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The sixth HAMP domain negatively regulates the activity of the group III HHK containing seven HAMP domains

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ABSTRACT

In fungi, the group III hybrid histidine kinases (HHK) act as important sensors to regulate osmoadaptation, hyphal growth, morphogenesis, conidia formation and virulence. They are molecular targets for antifungal agent fludioxonil. 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 orthologs from different genera. The orthologs from basidiomycetes harbor seven HAMP domains whereas those from yeast contain five HAMP domains. In order to understand the functioning of a seven-HAMP module, we have constructed a yeast-like chimera DhNik1–Tco1 containing seven HAMP domains. The functional characterization of this chimera in yeast *Saccharomyces cerevisiae* showed that the sixth HAMP domain played important regulatory role. Our results indicated that the negative regulation of histidine kinase activity by the penultimate HAMP domain could possibly be an evolutionarily conserved theme in the group III HHK containing different lengths of poly HAMP module.

1. Introduction

Genomes of fungi harbor large repertoire of two component hybrid histidine kinases (HHK) that are used to sense the fluctuations in the extracellular and the intracellular conditions. They are called hybrid histidine kinase because their histidine kinase domain and the receiver domain are fused in a single polypeptide [1–3]. Based on the domain organization, the fungal HHKs are classified into 11 groups [1]. HHKs are absent in the genomes of human and animals and therefore they are considered as ideal molecular targets for antifungal agent [2,4]. In fact, few antifungal agents e.g., fludioxonil and iprodione that are widely used in agriculture, target group III HHK [5]. Group III HHK constitutes an important signaling component in fungi that regulates osmo-adaptation. *NIK1* of *Neurospora crassa* was the first member of this group to be identified and the deletion of *NIK1* conferred osmosensitivity to the host [6,7], Subsequently, *NIK1* orthologs were also identified in many species [8–11].

The members of the group III HHK have been shown to regulate the HOG pathway, a MAP kinase pathway that mediates the osmotic and the oxidative stress responses in yeast and fungi [12–14]. The HOG pathway is regulated by the upstream HHK through a multi-step phosphor-relay mechanism. In *Saccharomyces cerevisiae*, this pathway is regulated by Sln1p which is surprisingly the lone HHK in this organism. Sln1p belongs to the group VI HHK and it regulates the HOG pathway negatively through a phosphorelay mechanism involving Sln1p-Ypd1p-Ssk1p [15–16]. Group III HHK complements *sln1* mutation in *S. cerevisiae* and also regulates the HOG pathway negatively through Ypd1p and Ssk1p [17–18]. Besides their role in the osmoadaptation, the members of the group III HHK are also involved in regulating hyphal growth, morphogenesis, conidia formation and virulence in fungi [19–22].

The members of the group III HHK are cytosolic proteins and the mechanism through which they sense and respond to the changes in the external osmolarity remained unclear. The presence of HAMP domain repeats in the NH_2-terminal region is a unique feature of this group of proteins. HAMP domains are composed of ~50 amino acid residues with two amphipathic helices joined by a connector [23,24]. HAMP domains are quite common in proteins such as histidine kinases, adenyl cyclases, methyl accepting chemotaxis proteins (MCP) and phosphatases that are involved in the signaling in prokaryotic organisms [24]. Many MCPs contain a single HAMP domain connecting the transmembrane domain and the cytosolic kinase control module. In MCP, this domain mainly functions as a signal conversion module [24]. HAMP domain repeats play an important role in the functionality of the group III HHK. Many osmosensitive and fludioxonil resistant mutants of *N. crassa* harbored point mutation in the HAMP domain repeats of Nik1p [25,26]. The regulatory role of the HAMP domain repeats were further elaborated in a recent study with a group III HHK (DhNik1p) from halotolerant yeast *Debaryomyces hansenii*. DhNik1p contained five HAMP domains which were functionally distinct and the positions and the arrangements of the HAMP domains were important

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for the activity of DhNik1p in vivo [17]. Most importantly the fourth HAMP domain negatively regulated the activity of DhNik1p in response to high osmolarity as well as the antifungal agent fludioxonil [17,18]. Although the HAMP domain repeat is a common feature among the group III HHKs, the number of repeat units varies across the genera [17,26]. For example, Nik1 ortholog Tco1p from basidiomycetes *Cryptococcus neoformans* which is a human pathogen, has seven HAMP domain repeats [14]. Tco1p functions as sensor kinase upstream to the HOG1 MAPK pathway in *C. neoformans*. It also controls virulence and sensitivity to the antifungal agent fludioxonil in this organism. The role of individual HAMP domain in the functionality of Tco1p is not known. It is also not clear how the poly HAMP unit of Tco1p which contains seven HAMP domains regulates the kinase activity in response to osmostress or the antifungal agent fludioxonil. To address this, we have carried out structure–function analysis of a chimeric group III HHK (DhNik1–Tco1p) containing the seven HAMP domains of Tco1p. Our result showed that the sixth HAMP domain played important regulatory role and in the absence of this domain DhNik1–Tco1p behaved like a constitutively active kinase which was unresponsive to fludioxonil. Thus, the negative regulation of the histidine kinase activity by the penultimate HAMP domain could possibly be a common theme in the group III HHK containing poly HAMP module of different length.

2. Materials and methods

2.1. Yeast strains and growth conditions

S. cerevisiae strains RJ1428 (*MATa sln1Δ::LEU2 his3-Δ200 leu2Δ1 ura3–52 trp1Δ63* with pRS-*PTP2 URA3*) [27] and NM2 (*MATa ura3 leu2 his3 sln1-ts4 ste11Δ::HPH*) [17] were used in this study. The media used were YPD (1% yeast extract, 2% peptone, 2% dextrose) and SD minimal medium (2% glucose and 0.67% yeast nitrogen base without amino acids) (Difco). Only supplements that were required by the strains were added.

2.2. Plasmid construction

DhNik1–Tco1 chimera was constructed by replacing the five-HAMP module of DhNik1p with the seven-HAMP module from Tco1p. It was constructed by combining three PCR fragments using overlap extension PCR [28]. Firstly, a 156 bp fragment corresponding to the NH_2-terminal region (preceding the five-HAMP module) of DhNik1p was amplified using forward AR5 (5′-GGGAATTCCATATGATGGGTACACCCGAATTAATG-3′) and reverse AR2 (5′-GAGAAGTTCATTGGCCGACTTGGAGGTATC-3′) primers. In the second PCR, a 1820 bp fragment corresponding to the seven-HAMP module of Tco1p was amplified from plasmid pTco1 [14] using forward AR1 (5′-CGGCCAATGAACTTCTCAAAGCTCAAGTTC-3′) and reverse AR3 (5′-GGTGTTTCTCTCGATGGACTCTCTGAGATTG-3′) primers. The third PCR was done to amplify the hybrid histidine kinase region comprising of 1926 bp from pDhNik1 using AR4 (5′-CCATCGAGAGAAACACCGCTGCAAGGG-3′) and AR-8 (5′-ACGCGTCGACTCAATCAAGATATTCAATCCTTGG-3′) as forward and reverse primers respectively. The three PCR products were taken as template for the subsequent overlap extension PCR to create DhNik1–Tco1 chimera using the forward AR5 (5′-GGGAATTCCATATGATGGGTACACCCGAATTAATG-3′) and the reverse AR-8 (5′-ACGCGTCGACTCAATCAAGATATTCAATCCTTGG-3′) primers respectively. The amplified PCR product was initially cloned in pGEM5Z vector at Sma1 restriction site to obtain pGEM-DhNik1–Tco1. The positive clone was confirmed by DNA sequencing. Finally, the 3.9 kb Nde1/Sal1 fragment from pGEM-DhNik1–Tco1 corresponding to the ORF encoding DhNik1–Tco1 (1300 aa) was cloned in plasmid pRS423 [29] along with the promoter of DhNik1 to obtain pDhNik1–Tco1. pRS423 is a multicopy yeast episomal vector with 2μ ori and *HIS3* marker.

Seven deletion mutants were generated using overlap extension PCR strategy, wherein individual HAMP domains were deleted. These mutants were named as ΔH1, ΔH2, ΔH3, ΔH4, ΔH5, ΔH6 and ΔH7. Another set of six different truncated constructs were generated where the HAMP domains were deleted sequentially such that the number of thesedomains were serially reduced (ΔH1–2, ΔH1–3, ΔH1–4, ΔH1–5, ΔH1–6 and ΔH1–7). For ΔH1, a ~2.3 kb fragment which carried a deletion in HAMP1 domain was made by overlapping extension PCR using mutagenic forward (5′- CAAGTCGGCCAATGAAGTCGAGCGTGTGTCTTTGG-3′) and reverse (5′- CGCTCGACTTCATTGGCCGACTTGGAGGTATC-3′) primers. This fragment was cloned into pDhNIk1–Tco1 at Nde1-EcoR1 site replacing the original fragment to obtain ΔH1. All other constructs were made in a similar way using different mutagenic primers the sequence of which will be made available upon request. Mutations in all the clones were confirmed by automated DNA sequencing.

2.3. Western blotting

Phosphorylated Hog1p in *S. cerevisiae* strain NM2 (*sln1-ts ste11Δ*) expressing DhNik1–Tco1 was detected by western blotting [30]. Cells were grown in SD medium at 28 °C until OD_{600} of 0.5–0.7 and then shifted to 37 °C for 1h. The total cell extract (~20 μg protein) from each sample was blotted onto nitrocellulose membrane and dually phosphorylated Hog1p was detected using anti-dually phosphorylated p38 antibody (Cell Signaling Inc.,). The level of Hog1p was detected in the same blot after re-probing with anti Hog1p antibody (Y-215; Santacruz Biotech).

3. Results and discussion

The presence of HAMP domain repeats at the NH_2-terminus is a unique feature of the group III HHK. The poly HAMP unit acts as sensor and regulates the histidine kinase activity of the group III HHK in response to osmostress or the antifungal agent fludioxonil. Interestingly, the number of the HAMP domain repeats varies among the orthologs from different genera. The orthologs from yeast, filamentous ascomycetes and basidiomycetes contain five, six and seven HAMP domain repeats respectively. Tco1p is one of the well characterized group III HHKs from the basidiomycetous human fungal pathogen *C. neoformans* [14]. Tco1p had been shown to regulate a plethora of cellular events, including stress responses, drug sensitivity, sexual reproduction, and virulence in this organism which was primarily through the HOG pathway. SMART analysis showed that Tco1p contained seven HAMP domain repeats (Fig. 1A). Each HAMP domain was interspersed with a linker region having HAMP like features [31]. To dissect the role of individual domain in the seven-HAMP module we have constructed a chimera DhNik1–Tco1 by swapping the five-HAMP module in DhNik1p (53–475 amino acid residues) with the seven-HAMP module (208–814 amino acid residues) of Tco1p (Fig. 1A). This construct was expressed under the regulation of *DhNIK1* promoter in *S. cerevisiae* strains RJ1428 carrying *sln1* deletion. In *S. cerevisiae* deletion of *sln1* gene is lethal as it causes constitutive activation of HOG pathway. In RJ1428, the lethality of *sln1* deletion was suppressed by expressing a phosphatase, *PTP2*, from a *URA3* based multicopy plasmid. Thus, the presence of this plasmid becomes essential for the survival of the strain RJ1428 and this can be assayed easily using 5-Fluoroorotic acid (5-FOA) plate as the *URA3* positive cells will not grow on this plate. RJ1428 transformed with plasmid harboring DhNik1–Tco1 showed good growth on 5-FOA plate (Fig. 1B). In comparison, the control strain containing the vector failed to

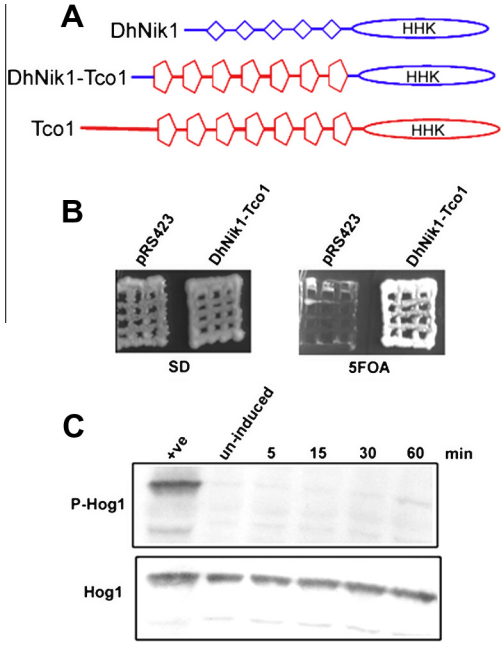

Fig. 1. Complementation of *sln1* mutation in *S. cerevisiae* by DhNik1–Tco1 chimera. (A) Diagrammatic representation of the domain architecture of DhNik1p, Tco1p and DhNik1–Tco1 chimera. Five-HAMP module of DhNik1p was replaced by the seven-HAMP module of Tco1p to create DhNik1–Tco1 chimera. HHK represents histidine kinase and receiver domains. (B) *S. cerevisiae* strain RJ1428 transformed with vector pRS423 or DhNik1–Tco1 was patched on SD plate and SD plate containing 1 mg/ml 5FOA. Growth of the cells incubated at 28 °C for two days is shown. (C) Immunoblots showing the level of Hog1p phosphorylation in *S. cerevisiae* strain NM2 (*sln1-ts*) expressing DhNik1–Tco1 at non-permissive temperature (37 °C). Cells were grown on SD medium with required nutrient supplement at 28 °C till exponential phase ($OD_{600} \sim 0.7$) before exposing them to 37 °C. The level of dually phosphorylated Hog1p (P-Hog1) was detected in the cell extracts with anti-phospho p38 antibody. Un-induced control – total extract from cells grown at 28 °C. +ve control – cell extracts from NM2 grown at 28 °C up to exponential phase and exposed to 0.7 M NaCl for 15 min. Blots were re-probed with anti Hog1 antibody to detect total Hog1. P-Hog1 or Hog1 indicate phosphorylated or total Hog1p in the blot.

grow on 5FOA plate as the presence of the plasmid expressing *PTP2* was essential for its growth. These results indicated that DhNik1–Tco1 could complement *sln1* mutation in *S. cerevisiae*.

In *S. cerevisiae*, Sln1p negatively regulates the HOG pathway. Earlier we have shown that DhNik1p also regulated the HOG pathway in a similar fashion [17,18]. Under normosmolarity conditions, the kinase activity of Sln1p prevents the activation of Hog1p. We therefore, wanted to check whether DhNik1–Tco1 was also proficient in preventing the HOG pathway activation in the absence of Sln1p. For this *S. cerevisiae* strain NM2 expressing DhNik1–Tco1 was grown to early logarithmic phase ($OD_{600} \sim 0.7$) and then exposed to 37 °C for different length of time. The levels of phosphorylated Hog1p in the total cell extract were determined by immunoblotting. As expected, in the un-induced control cells grown at 28 °C, no phosphorylated Hog1p was detected. *S. cerevisiae* strain NM2 harbors temperature sensitive allele *sln1-ts* and therefore, the incubation of the cells at 37 °C activates the HOG pathway. However, the phosphorylated Hog1p could not be detected in NM2 which was expressing DhNik1–Tco1 and exposed to 37 °C (Fig. 1C). The level of phosphorylated Hog1p remained unchanged even after 6 h of exposure to non-permissive temperature (data not shown). These results thus indicated that the chimeric construct was proficient in regulating the HOG pathway.

The members of the group III HHK are molecular target for the antifungal activity of fludioxonil and the heterologous expression of the group III HHK renders *S. cerevisiae* cells sensitive to this compound. Therefore, we checked whether DhNik1–Tco1 chimera could also confer fludioxonil sensitivity to *S. cerevisiae*. For this, DhNik1–Tco1 construct was transformed into *S. cerevisiae* and the fludioxonil sensitivity of the transformant was checked on SD plates containing 5 μg/ml fludioxonil by dilution spotting. *S. cerevisiae* expressing DhNik1–Tco1 did not show any growth and it was highly sensitive to fludioxonil (Fig. 2A). Earlier studies showed that the inhibition of the group III HHK by fludioxonil in *S. cerevisiae* resulted in the inappropriate activation Hog1p [5,11,18]. Therefore, we determined the level of phosphorylated Hog1p in *S. cerevisiae* expressing DhNik1–Tco1 by immunoblotting after exposing the cells to fludioxonil. In control untreated sample, the phosphorylated Hog1p could not be seen (Fig. 2B). In comparison, the phosphorylated Hog1p appeared within five minutes of exposure to fludioxonil. The level of phosphorylated Hog1p remained similar even after six hours indicating the constitutive activation of the HOG pathway by fludioxonil. Together these results suggested that DhNik1–Tco1 chimera could recapitulate group III HHK function in *S. cerevisiae* efficiently.

In prokaryotic sensor histidine kinases, HAMP domain has been shown to play active role in the intra-molecular signal transduction from the trans-membrane to the cytoplasmic kinase domain [24]. DhNik1–Tco1 possesses seven HAMP domains. Previous studies indicated that HAMP domain repeats were very important for the functionality of the group III HHKs and they appeared to act as both sensor and the regulatory modules in response to the changes in the external osmolarity [17,18]. The mutations in the HAMP domains of Nik1p orthologs conferred osmo-sensitive and fungicide resistance phenotype to the host [11,12,19,25]. In the case of DhNik1p which contained a five HAMP module, it was observed that the individual domains had distinct roles and moreover the positions and the arrangement of the HAMP domain repeats were crucial for its function. To investigate the role of each HAMP domain in DhNik1–Tco1, we constructed a set of mutants where

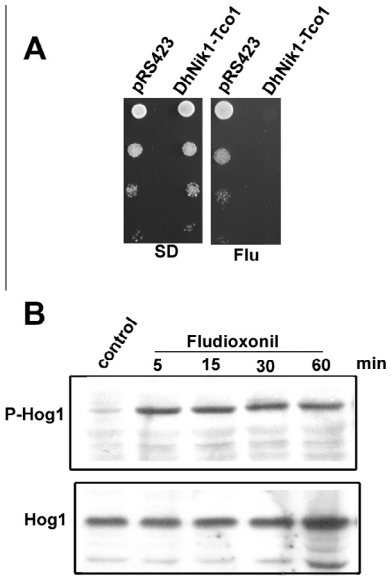

Fig. 2. DhNik1–Tco1 confers fludioxonil sensitivity to *S. cerevisiae*. (A) *S. cerevisiae* strain NM2 harboring vector pRS423 or DhNik1–Tco1 was grown at 28 °C up to exponential phase. 5 μl of the ten fold serial dilutions of these cultures (normalized to OD_{600} 1.0) was spotted onto SD plate or SD plate containing 5 μg/ml fludioxonil. Plates were incubated at 28 °C for two days before photographed. (B) *S. cerevisiae* strain NM2 harboring DhNik1–Tco1 was grown at 28 °C up to exponential phase before being exposed to fludioxonil (5 μg/ml). Total extracts of the cells were collected at different time points were subjected to immuno-blotting to detect the level of dually phosphorylated Hog1p (P-Hog1). Total extract from cells grown at 28 °C was loaded as control.

individual HAMP domains were deleted. These mutants were introduced into *S. cerevisiae* strain RJ1428 and the growth pattern of the transformants was checked on 5FOA plate. The deletion of HAMP1, HAMP2, HAMP3, HAMP4, HAMP5 or HAMP7 domain resulted in a nonfunctional allele as the cells carrying these mutants could not grow in the presence of 5FOA (Fig. 3A). In contrast, the growth pattern of ΔH6 on 5FOA plate was quite similar to DhNik1–Tco1 (Fig. 3A). This result clearly indicated that HAMP6 domain was dispensable for the kinase activity of DhNik1–Tco1 whereas the other HAMP domains were essential for it. Next we checked whether these HAMPdomain mutants could confer fludioxonil sensitivity to *S. cerevisiae*. For this, the mutant constructs were transformed into *S. cerevisiae* strain NM2 and the growth patterns of the transformants were checked on fludioxonil plates by dilution spotting. As expected, the mutants ΔH1, ΔH2, ΔH3, ΔH4, ΔH5 and ΔH7 showed fludioxonil resistance as they were nonfunctional. However, the mutant ΔH6 which could functionally complement *sln1* mutation, also showed resistance to fludioxonil (Fig. 3B). These results indicated that the histidine kinase activity of ΔH6 was unresponsive to fludioxonil. Earlier studies showed that the constitutive kinase mutants did not confer fludioxonil sensitivity to *S. cerevisiae* [18]. The mutant ΔH6 behaved in a similar fashion. To understand the role of each domain further, the HAMP domains were sequentially deleted in DhNik1–Tco1 to obtain ΔH1–2, ΔH1–3, ΔH1–4, ΔH1–5, ΔH1–6 and ΔH1–7. In ΔH1–2, the first two HAMP domains were deleted whereas the mutant ΔH1–7 was devoid of all seven HAMP domains. To examine the functional consequences of these deletions, these constructs were transformed into RJ1428 strain and the growth pattern of the transformants was examined on 5FOA plate. The mutants carrying deletion in first two, three or four HAMP domains did not grow 5FOA plate which indicated that they were non functional (Fig. 4A). Similarly the removal of all the HAMP domains (ΔH1–7) also resulted in a nonfunctional allele. In comparison, the growth of the mutant ΔH1–6 on 5FOA plate was quite similar to that of DhNik1–Tco1. With the mutant ΔH1–5, fewer colonies appeared on 5FOA plate, suggesting that this mutant also retained kinase activity albeit partially. Interestingly, the mutant ΔH1–6 also showed fludioxonil resistance phenotype and thereby indicating that it was also a constitutive kinase mutant (Fig. 4B).

Group III HHKs constitute an important class of molecular target for antifungal agents [5]. The presence of HAMP domain repeats is a unique structural feature in this group [5,23,17,25]. In the case of DhNik1p which contained five HAMP domains, the poly-HAMP module appeared to act as sensor as well as regulator for the C-terminal kinase domain [17,18]. In comparison to DhNik1p, orthologs from basidiomycetes harbor seven HAMP domains. How these structurally distinct organizations perform similar function is not very clear. Present study is the first documentation to understand the role of individual HAMP domain in a seven-HAMP module. Since the functional characterization was carried out in *S. cerevisiae*, we have made a chimeric yeast-like group III HHK having a seven-HAMP module. Compare to the yeast orthologs e.g., DhNik1p, Tco1p has much longer NH_2-terminal end (208 amino acid) preceding the seven-HAMP module which may have functional importance in their native host (Fig. 1A). In fact, *S. cerevisiae* strain expressing DhNik1–Tco1 exhibited fludioxonil sensitivity at much lower concentration than that was observed with Tco1p (data not shown). Moreover DhNik1–Tco1 showed much stronger phenotypic complementation of *sln1-ts* mutation at 37 °C than Tco1p. To determine the contributions of each HAMP domain in the functionality, we made several deletion mutants of DhNik1–Tco1. The phenotypic analysis of the deletion mutants showed that both ΔH6 and ΔH1–6 were functional and exhibited resistance to fludioxonil. Unlike DhNik1–Tco1, fludioxonil did not inhibit the activity of these mutants. In DhNik1–Tco1 the HAMP6 domain was not essential for kinase activity of DhNik1–Tco1 however, in the absence of this domain DhNik1–Tco1 became constitutively active kinase. Therefore, HAMP6 domain in DhNik1–Tco1 appeared to be crucial for responding to the environmental stress and fludioxonil sensitivity (Fig. 4C). In case of a yeast ortholog DhNik1p, the fourth HAMP domain was shown to be crucial for osmosensing and fludioxonil sensitivity [7,18]. The kinase activity of DhNik1p was regulated negatively by the fourth HAMP domain. The function of the HAMP6 domain in DhNik1p was analogous to that of the fourth HAMP domain in DhNik1p. Thus, the negative regulation by the penultimate HAMP domain, therefore, appeared to be a common, evolutionarily conserved theme in group III HHK.

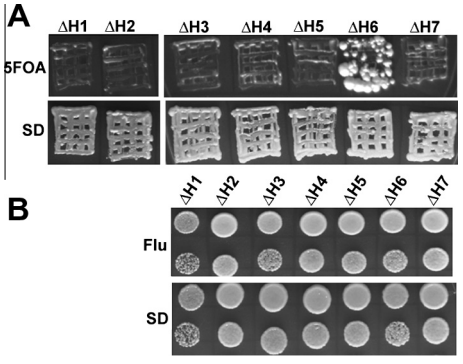

Fig. 3. Functional analysis of DhNik1–Tco1 mutants carrying deletion in HAMP domains individually. (A) *S. cerevisiae* strain RJ1428 transformed with HAMP domain mutants of DhNik1–Tco1 was patched on SD plate and SD plate containing 1 mg/ml 5FOA. Growth of the cells incubated at 28 °C for two days is shown. (B) Dilution spotting of *S. cerevisiae* strain NM2 transformed with HAMP domain mutants of DhNik1–Tco1 on SD plate or SD plate containing 5 μg/ml fludioxonil. Plates were incubated at 28 °C for two days and 10^{-1} and 10^{-2} dilutions are shown.

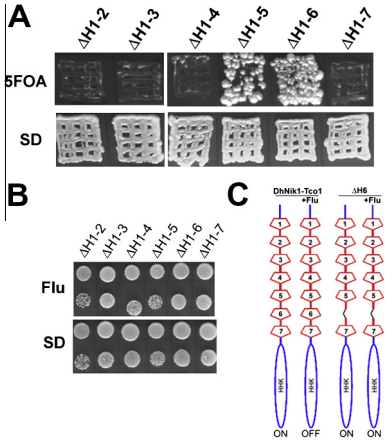

Fig. 4. Functional analysis of DhNik1–Tco1 mutants carrying serial deletion in HAMP domains. (A) *S. cerevisiae* strain RJ1428 transformed with mutants of DhNik1–Tco1 was patched on SD plate and SD plate containing 1 mg/ml 5FOA. Growth of the cells incubated at 28 °C for two days is shown. (B) Dilution spotting of *S. cerevisiae* strain NM2 transformed with HAMP domain mutants of DhNik1–Tco1 on SD plate or SD plate containing 5 μg/ml fludioxonil. Plates were incubated at 28 °C for two days and 10^{-1} and 10^{-2} dilutions are shown. (C) Cartoon showing the effect of fludioxonil on the activity of DhNik1–Tco1 and ΔH6 mutant.

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