

Identification of genetic interactors of Cdb4 by Yeast-2-Hybrid screening

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Abstract

Background/Objectives: Curved DNA binding protein (Cdb4) has unknown functions but binds to curved DNA *in vitro* in the fission yeast *Schizosaccharomyces pombe*. Previous study identified Nup184 as its synthetic lethal interactor. This study aims to further identify other interacting proteins of Cdb4 with a view to determining its biological function. **Materials and Methods:** Cdb4-containing bait plasmids were constructed by cloning the ORF (Cdb4_FL) or domain fragments (Cdb4_Nt, Cdb4_BDCt, Cdb4_Ct and Cdb4_t) into pGBKT7 backbone. The bait plasmids were first individually transformed into the *S. cerevisiae* host strain (AH109) and ~1 x 10⁹ of competent, bait-harboring AH109 cells were co-transformed with AD/cDNA library. Single colonies obtained from the low stringency plate were replica plated on medium and high stringency media and positive colonies further confirmed on a medium stringency medium containing 3-AT. cDNA fragments from prey plasmids were amplified by colony PCR, sequenced and the identity of interacting gene determined by a BLAST search. The function of the identified gene/protein was obtained from the pombe database (Pombase). **Results and Conclusion:** Of the five bait plasmids constructed, only Cdb4_t produced colonies on the high stringency confirmation. Eight proteins were found to interact with Cdb4_t and the interactions were sustained in the presence of 3-AT. The proteins were involved in diverse functional pathways including cell wall-related proteins (Psu1, Gto1), mitochondrial protein (Tdh1/Gpd3), transcription factors (Tmf1, Rep1), ubiquitin hydrolase (Ubp2), lipid metabolism (Plb1) and stress-related proteins (Pdr13). Cdb4 is potentially involved in several important processes in the cell. Its unique C-terminal region is responsible for interaction with proteins *in vivo*. Further genetic assays are required to confirm the biological significance of these identified interactions.

Keyword: Cdb4, *Schizosaccharomyces pombe*, Yeast-2-Hybrid, Cdb4_t, Cdb4_FL

Introduction

The curved DNA binding protein (Cdb4) is a non-essential protein with unknown biological function that binds curved DNA *in vitro* in the fission yeast *Schizosaccharomyces pombe* (1,2). It belongs to the clan MG, family M24, subfamily M24A of metallopeptidases, based on the classification of MEROPS database (3). Members of the subfamily M24A are typically methionyl aminopeptidases types I & II, and they are required for removal of the initiating N-terminal methionine of several newly synthesized proteins (4). A divalent metal (cobalt or manganese) center within its conserved catalytic site is pivotal to the

reactions of methionine aminopeptidases (MetAPs) but Cdb4 lacks this feature. Though absent in the budding yeast, homologues of *S. pombe* Cdb4 have been identified in a wide range of eukaryotes including *Tetrahymena thermophila*, *Dictyostelium discoideum*, *Leishmania major*, *Podospira anserine*, plants, mice and humans (5-7). The human Ebp1 and Cdb4 exhibit remarkable amino acid sequence conservation (41% identity, 63% similarity) and predicted secondary structures, implying the physiological importance of Cdb4 family among eukaryotes (8). Previously, it was reported that Cdb4 shows synthetic lethal interaction with Nup184 (8) but the precise

biological function of this well conserved protein is still elusive.

The yeast two-hybrid (Y2H) assay is a powerful tool to identify protein-protein interactions *in vivo* (9-11). In contrast to biochemical approaches such as affinity purification or co-immunoprecipitation of protein complexes, the Y2H presents handling protein interactions in their native environment. In addition, the *in vitro* techniques have restricted sensitivities as well as a marked bias for high affinity interactions (10). In this report, a Y2H library screen was conducted to identify the interacting proteins of Cdb4 in order to understand the biological pathways it is involved in and its role in such processes.

Materials and Methods

Strain and Media

The *Saccharomyces cerevisiae* strain, AH109 (*MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4A*, *gal80A*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*) was used as the host strain (12). The strain was grown in yeast peptone dextrose (YPD) medium composed of 1 % (w/v) yeast extract (Difco), 2 % (w/v) bactopectone (Difco), and 2 % (w/v) glucose or SD medium containing 0.145 % (w/v) yeast nitrogen base without amino acid and ammonium sulfate (Difco), 0.5 % (w/v) ammonium sulfate, and 2 % (w/v) glucose (13). A selective medium of synthetic dextrose (SD) supplemented with the required components was prepared by adding the following stock solutions to the SD medium as follows: 10 mM adenine (Ade), 40 mM tryptophan (Trp), 100 mM leucine (Leu), 20 mM uracil (Ura), 100 mM histidine (His), and 50 mM isoleucine. Solid medium was prepared by the addition of 2 % agar (Difco) before autoclave.

Construction of Cdb4 bait plasmids

Cdb4-containing bait plasmids were constructed by cloning Cdb4 ORF (Full-length Cdb4) or domain fragments of Cdb4 viz an N-terminus fragment (Cdb4_Nt), a fragment composed of the predicted DNA binding domain and the C-terminus (Cdb4_BDct), a C-terminus-containing bait plasmid (Cdb4_Ct) and a short fragment in the C-terminal region (Cdb4_t) which is well conserved in mouse and

mammals but absent in aminopeptidases from other species, into the bait plasmid (pGBKT7) backbone. Cloning was performed by the NEbuilder HiFi DNA assembly kit (New England) following the manufacturer's instruction. Oligonucleotides used in this study are listed in Table 1.

Yeast-2-hybrid library screening for Cdb4 interacting proteins.

The bait plasmids were individually transformed into the *Saccharomyces cerevisiae* host strain (AH109) and expression of bait protein as well as pseudo-positive effect was checked. Thereafter, several 2 – 3 mm colonies were inoculated in to 1 mL of SD/-Trp, vigorously vortexed and transferred to a 50 mL YES in a 200 mL conical flask and incubated for 16 – 18 hours to stationary phase ($OD_{600} > 1.5$). Appropriate volume of overnight culture was then transferred into 300 mL of yeast extract with supplements (YES) medium to achieve an $OD_{600} = 0.2 - 0.3$ and incubated with shaking at 30 °C for 3 hours until OD_{600} reaches about approximately 0.5. Yeast cells were collected by centrifugation (1,000 g for 5 minutes) at room temperature and washed twice with distilled water. The pellet was resuspended in freshly prepared 1.5 mL of 1X TE/LiAc solution in a new 50 mL tube and 1 mL of the competent cells then mixed with 20 µg activation domain (AD)/cDNA library (pTN-L1 – pGAD424 based library) and 2 mg Salmon Sperm Carrier DNA. Thereafter, 6 mL of PEG/LiAc/TE solution was added to the competent cell solution, vortexed to mix and incubated at 30°C for 30 minutes with shaking. DMSO (700 µL) was added and mixed by gentle inversion after which the solution was heat shocked for 15 minutes in a 42 °C water bath. This was then placed on ice for about 2 minutes and centrifuged at 1000 g for 5 minutes to collect the pellet. Cells were resuspended in 10 mL of YES and 100 µL of resuspended cells spread on SD/-Trp/-Leu plates and incubated at 30 °C for several days until colonies form. Single colonies were then re-streaked on a fresh SD/-Trp/-Leu (low stringency) plate and replica plated on both SD/-Ade/-His/-Leu/-Trp (high stringency) and SD/-His/-Leu/-Trp (medium stringency) media. Positive colonies were also

confirmed on an SD/-Ade/-His/-Leu/-Trp (high stringency) plate containing 5mM of 3-amino-1,2,4-triazole (3-AT). cDNA fragments contained in the prey plasmids derived from the library were amplified by colony PCR using prey-specific primers and sequenced. Identity of interacting gene was determined by a BLAST search of the sequence obtained. To confirm and replicate the identified interacting proteins, prey-derived plasmids were isolated from the yeast colony and co-transformed with the bait plasmid into AH109 strain.

Results

Construction of baits and library screening for interactors of Cdb4

In order to determine other possible interacting partners of Cdb4 proteins *in vivo*, five bait plasmids of Cdb4 were constructed (Figure 1). Interestingly, only the host strain harbouring the Cdb4_t plasmid produced colonies in the SD/-Ade/-His/-Leu/-Trp (high stringency) plate containing 5mM 3-AT. The result from the library screening using the Cdb4_t bait is summarized in Table 2.

Reproducibility and specificity of identified interactors

To eliminate false positives among the identified interaction, each gene was cloned into a prey plasmid and their interactions tested independently with the Cdb4_t and Cdb4_{Nt} baits (Table 2). Of the prospective interacting genes identified in Table 2, Mas5, Efla-c (Tef103) and SPAC637.03 did not produce a colony on the high stringency medium. Furthermore, the interaction between Plb1 and the bait was classified as 'strong' (denoted as ++) compared to the 'very strong' interactions (+++) seen between other genes and the bait plasmids. In addition, the specificity of each prey was tested with the negative control (p53 bait). Each prey plasmid did not interact with the negative control of p53. Under the screening conditions, p53 showed interaction with its cognate T protein confirming the specificity and reproducibility of the experiment.

Identification of the interacting gene function

Having confirmed the reproducibility of the identified interacting genes, the biological function(s) of each gene was sought in order to have an insight into the probable pathway(s) cdb4 is involved in. The gene names were used to query the dedicated knowledgebase of *Schizosaccharomyces pombe* genes, Pombase, and the Gene Ontology (GO) functions are summarized in Table 4. The screen identified two proteins involved in cell wall biosynthesis (Psu1 and Gto1), a mitochondria-related protein (Tdh1/Gpd3), two proteins with transcriptional activity (Tmf1 and Rep1), a stress-related proteins (Pdr13), one protein involved in lipid metabolism (Plb1) and a ubiquitin hydrolase (Ubp2).

Discussion

The curved DNA binding protein 4 (Cdb4) was purified by Yamada and co-workers due to its ability to bind synthetic curved DNA. The protein was subsequently shown to possess a higher affinity for curved DNA and low affinity to non-curved DNA (1). Identification of the precise biological function of the protein has been elusive due to its redundant, non-essential feature (2). Previous report showed a synthetic lethal relationship between Cdb4 and Nup184, an inner nuclear membrane protein. However, the functional significance of this synthetic lethal interaction is still unclear (8). Therefore, it is pertinent to unmask other interacting proteins to determine the major pathway(s) where Cdb4 is involved and ultimately elucidate its biological function.

The yeast-2-hybrid system is a highly sensitive method for detecting both stable interacting proteins as well as weak and transient protein interactions (11). In this study, it was shown that the full length bait (Cdb4_{FL}) was not a suitable bait for library screening. This could be attributed to the fact that the full length protein is not stably expressed or that its expression may be toxic to the host cell. In addition, the fused GAL4 domain may occlude the interaction site. A further explanation may be that the expressed bait protein is not properly

folded (14). A similar observation was made in the case of bait plasmids containing the N terminal truncation (Cdb4_Nt), the DNA binding domain and C terminal truncation (BDCt), as well as the construct containing only the C terminal truncation. However, the bait containing the unique 16 amino acid residues at the C terminal region (Cdb4_t) was proficient in forming stable interactions with prey plasmids.

The result of this study also revealed eight protein interactors of Cdb4 under 6 functional classes. Both Psu1 and Gto1 are cell wall related protein and are predicted to possess glycosidase activities in plant cell wall biogenesis. However, while Gto1 is a non-essential protein with an inferred biological function, Psu1 is an essential protein (deletion results in inviable vegetative cell population) implicated in fungal cell wall organization and in septum digestion after cytokinesis (15,16). Furthermore, Tdh1/Gpd3 is a proteins with mitochondrial-related functions and therefore connected to the energy status of the cell. *tdh1Δ* results in decreased cell population growth during glucose starvation as well as in the presence of several carbon and nitrogen sources (17). In addition, the MBF transcription factor activator (Rep1) is known to activate the transcription of cell cycle genes (*cdc10* and *cdc22*), meiotic cohesin complex genes (*rec11* and *rec8*) and also binds RNA polymerase II

cis-regulatory sequence (18). Also, *plb1Δ* cells exhibit decreased cell population at low temperature, glucose starvation and in the presence of other carbon sources (19) while the ubiquitin C-terminal hydrolase (Ubp2) deubiquitinates *S. pombe* Pcn1 during S phase (20). On the other hand, Gto1, Tmf1 and Pdr13 are among the many uncharacterized proteins in *S. pombe* and their biological roles are still unclear (2).

Conclusion

This study identifies eight proteins as genetic interactors of Cdb4 *in vivo*. The result shows that Cdb4 is involved in diverse important biological processes. However, the functional significance as well as the relationship of these interactor proteins with Cdb4 *in vivo* is still unknown. Further genetic assays are required to confirm the biological significance of these identified interactions.

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Conflict of interest

The authors declare no conflict of interest.

Table 1: Primer information for construction of bait plasmids

Gene Name	Primer Sequence (5' – 3')
Cdb4_FL_F	cggggatccgtcgacctgcacgactaaagaagcaactc
Cdb4_FL_R	ctagttatgcggccgctgcattattcattagctttgatggc
Cdb4_Nt_F	gggatccgtcgacctgcaatgtcgactaaagaagcaactt
Cdb4_Nt_R	atgctagttatgcggccgctgcacgtaaaagtatccatttcact
Cdb4_BDCt_F	cggggatccgtcgacctgcagatactttacgtttgagga
Cdb4_BDCt_R	ctagttatgcggccgctgcattattcattagctttgatggc
Cdb4_Ct_F	cggggatccgtcgacctgcagttgctgaattctattcaacc
Cdb4_Ct_R	ctagttatgcggccgctgcattattcattagctttgatggc
Cdb4_t_F	cggggatccgtcgacctgcagataaaaagtgaagacc
Cdb4_t_R	ctagttatgcggccgctgcattattcattagctttgatggc

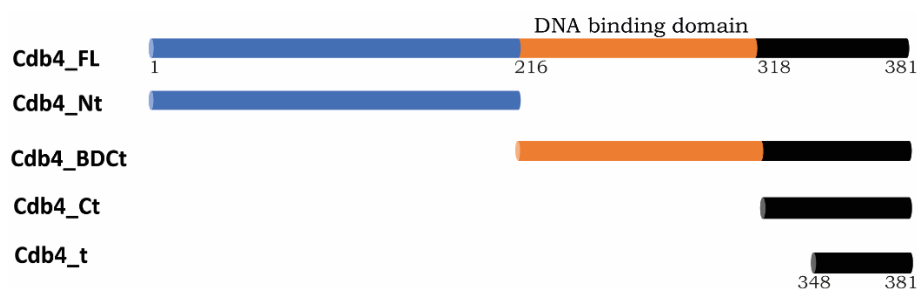


Figure 1: Construction of bait plasmids for yeast-2-hybrid assay. The Cdb4_FL consists of the N-terminus (residues 1 – 216; blue), DNA binding domain (residues 216 – 318; orange) and the C-terminal fragment (residues 318 – 381; black). Cdb4_t represents the unique C-terminal region (residues 348 – 381) conserved between Cdb4 and human erb3 binding protein (Ebp1).

Table 2: Interacting proteins detected more than once (frequency) in the library screen

Gene common name	Frequency
Psu1	16
Gto1	11
Tdh1/Gdp3	8
Tmf1	9
Pdr13	4
Plb1	4
Rep1	3
Ubp2	2
Efla-c (Tef103)	2
Pyr1	2
Mas5	2
Uncharacterized gene (SPAC637.03)	2

Table 3: Specificity of identified interactors. Interactions are denoted as absent (–), weak (+), strong (++) and very strong (+++) compared with the control (p53 and T).

Gene common name	Bait		
	Cdb4-t	Cdb4-Nt	pGBKT7-p53
Psu1	+++	+++	–
Gto1	+++	+++	–
Tdh1/Gdp3	+++	+++	–
Tmf1	+++	+++	–
Pdr13	+++	+++	–
Plb1	++	++	–
Rep1	+++	+++	–
Ubp2	+++	+++	–
Efla-c (Tef103)	–	–	–
Pyr1	–	–	–
Mas5	–	–	–
SPAC637. 03	–	–	–
pGAD423-T	–	–	+++

Table 4: Function of the interacting proteins derived from Pombase

Common name	Gene description	Gene Ontology (GO) molecular function
Psu1	Cell wall beta-glucosidase (predicted)	Hydrolase activity, acts on glycosyl bonds
Gto1	Alpha-glucosidase (predicted)	Hydrolase activity, acts on glycosyl bonds
Tdh1/Gdp3	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase activity and small molecule sensor
Tmf1	Golgi coiled-coil protein (predicted) (TATA element modulating factor-like protein)	Vesicle-mediated transport activity
Pdr13	Heat shock protein Ssz1 (predicted)	ATP-dependent protein chaperon activity
Plb1	Phospholipase B homolog.	Hydrolase activity, acts on ester bonds.
Rep1	MBF transcription factor activator	Molecular adaptor, transcription activator and transcription coregulatory activities
Ubp2	Ubiquitin C-terminal hydrolase	Possesses de-ubiquitinase activity.

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