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MARK2 phosphorylates KIF13A at a 14-3-3 binding site to polarize vesicular transport of transferrin receptor within dendrites

Yue Han[a,b,1], Min Li[a,b,1], Bingqing Zhao[a,b,1], Huichao Wang[a,b], Yan Liu[a,b], Zhijun Liu[c,2], Jiaxi Xu[b,2], and Rui Yang[a,b,d,2]

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Neurons regulate the microtubule-based transport of certain vesicles selectively into axons or dendrites to ensure proper polarization of function. The mechanism of this polarized vesicle transport is still not fully elucidated, though it is known to involve kinesins, which drive anterograde transport on microtubules. Here, we explore how the kinesin-3 family member KIF13A is regulated such that vesicles containing transferrin receptor (TfR) travel only to dendrites. In experiments involving live-cell imaging, knockout of KIF13A, BioID assay, we found that the kinase MARK2 phosphorylates KIF13A at a 14-3-3 binding motif, strengthening interaction of KIF13A with 14-3-3 such that it dissociates from TfR-containing vesicles, which therefore cannot enter axons. Overexpression of KIF13A or knockout of MARK2 leads to axonal transport of TfR-containing vesicles. These results suggest a unique kinesin-based mechanism for polarized transport of vesicles to dendrites.

neuron polarity | selective transport | TfR vesicle | KIF13A | MARK2

Significance

Our findings suggest that at least one type of vesicles, those containing transferrin receptor, travel exclusively to dendrites and are excluded from axons because the kinase MARK2 phosphorylates the kinesin KIF13A to promote its separation from vesicles at the proximal axon, preventing vesicle transport into axons, such that they travel only to dendrites. Future studies should explore how this mechanism of polarized vesicle transport supports neuronal function.

Neurons are polarized into axonal and soma-dendritic domains to ensure unidirectional information processing. This polarization arises, in part, through the selective trafficking of vesicles along microtubules into one domain or the other: Vesicles containing dendritic proteins are transported efficiently into dendrites but do not enter the axon, while vesicles containing axonal proteins preferentially travel to axon even if they are not actively excluded from dendrites (1, 2). Kinesins, which act as motors to drive anterograde transport along microtubules, appear to help mediate polarized vesicle transport in neurons (3–6), but the details of how they accomplish this remain unclear.

Neurons express at least 15 kinesins, and different kinesins appear to help transport different types of vesicles (1, 7). We previously showed while vesicles transported by the kinesin-1 family member KIF5A or KIF5C preferentially enter axons, the kinesin-3 family member KIF13A transported vesicles move largely within dendrites, but not axons. KIF13A transported vesicles containing mannose-6-phosphate receptor (8), viral proteins, serotonin type 1A receptor (9) or transferrin receptor (TfR) (1, 7). KIF13A also helps the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor translocate to dendritic spines during long-term potentiation (10). Given the observations that KIF13A drives anterograde transport along microtubules and that axons contain abundant microtubules initiated from the cell body with their "+" ends pointing to the distal axon, we wondered why KIF13A-transported vesicles do not enter axons.

Here, we examined this question by knocking out or overexpressing KIF13A in primary neuronal cultures and examining the effects on transport of vesicles containing TfR. TfR is a ubiquitous transmembrane glycoprotein that mediates iron uptake from circulating transferrin (Tf) at the plasma membrane. The TfR–Tf complex formed at the cell surface is then delivered to endosomes (11). In neurons, vesicles containing TfR are mainly formed and transported at the soma-dendritic area (12). Therefore, it is often used to study dendritic selective transport. In our study, we found that KIF13A is a major transporter for vesicles containing TfR at the soma-dendritic area. Microtubule affinity-regulating kinase 2 (MARK2/Par1) phosphorylation of KIF13A-tail at the 14-3-3 binding motif is required for keeping TfR vesicles dendritically. Overexpression of KIF13A or knockout of MARK2 leads to axonal transport of TfR-containing vesicles.

Results

KIF13A Transports Vesicles Containing TfR to Dendrites. Previously, we found that KIF13A-labeled vesicles do not cross the axon initial segment (AIS) to enter the axon. In addition, KIF13A vesicles were found overlapped with TfR vesicles in dendrites. As a confirmation of our previous work (1), GFP-fused KIF13A-tail with Halo-tagged TfR were coexpressed in cultured hippocampal neurons and the movement of vesicles were

Author affiliations: [a]Institute of Neuroscience, Translational Medicine Institute, School of Basic Medical Sciences, Health Science Center, Xi'an Jiaotong University, Xi'an 710061, China; [b]Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Health Science Center, Xi'an Jiaotong University, Xi'an 710061, China; [c]Department of Infectious Diseases, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province 710061, China; and [d]The Jungers Center for Neurosciences Research, The Department of Neurology, Oregon Health & Science University, Portland, OR 97239-3098

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[1]Y.H., M.L., and B.Z. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: zhijun_liu@xjtufh.edu.cn, xujiaxi@xjtu.edu.cn, or rui.yang@xjtu.edu.cn.

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recorded with time-lapse spinning disc microscopy (Fig. 1 *A* and *B* and Movie S1). Vesicles containing KIF13A-tail and vesicles containing TfR were overlapped and were bidirectionally moving in dendrites. Very few vesicles were observed entering axons.

To test whether KIF13A transports vesicles containing TfR, small-guide RNA (KIF13A-gRNA) was designed in KIF13A exons. With the presence of CRISPR-Cas9, the expression of KIF13A was suppressed (*SI Appendix*, Fig. S1 *A* and *B*). We then used KIF13A-gRNA to suppress KIF13A in neurons and recorded the trafficking of TfR vesicles. For a short period of time (1 d after transfection), we did not see an obvious change in the trafficking of TfR vesicles. After 3 d, we observed a decrease in the trafficking of TfR vesicles (Fig. 1 *C* and *D*). In the control neuron where scramble-gRNA were transfected, the trafficking of TfR vesicles was not disturbed. Although knockout of KIF13A largely impaired the transport of TfR vesicles, it did not change the polarization of TfR vesicles, which probably means that dynein aggregated in the AIS region recycle TfR vesicles to the soma. Analysis was represented by the dendrite-axon ratio of TfR intensity (Fig. 1 *C* and *E*).

Overexpression of KIF13A Allows Axonal Transport of Vesicles Containing TfR. To test how KIF13A may affect the polarized transport of vesicles containing TfR, we overexpressed KIF13A in primary hippocampal neurons and recorded the movement of vesicles containing TfR. Overexpressed KIF13A leads to substantial traffic of vesicles containing TfR into axons, reducing the dendrite-axon ratio of TfR intensity (Fig. 2 *A* and *B* and Movie S2). As expected for the direction of KIF13A migration along microtubules, most vesicles in the axon moved anterograde (Fig. 2*C*). The loss of purely dendritic polarity of vesicle transport was associated with reduced uptake of transferrin (*SI Appendix*, Fig. S2*A*), which may be a consequence of the mislocalization of TfR.

Polarity of Trafficking of Vesicles Containing TfR Depends on KIF13A Motor Activity. The special delivery of proteins to dendrites or axons is thought to begin with the separation of proteins into different carrier vesicle groups. Subsequently, these vesicles are delivered to dendrites or axons in a microtubule-dependent selective transport (5). Accordingly, overexpression of KIF13A caused axon miss-targeted TfR could result from disturbing the sorting process or from disturbing the transport. To test whether KIF13A overexpression disrupts the sorting of TfR, we labeled axon-targeted vesicles with neuron-glia cell adhesion molecule (NgCAM). Vesicles containing NgCAM at axon is 5- to 10-fold higher than at dendrite and showed no overlapping with TfR vesicles (12) (Fig. 3*A* and Movies S3 and S4). When KIF13A was overexpressed, vesicles containing TfR started moving into the axon, but still they did not colocalize with vesicles containing NgCAM, indicating that TfR was not wrongfully sorted into axonal vesicles. Therefore, it is highly possible that overexpressed KIF13A disturbed the transport phase, but not the sorting phase.

To test the hypothesis above, we first set up experiment to test whether the motor activity of KIF13A is required for sending TfR to axon when overexpressed. We introduced mutations (Ser221Ala, His222Ala) to the motor domain of KIF13A (KIF13A-mm) to unable to hydrolyze ATP (13, 14). As shown in Fig. 3*B*, TfR vesicles were not observed in the axon when KIF13A-mm was overexpressed. Moreover, it led to an even higher ratio of dendritic to axonal vesicle delivery than the wild-type neurons (Fig. 3*B*), confirming that it is transportation that mediates the axonal delivery of TfR vesicles in the condition of KIF13A overexpression.

The next question is whether it is the tail domain responsible for binding to vesicles, or the motor domain responsible for movement across microtubules, that plays a decisive role in sending TfR to axons upon KIF13A overexpression. We constructed a chimera containing the motor domain of KIF13A and the tail domain of KIF13B, another member of kinesin-3 family that is less responsible for dendritic trafficking of TfR vesicles (1). Expression of this chimera (KIF13A-B) did not alter the ratio of dendritic to axonal delivery of TfR vesicles in a manner like KIF13A. On the contrary, expression of a chimera containing the motor domain of KIF13B and the tail domain of KIF13A (KIF13B-A) did induce an axonal transportation of TfR vesicles in a similar manner as KIF13A overexpression, suggesting that it is the tail domain of KIF13A, not the motor domain, involved in the axonal transportation of TfR upon KIF13A overexpression (Fig. 3*B*).

MARK2 Phosphorylates KIF13A at a Binding Site for Protein 14-3-3. Next, we explored what upstream proteins might regulate the effect of KIF13A on polarized vesicle transport. We engineered a plasmid encoding the tail domain of KIF13A fused to *Escherichia coli* biotin ligase BirA (15), expressed the plasmid in HEK293T cells and screened the lysate for biotinylated proteins, which were candidate interactors of KIF13A (16). We detected numerous potential interactors (*SI Appendix*, Fig. S3 and Dataset S1), among which we focused on "microtubule affinity-regulating kinase 2" (MARK2), also known as Par1, because it has been implicated in neuronal polarity (17, 18). In addition, Par1b/MARK2 phosphorylates KIF13B at a binding site for protein 14-3-3 in order to regulate axonal formation (19).

First, we demonstrated that MARK2-GFP is enriched in the AIS in primary hippocampal cultures (Fig. 4*A*). The distribution of MARK2 in neuron is similar to ankyrin G, which specifically localizes in the initial segment of axon and Ranvier node. Coimmunoprecipitation of MARK2 using subfragments of the tail domain of KIF13A identified the region encompassing residues 915 to 1,749 in KIF13A as responsible for interaction with the kinase (Fig. 4*B*). This was also the only subfragment of KIF13A that colocalized with vesicles containing TfR in primary hippocampal neurons (Fig. 4 *C* and *D*). A different subfragment of KIF13A, encompassing residues 362 to 914, also colocalized with vesicles in neurons, but not with the subset of vesicles containing TfR.

Given that MARK2 has been shown to phosphorylate the tail domain of KIF13B at a binding site for the protein 14-3-3 ($_{1377}$RXXXSXP$_{1383}$) (19), we performed the kinase assay and confirmed that KIF13A is also phosphorylated by MARK2 (*SI Appendix*, Fig. S4*A*). Indeed, we identified a conserved binding site for protein 14-3-3 in the tail domain ($_{1367}$RSLSTP$_{1373}$) (Fig. 5*A*), which coincided with the fragment that we identified above as critical for interaction with MARK2. Then, we mutated the Ser1371 at the phosphorylation site to Asp in order to mimic phosphorylation, or to Ala to prevent phosphorylation (Fig. 5*B*). In HEK293T cells, the Ser1371Asp mutant of KIF13A bound to 14-3-3ε much more strongly than the Ser1371Ala mutant did, as well as isoform ζ or isoforms β (*SI Appendix*, Fig. S4*B*). These results suggest that the phosphorylation of KIF13A at the 14-3-3 binding site by MARK2 strengthens the kinesin's interaction with 14-3-3.

Phosphorylation of KIF13A by MARK2 Detaches the Kinesin from Vesicles Containing TfR. We examined the association of KIF13A with vesicles containing TfR and the subsequent trafficking of those vesicles in rat embryonic fibroblasts. These cells are flatter than neurons, which facilitates the detection of kinesin colocalization

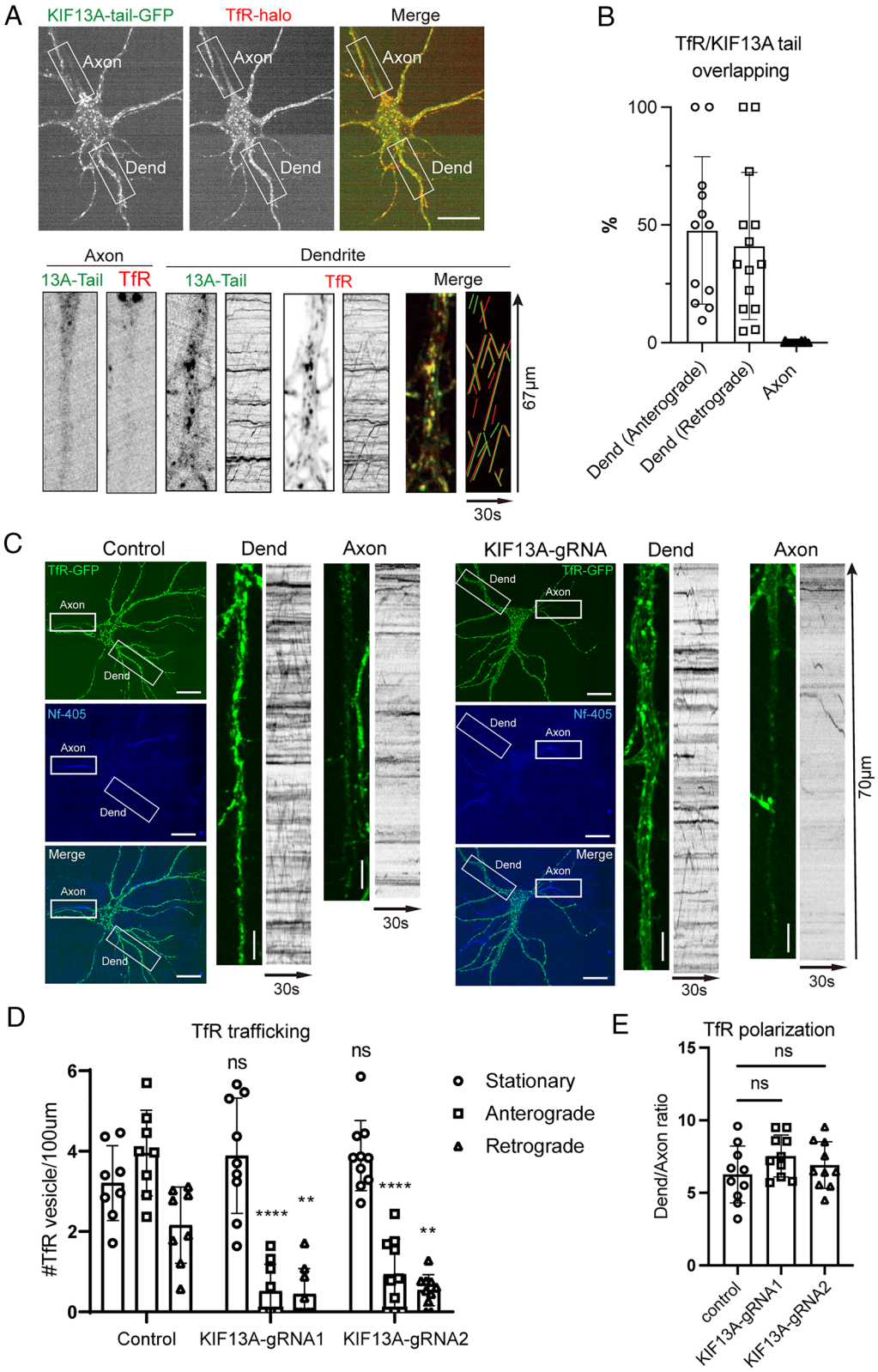

Fig. 1. KIF13A drives polarized transport of vesicles containing TfR into dendrites. (*A* and *B*) Primary rat hippocampal neurons that had been cultured for 5 d were cotransfected with a plasmid encoding a fusion of the tail domain of KIF13A with green fluorescent protein (GFP) and a plasmid encoding Halo-tagged TfR. At 6 h after transfection, cultures were imaged for 30 s at 2 frames/s. The Halo tag was visualized using dye JF594. (*A*) Single frames of the time series showing the distribution of the tail domain of KIF13A and TfR. In the kymograph at the *Bottom Right,* the green line corresponds to the tail domain; the red line, to TfR. AIS, axon initial segment; Dend, dendrite. (Scale bar, 25 μm.) (*B*) Quantification of colocalization between the tail domain of KIF13A and TfR (n = 12, 14, 22 neurons, respectively). (*C–E*) Primary rat hippocampal neurons that had been cultured for five days were cotransfected with a plasmid encoding guide RNA (gRNA1 or gRNA2) targeting KIF13A and a plasmid encoding a fusion of GFP to the TfR. (*C*) Single frames of the time series showing the distribution of TfR in axons and dendrites. Kymographs are shown next to the high magnified images. Horizontal scale bar, 25 μm; vertical scale bar, 5 μm. (*D*) Quantification of the transport of vesicles containing TfR. ns, not significant; $^{**}P < 0.01$, $^{****}P < 0.0001$ (two-way ANOVA, Tukey's multiple comparisons test; n = 8 to 10 neurons). (*E*) Quantification of the polarization of TfR. (ns, not significant, $P > 0.05$; Dunnett's multiple comparison was used after one-way ANOVA; n = 10 neurons for all conditions).

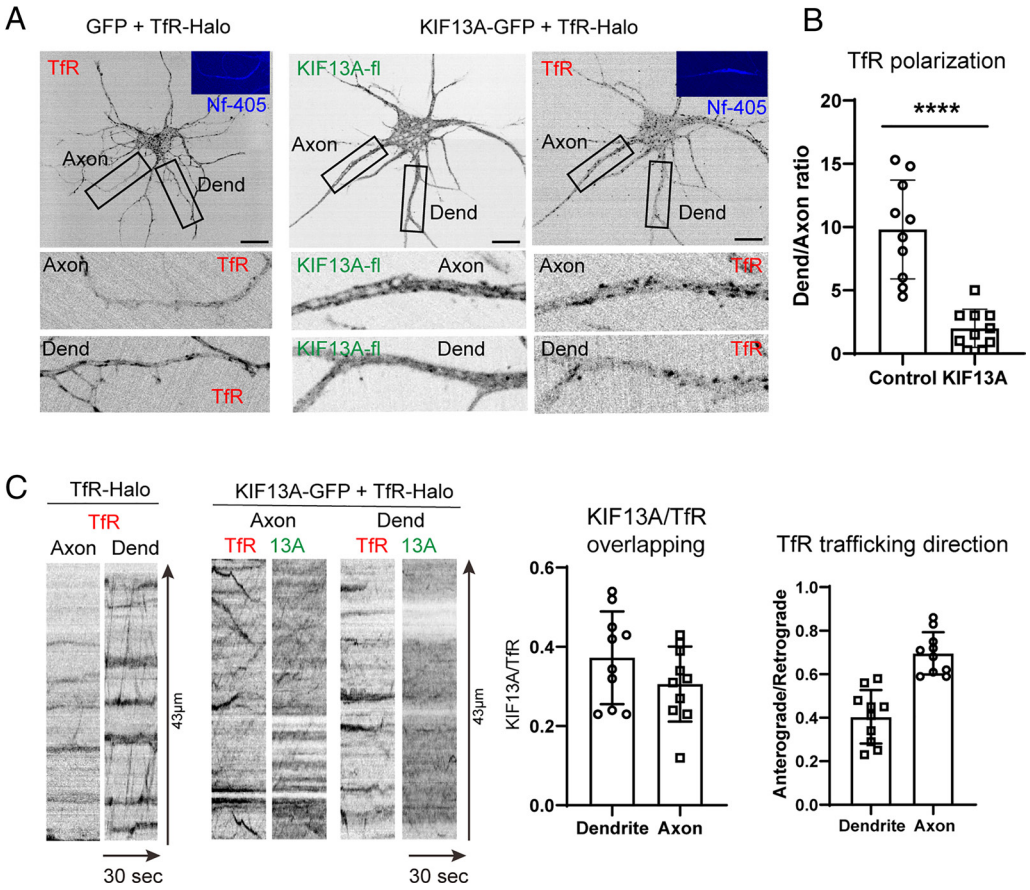

Fig. 2. Overexpression of KIF13A in primary hippocampal neurons allows vesicles containing TfR into axons. (*A–C*) Rat hippocampal neurons that had been cultured for five days were cotransfected with a plasmid encoding Halo-tagged TfR and a plasmid encoding either a fusion of GFP with full-length KIF13A or GFP on its own. At 6 h after transfection, cultures were imaged for 30 s at 2 frames/s. (*A*) Single frames of the time series. Some frames contain insets showing the staining of AIS marker neuronfascin (Nf-405) to indicate the axon. Dend, dendrite. (Scale bar, 20 μm.) (*B*) Quantification of the polarization of TfR. Control expresses GFP instead of KIF13A-GFP. (****$P < 0.0001$ based on Student's *t* test; n = 10 neurons) (*C*) Kymographs of indicated vesicles in dendrites or axons on the *Left,* and quantification of colocalization between KIF13A and TfR as well as TfR trafficking direction on the *Right* (n = 10 neurons).

with vesicles; and the microtubules in these fibroblasts radiate uniformly outward from the centrosome to the cell periphery, which facilitates observation of the direction of kinesin movement. Vesicles containing TfR in these fibroblasts were evenly distributed throughout the cytosol in the absence of KIF13A, while coexpression of the kinesin caused the vesicles to accumulate at the cell periphery (Fig. 5*D*). This accumulation at the cell periphery was partially reversed by coexpressing MARK2 with KIF13A, which also reduced colocalization between KIF13A and vesicles (Fig. 5*E*). These effects of MARK2 were abrogated when KIF13A contained the Ser1371Ala mutation preventing phosphorylation. These results suggest that phosphorylation of KIF13A by MARK2 inhibits the kinesin's ability to interact with, and traffic, vesicles containing TfR.

In support of this idea, we found that in HEK293T cells expressing separate motor and tail domains of KIF13A, the motor domain of KIF13A interacted more strongly with tail domains containing the wild-type 14-3-3 binding motif or the motif with the Ser1371Asp mutation mimicking phosphorylation than with a tail domain containing the nonphosphorylatable Ser1371Ala mutation (Fig. 5*F*). Since stronger interaction between the motor and tail domains of kinesin inhibits its binding to microtubules (20), our findings argue that phosphorylation of KIF13A by MARK2 detaches the kinesin from vesicles containing TfR.

Phosphorylation of KIF13A by MARK2 Is Required for Dendritic Trafficking of Vesicles Containing TfR. To clarify how phosphorylation of KIF13A by MARK2 affects polarized transport of vesicles containing TfR, we knocked out MARK2 using CRISPR-Cas9 (*SI Appendix*, Fig. S4) and analyzed the trafficking of such vesicles in primary cultures of hippocampal neurons (Fig. 6 *A* and *B*). Knockout increased the proportion of vesicles trafficked into axons rather than dendrites, consistent with what we observed in rat embryonic fibroblasts expressing the Ser1371Ala mutant of KIF13A. Indeed, again consistent with our results in fibroblasts, we found that overexpressing KIF13A in primary hippocampal neurons expressing endogenous MARK2 allowed a certain degree of axonal trafficking of vesicles containing TfR (Fig. 6 *C* and *D*). The strictly dendritic trafficking of these vesicles was partially restored when KIF13A and MARK2 were overexpressed together, but not when the overexpressed MARK2 contained the active-site mutation Ser212Asp (MARK2 kinase dead, MARK2-KD) to abrogate its kinase activity or when the overexpressed KIF13A contained the nonphosphorylatable mutation Ser1371Ala.

We then tested the role of KIF13A-Ser1371Ala or KIF13A-Ser1371Asp in TfR trafficking when endogenous KIF13A was eliminated (*SI Appendix*, Fig. S5 *B* and *C*). When KIF13A is knocked out, KIF13A-Ser1371Ala, which contains a nonsense mutation on the PAM sequence, can cause TfR to enter the axon. However, under the same conditions, KIF13A-Ser1371Asp did not cause TfR to enter the axon. To further confirm that the change of TfR polarization caused by MARK2 knock out is KIF13A dependent, we knock out both MARK2 and KIF13A in primary cultured hippocampal neurons and observed that TfR is removed from the axon (*SI Appendix*, Fig. S5 *D* and *E*).

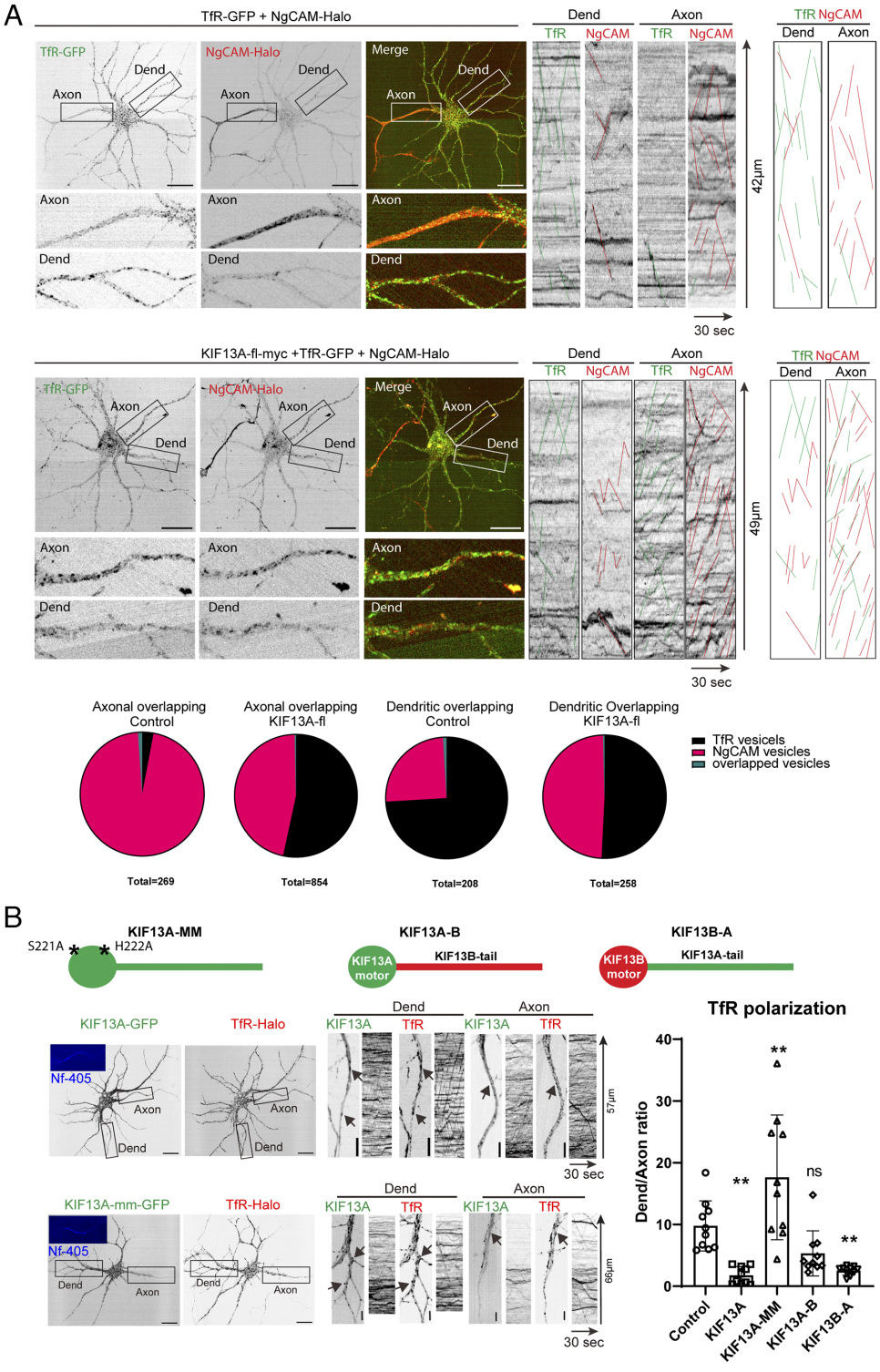

Fig. 3. A tail domain of KIF13A is required to shuttle vesicles containing TfR to axons. (*A*) Rat hippocampal neurons that had been cultured for five days were cotransfected with a plasmid encoding a fusion of GFP with TfR, a plasmid encoding Halo-tagged NgCAM and with or without a plasmid encoding myc-tagged full-length KIF13A. At 6 h after transfection, cultures were imaged for 30 s at 2 frames/s. The Halo tag was visualized using dye JF594. Single frames of the time series are displayed on the *Left*. Kymographs of vesicles are displayed on the *Right,* where green lines represent TfR vesicles and red lines represent NgCAM vesicles. Dend, dendrite. (Scale bar, 25 µm.) The overlapping between NgCAM and TfR was quantified by counting the moving events of NgCAM vesicles (red), TfR vesicles (black), and overlapped vesicles (green) in the kymograph (10 neurons were counted for each condition). (*B*) Rat hippocampal neurons that had been cultured for five days were cotransfected with a plasmid encoding Halo-tagged TfR and one of the indicated kinesin constructs: KIF13A-mm, containing two point mutations (Ser221Ala, His222Ala; labeled as asterisks) to abrogate ATPase activity of the motor domain; a chimera of the motor domain of KIF13A with the tail domain of KIF13B; or a chimera of the tail domain of KIF13A with the motor domain of KIF13B. As a control, some neurons expressed a fusion of GFP with wild-type, full-length KIF13A. At 6 h after transfection, cultures were imaged for 30 s at 2 frames/s. Typically micrographs are shown, and the staining of AIS by antibody against neurofascin (Nf-405) is displayed as an inset at the *Upper Left*. Horizontal scale bar, 25 µm; vertical scale bar, 5 µm. The extent of polarized trafficking of TfR vesicles is quantified on the *Right* (ns, not significant; $^{**}P < 0.01$, $^{*}P < 0.05$ based on one-way ANOVA with multiple comparison).

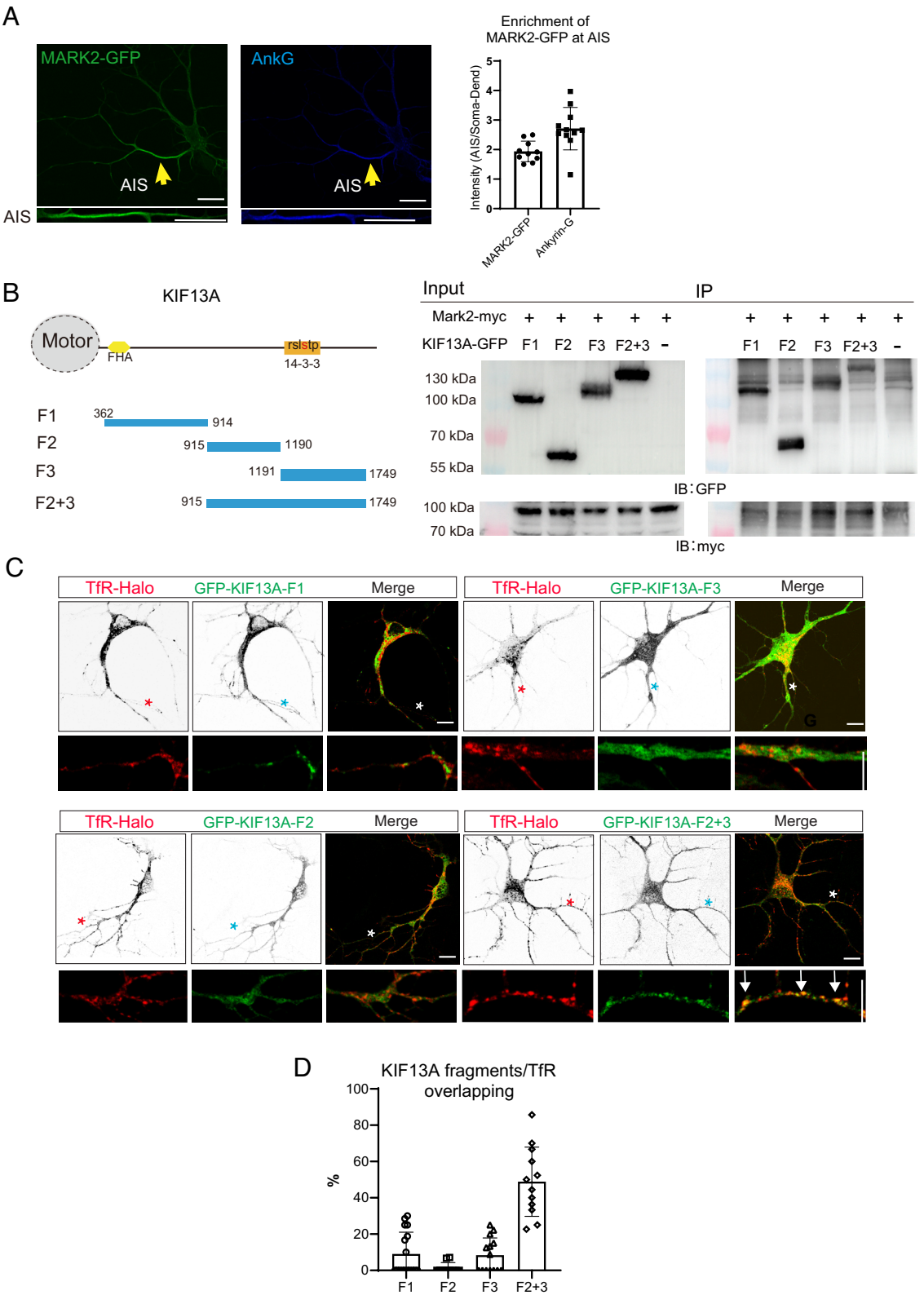

Fig. 4. MARK2 binds to and phosphorylates the tail domain of KIF13A. (*A*) Rat hippocampal neurons that had been cultured for 5 d were cotransfected with a plasmid encoding a fusion of GFP with MARK2. The AIS was stained with antibody against ankyrin-G (AnkG). The relative localization of each protein relative to the soma is shown to *Right*. AIS, axon initial segment. [Scale bar, 25 μm (*Upper* micrographs) or 5 μm (*Lower* micrographs).] (*B*) The indicated fragments of the tail domain of GFP-tagged KIF13A were coexpressed with myc-tagged MARK2 in HEK293T cells, then the tail domain was immunoprecipitated from cell lysates using an anti-GFP antibody. The immunoprecipitates were probed using an anti-myc antibody. FHA, forkhead-associated; 14-3-3, binding site for protein 14-3-3. (*C* and *D*) Rat hippocampal neurons that had been cultured for 5 d were cotransfected with a plasmid encoding Halo-tagged TfR and a plasmid encoding a fusion of GFP with one of the tail domain fragments in panel *B*. Asterisks mark dendrites shown at higher magnification in the *Lower* images, where white arrows indicate colocalization of KIF13A tail and TfR. Horizontal scale bar, 25 μm; vertical scale bar, 5 μm. (*D*) Quantification of colocalization.

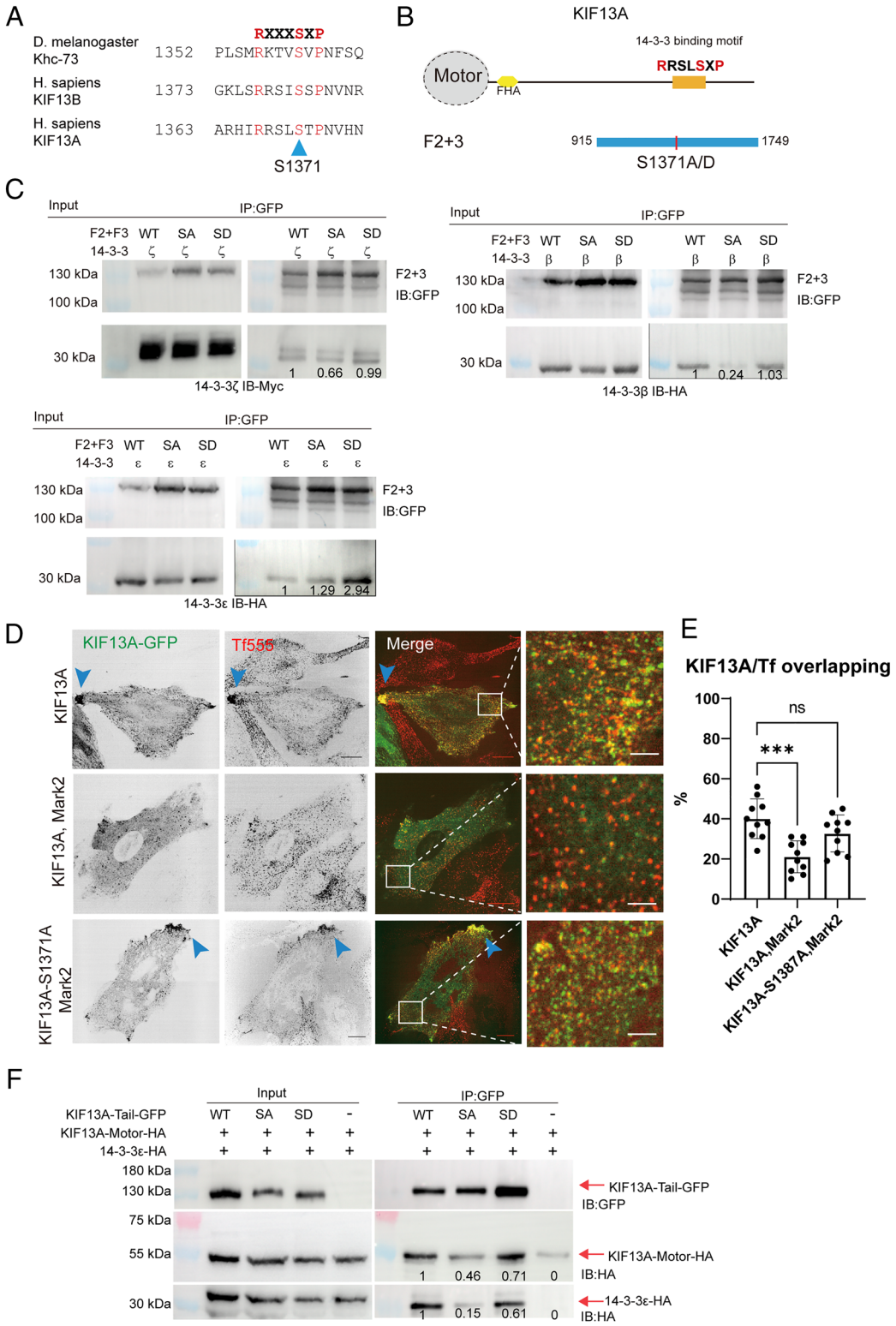

Fig. 5. Phosphorylation of Ser1371 in the tail domain of KIF13A by MARK2 is required for the kinesin to associate with vesicles containing TfR. (*A*) Conservation of the 14-3-3 binding motif across species. The consensus sequence is written at the *Top*. The Ser1371 in KIF13A that MARK2 phosphorylates is indicated at the *Bottom*. (*B*) Construction of a fragment of the tail domain of KIF13A that combines fragments F2 and F3 in Fig. 4*B* and contains either a mutation to remove the MARK2 phosphorylation site (Ser1371Ala) or a mutation to mimic phosphorylation (Ser1371Asp). (*C*) HEK293T cells were cotransfected with a plasmid expressing a fusion of GFP with Ser1371Ala or Ser1371Asp mutants of the tail domain of KIF13A; and with a plasmid expressing myc-tagged 14-3-3 isoform ζ or HA-tagged isoforms ε or β. KIF13A fragments were immunoprecipitated using anti-GFP antibody, and the immunoprecipitates were probed with anti-myc or -HA antibody. Relative intensity of the 14-3-3 band is shown *below* each lane. (*D* and *E*) Rat embryonic fibroblasts were transfected with a plasmid expressing wild-type KIF13A full-length or the Ser1371Ala mutant and, in some cases, with a plasmid expressing MARK2. Cultures were treated with transferrin conjugated to the fluorescent dye Alexa 555 (Tf555). (*D*) Representative fluorescence images showing the localization of KIF13A and TfR vesicles. The area boxed in white is shown at higher magnification at the *far right*. Scale bar, 25 μm or 5 μm (*far right*). (*E*) Colocalization of KIF13A and TfR vesicles. (ns, not significant; ***$P < 0.001$ based on one-way ANOVA. n = 10 cells). (*F*) HEK293T cells were cotransfected with a plasmid encoding a fusion of GFP with the wild-type or mutant version of the tail domain of KIF13A, along with a plasmid encoding the HA-tagged motor domain of KIF13A and the HA-tagged 14-3-3 isoform ε. KIF13A fragments were immunoprecipitated using anti-GFP antibody, and immunoprecipitates were probed using anti-HA antibody. Relative intensity of the band is shown *below* each lane.

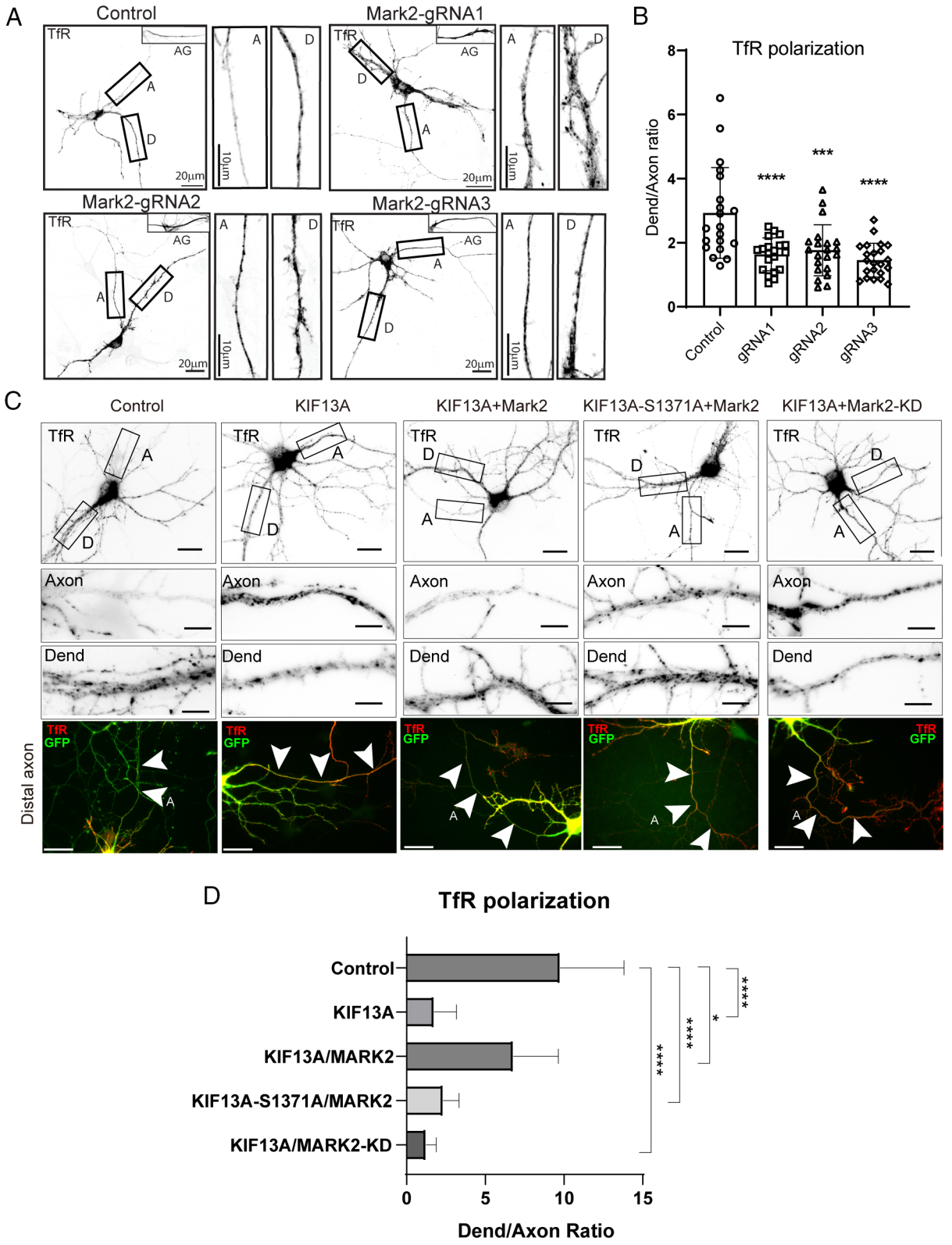

Fig. 6. Phosphorylation of KIF13A by MARK2 is required for polarized transport of vesicles containing TfR to dendrites. (*A* and *B*) Cultured hippocampal neurons at DIV5 were cotransfected with a plasmid expressing Halo-tagged TfR and a plasmid encoding the indicated small guide RNA (gRNA) targeting MARK2. (*A*) Representative photomicrographs. The immunostaining for AIS maker ankyrin-G (AG) is displayed in the small black box at the upright corner. The areas boxed in black on the *Left* are shown at higher magnification on the *Right*. D, dendrite. A, axon. Horizontal scale bar, 20 μm; vertical scale bar, 10 μm. (*B*) Extent of polarization of TfR vesicles. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 (based on one-way ANOVA, n = 20, 20, 21, 22 neurons for control, gRNA1, gRNA2, and gRNA3, respectively). (*C* and *D*) Cultured hippocampal neurons at five days were cotransfected with a plasmid expressing Halo-tagged TfR and a plasmid encoding a fusion of GFP with full-length wild-type KIF13A or the Ser1371A mutant. Some cultures were also transfected with a plasmid expressing wild-type MARK2 or the MARK2-Ser212Arg kinase-deficient (KD) mutant. The regions boxed in black above are shown at higher magnification below. A, axon; D or Dend, dendrite. A snapshot for the distal axon was displayed at the bottom. The white arrowheads point to the axon. Scale bar, 25 μm (above) or 10 μm (below). (*D*) Extent of polarization of trafficking of TfR vesicles. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 (based on one-way ANOVA, n = 10 neurons for each group).

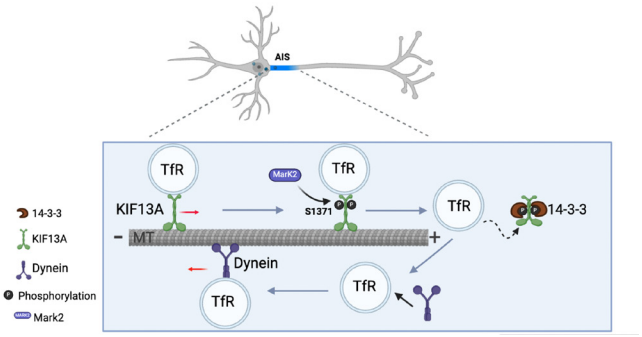

Fig. 7. Model for phosphorylation of the 14-3-3 binding motif in KIF13A by MARK2 in the AIS. A microtubule at AIS (blue) of a neuron (gray) is enlarged and displayed in the light blue box *below*. TfR vesicles (light blue circles) were transported along the microtubule to AIS by KIF13A. KIF13A was phosphorylated by MARK2 at AIS. The phosphorylation of KIF13A caused a recruitment of 14-3-3 to KIF13A and the release of KIF13A from TfR vesicles. TfR vesicles are then transported back to the soma by dynein.

These results suggest that phosphorylation of KIF13A by MARK2 is required for the strictly dendritic trafficking of vesicles containing TfR in neurons.

Discussion

Dendritic polarized vesicles were barely across the AIS and enter the axon. How neurons maintain dendritic vesicles is a key question in the neuron polarity field. Here, we provide evidence that vesicle subpopulations containing TfR trafficked by the kinesin KIF13A are excluded from axons of neurons because the kinesin is phosphorylated by MARK2 (Fig. 7). Our results add KIF13A to the list of motor proteins that mediate polarized vesicle transport within neurons, a list that already includes myosin V (21) and dynein (22–24), as well as the dense network of actin filaments within the AIS (25).

Since KIF13A shares high-sequence homology with KIF13B, which also associates with vesicles containing TfR in neurons (26), we expected that the two kinesins would perform the same in our experiments. However, overexpressing fusion of the motor domain of KIF13A with the tail domain of KIF13B affected the polarization of such vesicles to a much weaker extent than the corresponding changes in fusion of the motor domain of KIF13B with the tail domain of KIF13A, suggesting that KIF13A is the dominant transporter of this vesicle subpopulation. Indeed, our previous work with primary hippocampal neurons showed that few vesicles transported by KIF13B contained TfR and that an appreciable proportion of KIF13B-associated vesicles traveled to the axon, whereas most vesicles transported by KIF13A contained TfR and few traveled to the axon (1). We hypothesize that the different tail domains of kinesin-3 family members confer selectivity for different subpopulations of vesicles, which should be explored in future work.

In *SI Appendix*, Fig. S2, overexpression of KIF13A eliminated the transferrin uptake rather than results in transferrin uptake in axons. We think there are two possible explanation to this observation. The first explanation is that overexpression of KIF13A creates an additional force that prevents TfR-containing vesicles from docking and fusing to the cell membrane, resulting in a lack of TfR on the cell membrane. A second explanation is overexpressed KIF13A somehow disturbed the endocytosis, which prevented the transferrin uptake. Based on the fact that transferrin is completely absent in neurons, we are inclined to the first explanation. Because if the second explanation is true, we should at least see some transferrin on the cell membrane. This result also gives some hints about the source of axonal TfR in KIF13A overexpression or MARK2 knock down. TfR was synthesized and packed in vesicles at Golgi. Vesicles containing TfR were subsequently delivered to dendritic membrane, where TfR could bind with transferrin and been internalized. *SI Appendix*, Fig. S2 suggested that KIF13A bind to both the newly synthesized TfR and the ones in endocytic vesicles. Excess KIF13A pulls TfR containing endocytic vesicles in the cytoplasm to axon. In the meanwhile, excess KIF13A pulls the newly synthesized TfR vesicles directly to the axon before they could be delivered to the dendritic membrane. Therefore, by the time we took the image, due to the lack of TfR on dendritic cell membrane, there is no transferrin up taking.

We found that overexpressing MARK2 in primary hippocampal neurons that had been cultured for one week led to the protein's accumulation in the axon initial segment. In contrast, work of Hirokawa N and colleagues showed overexpressing MARK2 in primary neurons that had been cultured for 4 d led to its accumulation in soma and dendrites (27). This difference may reflect neurons at different developmental stages: The axon initial segment forms in primary neurons between four and seven days in culture (28). Future work should explore how MARK2 regulates polarized vesicle traffic at different stages of neuronal development.

14-3-3 is a conserved family of adaptor proteins that interact with diverse proteins and regulate their function. 14-3-3 family members were found interact with both dynein (29–31) and kinesin (32, 33). The interaction between 14-3-3 and dynein/kinesin could promote the dynein activity (29, 30) or inhibit kinesins activity. In this study, we showed phosphorylation of KIF13A by MARK2 enhanced the interaction between KIF13A and 14-3-3. We also showed that KIF13A-S1371A better colocalized with TfR vesicles. However, we didn't have direct evidence to show that the phosphorylation by MARK2 tips the balance between 14-3-3 and TfR vesicles for binding to KIF13A. We tried to look at 14-3-3 colocalization with KIF13A-S1371D in cells but didn't get convincing labeling, which may due to 14-3-3 interacts with diverse proteins. We predicted that MARK2 phosphorylated and inhibited KIF13A at AIS, which paused TfR vesicles based on MARK2-GFP is enriched at AIS. The ideal experiment should be to locally interfere with the kinase activity of MAKR2 at AIS to see whether it causes axonal entry of TFR.

Material and Methods

Detailed materials and methods can be found in *SI Appendix*.

Procedures involving animals were approved by Xi'an Jiaotong university, basic medical school (Approval number:2022-0046). Astroglia cells and pyramidal neurons were isolated from 1-d-old Sprague-Dawley rat pups as described (34). The CRISPR-Cas9 system was used to knockout KIF13A or MARK2 in primary hippocampal neurons using a short guide RNA targeting exon in KIF13A, and exon in MARK2. The short guide RNA was cloned into the vector lentiCRISPRv2 (35, 36). Knockout effect of KIF13A or endogenesis MARK2 were confirmed by western blot.

Time-lapse Imaging. We imaged primary cultures as described (1). Cells were incubated while imaging in Hibernate E medium without phenol red (Brain-Bits), then images were acquired with an sCMOS camera (Zyla 4.2+, Andor) under a Ti-E microscope (Nikon) equipped with a spinning-disk confocal head (model CSU-W1, Yokogawa). Vesicles containing TfR were imaged using an sCMOS camera through a CFI Apo 60× 1.49 objective (Nikon) on a Ti2 microscope (Nikon). The entire imaging stage and objectives were maintained at 37 °C in a closed system (customized by OkoLab). Image streams (two frames per sec) were acquired using a Plan-Apo 100× 1.49 NA objective (Nikon) with 2 × 2 binning, during which z axis movement was controlled using the Perfect Focus system on the Ti-E microscope (Nikon).

Statistical Analysis. Kymographs were generated from image series using Fiji 2.12. Only puncta that could be unambiguously assigned as vesicles were included in the analysis. Functional enrichment analysis of the biotinylated proteins was performed using RStudio version 2023.06.2+561. Missing values of area in list obtained after prequality control screening (Datasets S1 and S2) were imputed as the mean value of group. In order to ensure the comparability of proteins, the KIF13A area of each group were normalized, and the area of each protein was standardized according to this standard. Significantly enriched proteins (GFP-KIF13A Tail-BioID vs. GFP-BioID, *t* test unadjusted $P \leq 0.05$) were analyzed by the Go enrich program. In assays involving Primary pyramidal neurons in which MARK2 was knocked out, polarization between axons and dendrites was semiquantified in terms of the ratio of fluorescence in axons to the fluorescence in dendrites.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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1. R. Yang *et al.*, A novel strategy to visualize vesicle-bound kinesins reveals the diversity of kinesin-mediated transport. *Traffic* **20**, 851–866 (2019).
2. C. F. Huang, G. Banker, The translocation selectivity of the kinesins that mediate neuronal organelle transport. *Traffic* **13**, 549–564 (2012).
3. N. Hirokawa, Y. Noda, Y. Tanaka, S. Niwa, Kinesin superfamily motor proteins and intracellular transport. *Nat. Rev. Mol. Cell Biol.* **10**, 682–696 (2009).
4. S. E. Cason, E. L. F. Holzbaur, Selective motor activation in organelle transport along axons. *Nat. Rev. Mol. Cell Biol.* **23**, 699–714 (2022).
5. M. Bentley, G. Banker, The cellular mechanisms that maintain neuronal polarity. *Nat. Rev. Neurosci.* **17**, 611–622 (2016).
6. A. Serra-Marques *et al.*, Concerted action of kinesins KIF5B and KIF13B promotes efficient secretory vesicle transport to microtubule plus ends. *Elife* **9**, e61302 (2020).
7. M. A. Silverman *et al.*, Expression of kinesin superfamily genes in cultured hippocampal neurons. *Cytoskeleton (Hoboken)* **67**, 784–795 (2010).
8. T. Nakagawa *et al.*, A novel motor, KIF13A, transports mannose-6-phosphate receptor to plasma membrane through direct interaction with AP-1 complex. *Cell* **103**, 569–581 (2000).
9. R. Zhou, S. Niwa, L. Guillaud, Y. Tong, N. Hirokawa, A molecular motor, KIF13A, controls anxiety by transporting the serotonin type 1A receptor. *Cell Rep.* **3**, 509–519 (2013).
10. Y. Gutierrez *et al.*, KIF13A drives AMPA receptor synaptic delivery for long-term potentiation via endosomal remodeling. *J. Cell Biol.* **220**, e202003183 (2021).
11. B. D. Grant, J. G. Donaldson, Pathways and mechanisms of endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **10**, 597–608 (2009).
12. M. A. Burack, M. A. Silverman, G. Banker, The role of selective transport in neuronal protein sorting. *Neuron* **26**, 465–472 (2000).
13. N. Hirokawa, Y. Noda, Intracellular transport and kinesin superfamily proteins, KIFs: Structure, function, and dynamics. *Physiol. Rev.* **88**, 1089–1118 (2008).
14. F. J. Kull, E. P. Sablin, R. Lau, R. J. Fletterick, R. D. Vale, Crystal structure of the kinesin motor domain reveals a structural similarity to myosin. *Nature* **380**, 550–555 (1996).
15. D. I. Kim *et al.*, An improved smaller biotin ligase for BioID proximity labeling. *Mol. Biol. Cell* **27**, 1188–1196 (2016).
16. K. J. Roux, D. I. Kim, B. Burke, D. G. May, BioID: A screen for protein-protein interactions. *Curr. Protoc. Protein Sci.* **91**, 19.23.11–19.23.15 (2018).
17. T. Terabayashi *et al.*, Polarity-regulating kinase partitioning-defective 1/microtubule affinity-regulating kinase 2 negatively regulates development of dendrites on hippocampal neurons. *J. Neurosci.* **27**, 13098–13107 (2007).
18. Y. M. Chen *et al.*, Microtubule affinity-regulating kinase 2 functions downstream of the PAR-3/PAR-6/atypical PKC complex in regulating hippocampal neuronal polarity. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 8534–8539 (2006).
19. Y. Yoshimura, T. Terabayashi, H. Miki, Par1b/MARK2 phosphorylates kinesin-like motor protein GAKIN/KIF13B to regulate axon formation. *Mol. Cell Biol.* **30**, 2206–2219 (2010).
20. K. J. Verhey, J. W. Hammond, Traffic control: Regulation of kinesin motors. *Nat. Rev. Mol. Cell Biol.* **10**, 765–777 (2009).
21. T. L. Lewis Jr., T. Mao, K. Svoboda, D. B. Arnold, Myosin-dependent targeting of transmembrane proteins to neuronal dendrites. *Nat. Neurosci.* **12**, 568–576 (2009).
22. M. Kuijpers *et al.*, Dynein regulator NDEL1 controls polarized cargo transport at the axon initial segment. *Neuron* **89**, 461–471 (2016).
23. L. C. Kapitein *et al.*, Mixed microtubules steer dynein-driven cargo transport into dendrites. *Curr. Biol.* **20**, 290–299 (2010).
24. E. Klinman, M. Tokito, E. L. F. Holzbaur, CDK5-dependent activation of dynein in the axon initial segment regulates polarized cargo transport in neurons. *Traffic* **18**, 808–824 (2017).
25. A. H. Song *et al.*, A selective filter for cytoplasmic transport at the axon initial segment. *Cell* **136**, 1148–1160 (2009).
26. M. Bentley, H. Decker, J. Luisi, G. Banker, A novel assay reveals preferential binding between Rabs, kinesins, and specific endosomal subpopulations. *J. Cell Biol.* **208**, 273–281 (2015).
27. S. Ichinose, T. Ogawa, X. Jiang, N. Hirokawa, The spatiotemporal construction of the axon initial segment via KIF3/KAP3/TRIM46 transport under MARK2 signaling. *Cell Rep.* **28**, 2413–2426.e7 (2019).
28. R. Yang *et al.*, Neurodevelopmental mutation of giant ankyrin-G disrupts a core mechanism for axon initial segment assembly. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 19717–19726 (2019).
29. Z. Xu *et al.*, 14-3-3 protein targets misfolded chaperone-associated proteins to aggresomes. *J. Cell Sci.* **126**, 4173–4186 (2013).
30. D. E. Chapman *et al.*, Regulation of in vivo dynein force production by CDK5 and 14-3-3ε and KIAA0528. *Nat. Commun.* **10**, 228 (2019).
31. M. S. Lu, K. E. Prehoda, A NudE/14-3-3 pathway coordinates dynein and the kinesin Khc73 to position the mitotic spindle. *Dev. Cell* **26**, 369–380 (2013).
32. T. Ichimura *et al.*, Phosphorylation-dependent interaction of kinesin light chain 2 and the 14-3-3 protein. *Biochemistry* **41**, 5566–5572 (2002).
33. C. Dorner, A. Ullrich, H. U. Häring, R. Lammers, The kinesin-like motor protein KIF1C occurs in intact cells as a dimer and associates with proteins of the 14-3-3 family. *J. Biol. Chem.* **274**, 33654–33660 (1999).
34. S. Kaech, G. Banker, Culturing hippocampal neurons. *Nat. Protoc.* **1**, 2406–2415 (2006).
35. N. E. Sanjana, O. Shalem, F. Zhang, Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods* **11**, 783–784 (2014).
36. O. Shalem *et al.*, Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343**, 84–87 (2014).