Interaction of Voltage-gated Sodium Channel Na\textsubscript{v}1.6 (SCN8A) with Microtubule-associated Protein Map1b\textsuperscript{\*5}

Received for publication, December 23, 2011, and in revised form, March 15, 2012. Published, JBC Papers in Press, April 3, 2012, DOI 10.1074/jbc.M111.336024

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Background: Specific sodium channels have unique subcellular localizations within neurons.
Results: We identified a novel interaction of sodium channel Na\textsubscript{v}1.6 with a microtubule-associated protein.
Conclusion: Trafficking of Na\textsubscript{v}1.6 to the cell membrane is mediated by interaction with Map1b.
Significance: Correct localization of sodium channels is essential to prevent neurological disorders such as epilepsy and ataxia.

The voltage-gated sodium channel Na\textsubscript{v}1.6 is widely expressed in neurons of the central and peripheral nervous system and is highly concentrated on the axon initial segment and nodes of Ranvier (1, 2). Na\textsubscript{v}1.6 is required for repetitive firing and generation of resurgent currents in cerebellar Purkinje cells (3–5) and sensory neurons in dorsal root ganglia (6) and contributes to firing patterns in other types of neurons (for review, see Ref. 7). Spontaneous mutations of Na\textsubscript{v}1.6 in the mouse result in neurological disorders including tremor, dystonia, ataxic gait, paralysia, and juvenile lethality (8). Two mutations of human SCN8A have been described, an inherited protein truncation allele in a family with ataxia and cognitive impairment (9), and a de novo gain-of-function mutation in a child with epileptic encephalopathy (10).

Voltage-gated sodium channels interact with multiple binding partners that regulate gating properties and subcellular localization (11). Several protein interaction sites have been mapped to the intracellular loops and C terminus of the channels. Sequence analysis has identified putative protein-protein interacting motifs and sites for post-translational modification. The only previously described interaction of the N terminus with cytoplasmic proteins is the specific interaction of Na\textsubscript{v}1.8 with the annexin II light chain, which increases channel trafficking to the plasma membrane (12, 13).

We recently characterized the ethylnitrosourea-induced mouse mutant Scn8a\textsuperscript{ataxial}, in which the amino acid substitution S21P results in trapping of the Na\textsubscript{v}1.6 channel protein in the Golgi (14). The location of this mutation in the N terminus suggested that this region might be involved in protein-protein interactions required for trafficking of the channel protein to the cell surface. To test this prediction, we carried out a yeast two-hybrid screen of a mouse brain cDNA library to identify proteins that interact with the 117-residue N-terminal domain, we carried out a yeast two-hybrid screen of a mouse brain cDNA library. Three clones containing overlapping portions of the light chain of microtubule-associated protein Map1b (Map1b) were recovered from the screen. Interaction between endogenous Na\textsubscript{v}1.6 channels and Map1b in mouse brain was confirmed by co-immunoprecipitation. Map1b did not interact with the N terminus of the related channel Na\textsubscript{v}1.1. Alanine-scanning mutagenesis of the Na\textsubscript{v}1.6 N terminus demonstrated that residues 77–80 (VAVP) contribute to interaction with Map1b. Co-expression of Na\textsubscript{v}1.6 with Map1b in neuronal cell line N40T/23 resulted in a 50% increase in current density, demonstrating a functional role for this interaction. Mutation of the Map1b binding site of Na\textsubscript{v}1.6 prevented generation of sodium current in transfected cells. The data indicate that Map1b facilitates trafficking of Na\textsubscript{v}1.6 to the neuronal cell surface.

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The mechanism by which voltage-gated sodium channels are trafficked to the surface of neurons is not well understood. Our previous work implicated the cytoplasmic N terminus of the sodium channel Na\textsubscript{v}1.6 in this process. We report that the N terminus plus the first transmembrane segment (residues 1–153) is sufficient to direct a reporter to the cell surface. To identify proteins that interact with the 117-residue N-terminal domain, we carried out a yeast two-hybrid screen of a mouse brain cDNA library. Three clones containing overlapping portions of the light chain of microtubule-associated protein Map1b (Map1b) were recovered from the screen. Interaction between endogenous Na\textsubscript{v}1.6 channels and Map1b in mouse brain was confirmed by co-immunoprecipitation. Map1b did not interact with the N terminus of the related channel Na\textsubscript{v}1.1. Alanine-scanning mutagenesis of the Na\textsubscript{v}1.6 N terminus demonstrated that residues 77–80 (VAVP) contribute to interaction with Map1b. Co-expression of Na\textsubscript{v}1.6 with Map1b in neuronal cell line N40T/23 resulted in a 50% increase in current density, demonstrating a functional role for this interaction. Mutation of the Map1b binding site of Na\textsubscript{v}1.6 prevented generation of sodium current in transfected cells. The data indicate that Map1b facilitates trafficking of Na\textsubscript{v}1.6 to the neuronal cell surface.

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Na_{1.6} Interaction with Map1b

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Assay**—The cytoplasmic N terminus of Na_{1.6} (residues 1–117) was amplified from mouse brain cDNA (strain C57BL/6J) and cloned into the vector pGBKT7 for use as “bait” in the yeast two-hybrid screen. The prey consisted of the mouse brain cDNA library in the vector pGADT7 (630489; Clontech). The yeast two-hybrid screen and directed tests were performed according to recommendations except that yeast were prepared for transformation by placing a 2–3-mm colony into 50 ml of YPDA broth. The culture was incubated at 30 °C for 16–20 h until A_{600} > 1.5. The culture was diluted in YPDA to an absorbance of 0.2–0.3 and incubated, with shaking, at 30 °C, until an absorbance = 0.4–0.6 was reached. Transformation of yeast with 0.5 μg of each plasmid was performed using the Clontech Yeastmaker Yeast Transformation System 2 protocol (630439; Clontech). All transformed yeast grew on −Leu/−Trp medium, which selects for the presence of the bait and prey constructs. Interactants were identified by growth on selective −Leu/−Trp/−His/−Ade medium, which requires interaction between the transformed proteins. The cDNA fragment encoding Map1b (residues 1924–2464) was amplified from mouse brain cDNA and cloned into pGADT7. This fragment encodes the 541 C-terminal residues of the encoded protein fragment in transfected pGADT7. This fragment encodes the 374 residues of the light chain (15).

The stability of the encoded protein fragment in transfected cells was demonstrated by Western blotting. Hybrid N-terminal constructs were cloned by PCR fusion of cDNA residues 1–54 of Na_{1.1} and 55–117 of Na_{1.6} (1A/8A), or residue 1–54 of Na_{1.6} and 55–117 of Na_{1.1} (8A/1A). The fusion products were cloned into pGBK7. Deletion constructs based on NdeI and EcoRI restriction sites were generated in vector pGBK7 by Dr. W. Clay Brown at the High Throughput Protein Laboratory, Life Sciences Institute, University of Michigan. Alanine residues were introduced into the N-terminal domain of Na_{1.6} by QuikChange XL mutagenesis (Agilent) using the primers listed in supplemental Table S1.

**Cloning of Na_{1.6}-CD74 Fusion Protein**—A cDNA fragment encoding the N terminus plus the first transmembrane segment of Scn8a (residues 1–153) was amplified from the Na_{1.6} cDNA clone pcDNA3-mod-Na_{1.6}R (18). The pcDNA3-CD74 clone encoding full-length human CD74 (residues 1–232) was provided by Dr. Blanch Schwappach, University of Manchester, UK (19). Residues 1–71 of CD74 were replaced with residues 1–153 of Na_{1.6}, which removed the cell surface localization signal in the first transmembrane domain of CD74 (19). The ataxia3 mutation p.S21P was introduced into the Na_{1.6}-CD74 fusion protein by QuikChange XL mutagenesis. The coding regions of all constructs were analyzed by Sanger sequencing at the University of Michigan DNA Sequencing core before use in transfection experiments.

**Site-directed Mutagenesis of Nav1.6R cDNA Clone**—The V777A/V797A/P80A mutation was introduced into the tetradotoxin-resistant Na_{1.6} cDNA clone Nav1.6R (18) by QuikChange XL mutagenesis using the primers listed in supplemental Table S1. The entire 6-kb open reading frame was sequenced to confirm the absence of additional mutations prior to functional testing.

**Immunocytochemistry**—HEK293 cells were co-transfected with Na_{1.6}-CD74 and myc-Map1b as described above. Cell extracts were prepared and immunoprecipitated as described (20). 10-cm plates of confluent cells were lysed in 1 ml of buffer containing 60 mM Tris-HCl, pH 7.5, 180 mM NaCl, 1% Triton X-100, and 6 mM EDTA. Lysates were preincubated for 1 h at 4 °C with 5 μl of IgG and washed protein G-Sepharose beads. After centrifugation, the supernatant was incubated for 1 h at 4 °C with primary antibody, 25 μl of anti-CD74 (sc-5438; Santa Cruz Biotechnology) or 5 μl of monoclonal anti-c-myc (3631206; Clontech). Protein G-Sepharose beads were added and incubated for 1 h at 4 °C. Beads were centrifuged and washed three times; the final wash buffer included 0.1% Triton X-100 and 0.02% SDS. Proteins were eluted into 80 μl of electrophoresis sample buffer (0.125 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.025% bromphenol blue, 1 mM β-mercaptoethanol, and 22.5% glycerol in 0.5 × PBS). Western blotting was carried out with antibody to CD74 (1:200) and c-myc (1:500) as described previously (14).

**Brain membrane fractions** were prepared from wild-type and Scn8α<sup>−/−</sup>–null homozygous mice by homogenation in 50 mM Tris-HCl, pH 7.5, containing 10 mM EGTA and 5 tablets of Roche Complete Mini Protease Inhibitor Mixture/50 ml of buffer. After centrifugation at 3,500 rpm, membrane proteins were pelleted from the supernatant by centrifugation at 100,000 × g for 30 min. The membrane pellet was suspended in 0.2 ml of homogenization buffer by tituration, and 25–μl aliquots were stored at −80 °C. For immunoprecipitation, one aliquot of stored membrane protein was diluted to 1 ml in 60 mM Tris-HCl buffer containing 1% Triton X-100 (see above) and incubated with 5 μg of monoclonal pan-neuronal sodium channel antibody (S8809–1MG; Sigma) for 8 h at 4 °C. Western blotting was carried out with polyclonal antibody to Na_{1.6} (ASC-009, 1:100; Alomone) or polyclonal antibody to the light chain of Map1b (sc-8971, 1:100; Santa Cruz Biotechnology).

**Electrophysiology**—The DRG-derived cell line ND7/23 (21) was cultured on 12-mm glass coverslips coated with poly-D-
lysine/laminin (BD Biosciences) and transfected using Lipofectamine 2000 (Invitrogen) with 1 μg of DNA/well (0.6 μg of Na⁺,1.6 cDNA, 0.2 μg of pEGFP-C1 (Clontech), and 0.2 μg of either vector pcDNA3 (Clontech) or the Map1b cDNA construct. After 48 h, cells with robust green fluorescence were selected for recording. Whole cell voltage clamp recording was done essentially as described previously (14) with a few modifications: (i) EPC-9 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) was used in this study; (ii) data were filtered at 2.9 kHz and sampled at a rate of 20 kHz; (iii) current-voltage relationship was determined by recording from cells held at −120 mV and stepped to a range of potentials (−80 to 40 mV in 5-mV increments) for 100 ms each; (iv) steady-state fast inactivation was achieved with a series of 500-ms prepulses (−150 to 0 mV in 10-mV increments), and the fraction of nonactivated channels was measured by a 40-ms test pulse to 0 mV; (v) dextrose instead of sucrose was used to adjust the osmolarity of pipette solution (315 mosmol/liter) and external solution (323 mosmol/liter); (vi) data were analyzed using Pulsefit 8.74 software (HEKA Electronics) and OrigenPro 8.1 software (Microlab Software, Northampton, MA), and statistical significance was tested using unpaired Student’s t test because data followed a normal distribution.

RESULTS

Cell Membrane Localization of CD74 Reporter—To determine whether the N terminus of Na⁺,1.6 is sufficient to direct protein localization to the cell membrane, we used the extracellular domain of CD74 (residues 72–232) as a cell surface reporter (19). Transfection of HEK293 cells with the CD74 extracellular fragment alone yields diffuse cytoplasmic staining (Fig. 1A). We cloned a hybrid construct containing the N terminus and first transmembrane segment of Na⁺,1.6 (residues 1–153) upstream of the extracellular domain of CD74. This protein is localized to the cell surface (Fig. 1B). Introduction of the ataxia3 mutation p.S21P into the hybrid construct did not prevent surface localization (Fig. 1C). The S21P mutation does prevent surface localization of full-length Na⁺,1.6 in primary cultured neurons; the reason for the lack of effect on the CD74 construct in HEK cells is not clear. The experiments indicate that additional residues in the N terminus are involved in transport of Na⁺,1.6 to the cell surface.

Yeast Two-hybrid Screen—To identify proteins involved in surface localization of Na⁺,1.6, we screened a mouse brain cDNA library using the N-terminal fragment of Na⁺,1.6 (residues 1–117) as bait (Fig. 2A). Growth on selective medium identified three independent overlapping clones containing portions of the light chain of Map1b (Fig. 2B). The interaction was confirmed by a directed yeast two-hybrid assay using a 541-residue Map1b fragment (residues 1924–2464) including the light chain (Fig. 2C).

Interaction between Na⁺,1.6-CD74 Fusion Protein and Map1b—To determine whether the N terminus of Na⁺,1.6 interacts with Map1b in mammalian cells, we cloned the Map1b fragment into the myc-tagged mammalian expression vector pCMV-myc. myc-Map1b and the fusion protein Na⁺,1.6-CD74 were co-transfected into HEK293 cells. Lysates from co-transfected cells were immunoprecipitated with antibody against CD74, and Western blots were probed with anti-myc antibody. A band corresponding to the myc-tagged Map1b was detected in the immunoprecipitate, demonstrating interaction (Fig. 3A). The S21P mutation did not prevent co-immunoprecipitation (data not shown).

Interaction of Endogenous Full-length Na⁺,1.6 with Light Chain of Map1b—To assess in vivo interaction, we carried out immunoprecipitation of mouse brain membrane protein using a pan-sodium channel antibody followed by Western blotting with an antibody to the light chain of Map1b. Na⁺,1.6 was co-immunoprecipitated with the light chain of Map1b from wild-type brain (Fig. 3B). These results demonstrate that the interaction detected in the yeast two-hybrid system also occurs in vivo with full-length endogenous proteins. Map1b was not co-immunoprecipitated from Scn8a-null brain that lacks Na⁺,1.6 (Fig. 3B). Because the null brain extracts contain normal levels of the other major sodium channels Na⁺,1.1 and Na⁺,1.2 (22), the lack of immunoprecipitation of Map1b from null brain suggested that interaction with Map1b might be specific to Na⁺,1.6.

Map1b Does Not Interact with N Terminus of Na⁺,1.1—To evaluate the channel specificity of the interaction directly, we tested the binding of Map1b to the N terminus of Na⁺,1.1, which differs at 37 of 117 residues (30%) from Na⁺,1.6. No interaction of Na⁺,1.1 with Map1b was observed (Fig. 4A). To localize the Map1b binding site of Na⁺,1.6, we constructed hybrid clones consisting of residues 1–54 from one channel and residues 55–117 from the other. The construct containing residues 55–117 of Na⁺,1.6 (1A/8A) interacted with Map1b in the yeast two-hybrid assay, but the reciprocal construct (8A/1A) did not interact (Fig. 4B). This result localized the binding site to residues 55–117 of the N terminus of Na⁺,1.6, which differ at 16 of 62 positions from Na⁺,1.1 (Fig. 4C).

Localization of Map1b Binding Site in Na⁺,1.6—To further define the Map1b binding site in the distal half of the Na⁺,1.6 N terminus, we generated two sets of C-terminal deletion constructs, beginning either at residue 7 or at residue 13 relative to the first methionine in the N terminus (Fig. 5A). In both sets of constructs, deletion of residues 90–117 did not prevent binding of Map1b, but deletion to residue 80 did prevent binding (Fig. 5A, asterisks). The internal fragment containing residues 38–90 was sufficient for interaction with Map1b (Fig. 5A).

To identify the critical amino acids, we generated seven overlapping 4-residue alanine substitution mutations of the Na⁺,1.6

FIGURE 1. N terminus and first transmembrane segment of Na⁺,1.6 are sufficient to direct a reporter protein to the cell surface. Confocal images of HEK293 cells transfected with the reporter constructs and probed with anti-CD4 antibodies are shown. Green, anti-CD74; blue, DAPI. A, extracellular domain of CD74 lacking membrane-targeting N terminus. B, N terminus and first transmembrane segment of Na⁺,1.6 fused to the extracellular domain of CD74. C, ataxia3 mutation S21P not disrupting surface localization.
**Na,1.6 Interaction with Map1b**

N terminus between residues 73 and 90 of Na,1.6. Six of the 7 alanine substitution constructs retained interaction with Map1b (Fig. 5B). The only noninteracting construct resulted from substitution of AAAA for VAVP (residues 77–80) (Fig. 5B). The corresponding sequence of Na,1.1 (VSEP) differs from Na,1.6 at two residues. These experiments localized the Map1b binding site near the center of the cytoplasmic N terminus of Na,1.6.

The effect of three pathogenic missense mutations was examined: p.S21P (14), p.E82D (23), and p.S107G (23). None of these mutations altered interaction with Map1b, consistent with the mapping data above.

**Functional Effect of Map1b on Na,1.6 Current in ND7/23 Cells**—Measurement of sodium current provides a sensitive assay for the presence of functional sodium channels at the cell surface. To test the effect of Map1b on transport of full-length Na,1.6 to the cell surface, we measured sodium current density in neuron-derived cells transfected with Na,1.6 alone or co-transfected with Map1b. The transfected ND7/23 cells were analyzed using whole cell voltage clamp electrophysiology. Endogenous ND7/23 currents were blocked by addition of 300 nM tetrodotoxin to the culture medium (24). ND7/23 cells were transiently transfected with the tetrodotoxin-resistant construct Na,1.6+ alone or together with Map1b. Robust sodium
FIGURE 5. Localization of Map1b interaction site within N terminus of Nav1.6. A, 15 deletion constructs assayed for interaction with Map1b using the yeast-2-hybrid assay. C-terminal deletion to residue 80 or beyond prevented interaction with Map1b (asterisks). The internal residue 38–90 was sufficient for interaction. B, alanine-scanning constructs spanning the region between residues 73 and 90. Mutation of residues 77–80 prevented interaction with Map1b. +, growth on stringent selection plates; –, no growth.

FIGURE 6. Co-expression of Map1b increases Na,1.6 peak current density in ND7/23 cells transfected with Na,1.6p. A and B, representative sodium currents were recorded from ND7/23 cells transiently co-transfected with Na,1.6p, EGFP, and vector (n = 23) (A) or Map1b (n = 20) (B). Cells were held at −120 mV, and sodium currents were elicited by a series of step depolarizations from −80 to +40 mV in 5-mV increments. C, co-expression of Map1b significantly increases current density of Na,1.6 in ND7/23 cells (**, p < 0.01). D, Map1b does not alter activation or steady-state fast inactivation of Na,1.6.
**Na\textsubscript{\textit{1.6}} Interaction with Map1b**

### TABLE 1
Map1b increases the amplitude of Na\textsubscript{\textit{1.6}} current in ND7/23 cells

| Current density | Activation $V_{1/2}$ | Fast inactivation $V_{1/2}$ |
|-----------------|-----------------------|----------------------------|
| $\mu$A/pF       | $mV$                  | $mV$                       |
| Na\textsubscript{\textit{1.6}} | $65.5 \pm 8.7$ ($n = 23$) | $-14.2 \pm 1.1$ ($n = 14$) |
| Na\textsubscript{\textit{1.6}} and Map1b | $98.7 \pm 8.0$ ($n = 20^{**}$) | $-14.5 \pm 0.6$ ($n = 19$) |

$^{**}$, $p < 0.01$.

### DISCUSSION

**Novel Na\textsubscript{\textit{1.6}} Protein Interaction**—The functions of the cytoplasmic N terminus of voltage-gated sodium channels are currently not well understood. We demonstrate here that the N terminus of Na\textsubscript{\textit{1.6}} interacts with the adaptor protein Map1b, resulting in an increase in current density without a change in activation or fast inactivation of the channel. The N terminus in combination with the first transmembrane segment is also sufficient to direct the CD74 extracellular reporter to the cell surface. Interaction between the mature full-length Na\textsubscript{\textit{1.6}} protein and the light chain of Map1b was demonstrated by co-immunoprecipitation from brain extracts. Co-transfection with Map1b resulted in a 50% increase in the sodium current density generated by transfected Na\textsubscript{\textit{1.6}}, and mutation of the Map1b binding site prevented the generation of sodium currents. The data support a model in which interaction with the light chain of Map1b mediates transport of Na\textsubscript{\textit{1.6}} to the cell surface.

**Biological Role of Map1b**—Map1b contributes to trafficking of several channel and receptor proteins. It directly interacts with the ligand-gated serotonin channel 5-HT3a to mediate channel desensitization (16) and binds NMDA receptor subunit 3A (NR3A), which indirectly affects the conductance of the receptor (17). GABARAP, a molecule with homology to the light chain of Map1b, interacts with the GABA\textsubscript{\textit{A}} receptor and acts as an anchor protein (25). Our work suggests that Na\textsubscript{\textit{1.6}} is another neuronal protein that is trafficked along the microtubule network to the cell surface.

The nine paralogous mammalian sodium channel genes share a highly conserved tertiary structure and extensive sequence conservation within the transmembrane segments, but their cytoplasmic domains are more divergent (26, 27). Interestingly, the VAVP motif of Na\textsubscript{\textit{1.6}} required for interaction with Map1b is not conserved in the other channels, consistent with the experimental evidence that this interaction may be specific to Na\textsubscript{\textit{1.6}}. In vertebrate orthologs of Scn8a, the VAVP motif is conserved in reptiles, birds, and marsupials, but not in fish. Further work will be necessary to define a consensus binding motif for the light chain of Map1b.

**Consequences of Map1b Deficiency in Mouse**—Inactivation of Map1b in targeted knock-out mice results in juvenile lethality of 55% of homozygotes prior to 4 weeks of age (28). The surviving homozygotes have weakness and loss of body weight that resemble the effects of muscle atrophy in Na\textsubscript{\textit{1.6}} knock-out mice (28). Mice carrying a dominant negative allele of Map1b display a more severe phenotype, with embryonic lethality of homozygotes and a movement disorder in heterozygotes that resembles Scn8a mutants, including ataxia, hind limb tremor, and paralysis (29). Impaired trafficking of Na\textsubscript{\textit{1.6}} could contribute to the phenotype of these mice as well. Unfortunately, the mice are not available for further testing.

** Trafficking and Subcellular Localization of Voltage-gated Ion Channels**—The subcellular trafficking of voltage-gated potassium channels in neurons has been studied extensively (30). These channels appear to be selectively transported to their final locations, rather than randomly transported to the cell.
surface with subsequent selective removal. Vesicles containing dendritically localized potassium channels are trafficked by myosin V and/or dynein, which are unable to enter the axon due to steric or directional constraints. Neuronal activity appears to regulate the trafficking of voltage-gated potassium channels to specific subcellular locations (30).

Less is known about the clustering and trafficking of voltage-gated sodium channels. Two alternative models for clustering of sodium channels at the axon initial segment have been considered: transport to the AIS followed by direct insertion or nonspecific transport to the cell surface followed by lateral diffusion to tether points including the AIS (31). Sodium channels are stabilized at the AIS and at nodes of Ranvier by ankyrin G, which interacts with a binding site in cytoplasmic loop II–III (32–34). Adhesion proteins derived from glial cells are also thought to contribute to localization of sodium channels at nodes of Ranvier in myelinated axons. It has been suggested that voltage-gated ion channels are inserted directly into mature nodes, with diffusion limited by myelin and other proteins at the paranode (31). Analysis of axonal transport in transected sciatic nerve suggests that sodium channels reach the nodes by vesicular trafficking, possibly from the cell body (35). However, the molecular mechanism of transport along the axon to the nodes remains unclear. Because microtubules extend along the full length of the axon, Map1b could play a role in localization of Na+,1.6 to both the AIS and the nodes of Ranvier. Overall, our data support a model in which microtubular trafficking of Na+,1.6 to the cell surface is mediated by interaction with the adaptor protein Map1b (Fig. 8).

Acknowledgments—We thank Drs. Luis Lopez-Santiago and Lori Isom for advice and helpful discussions and Dr. W. Clay Brown for cloning the N-terminal deletion constructs.

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