AKAP350 at the Golgi Apparatus

I. IDENTIFICATION OF A DISTINCT GOLGI APPARATUS TARGETING MOTIF IN AKAP350*

The protein kinase A-anchoring proteins (AKAPs) are defined by their ability to scaffold protein kinase A to specific subcellular compartments. Each of the AKAP family members utilizes unique targeting domains specific for a particular subcellular compartment. AKAP350 is a multiply spliced AKAP family member localized to the centrosome and the Golgi apparatus. Three splicing events in the carboxyl terminus of AKAP350 generate the AKAP350A, AKAP350B, and AKAP350C proteins. A monoclonal antibody recognizing all three splice variants as well as a polyclonal antibody specific for AKAP350A demonstrated both centrosomal and Golgi apparatus staining in paraformaldehyde-fixed HCA-7 cells. Golgi apparatus-associated AKAP350A staining was dispersed following brefeldin A treatment. Using GFP-chimeric constructs of the carboxyl-terminal regions of AKAP350A, a Golgi apparatus targeting domain was identified between amino acids 3259 and 3307 of AKAP350A. This domain was functionally distinguishable from the recently described centrosomal targeting domain (PACT domain, amino acids 3308–3324) located adjacent to the Golgi targeting domain. These data definitively establish the specific association of AKAP350A with the Golgi apparatus in HCA-7 cells.

Scaffolding proteins are defined by their ability to target proteins to specific subcellular compartments in order to localize the effects of these proteins for specialized cellular functions. One family of scaffolding proteins, AKAPs, is defined by its ability to bind to the regulatory dimer of protein kinase A. Furthermore, AKAPs are capable of scaffolding other signaling proteins to specific subcellular compartments (1–4). The ability of AKAPs to target these complexes resides in unique targeting domains specific for different subcellular compartments. Interestingly, AKAPs are also capable of containing more than one targeting domain and therefore are able to differentially localize signaling proteins. D-AKAP1 contains dual targeting domains that are utilized based upon an amino-terminal splice that selects for the expression of either mitochondrial or endoplasmic reticulum targeting domains (5). Therefore, AKAPs are capable of utilizing one or more targeting domains to localize scaffolded proteins to specific subcellular compartments.

Keryer et al. (6) originally reported an R<sub>G</sub> binding protein of 350 kDa localized exclusively at the centrosome in lymphoblasts. We and others have subsequently obtained cDNA sequences coding for 400–450-kDa splice variants of this centrosomal AKAP (7–9). A monoclonal antibody developed against AKAP350 recognized a 350–450-kDa band, but with consideration to the original report we have used the name AKAP350 (7). This antibody also confirmed the centrosomal localization of AKAP350 (7). Subsequent reports also identified AKAP350 as a centrosomal protein (8, 9). Gillingham and Munro (10) reported that AKAP350 contains a pericentrin homology region in its carboxyl terminus. This AKAP350 domain is able to target specifically to the centrosome in COS cells. This pericentrin-AKAP450 centrosomal targeting (PACT) domain demonstrated homology with a region of pericentrin that was also able to target to the centrosome (10). No Golgi apparatus expression was observed by these investigators, leading to the supposition that Golgi apparatus staining may be the result of artificial cross-reactive immunostaining.

Nevertheless, an anti-AKAP350 monoclonal antibody (7), and two anti-AKAP350 polyclonal antibodies (9) demonstrated noncentrosomal staining in several cell lines and tissues (7). Two anti-AKAP350 polyclonal antibodies utilized by Takahashi et al. (9) demonstrated Golgi apparatus staining and led to their designation of this protein as CG-NAP (centrosome and Golgi-localized PKN-associated protein) (9). Previous studies have reported that AKAP350 can bind to protein kinase N (PKN), proteins phosphatase 2a (PP2a), protein phosphatase 1 (PP1), phosphodiesterase 4D3 (PDE4D3), and protein kinase C epsilon (PKCe) (6, 9, 11–13). AKAP350 could therefore scaffold these proteins to the Golgi apparatus or the centrosome to perform specific functions.

The centrosomal targeting domain, PACT, is found in two carboxyl-terminal splice variants of AKAP350 that we have now designated AKAP350A and AKAP350B (7). This domain is not found in the novel AKAP350 carboxyl-terminal splice variant, AKAP350C. We now provide definitive demonstration of AKAP350 association with the Golgi apparatus. Antibodies specific for one splice variant, AKAP350A, prominently stain the Golgi apparatus in HCA-7 human colon adenocarcinoma cells. The localization of AKAP350A to the Golgi apparatus is dependent upon a distinct carboxyl-terminal Golgi apparatus targeting domain located adjacent to the PACT domain. These results suggest that the differential subcellular targeting of

* This work was supported by Grants DK48370 and DK43405 from NIDDK, National Institutes of Health and a Department of Veterans Affairs Merit Review (to J. R. G.) as well as a National Institutes of Health National Research Service Award postdoctoral fellowship (to P. H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF247727.

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‡ The abbreviations used are: AKAP, protein kinase A-anchoring protein; PACT, pericentrin-AKAP450 centrosomal targeting; GFP, green fluorescent protein.

This paper is available on line at http://www.jbc.org
AKAP350 is determined by the presence of a centrosomal PACT domain and a distinct Golgi apparatus targeting domain.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by the Medical College of Georgia Molecular Biology Core Facility. Cy3- or Cy5-conjugated species-specific secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Prolong Antifade and Alexa 488- or 568-conjugated species-specific secondary antibodies were from Molecular Probes, Inc. (Eugene, OR).

Antibodies—The 14G2 monoclonal anti-AKAP350 antibody was developed as described previously (7). For production of AKAP350A-specific antibodies, a peptide corresponding to the unique region in the carboxyl terminus of the human gastric AKAP350A splice variant was conjugated to keyhole limpet hemocyanin using an Inject Immunogen EDC Kit (Pierce). The AKAP350A peptide (GSTQPGH4GMR) was synthesized in the Medical College of Georgia Molecular Biology Core Facility. Rabbit polyclonal antibodies were developed in the Antibody Facility at the University of Georgia. The antibody was affinity-purified using the Amino-Link system (Pierce) with the AKAP350A peptide. 100 μg/ml glycine, pH 2.5, was used to elute the AKAP350A antibody from the Amino-Link column. Immunostaining in Western blots of HCA-7 cell lysates showed one band at an estimated 350–450 kDa, which was blocked upon preincubation with 10 μM AKAP350A peptide (data not shown). Immunocytochemical staining with the AKAP350A polyclonal antibody was also blocked by preincubation with 10 μM peptide.

Immunocytochemistry—HCA-7 cells were grown to confluence on glass coverslips. The cells were then incubated in the absence or presence of 10 μg/ml brefeldin A for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 17% donkey serum in phosphate-buffered saline and then incubated with 14G2 (mouse anti-AKAP350) (1:85) for 2 h at room temperature. Separate slides were incubated simultaneously with mouse anti-p58 (1:100) or mouse anti-α-tubulin (1:100), and rabbit anti-AKAP350A (1:300) for 2 h at room temperature. The cells were then simultaneously incubated with Alexa-488-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG for 60 min. Coverslips were inverted and mounted with Prolong antifade (Molecular Probes). Cells were examined for triple labeling by confocal microscopy (Amersham Biosciences) with maximum intensity projections of 0.3-μm optical sections in the apical region of the cell or epifluorescence microscopy utilizing an Axioshot digital imaging system (Zeiss).

GFP-Chimera Expression—Targeting construct sequences were amplified using primers specific for the development of the GFP-chimera proteins: GFP-AKAP350A (amino acids 3299–3524), GFP-C (amino acids 3308–3424), and GFP-G (amino acids 3259–3307). EcoRI and SalI sites were utilized to clone into pEGFP-C2 (Clontech). These constructs were transfected with Effectene (Qiagen) into HCA-7 cells, which were then grown overnight on glass coverslips. Cells were fixed and stained as described above.

RESULTS

AKAP350 Carboxyl-terminal Splice Variant, AKAP350A, Is Associated with the Golgi Apparatus in HCA-7 Cells—AKAP350 was originally identified as a centrosomal AKAP that was multiply spliced at the carboxyl terminus (Fig. 3) (7). Although our original immunocytochemical studies were performed with methanol fixation (7), utilization of 4% paraformaldehyde fixation revealed additional staining in HCA-7 cells reminiscent of the Golgi apparatus with the monoclonal anti-AKAP350A antibody, 14G2 (Fig. 1). Treatment with brefeldin A disrupted the noncentrosomal staining pattern but had no effect on the 14G2 staining of the centrosome (Fig. 1). The Golgi apparatus localization with the 14G2 antibody was also observed in several other cell lines including HTC116, Madin-Darby canine kidney cells, rabbit gastric fibroblasts, and rabbit gastric parietal cells (data not shown). These data established the dual staining of AKAP350 at the Golgi apparatus and the centrosome with the 14G2 antibody, confirming the dual localization of AKAP350 as observed by Takahashi et al. (9).

To investigate further the distribution of AKAP350 in HCA-7 cells, we performed immunocytochemistry with an affinity-purified polyclonal antibody specific for the carboxyl-terminal end of the human gastric clone of AKAP350, AKAP350A (unique sequence displayed in Fig. 3). The AKAP350A carboxyl-terminal sequence is also contained in the sequences of CG-NAP and AKAP450. Dual immunocytochemistry staining with the Golgi apparatus marker p58 and anti-AKAP350A showed colocalization of these antibodies at the Golgi apparatus in paraformaldehyde-fixed HCA-7 cells (Fig. 2A). The anti-AKAP350A staining also colocalized with Golgi apparatus markers giantin and γ-adaptin (AP-1) (data not shown). AKAP350A antibodies also stained the centrosomes of HCA-7 during interphase and mitosis (Fig. 2). To confirm localization with the Golgi apparatus, we treated HCA-7 cells with brefeldin A and stained with the anti-AKAP350A and anti-p58. The staining of both p58 and AKAP350A were dispersed following brefeldin A treatment (Fig. 2A). Interestingly, there was a loss of colocalization of anti-AKAP350A with the Golgi apparatus marker (Fig. 2A). The p58 and AKAP350A staining were also dispersed by either nocodazole treatment or in dividing cells (data not shown). These results suggest that the noncentrosomal staining of AKAP350A/AKAP450/CG-NAP in HCA-7 cells reflects targeting of AKAP350A to the Golgi apparatus (7).

AKAP350A staining of the centrosomes with anti-AKAP350A antibodies was considerably weaker than staining observed with the 14G2 anti-AKAP350 monoclonal antibody. To confirm the localization AKAP350A at the centrosome, HCA-7 cells were dual stained with anti-AKAP350A and the centrosomal marker, α-tubulin. Fig. 2B demonstrates that anti-AKAP350A stained structures associated with the centrosome. As we have previously noted for 14G2 staining of Madin-Darby canine kidney cells (7), AKAP350A stained discrete extensions from one centriole.

The Carboxyl Terminal of AKAP350A Demonstrates Centrosome and Golgi Apparatus Targeting in HCA-7 Cells—Previous studies have characterized a carboxyl-terminal region responsible for the centrosomal targeting of AKAP350 (10). This region, designated the PACT domain, is shared by two AKAP350 carboxyl-terminal splice variants, AKAP350A and AKAP350B (Fig. 3). AKAP350C (GenBank™ accession number AF247727),
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Fig. 2. AKAP350A localizes to the Golgi apparatus. A, confocal fluorescence microscopy of HCA-7 cells stained with anti-AKAP350A, the Golgi apparatus marker (anti-p58), and an overlay of the two with anti-AKAP350A staining in green and anti-p58 staining in red (right column). The top row represents control HCA-7 cells (−BFA), and the bottom row represents brefeldin A-treated cells (+BFA). Arrowheads indicate the staining of the Golgi apparatus. B, confocal fluorescence microscopy of HCA-7 cells stained with anti-AKAP350A and anti-γ-tubulin (a centrosomal marker). An overlay of the two with anti-AKAP350A staining in green and anti-γ-tubulin staining in red is shown in the right column. Arrowheads indicate the position of AKAP350A staining associated with centrosomes.

Fig. 3. Location of the centrosomal targeting sequence in AKAP350. A schematic diagram of the AKAP350 splice variants AKAP350A, AKAP350B, and AKAP350 C depicts the centrosomal targeting domain/PACT domain (P). The domain is confined to the AKAP350A and AKAP350B splice variants. Also shown are the Yotiao homology region (YHR), unique carboxyl tails A, B, and C, two RII binding domains, and two carboxyl-terminal leucine zipper regions (hatched areas) as well as the position of the site recognized by the 14G2 monoclonal antibody.

Fig. 4. GFP deletion constructs of the carboxyl terminus of AKAP350A. The diagram depicts the full-length AKAP350A with carboxyl-terminal leucine zipper regions (LZ) and RII binding sites. The constructs represent the following amino acids in AKAP350A: GFP-AKAP350A (amino acids 3259–3524), GFP-L100 (amino acids 3424–3524), GFP-C (amino acids 3308–3424), and GFP-GB (amino acids 3259–3307). The PACT domain constructed, designated ΔN/ΔC by Gillingham and Munro (10) (amino acids 3321–3402 of AKAP350A), is located within the GFP-C construct.

a novel carboxyl-terminal splice variant found by 3’-rapid amplification of cDNA ends (RACE) analysis of human lung cDNA, codes for a large carboxyl-terminal truncation of the AKAP350 protein. This splice variant does not contain the centrosomal targeting region (PACT domain) (Fig. 3) (14). Because the immunocytochemical studies with anti-AKAP350A demonstrated that AKAP350A is present on the Golgi apparatus in HCA-7 cells, we utilized deletion constructs of the regions surrounding the known centrosomal targeting sequence (PACT domain) in AKAP350A to determine whether the carboxyl terminus of AKAP350 also contained a Golgi apparatus targeting region (Fig. 4). We developed a GFP chimeric fusion protein, which coded for the carboxyl terminus of AKAP350A just distal to the last leucine zipper region (GFP-AKAP350A) (Fig. 4). HCA-7 cells were transiently transfected with GFP-AKAP350A, fixed with paraformaldehyde, and separately costained with the anti-AKAP350A antibody and either 14G2 or the Golgi apparatus marker antibody, anti-p58 (Fig. 5). GFP-AKAP350A colocalized with both 14G2 and anti-p58 staining. Therefore, GFP-AKAP350A was capable of targeting to both the Golgi apparatus and the centrosome (Fig. 5). The localization of the GFP-AKAP350A at the Golgi apparatus in HCA-7 cells led us to conclude that the carboxyl-terminal region of AKAP350A contained both Golgi apparatus and centrosome localization domains.

Characterization of a Golgi Apparatus Targeting Sequence in AKAP350—To define more precisely the region of AKAP350A responsible for Golgi targeting, we constructed further truncations of the AKAP350A carboxyl-terminal sequence. We confirmed in HCA-7 cells the observation of Gillingham and Munro (10) that the last 100 amino acids of AKAP350A (GFP-L100) did not specifically target to either centrosomes or Golgi (Fig. 4, data not shown). Therefore, we expressed GFP-AKAP350-(3259–3307) (GFP-GB) representing the sequence of AKAP350A found between the last leucine zipper region and the centrosomal targeting region (PACT domain) (Fig. 4). HCA-7 cells transiently transfected with GFP-GB were fixed with paraformaldehyde and subsequently costained with either the monoclonal anti-AKAP350 antibody, 14G2, or the Golgi apparatus marker, anti-p58 (Fig. 4). GFP-GB colocalized with both the AKAP350 and p58 staining on the Golgi apparatus, but this GFP-chimera did not colocalize with the anti-AKAP350 (14G2) centrosomal staining (Fig. 6). The cells transiently transfected with GFP-GB did display nuclear staining, but this staining was similar to that seen in transfections with GFP alone (data not shown). As a comparison, we transiently transfected HCA-7 cells with the centrosomal targeting region (PACT domain) (GFP-C) (Fig. 4). In COS-7 cells, Gillingham and Munro (10) demonstrated that a construct containing the PACT domain (designated ΔN/ΔC) construct in their study, corresponding to amino acids 3321–3402 in AKAP350A targeted to the centrosome. Similarly, GFP-C (amino acids 3308–3424), also targeted exclusively to the centrosome in HCA-7 cells (Fig. 7). Transfected cells also displayed nuclear staining similar to cells transiently transfected with the GFP-GB
or GFP alone, but no targeting to the Golgi apparatus was observed (Fig. 7).

DISCUSSION

Using RII overlays, Keryer et al. (6) originally defined AKAP350 as a 350-kDa centrosomal, RII-binding protein in KE-37 lymphoblast cells. Following the cloning of this protein, Western blot analysis utilizing an anti-AKAP350 monoclonal antibody, 14G2, recognized a group of 350–450-kDa immunoreactive bands. Immunocytochemistry performed with the 14G2 antibody revealed that AKAP350 was localized to the centrosome (7). However, the 14G2 antibody also displayed a noncentrosomal staining pattern, especially in epithelial cell types. Using paraformaldehyde fixation, we have now demonstrated that the noncentrosomal staining with the 14G2 antibody represents staining of the Golgi apparatus. Furthermore, antibodies specific for the AKAP350A splice variant also showed both Golgi and centrosomal staining in HCA-7 cells. These observations confirm the localization at the Golgi apparatus of CG-NAP, which is identical to AKAP350A except for an alternate 5’ start site coding for the entirety of the Yotiao protein in the amino terminus (9). Indeed, two polyclonal an-
tibodies developed against Yotiao, which recognize a 350–450-kDa immunoactive band on Western blots of HCA-7 cells, also demonstrate Golgi apparatus staining in several polarized epithelial cell lines (data not shown). Therefore, antibodies directed toward several different epitopes in AKAP350 demonstrate Golgi apparatus staining. These results all support the association of AKAP350 with the Golgi apparatus.

Carboxyl-terminal splicing events in the AKAP350 gene lead to at least four different sequences: AKAP350A, AKAP350B, AKAP350C, and Yotiao. Yotiao, the smallest AKAP350 splice variant, is localized to the post-synaptic densities of neurons and is not observed at the Golgi apparatus in neurons (15). Therefore, carboxyl-terminal splicing events could provide a means of differential localization of AKAP350 in the cell. We developed an antibody specific for a unique region of the AKAP350A carboxyl-terminal splice variant. This report suggests that the anti-AKAP350 Golgi apparatus staining observed by Takahashi et al. (9) and Schmidt et al. (7) may reflect the distribution for AKAP350A. This result contrasts with the reports in KE-37 lymphoblasts (6) and COS cells (10) that suggest exclusive localization of AKAP350 to the centrosomes. Indeed, we have performed immunocytochemistry with the monoclonal anti-AKAP350 antibody and the polyclonal anti-AKAP350A antibody in Jurkat cells, a T-lymphoblast line. Interestingly, both antibodies demonstrated exclusive staining of the centrosome in Jurkat cells (data not shown). Therefore, AKAP350A is distributed differentially in individual cell types to the centrosome and/or the Golgi apparatus. Thus, the lymphoblast cell line or COS-7 cells used in some investigations may have introduced a bias toward exclusive centrosomal localization. Furthermore, because 14G2 does stain centrosomes in HCA-7 cells more prominently than the AKAP350A antibodies, other AKAP350 splice variants such as AKAP350B are likely to be localized to this organelle.

Localization of AKAP350 to both Golgi apparatus and centrosomal compartments suggested that AKAP350 contained more than one targeting domain. Gillingham and Munro (10) recently described a centrosomal targeting region (the PACT domain, designated as the ΔN/ΔC construct). We utilized a GFP construct (GFP-C) with amino acid sequence similar to the ΔN/ΔC construct and confirmed its ability to target to the centrosome in HCA-7 cells (Fig. 7). Because we had localized AKAP350A to the Golgi apparatus, the carboxyl-terminal end of AKAP350A was utilized to construct a GFP chimeric protein to determine whether there was also a Golgi apparatus targeting sequence. In this report, we have found that GFP-AKAP350A, similar to the GFP-ΔN-3463 construct utilized by Gillingham and Munro (10) (corresponding to amino acids 3321–3524 in the AKAP350A sequence), demonstrated not only centrosomal but also Golgi apparatus targeting in HCA-7 cells (Fig. 5). Deletion constructs of the GFP-AKAP350A construct determined that a 48-amino acid region adjacent to the recently defined centrosomal targeting region (PACT domain) was sufficient for Golgi apparatus targeting. The Golgi apparatus targeting domain, GFP-GB, localized to the Golgi apparatus and not to the centrosome. Taken together, these data indicate that AKAP350A localizes to the centrosome and the Golgi apparatus via two distinct targeting domains located in its carboxyl terminus.

The data presented here contradict the findings of Gillingham and Munro (10), who reported an exclusive targeting of the AKAP450 sequence to the centrosome. Golgi apparatus localization of GFP-ΔN-3463 (corresponding to GFP-AKAP350A in our report) in COS-7 cells was not observed with the techniques utilized by Gillingham and Munro (10). Two possible explanations exist for this discrepancy. First, in comparison with HCA-7 cells, COS7 cells maintain a more diffuse organization of the Golgi apparatus. Therefore, Golgi apparatus targeting of a GFP construct could prove more difficult to identify. Second, and more likely, different cell types may display differential targeting of AKAP350A. Four constructs produced by Gillingham and Munro included all but six amino acids on the amino-terminal side of GFP-GB, but no Golgi apparatus localization was observed (10). When we transfected the GFP-AKAP350A construct into Jurkat cells, only centrosomal targeting was identified (data not shown). We also did not observe GFP-GB localization with the Golgi apparatus in Jurkat cells (data not shown). These results correlate with our localization of AKAP350A immunostaining exclusively to the centrosome in Jurkat cells. Thus, these data suggest that the localization of AKAP350 is cell type-specific.

AKAPs are known to bind to and scaffold proteins, facilitating the formation of large macromolecular complexes (16). The unique targeting domains found in AKAP350A may direct the assembly of such complexes. For example, the structural integrity of the Golgi apparatus is dependent upon a family of proteins designated golgins (17, 18). Although AKAP350A is localized to the Golgi apparatus, brefeldin A treatment displaced AKAP350A from Golgi apparatus remnants (Fig. 2). Therefore, the Golgi apparatus targeting is contingent upon a structural component that is lost when the Golgi apparatus is disrupted. AKAP350A could interact with a structural component of the Golgi apparatus via its Golgi apparatus targeting domain. It seems likely that the Golgi apparatus targeting and centrosomal targeting regions interact with specific binding partners on their respective organelles.

This report provides discrete mechanisms for the targeting of AKAP350 scaffolded signaling complexes within the cell. Previous studies have reported that AKAP350 can bind to protein kinase A (PKA), protein kinase N (PKN), proteins phosphatase 2a (PP2a), protein phosphatase 1 (PP1), phosphodiesterase 4D3 (PDE4D3), and protein kinase C epsilon (PKCe) (6, 9, 11–13). Therefore, these independently utilized targeting domains could provide the cell with the means to control signaling events at both the centrosome and the Golgi apparatus. In an accompanying paper (19), we have demonstrated the interaction of AKAP350 with a novel CLIC family member, CLIC5B, at the Golgi apparatus. Still, this interaction occurs at a site distinct from the Golgi apparatus targeting domain of AKAP350. Further targeting motifs likely exist because AKAP350C and Yotiao lack both the PACT domain and the Golgi apparatus targeting region. Targeting of these splice variants could be accomplished via dimerization domains capable of interacting with other AKAP350 splice variants or through distinct targeting domains coded for by the unique carboxyl-terminal sequences of AKAP350C and Yotiao.

In summary, we have demonstrated definitively that AKAP350A is associated with the Golgi apparatus in HCA-7 cells. In addition, we have identified a targeting sequence in AKAP350 sufficient for association with the Golgi apparatus in HCA-7 cells. The existence of a distinct Golgi apparatus and centrosomal targeting sequences suggests that the intracellular localization of AKAP350 is regulated by the interaction of these domains with organelle-specific targets.

Acknowledgments—We thank Dr. Lynne A. Lapierre for assistance with the yeast two-hybrid work and for her intellectual input into this manuscript. We also thank Dr. John Scott for providing the anti-Yotiao antibodies.

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