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**Identification of fortilin's interacting proteins in a skeletal muscle cDNA library using the yeast two-hybrid system**

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สังกัด

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Study of fortilin interacting protein in a skeletal muscle using the yeast two-hybrid system

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## Abstract

Fortilin is implicated in development, cellular processes and malignant transformation. However, a unifying picture of proteins in key processes pertaining to fortilin function is not yet emerging. To investigate the potential interactions, human fortilin was expressed in the yeast cells, and used to screen for fortilin binding proteins in a human skeletal muscle. Yeast cells were transformed in the sequential procedure using yeast two-hybrid expression vector (pAS2-1) containing full-length human fortilin as “bait” for the first transformation, and pGAD10 vector containing a muscle cDNA library for the second transformation. In addition, a direct interaction of fortilin with known proteins, Ca<sup>2+</sup>-ATPase, creatine kinase, glycogen phosphorylase and troponin C was evaluated.  $\beta$ -Galactosidase activity was assayed as an index of interaction between fortilin and the potential target proteins whereas yeast mating strategy was used to eliminate false positive and to reconfirm the actual binding. From this analysis, eukaryotic translation elongation factor-1 delta (guanine nucleotide exchange protein) was identified as a putative fortilin interacting protein.

**Keywords:** Fortilin, yeast two-hybrid system, skeletal muscle cDNA library, eukaryotic translation elongation factor-1 delta

## 1. Introduction

Fortilin, a 19 kDa polypeptide, also known as a translationally controlled tumor protein (TCTP) and histamine releasing factor (HRF) (MacDonald *et al.*, 1995), is a highly conserved protein ubiquitously expressed in normal cells and tissues of eukaryotes. It involves in important processes such as cell growth, cell cycle progression, apoptosis and malignant transformation (Bommer and Thiele, 2004). Fortilin is a critical molecule in early mammalian development (Koide *et al.*, 2009). Intracellular fortilin is capable of various functions including a guanine nucleotide dissociation inhibitor regulating protein translation (Cans *et al.*, 2003), calcium binding and calcium transport (Arcuri *et al.*, 2004; Arcuri *et al.*, 2005). Previous data on fortilin mediating cell growth control and anti-apoptosis function include: 1.) Overexpression of fortilin has been shown to exert an inhibitory effect on caspase-3 activation, and could prevent HeLa and U2OS cells from undergoing etoposide-induced apoptosis; 2.) Fortilin depletion by antisense is able to induce MCF-7 cells to die spontaneously; 3.) Fortilin binds to and modulates the stability of the anti-apoptotic protein MCL1 (Li *et al.*, 2001; Zhang *et al.*, 2002; Liu *et al.*, 2005); and 4.) Fortilin and Bcl-xL are highly upregulated during T-cell activation induced by T-cell antigen receptor and CD28 costimulation, suggesting a role for fortilin in unifying the cell survival function of Bcl-xL with certain pathways in T-cell activation (Yang *et al.*, 2005). Furthermore, fortilin is secreted, and has extracellular function as histamine-releasing factor (HRF) and cytokine (MacDonald *et al.*, 1995; Bheekha-Escura *et al.*, 2000).

Protein-protein interactions elucidating by the yeast two-hybrid system provides useful information for functional characterization of proteins, and for understanding the molecular relationship of the interacting proteins. Through protein-protein interaction strategies, fortilin is identified as the interacting partner of MCL1 (Zhang *et al.*, 2002), mitotic polo-like kinase (Yarm, 2002), and microtubules and tubulin (Gachet *et al.*, 1999; Arcuri *et al.*, 2004). By screening a rat skeletal muscle cDNA library using the Na,K-ATPase  $\alpha$  subunit as bait, fortilin (TCTP) is found and later is shown to act as a cytoplasmic repressor of Na,K-ATPase (Jung *et al.*, 2004). Self-interaction of fortilin (HRF) which is essential for its cytokine-like activity has been demonstrated (Yoon *et*

*al.*, 2000; Kim *et al.*, 2009). In addition, fortilin (TCTP) is one of the 54 most abundant transcripts identified in a collection of cDNA library probes prepared from several types of bovine muscles (Sudre *et al.*, 2005). If consider its wide-ranging biological importance, the involvement of more proteins in key processes pertaining to fortilin's role is expected.

In the present study, we employed the yeast two-hybrid system to screen the human skeletal muscle protein library for protein interactions with fortilin. Investigation of direct interaction of fortilin with known target proteins (Ca<sup>2+</sup>-ATPase, creatine kinase and glycogen phosphorylase) was also included. The putative binding partner of fortilin was proved to be eukaryotic translation elongation factor-1 delta.

## 2. Methods

### 2.1 Bait and pray plasmids

Yeast two-hybrid expression vectors (pAS2-1, pACT2 and pGAD10) were obtained from Clontech Laboratories (Palo Alto, CA). Full-length cDNA of human fortilin was amplified by polymerase chain reaction (PCR) with a *EcoRI*-containing forward primer (5' CG GAA TTC ATG ATT ATC TAC CGG GAC CTC 3') and a *BamHI*-containing reverse primer (5' CG GGA TCC TTA ACA TTT TTC CAT TTC TAA 3'), and cloned into pAS2-1, a GAL4 DNA-binding domain (DB)-containing vector to create pAS-For. Full-length cDNA of human Ca<sup>2+</sup>-ATPase, creatine kinase and glycogen phosphorylase in pGAD10, a GAL4 activation domain (AD)-containing vector were recovered from human skeletal muscle cDNA library (Purintrapiban *et al.*, 2001). Ca<sup>2+</sup>-ATPase cDNA was subcloned into pACT2 in-frame with the AD. The correct orientation and composition of the insert were confirmed by DNA sequencing. All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). A pre-made human skeletal muscle cDNA library (Clontech Laboratories) was constructed in pGAD10.

## 2.2 Library plasmid DNA

The skeletal muscle cDNA plasmid library in pGAD10 (pGAD-Lib) was amplified according to manufacturer's instructions. Briefly, the *E. coli* library transformants were plated directly onto Luria Bertani broth (LB)/ampicillin plates at high density (20,000 colonies/150 mm-plate). Following overnight incubation at 37°C, the colonies were scraped into 2 L of LB/amp broth and incubated at 37°C for 3 h with shaking. The cells were collected by centrifugation. A CsCl gradient-purification was used to isolate the plasmid libraries (Heilig *et al.*, 1994).

## 2.3 Yeast competent cells

The modification of lithium acetate (LiAc) method developed by Ito *et al.* (1983) and a large-scale preparation were used for preparing yeast competent cells. *Saccharomyces cerevisiae* cells strain Y190 (MAT<sub>a</sub>, *his*<sup>-</sup>, *trp*<sup>-</sup>, *leu*<sup>-</sup>) were inoculated into 1 ml of Yeast-Peptone-Dextrose (YPD; 10 g of yeast extract, 20 g of peptone, 20 g of dextrose per liter), vortexed to disperse any clumps, and transferred into 50 ml of YPD. The culture media were incubated at 30°C for 20 h with shaking at 250 rpm. The overnight culture was transferred into 300 ml of YPD to produce an OD<sub>600</sub> = 0.2-0.3, incubated at 30°C with shaking at 230 rpm for 3 h (Y190 and Y187) or 4-5 h (Y190 carrying pAS-For). The cell pellet was collected by centrifugation at 1,000xg for 5 min, washed once in water, and re-suspended in 1.5 ml of freshly made TE/LiAc (TE; 10 mM Tris.Cl with 1 mM EDTA)/0.1 M lithium acetate (LiAc, 1x) solution.

## 2.4 Yeast transformation

The LiAc-mediated method of Gietz *et al.* (1992) was used to introduce DNA into yeast. The sequential transformations were adopted for screening a library. Firstly, the pAS-For constructs were transformed into Y190 cells, and the resulting transformants were then used in large-scale transformations with pGAD-Lib plasmids as following: 1 ml of yeast competent cells in TE/LiAc solution was mixed with 200 µg of library plasmids and 2 mg of herring testes carrier DNA. Six ml of polyethylene glycol

(PEG)/LiAc solution (40% PEG, 1x TE/LiAc) was then added, and the mixture was incubated at 30°C with shaking. After the incubations, DMSO was added to 10% (v/v) and mixed gently by inversion. The cells were heat-shocked for 15 min in a 42°C water bath, placed on ice for 5 min, and collected by centrifugation for 5 min at 1,000xg. The cells were re-suspended in 10 ml of TE buffer (pH 7.5) and then plated on the synthetic dropout (SD) medium (SD/-his, -leu, -trp, +3-amino 1,2,4-triazole (3AT, a competitive inhibitor of *His3* gene product)) to select for transformants containing the plasmid(s) that expressed  $\beta$ -galactosidase ( $\beta$ -gal) and histidine-3 genes. The whole library was accomplished in five large-scale experiments and screened twice. The transformation efficiency was determined by the number of colonies growing on the SD/-leu medium that selected for the library plasmid only. The co-transformation efficiency was determined by the number of colonies growing on SD/-leu, -trp medium that selected for both plasmids. To test other interacting proteins and controls, small-scale transformations (one-tenth scale-down) was used.

## 2.5 $\beta$ -Galactosidase assays

The colony-lift filter assay was used to screen for a  $\beta$ -gal-producing colonies that survived the *His3* growth selection in a GAL4 two-hybrid library screening, and also was used to assay for interactions between two known proteins. A filter was placed over the surface of the plate of fresh colonies to be assayed (grown at 30°C for 2-4 days), then carefully lifted off the agar plate and was placed in a -80°C freezer with the colonies facing up for at least 1 hr. The filter was allowed to thaw at room temperature. The filter was then placed, colony side up, on X-Gal-pres soaked filters. X-gal solution composed of 0.27 ml  $\beta$ -mecaptoethanol, 1.67 ml X-gal stock solution (20 mg/ml) and 100 ml Z buffer pH 7.0 ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  16.1 g/l,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  5.5 g/l, KCl 0.75 g/l,  $\text{MgSO}_4 \cdot \text{H}_2\text{O}$  0.246 g/l). The filter was incubated at room temperature until the appearance of blue colonies. The blue color indicates *lacZ* reporter gene expression.

## 2.6 Cycloheximide selection

A colony of yeast cell carrying two different plasmids (the pAS-For plasmid and the pGAD-Lib plasmid) was re-suspended in 200  $\mu$ l of water, and 10  $\mu$ l of the cell suspension was spread on SD/-leu medium containing 10  $\mu$ g/ml cycloheximide. The plates were incubated at 30°C for 3-5 days until individual  $Cyh^R$  colonies appeared. The pAS-For plasmid that contained the wild-type *CYH<sup>S2</sup>* gene would render yeast host strains Y190 to become sensitive to cycloheximide (Guthrie and Fink, 1991). Therefore, the pAS-For plasmid had spontaneously been lost from the cell while the pGAD-Lib plasmid would be retained. The loss of GAL4-DB plasmid was verified by transferring the  $Cyh^R$  colonies to SD/-trp plates. Colonies that had lost GAL4-DB plasmids would not grow.

## 2.7 Yeast mating

Introducing two different plasmids into the same host cells induced by mating of a haploid state of yeasts to produce a diploid organism was used to verify that the candidate AD-library proteins identified in the two-hybrid library screen can activate the reporter genes only in the presence of the DB-target protein. Various control plasmids and candidate plasmids to be tested were transformed into proper hosts (strain Y187 or Y190), and the mating pairs were set up. One colony of each type was placed in a 1.5 ml microtube containing 0.5 ml of YPD medium. The medium was vortexed and incubated at 30°C with shaking at 250 rpm for 6 hr. The mating culture (100  $\mu$ l) was then spreaded on SD/-leu, -trp, and SD/-his, -leu, -trp, +3-AT plates. The plates were incubated at 30°C for 4-5 days or until diploid cells appeared.

## 2.8 Yeast plasmid extraction

Yeast plasmid was isolated using the PrepEase<sup>®</sup> yeast plasmid isolation kit (USB Corporation, USA) with slight modification. In brief, the yeast cells from 5 ml of overnight culture was re-suspended in spheroplast solution, mixed with enzyme solution, incubated at 37°C for 2 h, and then centrifuged to remove the supernatant. The cell pellet



was lysed in the lysis buffer at 95°C for 10 min. After chilling on ice, the precipitation solution was added. The mixture was incubated on ice for 20 min and then centrifuged. To the supernatant, an equal volume of chilled 100% isopropanol was added. The tube was incubated at -80°C for at least 1 h and centrifuged at 14,000 rpm for 20 min at 4°C to collect the pellet. The pellet was washed once with 70% ethanol. The plasmid was dissolved in distilled water and used for PCR, transformation or DNA sequencing.

## 2.9 Protein extraction for Western blot

Five ml of yeast overnight culture in SD selection medium was prepared from a single isolated colony. The cultures were vortexed and inoculated into 50 ml of YPD medium. After incubation at 30°C with shaking until the OD<sub>600</sub> had reached 0.4-0.6, the cultures were quickly chilled and centrifuged at 1,000xg for 5 min at 4°C. Cell pellets were washed in 50 ml ice-cold water. The pellets recovered by centrifugation were resuspended in cracking buffer/inhibitor solution. The cracking buffer (7.08 M urea, 4.4% w/v SDS, 35.4 mM Tris-HCl pH 6.8, 0.09 mM EDTA, 0.35 mg/ml bromophenol blue, 0.88% v/v β-mercaptoethanol) containing protease inhibitors (protease inhibitors cocktail, Sigma) prepared immediately before needed and pre-warmed to 60°C. The ratio of 100 μl of cracking buffer to 7.5 OD<sub>600</sub> units of cells was used. Total number of OD<sub>600</sub> units was equal to OD<sub>600</sub> of 1 ml sample x the culture volume. Proper amount of cracking buffer was added to cell pellets. The cell suspension was then transferred to a microtube containing 80 μl of glass beads per 7.5 OD<sub>600</sub> units of cells. The sample was heated at 70°C for 10 min and then vortexed for 1 min. The supernatants were collected by centrifugation at 14,000 rpm for 5 min in 4°C. The remaining pellet debris was placed in a boiling water bath for 5 min, vortexed for 1 min and centrifuged to collect the supernatants. Supernatants were combined and stored in -70°C freezer.

## 2.10 SDS-PAGE and Western blot

Proteins were subjected to 10% SDS-PAGE according to Laemmli (1970). After electrophoresis was completed, the gel was transferred to the electroblotter apparatus. The proteins were electrotransferred to PVDF membranes for 0.5 h at 50V, followed by

1.5 hr at 100V. Non-specific sites on the membrane were blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% v/v Tween 20) for 1 hr. The membrane was then incubated for 2 hr with monoclonal antibody against GAL4-DB or GAL4-AD (Clontech Laboratories, 1:5,000 dilution). The bound antibody was detected by 1 hr incubation with conjugated secondary antibody. Detection of bands was carried-out with the alkaline phosphatase colorimetric detection system.

### 3. Results and Discussion

The yeast two-hybrid analysis (Fields and Song, 1989) takes advantage of the properties of the GAL4 protein required for the expression of genes encoding enzymes for galactose utilization. The system of two-hybrid proteins contains portions of GAL4: the GAL4 DNA-binding domain (DB) fused to a protein 'X' and a GAL4 activating domain (AD) fused to protein 'Y'. If 'X' and 'Y' can form a protein-protein complex and reconstitute the proximity of the two GAL4 domains, the transcription of a gene regulated by GAL1 upstream activating sequence will be initiated. The yeast two-hybrid system was employed to screen muscle cDNA library for a gene encoding a protein that interacts with fortilin. In this study, the pAS-For (bait) plasmid in which fortilin gene was inserted in-frame with the GAL4-DB (Figure 1A) was transformed into yeast Y190 host strain for protein expression. The expression of DB/fortilin fusion proteins was verified by Western blot analysis with GAL4-DB monoclonal antibody, and correctly confirmed as a ~39 kDa protein (Figure 1B).

To test for nonspecific activation function of the DB/fortilin, Y190 cells were co-transformed with pAS-For and pACT2, and assayed for *lacZ* gene expression by the colony-lift  $\beta$ -galactosidase filter method. As shown in Table 1, co-expression of fortilin with GAL4-AD did not cause *lacZ* expression (yield white colonies) whereas  $\beta$ -gal positive blue signal was demonstrated with colonies carrying either pCL1 (positive control plasmid that encodes and expresses the full-length, wild type GAL4 protein) or pTD1-1 (positive control plasmid that encodes DB/murine p53 protein) plus pVA3-1 (positive control plasmid that encodes AD/SV40 large T-antigen protein). This suggested that the bait protein did not autonomously activate the reporter gene.

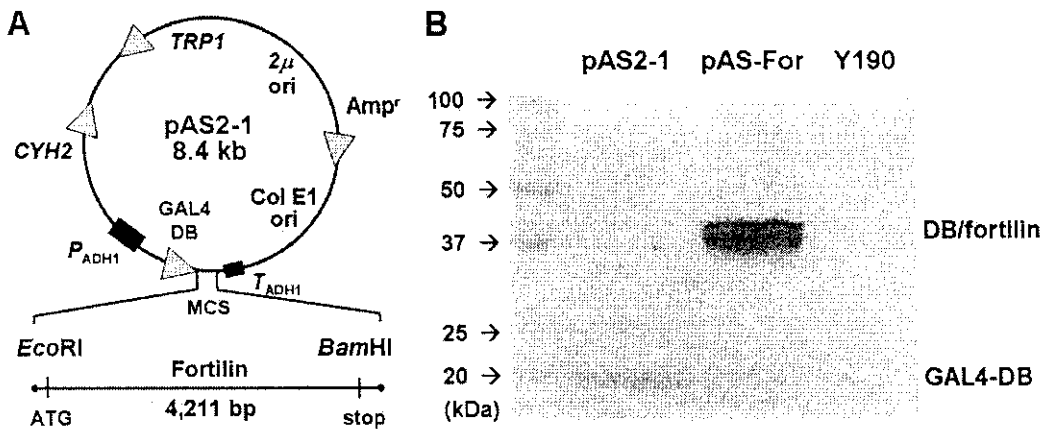


Figure 1. Schematic diagram of bait plasmid and its expressed proteins in yeast.

A: Yeast two-hybrid vector, pAS2-1, contains sequences encoding GAL4 DNA-binding domain (DB). Expression of the fusions in yeast is driven from *ADH1* promoter. Fortilin cDNA was cloned into *EcoRI* and *BamHI* as shown.

B: Expression of DB/fortilin was verified by Western blot. Protein lysates from yeast Y190, Y190 carrying pAS2-1 or pAS-For plasmids were prepared by urea/SDS method, separated on 10% SDS-PAGE, probed with antibodies for GAL4-DB.

Table 1. Test for autonomous reporter gene activation

Transformations		Selection	LacZ phenotype	To serve as
Plasmid 1	Plasmid 2	medium	(Colony color)	
pAS-For (DB/fortilin)	pACT2 (AD)	SD/-leu, -trp	White	Test
pVA3-1 (AD/T-antigen)	pTD1-1 (DB/p53)	SD/-leu, -trp	Blue	+ve control
pCL1 (GAL4)	-	SD/-leu	Blue	+ve control

To recover fortilin interacting proteins, approximately  $2 \times 10^6$  clones were screened for specific binding between fortilin and skeletal muscle library. Fusion of muscle cDNA library to the GAL4-AD in pGAD10 led to the appearance of twelve double-positive colonies (expressing histidine and lacZ) when transformed into the yeast pre-transformed with pAS-For (Figure 2). The transformation efficiency was  $0.9 \times 10^3$  colony-forming unit (cfu)/ $\mu\text{g}$  DNA. The presence of two-hybrid plasmids in the positive colonies was determined by PCR as shown in Figure 3.

The pGAD10 plasmids encoding cDNAs interacting with fortilin were recovered by spreading the positive two-hybrid interaction clones on SD/-leu, +cyh plates to permit a cycloheximide counterselection. This process eliminates the pAS-For plasmid which contains the wild-type *CYH<sup>S2</sup>* gene. Of the 12 positive clones analyzed by DNA sequencing, 10 clones had DNA sequences (differ in length) matched to the nucleotide sequences within the NCBI database for eukaryotic translation elongation factor 1 delta (EF-1 $\delta$ ) (GenBank accession, NM 001960). The remaining clones were identified as K (lysine) acetyltransferase 5 (KAT5) (GenBank accession, NM 182710) and troponin C type 2 (GenBank accession, NM 003279).

To eliminate false positive and reconfirm that the candidate AD/Library proteins identified in the two-hybrid library screen could activate the reporter genes only in the presence of the DB/fortilin, yeast strain Y190 and Y187 which differed physiologically in mating types were used in the mating strategy. In these experiments, yeasts carrying different plasmid were reproduced sexually by cellular and nuclear fusion to produce a diploid organism. The His3 and LacZ phenotypes of diploids were determined, and the results were shown in Table 2. Only EF-1 $\delta$ -producing clones strongly expressing lacZ were verified as a putative fortilin binding protein. Sequencing indicated that two of the clones (KAT5 and troponin C) were not inserted in-frame, thus, they either expressed reporter gene without DB/fortilin or encoding short sequences with no identity. In this study, we select troponin C for further analysis.

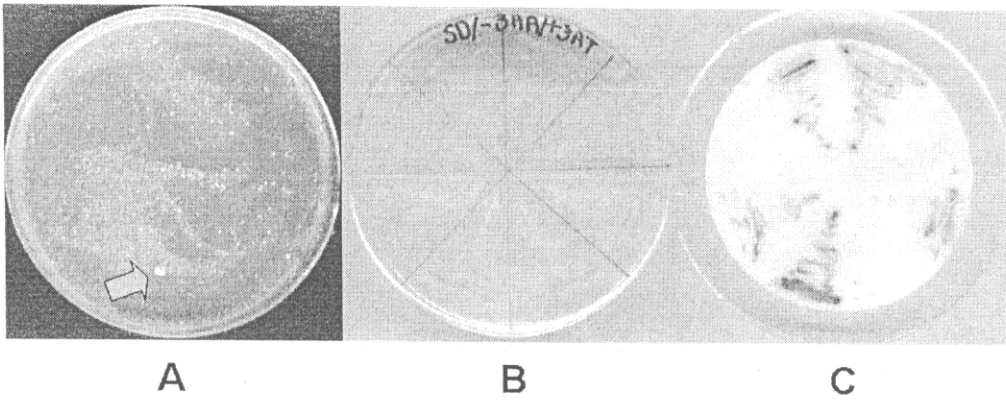


Figure 2. Screening muscle cDNA library for fortilin interacting proteins by the yeast two-hybrid system. A: Y190 cells carrying pAS-For were transformed with muscle cDNA library plasmids and spread on SD/-his, -leu, -trp, +3AT plates. Arrow, positive AD/library clone ( $\text{His}^+$  colonies). B: Example of 8  $\text{His}^+$  clones was streaked out on fresh plates. C: Expression of lacZ reporter was assayed and demonstrated by the colony-lift filter method.

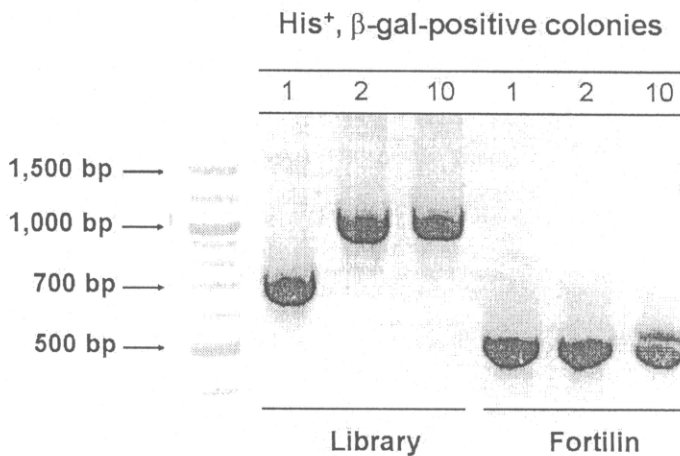


Figure 3. Yeast two-hybrid cDNA inserts in  $\text{His}^+$ ,  $\beta$ -gal-positive colonies. Library and fortilin cDNAs from three positive colonies were PCR-amplified using specific primers for either GAL4-AD or fortilin.

Table 2. Yeast mating experiments of the two-hybrid interactive proteins

Protein in		Phenotype		To serve as
Y187 ( <i>MAT<sub>α</sub></i> )	Y190 ( <i>MAT<sub>a</sub></i> )	<i>His3</i> (Growth on SD/ -his,-leu,-trp,+3AT)	<i>LacZ</i> (β-gal assay)	
DB /p53	AD/T antigen	Growth	Blue	+ve control
DB /lamin C	AD/library	No growth	White	- ve control
DB	AD/library	No growth	White	- ve control
DB /fortilin	AD/library 1, 2, 10 (EF-1δ)	Growth	Blue	Test
	AD/library 6 (KAT5)	Growth	White	Test
	AD/library 46 (Troponin C)	Growth	White	Test

In order to investigate potential interaction of troponin C, complete sequence of troponin C gene was adjusted for correct reading frame in pACT2, and determined for the expression as a ~37 kDa AD/troponin C fusion protein by Western blotting (data not shown). Interactions between fortilin and troponin C along with other known proteins, i.e. Ca<sup>2+</sup>-ATPase, creatine kinase and glycogen phosphorylase, previously obtained from this library using a different bait (Purintrapiban *et al.*, 2001) were analyzed. Again, we could not detect any clone strongly expressing lacZ. These results indicated that the test proteins do not interact with fortilin in the yeast two-hybrid system.

Despite protein-protein interactions in the yeast two-hybrid system influenced by many cellular factors leading to false-positive clones caused by non-specific binding have been known to be a major problem in cDNA library screening (Luo *et al.*, 1996). It was surprising that we could only detected a total of 12 double-positive clones by repeatedly screening the library, and EF-1δ was the only protein selected for interacting

with fortilin. Considering that two-hybrid selection is done with the fusion molecules, this may result in limited accessibility of bait by folding of the moiety of the fusion molecule. Some protein molecules need posttranslational modifications necessary for binding. Hence, interactions which are dependent on protein phosphorylation (i.e. tyrosine phosphorylation) that does not occur in yeast cells become impossible (Fields and Sterglanz, 1994). In addition, many proteins need factors to bind to their partner, and these cofactors might not be available in the nucleus. Therefore, some proteins either will not be selected or preferably selected above all other.

EF-1 $\delta$ , a guanine nucleotide exchange protein, is a part of the EF-1 protein complex (composed of four subunits: alpha, beta, gamma and delta) that mediates the elongation step of protein synthesis involved the hydrolysis of guanosine 5'-triphosphate (GTP) (Riis *et al.*, 1990). Considerable evidence reveals overexpression of EF-1 mRNA or protein of alpha, gamma, and/or delta subunits is correlated with oncogenic transformation and progression of malignant tumors (Grant *et al.*, 1992; Mimori *et al.*, 1995; Mathur *et al.*, 1998; Ogawa *et al.*, 2004) suggesting an important role of EF-1 in regulating cellular growth and tumorigenesis. We are not the first case of identification of EF-1 $\delta$  as a fortilin binding partner. The interactions between TCTP (fortilin) and either EF-1A (EF-1 $\alpha$ ) or EF-1B $\beta$  (EF-1 $\delta$ ) resulting from yeast two-hybrid hunt from human monocytic leukemia U937 cells have been described by Cans *et al.* (2003). Langdon *et al.* (2004) has uncovered EF-1 $\delta$  in a human T cell leukemia (Jurket cell) cDNA library, and determined its actual binding interaction with HRF (fortilin) using co-immunoprecipitation. Additional studies (Cans *et al.*, 2004) indicate that TCTP preferentially stabilizes the GDP-bound EF-1A, and impairs the GDP exchange reaction promoted by EF-1B $\beta$ . These suggest the intracellular role of fortilin in the elongation step of protein synthesis.

In conclusion, we have used the yeast two-hybrid system to analyze the interactions of fortilin with four known proteins, Ca<sup>2+</sup>-ATPase, creatine kinase, glycogen phosphorylase, and troponin C, and to search for fortilin binding partner in skeletal muscle cells. We are able to recover and confirm EF-1 $\delta$  as fortilin interaction protein. Other test proteins do not interact with fortilin in the system of yeast two-hybrid.

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