susceptibility in AMY1000. Unlike Hik1p, the functional HAMP domain deletion mutants (ΔH2, ΔH3, ΔH4, ΔH5, ΔH1-3, ΔH1-5 and ΔH1-6) conferred fludioxonil resistant phenotype although they suppressed growth defect of *sln1* mutation in the earlier assay. Among the functional HAMP-like linker deletion mutants, only ΔH2a mutant conferred fludioxonil sensitive phenotype on the host strain AMY1000 whereas ΔH3a and ΔH4a mutants exhibited fludioxonil resistant phenotype. However, the lone functional 92-residue repeat unit mutant Δ5+5a showed fludioxonil sensitive phenotype. On the other hand, as expected, AMY1000 cells harbouring any of the non-functional Hik1p mutants did not exhibit sensitivity to fludioxonil. β-galactosidase activity assay was also carried out with the Hik1p functional mutants to measure their *in-vivo* kinase activity and to gain insights into their fludioxonil induced phenotypes. Irrespective of the domain/s deleted, all Hik1p functional mutants (which included individual HAMP domain or HAMP-like linker deletion mutants as well as 92-residue repeat unit mutant Δ5+5a) had ~ 5.0-7.0 fold elevated kinase activity compared to Hik1p. Except ΔH2a and Δ5+5a mutants that had highly compromised kinase activity, rest of the mutants retained still retained more than 50% kinase activity upon fludioxonil treatment. The assay thus provided an evidence that the fludioxonil resistant phenotype is conferred by the Hik1p mutants that completely or partially lack a negative regulation of their HK activity.