The A20-binding protein ABIN-2 exerts unexpected function in mediating transcriptional coactivation

Chia-Yi Chien[a,b], Wei-Kuang Liu[b], Chen-Kung Chou[a,b], Jin-Yuan Su[a,*]

[a] *Department of Life Science, National Yang-Ming University, Taipei 112, Taiwan, ROC*
[b] *Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan, ROC*

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Abstract The human ABIN-2 was originally identified as an A20-associating cytosolic protein to block NF-κB activation induced by various stimuli. Here we report that ABIN-2 has the potential to enter the nucleus and plays a role in mediating transcriptional activation in both yeast and mammalian cells. The Gal4BD–ABIN-2 fusion protein is able to drive the expression of the *GAL4*-responsive reporter gene in yeast efficiently without the need of the Gal4p activation domain, suggesting that ABIN-2 functions as a transcriptional coactivator and facilitates transcription in yeast. In contrast to the activity in yeast, however, only the C-terminal fragment of ABIN-2 exerts the transactivating activity in mammalian cells but not the full-length ABIN-2 protein. This observation has led to the identification of the N-terminal 195 amino acids of ABIN-2 as a regulatory domain, which retains the full-length ABIN-2 in the cytoplasm of mammalian cells and thus cannot transactivate. We have also found that BAF60a, a component of chromatin-remodeling complex, interacts with ABIN-2 by the yeast two-hybrid analysis. Together, our results suggest that the nuclear ABIN-2 defines a novel transcriptional coactivator and acts presumably by recruiting a chromatin-remodeling complex to the site of the target gene.

Key words: Transcriptional coactivator; ABIN-2; FLIP1; Chromatin-remodeling complex; BAF60a; Yeast two-hybrid

1. Introduction

Elucidating the effects of an inhibitor, called A20, has provided a key aspect to unravel the regulation of NF-κB activity in the last decade [1]. A20 is a cytosolic zinc finger protein and was first identified as a tumor necrosis factor (TNF) responsive gene in human endothelial cells [2–4]. It was subsequently found that A20 inhibits NF-κB activity and TNF-mediated programmed cell death [5–7]. The A20-dependent inhibition of NF-κB requires a number of A20-associating proteins called ABINs (A20-binding inhibitor of NF-κB activation). Among these proteins are ABIN-1 and ABIN-2, which have been characterized previously [1,8]. ABIN-1 is the murine homolog of human immunodeficiency virus Nef-associated factor, which has a canonical leucine zipper structure [8,9]. ABIN-2 is a novel cytosolic protein that shows no homology with any other known protein [10]. Overexpression of ABIN-1 and ABIN-2 was sufficient to inhibit NF-κB activation induced by TNF or interleukin-1 (IL-1). The step where ABINs inhibited NF-κB activation was predicted to be upstream of the IKK complex, since ABINs failed to inhibit NF-κB activation induced by overexpression of IKK directly [8,10].

LKB1 has been proposed as a tumor suppressor and the causative gene of Peutz–Jeghers syndrome [11–13]. To investigate the cellular function of LKB1, we have previously cloned ABIN-2, also known as FLIP1, as one of the LKB1-interacting proteins by yeast two-hybrid screening [14]. In an effort to further explore the function of ABIN-2/FLIP1, we have unexpectedly discovered that ABIN-2 has the potential to enter the nucleus and possesses transactivating activity. Thus, ABIN-2 may define a dual-functional protein that regulates NF-κB activation in the cytoplasm and behaves as a novel transcriptional coactivator in the nucleus.

2. Materials and methods

2.1. Yeast two-hybrid analysis

The yeast two-hybrid system, including pPC62 (containing Gal4BD (Gal4 binding domain)) and pPC86 (containing Gal4AD (Gal4 activation domain)) plasmids, used in this study has been described previously [15]. The yeast strain, PCY2 (*MATα*Δ*gal4*Δ*gal80 URA3*::*GAL1–LacZ lys2 his3 trp1 leu2 ade2*), was routinely used in the two-hybrid analysis and transactivation assay except the screening of ABIN-2-interacting proteins. To screen for the ABIN-2-interacting proteins, the host strain PCY2* was obtained by mutagenesis of PCY2 with ethyl methanesulfonate (EMS; Sigma, St. Louis, MO, USA) according to the published protocol [16]. The human cDNA synthesized from hepatoma cell line, Huh7, was constructed into pPC86 and used as prey in the two-hybrid screening. Yeast transformation was performed by the lithium acetate procedure [16]. The β-galactosidase activity was analyzed by the colony-lift filter method [17]and the appearance of blue color on the colonies within 2 h after the addition of substrate was considered a positive reaction in this assay. The positive reaction by the colony-lift filter method was routinely reconfirmed by the liquid assay [16].

2.2. Plasmid constructions and recombinant DNA technique

The full-length ABIN-2 DNA was cloned as described in a previous study [14]. Various truncated ABIN-2 DNA fragments were generated by polymerase chain reaction (PCR) amplification from the full-length ABIN-2 DNA using specific primers designed to match the sequence at the truncation sites. A *Sal*I-recognition sequence and a *Not*I-recognition sequence were always incorporated into the 3′ and the 5′ primers, respectively, to allow a convenient cloning procedure following the PCR reaction. The PCR products were digested with *Sal*I and *Not*I, and then ligated into pPC62 to form Gal4BD-fused ABIN-2 constructs. The mammalian expression constructs of the Gal4BD-fused ABIN-2 equivalent to the yeast expression clones were carried out by *Hin*dIII and *Not*I digestion of each pPC62–ABIN-2 and then religated into the pcDNA3.0 vector (Invitrogen, CA, USA). For two

*Corresponding author. Fax: (886)-2-2823 4898.
E-mail address: jysu@ym.edu.tw (J.-Y. Su).

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truncated forms of BAF60a, BAF60a$_{1-380}$ was cut out by *Sal*I and *Xba*I digestion and BAF60a$_{1-304}$ was cut out by *Sal*I and *Pst*I digestion from pPC62–BAF60a and religated into pPC62. The BAF60a$_{176-476}$ fragment, on the other hand, was obtained by PCR as described above.

2.3. Cell culture, transfection and luciferase assay

Human embryonic kidney cells, HEK 293T, were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, MD, USA) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 3.7 mg/ml sodium bicarbonate, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 100 μM non-essential amino acids. Transfection of 293T cells was performed using the standard calcium phosphate precipitation method [18].

For mammalian transactivation assay, 293T cells were first seeding at 2.5×10^5 cells/well in a 12-well plate. The Gal4BD-fused ABIN-2 constructs were then cotransfected with *GAL4*-responsive luciferase reporter plasmid (pFR-Luc; Stratagene, La Jolla, CA, USA). 22 h after transfection, cells were lysed in lysis buffer (100 mM potassium phosphate, pH 7.4, 1% Triton X-100, 1 mM dithiothreitol, and 20 mM EDTA). Cell debris was removed by centrifugation and 10 μl of cell extract was then used to measure the luciferase activity using the Luciferase Assay kit (Promega, Madison, WI, USA) and an automatic microplate luminometer. The luciferase activity was routinely normalized by the amount of protein used in the assay. For Western blotting analysis of these cell lysates, aliquots of cell lysate were resolved by SDS–PAGE and transferred onto nitrocellulose membranes. Western blotting with anti-ABIN-2 antibodies on these membranes was performed according to standard procedure [19].

2.4. Immunofluorescence microscopy

Transfected 293T cells were seeded onto glass coverslips coated with poly-L-lysine. Slides were placed in DMEM containing 10% fetal calf serum, and cells were allowed to grow overnight until they reached a semi-confluent state. The cultured cells were washed and fixed with 3% formaldehyde in phosphate-buffered saline (PBS). Fixed cells were first incubated with anti-ABIN-2 antibodies, washed twice in PBS, and reacted with fluorescein-conjugated secondary antibodies. Cells were washed again with PBS, analyzed under a fluorescence microscope, and photographed.

3. Results and discussion

3.1. ABIN-2 exerts transactivation activity in yeast

ABIN-2 was previously identified as one of the LKB1-interacting proteins by yeast two-hybrid screening in our laboratory [14]. While we were in the process of reconfirming the protein–protein interaction between ABIN-2 and LKB1 in the yeast two-hybrid assay, we discovered that ABIN-2 fused to the DNA BD of Gal4p could drive the expression of reporter gene, *LacZ*, without the presence of the Gal4pAD. On the other hand, the fusion of ABIN-2 with the Gal4pAD did not cause the expression of *LacZ*, suggesting that the transactivation by ABIN-2 required a specific DNA-binding activity toward the target gene.

There are four potential coiled-coil domains predicted in the sequence of ABIN-2 [14]. Sequential deletion of these coiled-coil domains from its N-terminus was generated and tested for the transactivation activity in yeast. The result shown in Fig. 1 indicated that the transcriptional activation of ABIN-2 was mainly located in the C-terminal half of the protein, from amino acids 196 to 429. From the analysis of these overlapping constructs, two regions in the sequence of ABIN-2, amino acids 196–253 and 346–429, appeared to be essential for transactivating activity (Fig. 1). In addition, the observed transcriptional activation by ABIN-2 was not limited to the fusion of Gal4pBD. When the proper DNA BD was provided, ABIN-2 exerted its transcriptional activation. This was demonstrated by fusing ABIN-2 to the bacterial DNA-binding protein LexA; ABIN-2 was able to cause the expression of reporter gene under the regulation of LexA operator sequence as well (data not shown). Judged by its lack of DNA-binding activity, as shown above, and no predicted sequence or structural features which resemble known transcription factor, ABIN-2 is probably not a transcription factor by itself but may be a novel transcriptional coactivator.

3.2. Regulation of ABIN-2 transactivation in mammalian cells

To test whether ABIN-2 could behave as a transcriptional coactivator in mammalian cells, we performed a similar transactivation assay in the mammalian expression system using luciferase as a reporter gene under Gal4-responsive regulation. A mammalian expression construct of Gal4BD–ABIN-2 was transfected into 293T cells together with the reporter gene construct. The successful expression of the Gal4BD–ABIN-2 fusion protein in 293T cells was first confirmed by Western analysis (Fig. 2A). To our surprise, unlike in the case of yeast expression, the full-length ABIN-2 fused with Gal4BD was not able to drive the expression of reporter gene after transfection (Fig. 2B). We then asked the reason why the full-length ABIN-2 failed to activate in the mammalian cells. Since

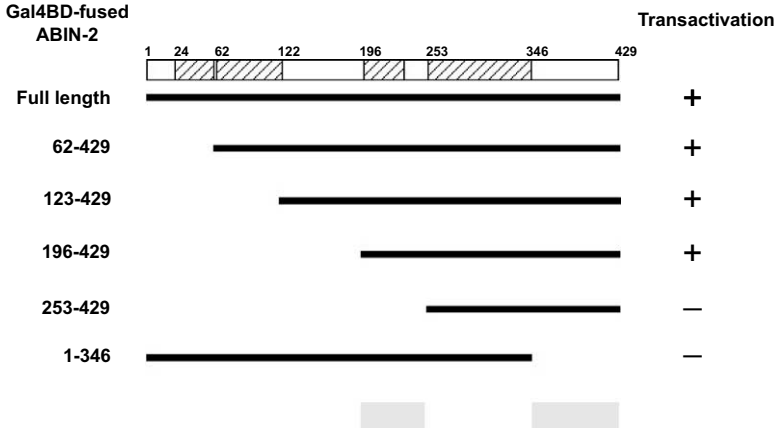


Fig. 1. Transactivation assay of ABIN-2 in yeast. Various lengths of truncated ABIN-2 as indicated by the amino acid numbers were fused to Gal4pBD and transformed into yeast. The transactivation was measured by the expression of the reporter gene, *LacZ*. A positive sign indicated the expression of active β-galactosidase analyzed both by colony-filter lift and liquid assays. The hatched boxes shown on the sequence of ABIN-2 are the potential coiled-coil regions. The boxes shown at the bottom are two domains of ABIN-2 important for transactivation.

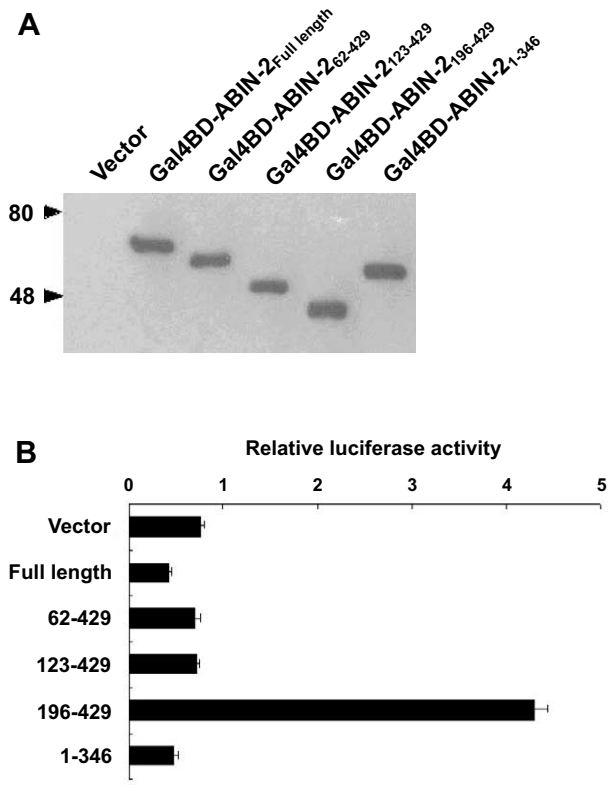


Fig. 2. Transactivation of ABIN-2 in mammalian cells. A: Western analysis of the expressed ABIN-2s in mammalian cells by anti-ABIN-2 serum. Each of the truncated ABIN-2s was fused to Gal4pBD as indicated and transfected into 293T cells. The protein molecular weight markers are shown on the left. B: The transactivation activity of ABIN-2 in mammalian cells. The activity is shown by the relative activity of luciferase measured from each cell lysate against the basal activity from cells without transfection. All assays were performed in triplicate and repeated twice.

our previous work has demonstrated that the full-length ABIN-2 was localized exclusively in the cytoplasm when expressed in 293T cells [14], we suspected that the Gal4BD–ABIN-2 protein might not be present in the nucleus and, thus, failed to activate transcription. When examined by indirect fluorescent microscopy, it was found that the expressed Gal4BD–ABIN-2 was localized in the cytoplasm (Fig. 3A). This result was unexpected, as the yeast Gal4BD, which contains a conserved nuclear localization signal, would drive its fusion protein into the nucleus and has been successfully used in the transactivation analysis in several mammalian systems [20–22]. It appeared that other factors might be involved in preventing Gal4BD–ABIN-2 from entry into the nucleus and hence failed to activate transcription.

Since the first 195 amino acids of ABIN-2 were shown to be dispensable for the transactivation in yeast (Fig. 1), we then examined the transactivation activity of Gal4BD–ABIN-$2_{196-429}$, in which its N-terminal 195 amino acid were deleted, in 293T cells. Surprisingly, the truncated Gal4BD–ABIN-$2_{196-429}$ protein readily stimulated the reporter gene expression to a great extent (Fig. 2B). From indirect fluorescent microscopic examination, most Gal4BD–ABIN-$2_{196-429}$ was shown to localize in the nucleus (Fig. 3G). It seems that the transactivation of ABIN-2 in mammalian cells was dependent on its subcellular localization, and the N-terminal portion of ABIN-2 likely played a role in regulating its localization. The truncation of 62 or 123 amino acids from the N-terminus of ABIN-2 was not sufficient to allow the Gal4BD-fused proteins to enter the nucleus, suggesting that the region responsible for ABIN-2 localization is between amino acids 123 and 196 (Fig. 3C,E). Furthermore, the efficiency of nuclear localization of the fusion protein Gal4BD–ABIN-$2_{196-429}$ was relatively high: about 80% was found in the nucleus (Fig. 3G), compared to about 50% of the protein localized in the nucleus when ABIN-$2_{196-429}$ was expressed without Gal4BD fragment [14]. This study supported that the nuclear localization potential by the Gal4BD-fused protein was functional in 293T cells, but was subjected to the influence of the N-terminal half of ABIN-2.

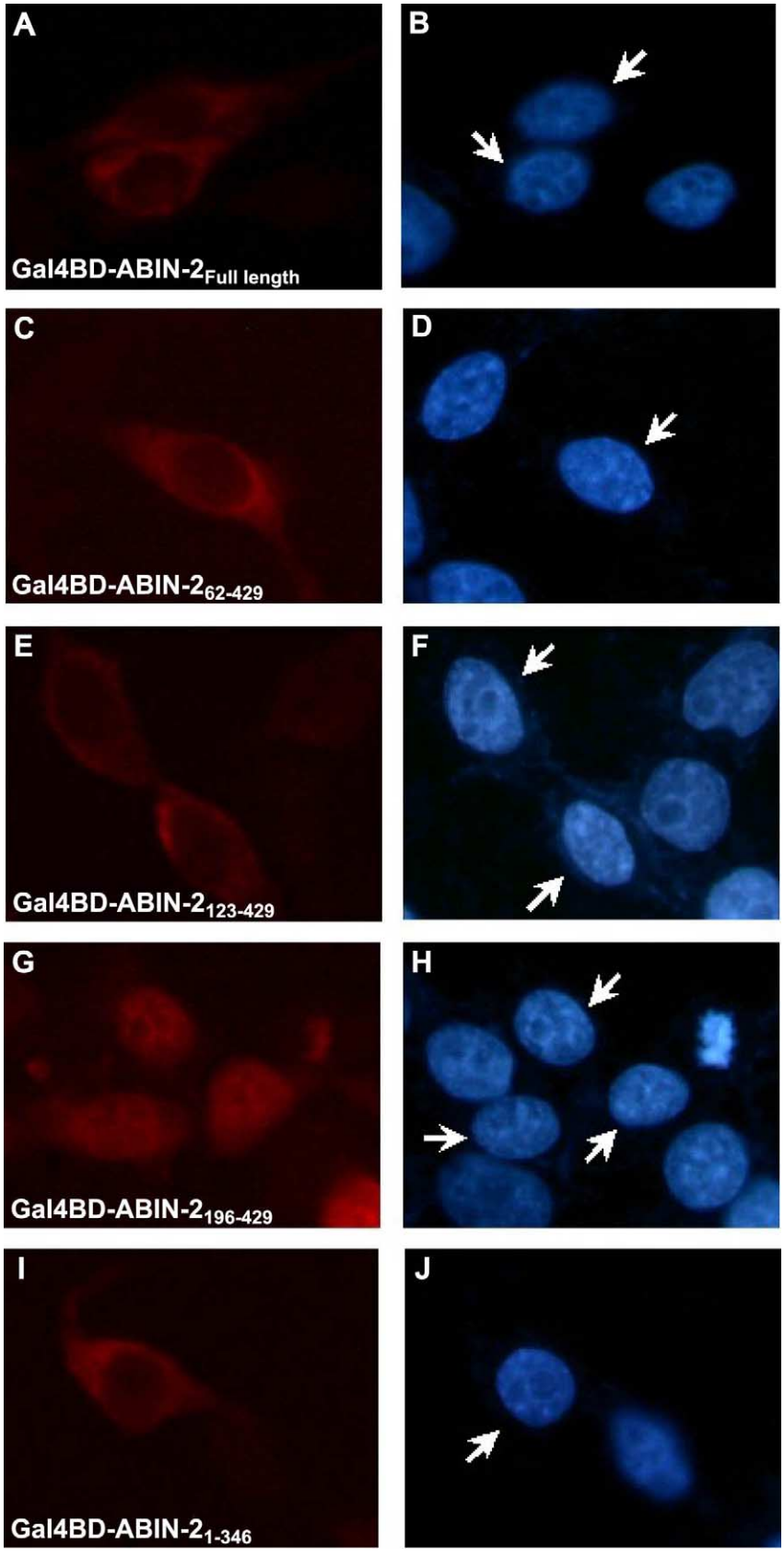
It is possible that the N-terminal 195 amino acids of ABIN-2 could bind to a protein in the cytoplasm, thereby preventing the entry of Gal4BD–ABIN-2 into the nucleus. We have searched for such proteins that associate with the N-terminus of ABIN-2 and are responsible for the retention of ABIN-2 in the cytoplasm by yeast two-hybrid screening. Our preliminary result has discovered that filamin (actin-binding protein-280), which is a cytoplasmic protein, has strong interaction with ABIN-2_{1-196} (our unpublished results). The significance of the interaction awaits further characterization.

Several distinct mechanisms have been identified for the post-translational activation of transcription factors in response to multiple cellular stimuli [23]. One particular mode of activation is proteolytic cleavage of these factors that allows them to enter the nucleus [23]. We speculate that a proteolytic mechanism could also be involved in generating a nucleus-bound and active ABIN-2 molecule. This cleavage may be executed under certain physiological circumstances. Whether the truncated form of ABIN-2 is present endogenously is still unclear at present. Alternatively, the full-length ABIN-2 may still have the transactivating activity in mammalian cells without any cleavage event. This is consistent with the finding that the full-length ABIN-2 transactivates in yeast. To accomplish the transactivation, ABIN-2 could access the genome during the mitotic process when the nuclear envelope breaks down. The notion that ABIN-2 plays a role in regulating gene expression during mitosis is tempting. Several reports have shown that there is unusual gene activation during the process of mitosis in various organisms [24–26]. In one particular case, the recruitment of a chromatin-remodeling complex was involved in the expression of certain genes that were essential for mitotic exit [27]. This correlates with the finding that ABIN-2 has the ability to interact with the component of the chromatin-remodeling complex as described below. Finally, we cannot rule out the possibility that other specific

Table 1
Loss of the transactivation activity of ABIN-2 in PCY2*

Gal4BD–	Gal4AD–	Yeast	β-galactosidase (nmol/min per mg protein)
LKB1	ABIN-2	PCY2	800 ± 35
ABIN-2	–	PCY2	250 ± 22
LKB1	ABIN-2	PCY2*	780 ± 40
ABIN-2	–	PCY2*	10 ± 3

The measurement of β-galactosidase activity was performed by liquid assay [16]. Data shown represent mean values of the specific activity of β-galactosidase per mg of protein ± S.E.M. of triplicate tubes from two independent experiments.


A
B
Gal4BD-ABIN-2Full length
C
D
Gal4BD-ABIN-262-429
E
F
Gal4BD-ABIN-2123-429
G
H
Gal4BD-ABIN-2196-429
I
J
Gal4BD-ABIN-21-346

factors might be required to transport ABIN-2 into the nucleus more efficiently.

That the full-length ABIN-2 fused to Gal4BD had a significant transactivation activity in yeast was somewhat puzzling (Table 1; second row). When the subcellular localization of Gal4BD–ABIN-2 was examined in yeast, the expressed protein was distributed exclusively in the nucleus as expected (data not shown). We speculate that the yeast cell could lack a similar ABIN-2-interacting protein to retain ABIN-2 in the cytoplasm. Or, given the fact that Gal4p is a genuine yeast transcription factor, Gal4BD could possess a distinct nuclear localization mechanism in yeast as opposed to that of mammalian cells.

3.3. *ABIN-2 interacts with a component of the chromatin-remodeling complex*

In order to further characterize the function of ABIN-2, we were interested in searching for other cellular factors which are associating with ABIN-2 and, thus, would provide information on how ABIN-2 could participate in transcriptional regulation. Since ABIN-2 alone fused to Gal4BD possesses transactivating activity in yeast, it cannot be used directly as bait in the standard two-hybrid screening system. Therefore, we adopted a genetic approach by first chemically mutagenizing the yeast strain, PCY2, with EMS in order to find a mutant strain which would allow us to screen for the ABIN-2-interacting proteins. This strategy was based on the assumption that the mediators for the transactivation by ABIN-2 and for the Gal4p-activated transcription in the two-hybrid reaction were different. Thus, mutational inactivation of one of the mediators exclusively involved in the ABIN-2 transactivation may not interfere with the activity of Gal4p required for a standard yeast two-hybrid analysis.

After screening 20 000 EMS-mutagenized colonies of PCY2, we obtained a mutant strain, designated as PCY2*, that showed a specific defect for the ABIN-2 transactivation (Table 1). In the PCY2* strain, unlike in PCY2, Gal4BD-fused ABIN-2 alone no longer showed any transactivation activity. However, the interaction between Gal4BD–LKB1 and Gal4-AD–ABIN-2 in PCY2* occurred normally in the two-hybrid analysis. This suggested that the transcriptional activity by the complex between Gal4BD and Gal4AD through the interaction of Gal4BD–LKB1 and Gal4AD–ABIN-2 was still functional in this mutant. Thus, PCY2* was chosen as the host strain for the subsequent two-hybrid screening work. The molecular basis of the PCY2* mutation remains to be determined in the future.

To search for ABIN-2-interacting proteins, we chose to use the ABIN-$2_{196-429}$ DNA fragment as bait in our two-hybrid screening to avoid getting redundant clones, which were associated with ABIN-2_{1-196}, collected in our previous screening. After screening of 55 000 colonies, a positive clone was obtained and subjected to further analysis. The recovered plasmid was later found to contain a complete cDNA insert, which was sequenced and shown to be identical to the human BAF60a gene. BAF60a is a component of the SWI–SNF chromatin-remodeling complex that has been shown to activate transcription by remodeling nucleosomes to permit access of transcription factors and plays important roles in gene expression throughout eukaryotes [28,29]. Thus, it is likely that ABIN-2 could exert its transcriptional activation by recruiting a chromatin-remodeling complex to the site of the target gene through the interaction with BAF60a. Using a deletion analysis of ABIN-2 in yeast, we further mapped the BAF60a-interacting region of ABIN-2 within the stretch of amino acids ranging from 196 to 253 (Fig. 4A). The fact that ABIN-2_{1-346} bound BAF60a but failed to transactivate the reporter gene demonstrated that another domain, from amino acids 346 to 429, is required for transactivating activity (Fig. 1). It is likely this second domain could also mediate the binding with other factors, in addition to BAF60a, essential for transactivation. The region of BAF60a responsible for interacting with ABIN-2 was also mapped at two areas, ranging from amino acids 1 to 175 and 305 to 380 (Fig. 4B).

The finding that ABIN-2 has transactivating activity and associates with a component of chromatin-remodeling com-

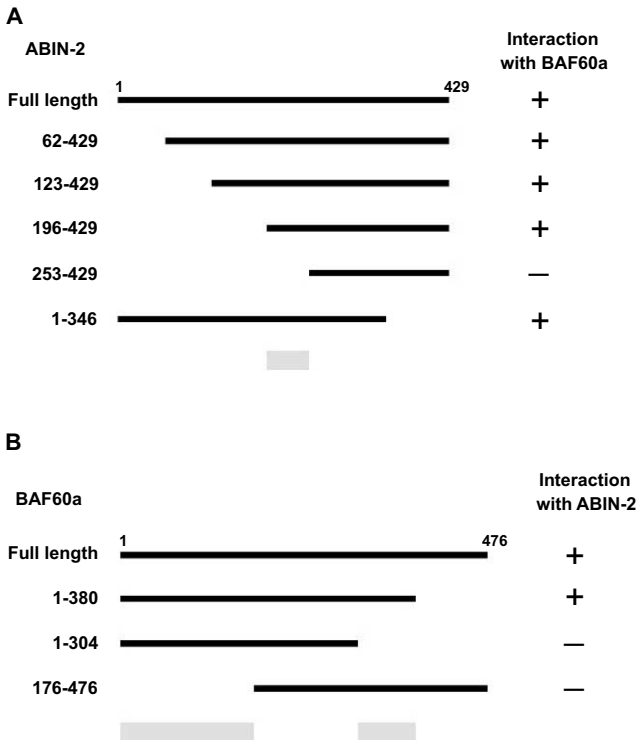


Fig. 4. Mapping the interacted regions between ABIN-2 and BAF60a in yeast. A: Domain mapping of ABIN-2 interacting with BAF60a. B: Domain mapping of BAF60a interacting with ABIN-2. Both interactions were performed by the standard yeast two-hybrid method. Boxes shown at the bottom of each panel are the regions mapped to be essential for the interaction in each analysis.

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Fig. 3. Subcellular localization of various ABIN-2 constructs expressed in 293T cells by transient transfection. The ectopically expressed Gal4BD-fused ABIN-2 constructs were detected by indirect immunofluorescent microscopy. Detection of expressed proteins was performed using mouse polyclonal antisera against ABIN-2, followed by immunofluorescent staining with Rhodamin-conjugated secondary antiserum to examine the localization of ABIN-$2_{full-length}$ (A), ABIN-2_{62-429} (C), ABIN-$2_{123-429}$ (E), ABIN-$2_{196-429}$ (G) and ABIN-2_{1-346} (I), respectively. Cells were also stained with Hoechst to show the location of nuclei (B,D,F,H,J). Arrows indicate the corresponding cells shown on the left panel with positive immunostaining.

plexes, BAF60a, is particularly intriguing. BAF60a has been illustrated as a determinant of the transactivation potential of the transcription factor c-Fos/c-Jun dimers in mouse [30]. Deletion analysis of the mouse BAF60a revealed the location of two regions spanning amino acids 1–175 and 307–366, which is responsible for binding with either c-Fos or c-Jun [30]. Here we demonstrate that the human BAF60a, which shares high homology with the mouse counterpart, is an interacting protein of ABIN-2. More importantly, the protein domains of the human BAF60a responsible for binding to ABIN-2 overlapped precisely with the areas that bind to c-Fos/c-Jun in mouse (Fig. 4B). These areas of BAF60a should potentially represent important domains in association with cellular activators leading to transcriptional activation.

In the field of gene regulation, transcriptional coactivator complexes that facilitate the action of sequence-specific gene activators have emerged over the last decade [31,32]. Many of these factors are pivotal to our current understanding of mechanisms in cell growth, differentiation, development, and tumor formation [31,32]. We report here that ABIN-2 is likely to represent such a novel transcriptional coactivator.

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