SUMMARY

In fungi, the group III hybrid histidine kinases (HHK) regulate osmoadaptation, hyphal growth, morphogenesis, conidia formation and virulence (Bahn *et al.*, 2006). They are widely found among different fungal genera and are also called NIK1 orthologs. NIK1 orthologs act as cytosolic osmosensors upstream of the HOG pathway in fungi and are molecular targets for antifungal agent fludioxonil (Catlett *et al.*, 2003). They typically have HAMP domain repeats at the NH_2–terminus that are important for their activity. Interestingly, the numbers of HAMP domain vary among the othologs from different genera. The orthologs from basidiomycetes harbour seven HAMP domains whereas those from yeast contain five HAMP domains.

Present study is the first of its kind to understand the role of individual HAMP domain in a seven-HAMP module. Since the functional characterization was carried out in *S. cerevisiae* host, a chimeric yeast-like group III HHK having a seven-HAMP module was constructed by swapping five-HAMP module by seven-HAMP module of Tco1p in DhNik1p. *S. cerevisiae* strain expressing DhNik1-Tco1 showed phenotypic complementation of *sln1-ts* mutation at 37°C and exhibited fludioxonil sensitivity to *S. cerevisiae*. Several HAMP deletion mutants of seven-HAMP module were made and the contribution of each HAMP domain was determined in the functionality of DhNik1-Tco1. The phenotypic analysis of the deletion mutants showed that both ΔH6 and ΔH1-6 were functional and exhibited resistance to fludioxonil. This showed that the HAMP6 domain was not essential for kinase activity of DhNik1-Tco1. Moreover, fludioxonil did not inhibit the activity of these mutants, unlike DhNik1-Tco1. It seemed that DhNik1-Tco1 became constitutively active kinase in its absence. HAMP6 domain in DhNik1-Tco1, therefore, appeared to be crucial for the regulation of its kinase activity under environmental stress and fludioxonil treatment. The function of the HAMP6 domain in DhNik1-Tco1p was found to be analogous to that of the

fourth HAMP domain in DhNik1p. Thus, the negative regulation by the penultimate HAMP domain appeared to be a common, evolutionarily conserved theme in group III HHK.

ClNik1p has a unique N-terminal region with five HAMP domains and four HAMP like linkers. In order to examine whether the similar mechanism as was found in case of DhNik1p, also exists in NIK1 orthologs from pathogenic yeast, functional study of HAMP deletion mutants of ClNik1p was done in *S. cerevisiae*. *ClNIK1* gene was cloned and expressed in *S. cerevisiae*. Heterologous expression of *ClNIK1* was able to complement the *sln1* deletion in *S. cerevisiae* by maintaining the Hog1p phosphorylation at basal level under normal growth conditions. ClNik1p was also found to respond to change in osmolarity of the growth medium. Hence, Clnik1p negatively regulated the HOG pathway and established as bonafide osmosensor in *S.cerevisiae*. Sln1p negatively regulates the HOG pathway through a phosphor-transfer protein Ypd1p in *S. cerevisiae*. NIK1 orthologs has also been thought to function through Ypd1p in *S. cerevisiae*. Through yeast two hybrid assay ClNik1p was shown to interact with Ypd1p, though with lesser affinity as compared to Sln1p.

To illustrate the role of the HAMP domains in the kinase activity of ClNik1p, HAMP domain deletion mutants were constructed and their ability to complement *sln1*deletion in *S. cerevisiae* was examined. It was observed that HAMP1, HAMP2, HAMP3 and HAMP5 domains were essential for the activity of ClNik1p as deletion of any one of them resulted in a non-functional allele. Phenotypic analysis showed that ΔH4 and ΔH1-4 retained histidine kinase activity. Like DhNik1p, the fourth HAMP domain of ClNik1p was found to be not essential for the kinase activity, but had regulatory role. The kinase activity of ClNik1p was regulated negatively by the fourth HAMP domain.

Bioinformatic analysis of ClNik1p by traditional SMART analysis predicted four linkers with discrete boundaries whereas HMM analysis predicted four HAMP like linkers having overlapping boundaries with adjacent HAMP domains. In the case of prokaryotic

soluble receptor Aer2, HAMP like linkers were shown to have structural similarity with HAMP domain. It was not clear whether the linkers regions in ClNik1p functions as only connector or participate in signal conversion. The linkers a, b or c were dispensable as the deletion of these linkers did not abolish the kinase activity of ClNik1p. The replacement of these linkers with Gly-rich flexible peptide sequences also had the similar effect which indicated that the structural integrity of a, b and c linkers were not essential for its function. In contrast, the linker present between HAMP4 and HAMP5 was found to be indispensable for the kinase activity of ClNik1p. Our study also showed that difference in boundaries had no significant role in the functionality of ClNik1p. Moreover, the linkers a, b and c could be functioning only as connector and HAMP like structural feature of the linkers was not essential for the activity of ClNik1p.

Functional studies of ClNik1p in *S. cerevisiae* also shed some light on its role in the antifungal activity of fludioxonil. Heterologous expression of *ClNIK1* rendered *S. cerevisiae* cells sensitive to fludioxonil. Further, sensitivity to fludioxonil was conferred in a dominant negative manner as wild type BY4741 with functional Sln1p was also found to be sensitive to it. Like previous studies we have also observed that fludioxonil induced phosphorylation of Hog1p. However, the activation kinetics of Hog1p was quite distinct from that observed with osmostress. We have observed that Hog1p was phosphorylated within five minutes of fludioxonil treatment and remained activated even after twelve hours. In the case of high osmolarity stress, there was an initial outburst of Hog1p phosphorylation within five minutes which gradually decreased over within a short period of time (depending upon the magnitude of stress). However there was no such outburst of Hog1p phosphorylation noticed in case of fludioxonil and the level of Hog1p phosphorylation remained same. So the activation of the HOG pathway in response to fludioxonil treatment was different both in magnitude and duration from that observed with osmotic stress. Further we wanted to check whether the

fludioxonil activated Hog1p translocated to nucleus to elicit the adaptive response. For this, Hog1p-GFP localization was observed in presence of fludioxonil and osmotic shock in BY4741/pClNik1. It was found that unlike nuclear translocation of Hog1p within five minutes upon osmotic shock, Hog1p was not localized to nucleus upon fludioxonil treatment even after three hours. Hence the fludioxonil activated Hog1p behaved differently from the way it acted in case of osmotic shock and cytoplasmic role of Hog1p could be more significant than nuclear role in conferring fludioxonil sensitivity.

Phospho-transfer reactions between Sln1p and Ypd1p have been studied extensively in *S. cerevisiae* (Kaserer *et al.*, 2009). Sln1p-Ypd1p interaction was found to be transient and reversible whereas Ypd1-Ssk1p interaction was stable and irreversible. This was necessary for keeping the Ssk1p in inactive form. Next we hypothesized that the inhibition of Clnik1p by fludioxonil decreased the intracellular pool of phosphorylated of Ypd1p. For this, we checked the level of phosphorylated Ypd1p under normal growth conditions and in presence of fludioxonil. Phosphorylated fraction of Ypd1p was separated from unphosphorylated fraction using phos-tag acrylamide gel and surprisingly it was observed that there was no major difference in the phosphorylated fractions of Ypd1p under normal conditions as well as upon fludioxonil treatment. To understand further the mode of action fludioxonil, genome wide screenings like deletion sensitivity profiling (DSP) was undertaken.

The availability of genome wide deletion mutant collection of *S. cerevisiae* allows the systematic analysis of their phenotypes in a given set of conditions to understand drug-gene interactions. In this study, we exploited *S. cerevisiae* model to understand the mode of action of fludioxonil and generated DSP for fludioxonil using complete set of yeast haploid deletion mutants. In order to carry out deletion sensitivity profiling for fludioxonil, a dose of fludioxonil that causes 10-20% decreased growth rate on wild type *S. cerevisiae* having ClNik1p was determined and 0.76 µg/ml fludioxonil was found to be optimum which

inhibited the growth of BY4741/pClNik1 by 10-20%. Each deletion strain (BY4741 genetic background) was individually transformed with plasmid pClNik1 in 96 well plate formats. The growth pattern of transformants from each deletion strain was evaluated in the presence of 0.76 µg/ml fludioxonil. Deletion sensitivity profiling for fludioxonil identified 116 genes whose deletion caused hypersensitivity to fludioxonil in *S. cerevisiae* harboring ClNik1p. Web based tools like Func Associate 2.0 and GO Term Slim Mapper were used to analyze this list of genes. Analysis by Func Associate highlighted two processes: cytokinesis and secretory pathway mainly vacuole fusion. GO term Slim Mapper also suggested that the genes involved in endosomal transport, secretory pathway and cytokinesis were over represented in this list.

We next investigated the effect of fludioxonil on the secretory pathway, vacuolar morphology and cytokinesis by examining the localization of Sec13-GFP, Chc1-GFP and Hof1-GFP. Sec13p is structural component of COP II vesicles responsible for transfer of protein cargo from ER to Golgi. So Sec13p is generally found at ER-Golgi interface under normal growth conditions. However, Sec13p was found to be scattered all over cytoplasm in the form of brighter puncta. Detailed analysis of Sec13p would further shed light on the reason of its altered localization upon fludioxonil shock.

Chc1p (Clathrin Heavy Chain) forms the structural component of clathrin which is involved in vacuolar protein sorting and trafficking, and endocytosis (Costaguta *et al.*, 2001). So Chc1p is mainly found at TGN. Although we didn't find any significant difference in the localization pattern of Chc1p-GFP upon fludioxonil treatment, there was twofold increase in its fluorescence intensity upon fludioxonil shock in *S. cerevisiae* harboring ClNik1p. Increase in intensity of Chc1-GFP might be due to accumulation of clathrin coated vesicles at TGN interface. Vacuolar protein sorting and trafficking might be arrested in presence of fludioxonil in yeast harboring ClNik1p.

Effect of fludioxonil on endocytic pathway and vacuole morphology in *S. cerevisiae* having ClNik1p was also examined by FM4-64 staining. Endocytosis was found to be as efficient in fludioxonil treated yeast cells as in normal growth conditions. However, vacuolar morphology was altered to a great extent upon fludioxonil treatment. Most of the budded and unbudded cells were found to have highly fragmented vacuole. FM4-64 staining results together with the localization results suggests that fludioxonil might cause fusion defect in the endosomes and vesicles involved in vacuolar protein sorting and vacuole itself.

There must be some interaction between Hog1p and proteins of secretory pathway as fludioxonil sensitivity is caused by Hog1p activation. Search of BIOGrid database showed genetic interactions of Hog1p with Vps9p, Vps24p and Scs7p those were known to be involved in secretory pathway. Detailed studies are needed to determine that whether these proteins are downstream effectors of Hog1p and bring about changes in the secretory pathway in response to the activation of the HOG pathway.

DSP for fludioxonil also revealed that cytokinesis might also get affected in yeast harboring ClNik1p. To check this, we did localization studies of Hof1p, a protein involved in cytokinesis. Hof1p was also one of the fludioxonil hypersensitive genes. Hof1p is generally localized at bud neck during late anaphase and is degraded at the onset of new cell cycle (Vallen *et al.*, 2000). However, we found that Hof1p-GFP was not localized at bud neck upon fludioxonil treatment and Hof1-GFP appeared to be scattered in the cytoplasm away from bud neck. Together these results indicate the impairment of secretory pathway and cytokinesis in yeast upon fludioxonil treatment. Thus fludioxonil targets multiple cellular processes to exert its antifungal activity.