

RESEARCH LETTER

***Aspergillus nidulans* translationally controlled tumor protein has a role in the balance between asexual and sexual differentiation and normal hyphal branching**

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Keywords

Aspergillus nidulans; translationally controlled tumor protein; differentiation.

Introduction

The translationally controlled tumor protein (TCTP), also called p21, p23, fortilin, histamine releasing factor and MMI1, which is initially identified in Ehrlich ascites tumor cells, is highly conserved and abundantly expressed across a wide range of eukaryotes (Chitpatima *et al.*, 1988; Bommer & Thiele, 2004). TCTP functioning is related to anti-apoptosis, tumor reversion, microtubule organization, ion homeostasis, oxidative stress resistance and histamine releasing factor (Bommer & Thiele, 2004). Cellular proteins, including polo kinase, Na⁺/K⁺-ATPase, protein synthesis elongation factor eEF1A, and small GTPase Rheb, have been identified to interact with TCTP (Cans *et al.*, 2003; Bommer & Thiele, 2004). The human TCTP has been suggested to participate in the control of the cell cycle through TCTP phosphorylation by polo kinase (Yarm, 2002). It also has been identified as a guanine nucleotide dissociation inhibitor of the translation elongation factor eEF1A (Cans *et al.*, 2003). In *Arabidopsis*

Abstract

Translationally controlled tumor protein (TCTP) is a highly conserved and ubiquitously expressed protein present in all eukaryotes. Cellular functions of TCTP include growth promoting, allergic response and responses to various cellular stresses, but the functions in filamentous fungi have not been reported. In this report, we characterized an *Aspergillus nidulans* TCTP (TcPA) with high similarity to TCTP. The level of *tcpA* mRNA was relatively high, both during vegetative growth stage and at early phases of development. TcPA was found predominantly in the nucleus during germination and mycelial growth, and was localized in cytoplasm and nuclei of vesicles on stipes during conidia development. Deletion of *tcpA* resulted in abnormal hyphal branch formation during vegetative growth. The *tcpA* deletion inhibited sexual development, but enhanced asexual development via induction of *brlA* expression. These results imply that TcPA is involved in normal hyphal branch establishment during vegetative growth and also has a role in the balance between asexual and sexual differentiation.

thaliana, TCTP has been implicated as an important regulator of growth as a mediator of the target-of-rapamycin (TOR) activity (Berkowitz *et al.*, 2008). It has been identified as a transcription factor activating the pluripotency genes *oct4* and *nanog* (Kozioł *et al.*, 2007; Johansson *et al.*, 2010), implying a role as a regulator of early development and stem cell proliferation.

The filamentous fungus, *Aspergillus nidulans* is an important model organism used to investigate fundamental biological questions in fungal development, gene regulation, and the regulation of secondary metabolites (Morris & Enos, 1992; Adams *et al.*, 1998; Osmani & Mirabito, 2004). Previously, we reported proteins involved in early phase of conidial germination in *A. nidulans* by proteomic approach and observed up-regulation of *A. nidulans* TCTP (TcPA) after initiation of germination (Oh *et al.*, 2010). To further investigate functions of TcPA, we examined the expression and localization of TcPA and analyzed the phenotypic characteristics of a *tcpA* deletion mutant strain.

Materials and methods

Strain and growth conditions

Aspergillus nidulans strains used in this study are listed in Table 1. For submerged cultivation, 10^7 spores were inoculated in 100 mL glucose minimal medium (MM) with essential supplements (Käfer, 1977) or complete medium (CM) with 0.5% (w/v) yeast extract and cultured at 37 °C with shaking. For Northern blot analysis, fungal samples from liquid-submerged and developmentally induced cultures were collected at designated time points. For induction of asexual and sexual development, vegetative mycelia grown for 18 h or 10^6 conidia were spread onto a solid MM. The plates were then incubated under air-exposed light conditions for asexual development induction or under air-tight dark conditions for sexual development induction as described (Adams *et al.*, 1988; Han *et al.*, 2004; Seo *et al.*, 2004).

Nucleic acid isolation and manipulation

To obtain the open reading frame (ORF) of *tcpA* coding region, total RNA from the mycelia was applied to a reverse transcription reaction and then cDNA was amplified by PCR using YTA01; YTA02 primer pair (Supporting Information, Table S1). The amplicon was sequenced and compared with the DNA sequence of *tcpA*. Southern and Northern blot were performed as given in detail in Sambrook & Russell (2001) and Yu *et al.* (2004). For Northern blot analysis, individual probes were prepared by PCR amplification of the coding regions of *tcpA* (amplified by YTA11; YTA12) and *brlA* (amplified by YTA13; YTA14).

Deletion and localization of TcPA

For the deletion of *tcpA*, the DJ-PCR method was used (Yu *et al.*, 2004). Both flanking regions of *tcpA* were amplified from FGSC A4 genomic DNA using the YTA03; YTA04 and YTA05; YTA06 primer pairs. The *A. nidulans* *argB* marker gene was PCR-amplified from FGSC A4 genomic DNA using the YTA1-1; YTA1-2 primer pair. The three fragments were fused, and the final PCR product for

the *tcpA* deletion was amplified using the YTA07; YTA08 primer pair, and introduced into FGSC A850 protoplasts generated by lysing enzymes (L1412; Sigma-Aldrich; Szewczyk *et al.*, 2006). The successful transformation was confirmed by PCR and Southern blot (Fig. S1B).

For the construction of TcPA with C-terminal GFP tag, the *tcpA* ORF without a stop codon was amplified using the YTA09; YTA10 primer pair. The PCR product then was digested with *AscI* and *NheI*, and cloned into the pAT-sGFP vector, which was modified by exchange of appropriate multiple cloning sites from pMT-sGFP (FGSC) vector. The construct was integrated into the genomic DNA locus of *tcpA*. The single locus integration by homologous recombination was confirmed by PCR and Southern blot analyses (Fig. S1D).

Microscopic analysis

Samples of conidia and mycelia were fixed in 3.7% formaldehyde, 50 mM Na_2HPO_4 (pH 7.0) and 0.2% Tween 80 for 30 min. Samples were washed in deionized water and stained for 5 min with $0.1 \mu\text{g mL}^{-1}$ 4,6-diamidino-2-phenylindole (DAPI), and the preparations were examined under a fluorescent microscope (Olympus BX51) to detect DAPI (equipped for fluorescence with a fluorescein iso-thiocyanate filter for DAPI) and GFP (samples were excited with a 488 nm and emitted light was bandpassed with a 522–532 nm filter) fluorescence.

Results

Gene structure of *tcpA*

The ORF of *tcpA* (chromosome VIII; AN0641.2) was 809 bp in length and consisted of four exons (15, 280, 160 and 85 bp) and three introns (Fig. 1a). The predicted TcPA protein revealed 179 amino acid-length polypeptides, and including two TCTP signatures, a Ca^{2+} /microtubule binding domain and a polo kinase interaction domain (Fig. S2). Multiple sequence alignment revealed that TcPA had between 23% and 46% identity with TCTPs from other eukaryotic organisms. A phylogenetic tree showed that the fungal and eukaryotic TCTPs were clearly separated (Fig. 1b). TCTP was highly conserved in most aspergilli including *Aspergillus terreus* (87% amino acid sequence identity), *Aspergillus niger* (77%), *Aspergillus clavatus* (84%), and *Aspergillus fumigatus* (82%) (data not shown).

Expression and subcellular localization of TcPA

Northern blot analysis revealed that the level of *tcpA* mRNA peaked during active vegetative growth and decreased rapidly during the stationary phase (Fig. 2a).

Table 1. *Aspergillus nidulans* strains used in this study

Strains	Genotype	Source
FGSC A4	veA ⁺ (wild-type)	FGSC*
FGSC A850	biA1; argB; methG1; veA1 (wil-type)	FGSC*
ATD5-2	biA1; argB; methG1; tcpA::argB ⁺ veA1	This study
TF3-2	biA1; argB; methG1; tcpA::gfp::argB ⁺ veA1	This study

*Fungal genetics stock center.

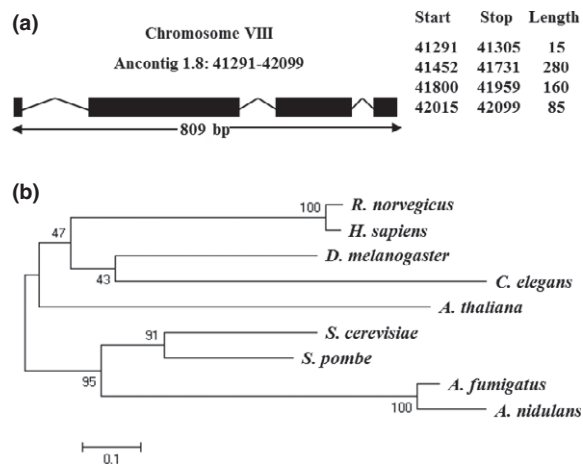


Fig. 1. Structure of *tcpA*. (a) Schematic presentation for the *tcpA* gene. The exons and introns were determined by comparison of genomic DNA and cDNA sequences. Filled blocks and lines represent exons and introns, respectively. Length of the three introns (from 5' to 3') are 147, 69, and 54 bp, respectively. (b) Phylogenetic tree of TCTP. The tree was derived from multiple sequence alignment (Fig. S2).

At early stages of (a)sexual development, the mRNA level was relatively high (Fig. 2a). This indicates a potential role of TcpA in germination, early hyphal growth and the developmental stage.

To examine the localization of TcpA, we generated a strain TF3, which carries a *GFP::tcpA* allele replacing the wild-type gene. Successful construction of the strain was confirmed by Southern blot analysis (Fig. S1D). Fluorescence microscopic analysis revealed that GFP::TcpA was predominantly localized in the nucleus and dispersed throughout the cytoplasm in a relatively low concentration in vegetative cells (Fig. 2b). To understand the function of TcpA in spore germination, we investigated the localization of TcpA in dormant (0 h), activated (1 h),

swelling (2 h), and germ-tube formation (4–6 h) stages of germination. As shown in Fig. 2c, GFP::TcpA was not present in dormant spores. After the activated phase of germinating conidia, GFP::TcpA was uniformly distributed upon activation of conidia (Fig. 2c, 1 h). GFP::TcpA was then localized predominantly in the nucleus in the swelling and the germ-tube formation stages (Fig. 2c, 2–6 h). Because, after early phase of asexual development the mRNA level of *tcpA* decreased (Fig. 2a), we investigated the localization of TcpA in early (vesicle), middle (metulae, phialide), and late (basipetal structure) asexual developmental structures (Fig. 2d). As expected, the GFP signal was high in the cytosol and nuclei of vesicles on stipes, but decreased notable in phialides and could not be observed in newly formed conidia (Fig. 2d).

TcpA is required for hyphal branching

In our previous proteomic study, TcpA was abundantly present during conidial germination (Oh *et al.*, 2010). *TcpA* was also highly expressed in the spore germination and hyphal growth stages, confirming our previous observation (Fig. 2a). To investigate functions of TcpA, we constructed a *tcpA* deletion mutant (Fig. S1A and B) and its phenotypic characteristics during germination and hyphal stages were examined. Unexpectedly, deletion of *tcpA* resulted in accelerated conidial germination that was completed 1 h earlier compared to the wild type (Fig. 3a and b). Moreover, the second germ tube was not formed and aberrant (hyper) branching was observed on primary germ-tube in *tcpA* deletion mutant (Fig. 3a).

In hyphae, side branches generally emerge from subapical cells (Momany, 2002). However, in the *tcpA* deletion mutant, the side branch showed patches with disturbed side branch patterning (Fig. 3c). The multiple side branches emerged at septa without distinct primary hyphae or side branch in one direction (Fig. 3c).

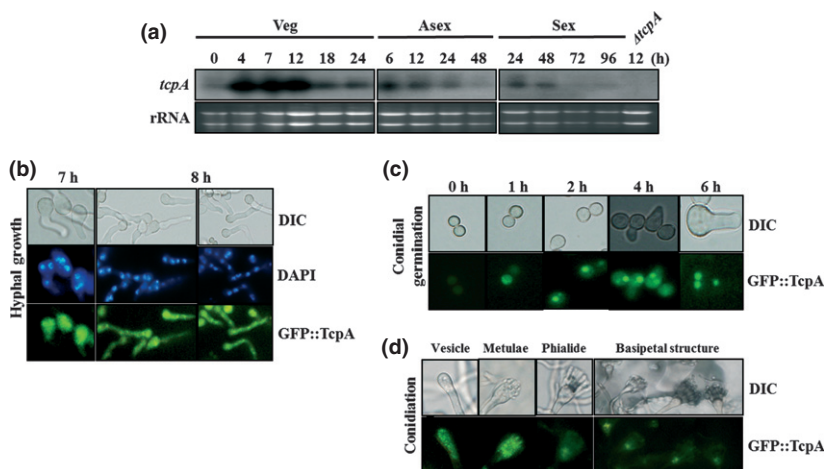
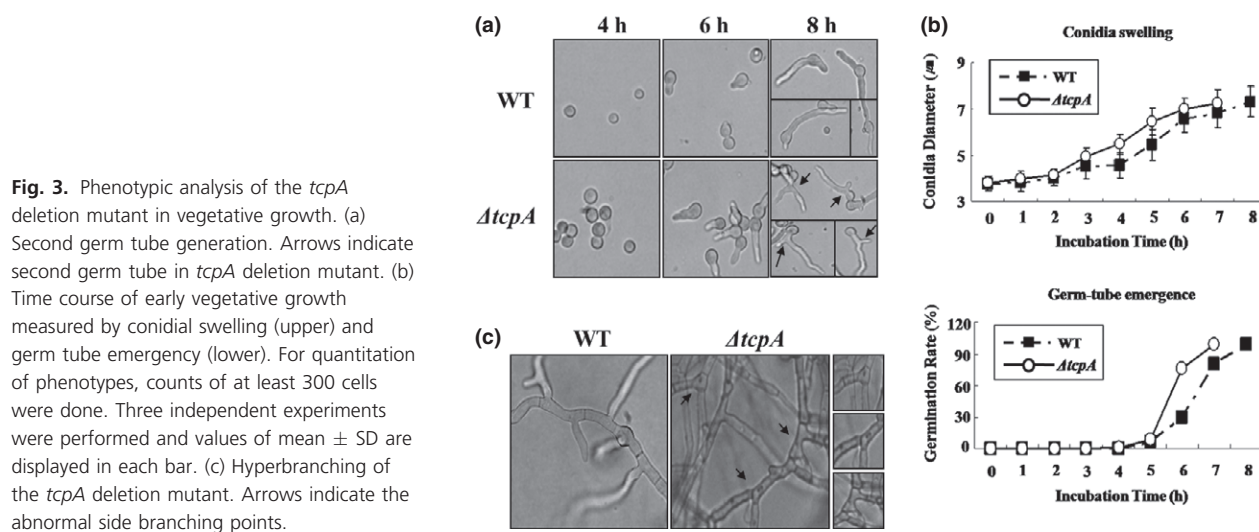


Fig. 2. Expression and localization of TcpA. (a) Expression of *tcpA* during the life cycle of *Aspergillus nidulans*: Veg, vegetative growth; Asex, asexual induction; Sex, sexual induction; $\Delta tcpA$ indicated negative control. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA. (b) Localization of GFP::TcpA in vegetative hyphae. Conidia from strain TF3-2 (GFP::TcpA) were inoculated on a coverslip submerged in liquid minimal medium. (c) Localization of GFP::TcpA in germination process. (d) Localization of GFP::TcpA in conidation.



Functions of TcpA in the regulation of sexual and asexual development

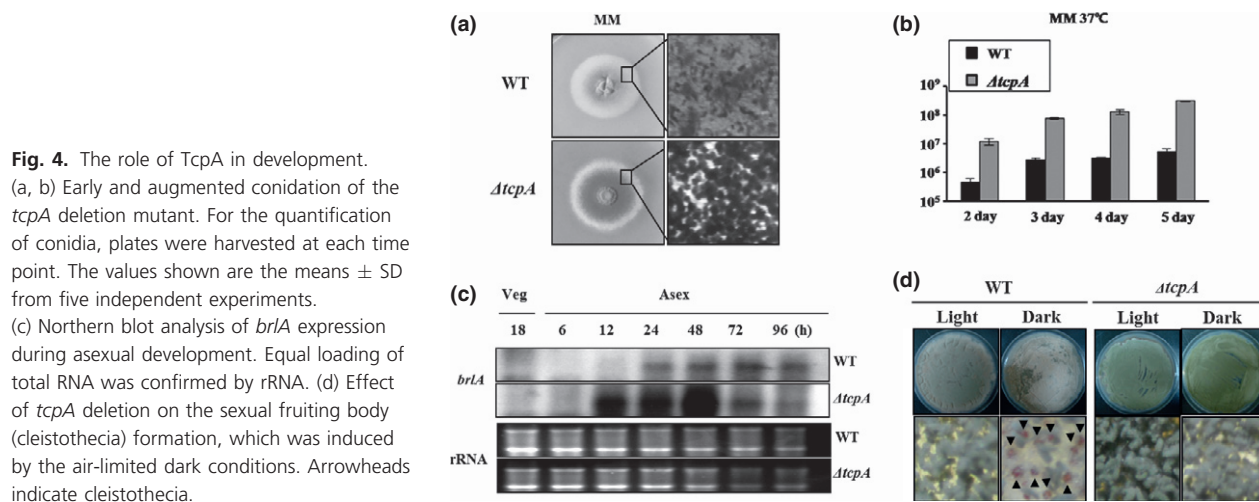
The deletion of *tcpA* mutant strain showed a clear overproduction of conidia compared to the wild type. The mutant produced up to 3.2×10^8 conidia/plate after 5 days compared to 5.0×10^6 in case of the wild type (Fig. 4b). This suggests a role of TcpA in the regulation of asexual development. The transcription factor *brlA* is a key regulator of asexual development and its activation is indispensable for conidiation (Adams *et al.*, 1988). The expression of *brlA* mRNA in the wild type was induced after 24 h and the amount of transcript gradually increased and peaked at 72 h (Fig. 4c). However, in the *tcpA* deletion mutant, a huge amount of the *brlA* mRNA started to accumulate at only 12 h after induction and the level peaked after 48 h (Fig. 4c).

Because asexual and sexual development pathways are mutually antagonistic, we examined whether *tcpA* deletion

reduces the formation of the sexual fruiting body (cleistothecium) formation. We spread 10^6 conidia of wild-type (WT) and *tcpA* deletion mutant on a solid medium and incubated in the air-limited dark condition for sexual fruiting body formation. As shown in Fig. 4d, the *tcpA* deletion mutant was unable to produce cleistothecium while WT accumulated high amount of cleistothecium (Fig. 4d, arrow heads). The results suggest that TcpA has a role in the balance between asexual and sexual differentiation.

Discussion

The mRNA of *tcpA* was abundantly present during germination, germ tube elongation and early vegetative growth and subsequently decreased in the stationary phase. It was also present during early (a)sexual developmental stages, but became absent at later sporulation stages. GFP::TcpA was absent from dormant conidia, became formed in the cytoplasm and then transferred to the nuclei during early



germination and It was clearly observed in the vesicles on stipes during conidiophore formation and then disappeared in phialides and newly formed conidia. Deletion of *tcpA* resulted in an acceleration of swelling of conidia and germination, but no second germ tube was formed and aberrant branching was observed. Very notable, while asexual development was much enhanced after deletion of *tcpA*, the formation of cleistothecium was inhibited.

TCTP has been implicated to participate in cell growth and cell division. Overexpression of a mutant TCTP, in which the phosphorylation sites for the mitotic polo-like kinase (Plk) was mutated, disrupted the completion of mitosis (Yarm, 2002). In *A. nidulans*, spore polarization is coupled to cell cycle progression (Harris, 1999; Momany & Taylor, 2000). It has been suggested that coordination between the polarity establishment and the nuclear division is important for ensuring an appropriate volume of cytoplasm per nucleus in the dividing hyphae (Harris, 2006). Recently, the function of *A. nidulans* PLK (PLKA) was reported that PLKA is not essential for hyphal growth but may be important for aspects of hyphal morphogenesis and polar axis formation (Mogilevsky *et al.*, 2012). On this basis, our findings that include translocation of TcpA to nucleus during germination and hyphal growth (Fig. 2b) and hyperbranching (Fig. 3a and c) of the TcpA deletion strain, may imply a role of TcpA in cell cycle progression. This is further justified by the fact that TcpA contains a Plk interaction domain (Fig. S2).

Sexual fruiting body formation occurs preferentially in darkness in *A. nidulans* and is inhibited by light as an external signal (Bayram *et al.*, 2008; Calvo, 2008). In contrast, formation of asexual spores is promoted by light. The molecular mechanism of light signal transduction is yet to be fully understood, but a nuclear protein VeA (velvet A) has been known to regulate the developmental process by activating sexual development and inhibiting asexual development (Champe *et al.*, 1981; Yager, 1992). *Aspergillus nidulans* VeA localizes in the nucleus in dark conditions but is predominantly retained in the cytoplasm in light conditions. VeA interacts with α -importin KapA, a nuclear transporter (Stinnett *et al.*, 2007) and regulates the expression of the asexual transcription factor gene *brlA* (Kato *et al.*, 2003). Deletion of *veA* (Δ veA) caused failure in fruiting body formation while overexpression of VeA increased formation of sexual structures with reduced conidiation. These indicate that *veA* is a positive regulator of sexual development and simultaneously a negative regulator of asexual development (Kim *et al.*, 2002). Additionally, the function of GanB ($G\alpha$ -protein) was indicated that negative regulation of asexual sporulation by active GanB maybe mediated through repression of *brlA* expression at the transcriptional level (Chang *et al.*, 2004). According to our data,

tcpA deletion induced asexual development and caused failure in fruiting body formation even under the conditions that promote sexual development (Fig. 4). Moreover, the *tcpA* deletion resulted in premature and augmented induction of the *brlA* mRNA. The presented data suggest that the function of TcpA has a close relationship with that of VeA and GanB proteins during development.

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Authors' contribution

Y.T.O. and C.-S.A. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

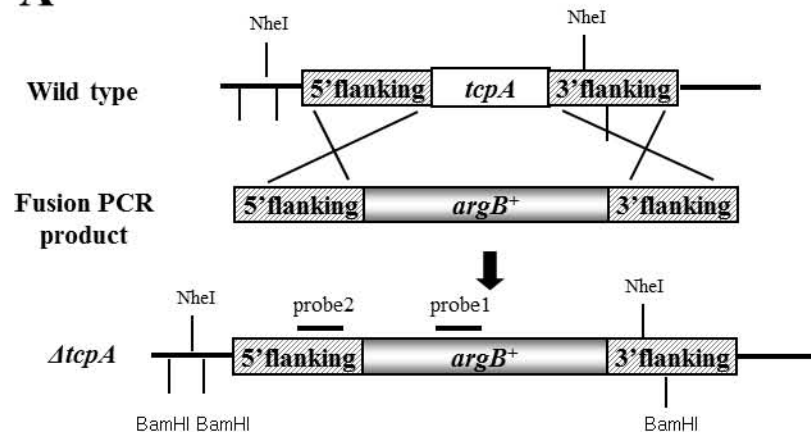
Fig. S1. Deletion of the *tcpA* gene and construction of the GFP::TcPA line.

Fig. S2. Multiple sequence alignment of TCTP proteins in various organisms.

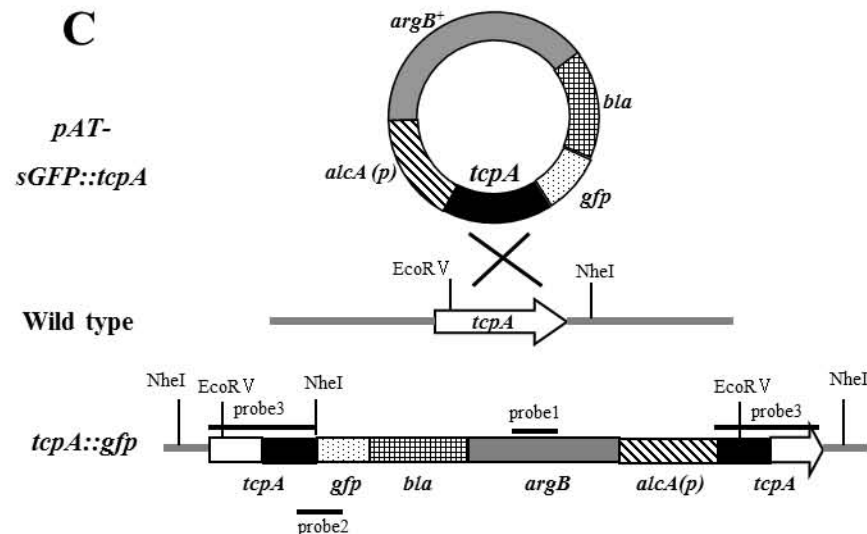
Table S1. The primers used in this study.

Supplementary figure. 1

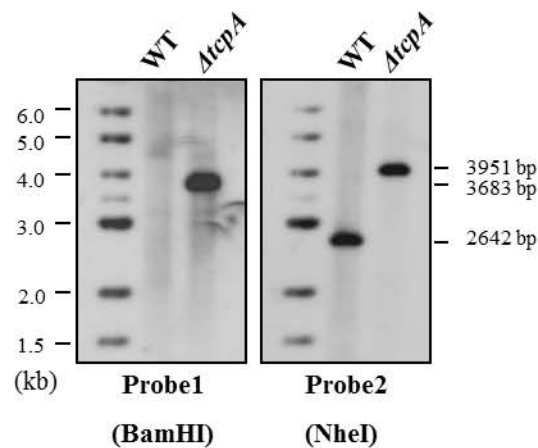
A



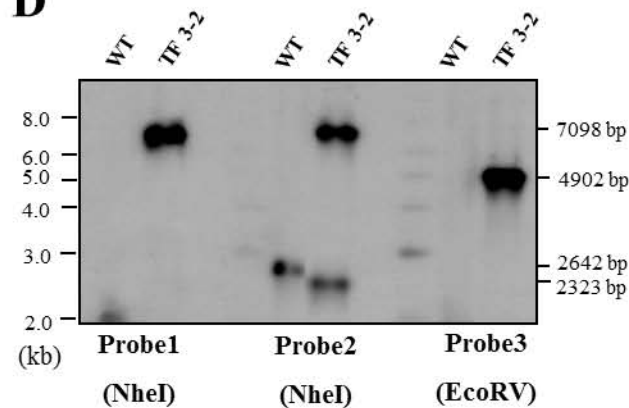
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B



D

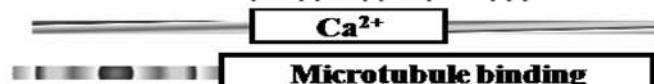


Supplementary figure. 2

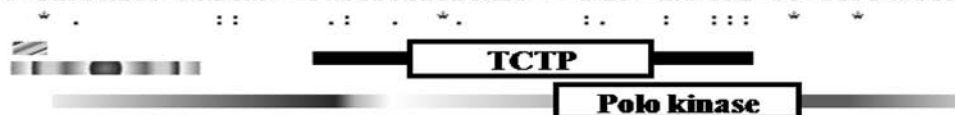
<i>A. nidulans</i>	MIIYKDIISGDEVLSDTYNIKTVDG-VLYECDCKRYLKKT-NEDFELEGANPSAEGGDDE	58
<i>A. fumigatus</i>	MIIYKDIISGDEVLSDNFKIKEVDG-VLYECDCKRYLKRR-NEDIQLEGANPSAEEGDDD	58
<i>R. norvegicus</i>	MIIYRDLISHDELFSDIYKIREIADGLCLEVEGKMVSRTEGAIDDSLIGGNASAEG-PEG	59
<i>H. sapiens</i>	MIIYRDLISHDEMFSDIYKIREIADGLCLEVEGKMVSRTEGNIDDSLIGGNASAEG-PEG	59
<i>D. melanogaster</i>	MKIYKDIITGDEMFADTYKMKLVDD-VIYEVYKGLITRQGGDIK--LEGANASAE--AD	55
<i>C. elegans</i>	MLIYKDIIFTDDELSSDSFPMKLVDD-LVYEFKKGHVVRKEGEIV--LAGSNPSAEEGAED	57
<i>A. thaliana</i>	MLVYQDLLTGDELLSDSFYKEIENGILWEVEGKWVTVGAVDVN--IGANPSAEEGGED	57
<i>S. cerevisiae</i>	MIIYKDIIFSNDLLSDAYDAKLVD--VIYEADCAMVNVG--GDNIDIG-ANPSAEGGDDD	56
<i>S. pombe</i>	MLLYKDVISGDELVS DAYDLKEVDD-IVYEADCMVTVKQ--GGDVDIG-ANPSAEDAEN	57
	* * * * *	



<i>A. nidulans</i>	GGAEGGEVMVHDIEDQFRLVWLKAEDGAKPPKDAFKGHLKTYMKKVLKALQDKGAPDETI	118
<i>A. fumigatus</i>	AGGDGEEVMVHDIEDQFRLVWLKTEEGMKPSKDAFKSHLKTYMKKVLAKLQEKGVPEAEI	118
<i>R. norvegicus</i>	EGTESTVTGVDIVMNNHHLQETSFTK-----EA--YKKYIKDYMKSLKGKLEEQKPE--RV	111
<i>H. sapiens</i>	EGTESTVITGVDIVMNNHHLQETSFTK-----EA--YKKYIKDYMKSIKGLKEEQKPE--RV	111
<i>D. melanogaster</i>	EGDITSESQGVVVLNHLRTECFAGF-----DKKSYTLYLDKMKKVLAKLLEEKSPD--QV	109
<i>C. elegans</i>	DGSDHEHVERGIDIVLNHKLLEMNCYE----DASMFKAYIKKFMKNVIDHMEKNNRDKADV	113
<i>A. thaliana</i>	EGVDDSTQKVVDIVDTFRLQEQTPTD----KKG-FIAYIKKYIKLLTPKLSEEDQA----	108
<i>S. cerevisiae</i>	--VEEGAEMVNNVVHSFRLQQTAFD-----KKSFLTYIKGYMKAVKAKLQETNP--EEV	106
<i>S. pombe</i>	--AEEGTETVNNLVYSFRLSPTSFD-----KKSYSYIKGYMKAIKARLQESNP--ERV	107



<i>A. nidulans</i>	NEFKKGAPAAVNKILKN--YDNYDVLMGESMDG---DAMHILIDFRE-DG-VTPYATLWK	171
<i>A. fumigatus</i>	DAFKKGAPAAVKKILAN--YDNYDVLMGHSMDG---DAMHVLIDFRE-DG-VTPYATLWK	171
<i>R. norvegicus</i>	KPFMTGAAEQIKHILAN--FNNYQFFIGENMNP---DGMVALLDYRE-DG-VTPFMIFFK	164
<i>H. sapiens</i>	KPFMTGAAEQIKHILAN--FKNYQFFIGENMNP---DGMVALLDYRE-DG-VTPYMIFFK	164
<i>D. melanogaster</i>	DIFKTNMNAKMDILG--RFKELQFFTGESMDC---DGMVALVEYREINGDSVPVLMFFK	164
<i>C. elegans</i>	DAFKKKIQGWVVSLLAKDRFKNLAFFIGERAAEGAENGQVAIEYRDVDGTEVPTLMLVK	173
<i>A. thaliana</i>	-VFKKGIEGATKFLLLPR--LSDQFFVFVGEGMHD---DSTLVFAYYKE--GSTNPTFLFYA	160
<i>S. cerevisiae</i>	PKFEKGATYVKKVIGS--LKDQEFFTGESMDP---DAMVVMNLNYRE-DG-TTPFVAIWK	159
<i>S. pombe</i>	PVFEKNAIGFVKKILAN--FKDYDFYIGESMDP---DAMVVMNLNYRE-DG-ITPYMIFFK	160



<i>A. nidulans</i>	HGLEEMKV	179
<i>A. fumigatus</i>	HGLEEMKV	179
<i>R. norvegicus</i>	DGLEMEKC	172
<i>H. sapiens</i>	DGLKMEKC	172
<i>D. melanogaster</i>	HGLEEEKC	172
<i>C. elegans</i>	EAIIEEKC	181
<i>A. thaliana</i>	HGLKEVKC	168
<i>S. cerevisiae</i>	HGIVEEKI	167
<i>S. pombe</i>	DGLVSEKF	168



Supplementary table. The primers used in this study

Name	Sequence (5' → 3')	Description
YTA01	ACA TGA CTT GCA TAT GAT TAT TTA CAA GGA TAT	<i>tcpA</i> ORF 5'-forward for cDNA amplification and sequencing
YTA02	CAA GTC ATG TGG ATC CTT AAA CCT TCA TCT CCT CAA	<i>tcpA</i> ORF 3'-reverse for cDNA amplification and sequencing
YTA11	GGT CCT CTC GGA CAC CTA CA	<i>tcpA</i> probe 5'-forward
YTA12	ACA CCA TCC TCA CGG AAG TC	<i>tcpA</i> probe 3'-reverse
YTA13	CCA GGC AGC TGC TTA CCT AC	<i>brlA</i> probe 5'-forward
YTA14	GAA CTG CAC CTG CTT CAT GA	<i>brlA</i> probe 3'-reverse
YTA03	AGG TTT GAC AGT TAA CTT CGT	5' flanking region of <i>tcpA</i>
YTA04	GGT CTG TTT CAT TAG ATG ACT TTT GAA AGA TGG AGA TGG AGA	5' flanking reverse with 5' <i>argB</i> tail
YTA05	ATC CAG CAC ATT CTT CTG AGG GTT CCA CGA ATT ATG AAG AAT	3' flanking forward with 3' <i>argB</i> tail
YTA06	ACC ACA GTC TCT CAA TCT CCT	3' flanking region of <i>tcpA</i>
YTA1-1	AGT CAT CTA ATG AAA CAG ACC	Forward primer for <i>argB</i>
YTA1-2	CCT CAG AAG AAT GTG CTG GAT	Reverse primer for <i>argB</i>
YTA07	AGT CAT CTA ATG AAA CAG ACC	5' nested of <i>tcpA</i>
YTA08	CCT CAG AAG AAT GTG CTG GAT	3' nested of <i>tcpA</i>
YTA09	CAG CTA GCA ACC TTC ATC TCC TCA AGA CC	<i>tcpA</i> ORF 5'-forward with <i>AscI</i>
YTA10	ACA CAC GGC GCG CCT GCA GGA TCT CCA TCT CCA	<i>tcpA</i> ORF 3'-reverse with <i>NheI</i>