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Oatp1a1 Requires PDZK1 to Traffic to The Plasma Membrane by Selective Recruitment of Microtubule-based Motor Proteins

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Running title: PDZK1 selectively recruits microtubule-based motor proteins

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Non-standard abbreviations: AMP-PNP – Adenosine 5′-(β,γ-imido) triphosphate lithium salt hydrate; BSP – sulfobromophthalein; HDL – High Density Lipoprotein; MEPS buffer – 5mM $MgCl_2$, 5mM EGTA, 35mM PIPES, pH7.4; OATP – organic anion transport protein; PDZ -- post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), zonula occludens-1 protein (ZO-1); PMEE buffer – 35mM PIPES-K_2, 2mg/ml BSA, 5mM $MgCl_2$, 1mM EGTA, 0.5mM EDTA, 4mM DTT, 5mg/ml casein, pH7.4; PMSF – phenylmethylsulfonyl fluoride; SR-B1 – Scavenger Receptor class B type 1

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Figures: 7
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Introduction: 479 words
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Abstract

Previous studies identified a family of organic anion transport proteins (OATPs), many of which have C-terminal PDZ binding consensus sequences. In particular, the C-terminal four amino acids of oatp1a1, a transporter on rat and mouse hepatocytes, comprise a consensus binding site for PDZK1. In PDZK1 knockout mice and in transfected cells where PDZK1 expression was knocked down, oatp1a1 accumulates in intracellular vesicles. The present study tests the hypothesis that oatp1a1 traffics to and from the cell surface in vesicles along microtubules and that PDZK1 guides recruitment of specific motors to these vesicles. Oatp1a1-containing vesicles were prepared from wild type and PDZK1 knockout mice. As seen by immunofluorescence, kinesin-1, a microtubule plus-end directed motor, was largely associated with vesicles from wild type mouse liver, while dynein, a minus-end directed motor, was largely associated with vesicles from PDZK1 knockout mouse liver. Quantification of motility on directionally marked microtubules following addition of 50 μM ATP showed that wild type vesicles moved equally towards the plus and minus ends while PDZK1 knockout vesicles moved predominantly towards the minus end, consistent with net movement towards the cell interior. These studies provide a novel mechanism by which PDZK1 regulates intracellular trafficking of oatp1a1 by recruiting specific motors to oatp1a1-containing vesicles. In the absence of PDZK1, oatp1a1-containing vesicles can't recruit kinesin-1 and associate with dynein as a predominant minus-end directed motor. Whether this is a result of direct interaction of the oatp1a1 cytoplasmic domain with dynein or with a dynein-containing protein complex remains to be established.

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Introduction

The hepatocyte serves as a major site of organic anion clearance from the circulation (Wolkoff, 2012;Scharschmidt, et al., 1975). Many of these compounds are poorly soluble in aqueous solution, and circulate bound to proteins such as albumin (Wolkoff, et al., 1987;Choi, et al., 2009). Uptake involves extraction from the protein carrier and is mediated by a specific transporter(s) on the basolateral (sinusoidal) surface of the cell (Meier, et al., 1997;Wolkoff, 2012). Past studies have identified a family of organic anion transport proteins (OATPs) that can mediate this uptake process (Jacquemin, et al., 1994;Wolkoff, 2012). These proteins have been shown to play a role in clearance of anionic drugs from the circulation, and mutations have been associated with several reports of toxicity in patients due to reduced clearance of drug by the liver (Link, et al., 2008;Nakanishi and Tamai, 2012;Clarke and Cherrington, 2012). Many of the members of the oatp family have been found to have PDZ binding consensus sequences at their C-termini (Choi, et al., 2009). Previous studies of the rat oatp1a1 transporter showed that its C-terminal 4 amino acids (KTKL) comprise a consensus site that binds PDZK1 (Wang, et al., 2005;Choi, et al., 2011). The mouse homolog has the same C-terminal sequence, and traffics poorly to the hepatocyte surface in PDZK1 knockout mice, accumulating in vesicular structures within cells (Wang, et al., 2005). These mice have no change in total expression of the transporter, but the altered subcellular distribution correlated with reduced uptake of the organic anion, sulfobromophthalein (BSP),as compared to wild type mice (Wang, et al., 2005).

Further studies were performed in 293T cells expressing oatp1a1 in the presence or absence of PDZK1 (Choi, et al., 2011). Similar to results in mice, in the absence of PDZK1, distribution of oatp1a1 was largely intracellular. This contrasted with its abundant plasma

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membrane expression in the presence of PDZK1 as seen by immunofluorescence as well as cell surface biotinylation and immunoanalysis (Choi, et al., 2011). Based on these findings, we hypothesized that oatp1a1 in vesicles could traffic to and from the cell surface along microtubules (Sarkar, et al., 2004;Sarkar, et al., 2006) and that motor activity was regulated by its interaction with PDZK1. This was tested in the present study in which intracellular vesicles were prepared from wild type and PDZK1 knockout mouse livers (Nath, et al., 2007). Vesicles that were associated with oatp1a1 were identified using oatp1a1 antibody and fluorescent secondary antibody. Motility chambers with a volume of approximately 5 µl were coated with polymerized taxol-stabilized fluorescent microtubules. Vesicles were flowed into the chambers and attached to the microtubules. Motors were activated by addition of ATP and motile oatp1a1-associated vesicles were quantified (Murray, et al., 2000;Murray and Wolkoff, 2005). The direction of movement towards the plus- or minus-ends of microtubules was quantified by using directionally labeled microtubules (Nath, et al., 2007). Immunofluorescence co-localization permitted identification of vesicle associated motors.

Materials and Methods

Animals

Wild type C57BL/6J male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PDZK1 knockout mice on a C57BL background were provided by Dr. David L. Silver (formerly of the Albert Einstein College of Medicine, now at the Duke-National University of Singapore Graduate Medical School) and bred in the animal facility at the Albert Einstein College of Medicine. Animals were studied at approximately 14 weeks of age and all

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procedures were approved by the Animal Use Committee of the Albert Einstein College of Medicine.

Reagents and Antibodies

Rabbit antibody raised against the N-terminal 14 amino acids of rat oatp1a1 and previously shown to react with mouse oatp1a1 was affinity purified as reported previously (Wang, et al., 2005). Mouse monoclonal antibodies against dynein (SC-13524), non-immune mouse IgG (SC-2025), and non-immune rabbit IgG (SC-2027) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to KifC1 (MCA4040Z) was purchased from AbD Serotec (Oxford, UK). Mouse monoclonal antibody to Kinesin-1 heavy chain (Kif5B) (MAB1613) was obtained from Chemicon International (Temecula, CA) now a subsidiary of EMD Millipore (Boston, MA). Mouse monoclonal antibody against KifC3 (SAB1406060) was purchased from Sigma (St. Louis, MO). Mouse monoclonal antibody against PDZK1 (AB89452) was purchased from Abcam (Cambridge, MA). Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Tubulin (TL238) and rhodamine tubulin (TL331M) were purchased from Cytoskeleton Inc. (Denver, CO).

Isolation of Endocytic vesicles

Endocytic vesicles were prepared from groups of three to five male mouse livers as previously described (Nath, et al., 2007). In brief, mice were anesthetized with 100mg/kg of ketamine-HCl. The livers were removed and Dounce homogenized in 0.25M sucrose MEPS buffer pH7.4 containing 5mM $MgCl_2$, 5mM EGTA, 35mM PIPES. Protease inhibitor cocktail from Sigma (St. Louis, MO) was added at a dilution of 1:50 along with cOmplete mini EDTA

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free tablet from Roche (Basel, Switzerland) at 1 tablet per 10ml of sucrose MEPS buffer. After a 10 minute centrifugation at 1,800g, the postnuclear supernatant was subjected to Sephacryl S200 (Pharmacia, Uppsala, Sweden) column chromatography. Vesicle enriched fractions were adjusted to 1.4M sucrose using 2.5M sucrose MEPS buffer with 2mM PMSF and protease inhibitors. Samples were layered at the bottom of a discontinuous sucrose gradient composed of 1.2M and 0.25M sucrose in MEPS buffer. Following centrifugation at 100,000g for 2 hours, vesicles were collected from the 1.2M to 0.25 M sucrose interface. Aliquots of vesicles were stored at -80°C until use.

Immunofluorescence Analysis of Vesicles

Fluorescent vesicles were flowed into an optical chamber. In previous studies, we found that vesicles bound avidly to the unprocessed glass surface (Murray and Wolkoff, 2005). Unattached vesicles were removed by washing with PMEE buffer (35mM PIPES-K_2, 2mg/ml BSA, 5mM $MgCl_2$, 1mM EGTA, 0.5mM EDTA, 4mM DTT, 5mg/ml casein, pH7.4). Vesicles were incubated on ice for 6 min with oatp1a1 antibody diluted 1:100 in PMEE buffer and washed in PMEE buffer. This was repeated with the second primary antibody (e.g. dynein antibody). Following the final wash, appropriate fluorescent secondary antibodies were flowed in, incubated for 5 min, and washed in PMEE buffer. Final washes were done in PMEE buffer with 2mg/ml ascorbic acid in the absence of casein.

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Analysis of Microtubule-based Motility of Oatp1a1-associated Vesicles

Preparation of Microtubules

Fluorescent microtubules were polymerized from tubulin in buffer containing 80mM PIPES-K2, 1mM $MgCl_2$, 1mM EGTA, 1mM GTP, 3% glycerol, pH 7.0 in a ratio of 7:1 mixture of unlabeled tubulin to rhodamine-tubulin at 37°C. To prepare polarity marked fluorescent microtubules, a dim seed with a 75:1 ratio of tubulin to rhodamine-tubulin was polymerized for 5 minutes at 37°C. These seeds were then sheared by rapidly pipetting 2µl of the mixture up and down 5 times in a 10 µl Eppendorf pipette. Microtubules were then polymerized at 37°C for 6min with a 6:1 mixture of unlabeled to rhodamine labeled tubulin, then stabilized with 20 µM taxol. Polymerized microtubules were centrifuged at 15 psi for 3 minutes in a Beckman (Brea, California) Airfuge to remove unpolymerized tubulin.

Vesicle motility assay

Fluorescent microtubules were flowed into a DEAE-dextran coated optical chamber (Bananis, et al., 2000). Unattached microtubules were removed by washing with PMEE buffer containing 20 µM Taxol. Vesicles were then flowed into the chamber and allowed to bind to microtubules at room temperature for 10 minutes. Unattached vesicles were removed by washing. Vesicles were labeled with oatp1a1 antibody as described above except that 20 µM taxol was added to the PMEE buffer. The motility chamber was then placed on the fluorescence microscope stage maintained at 37°C. Addition of 50 µM ATP was used to initiate vesicle motility. In studies of inhibition of motility, antibodies to motor proteins were flowed into the chamber prior to the addition of ATP while drug inhibitors were added with ATP.

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Image and Statistical Analysis

Optical chambers were imaged using a 60x 1.4 numerical aperture Olympus objective on an inverted Olympus IX71 microscope. Images were captured by a CoolSNAP HQ cooled charge-coupled device camera (Photometrics, Roper Scientific, Tuscon, AZ) controlled by MetaMorph software (Molecular Devices, Sunnyvale, CA). Data were analyzed using ImageJ 1.39u (National Institutes of Health public domain; htpp://rsb.info.nih.gov/ij/) and Adobe Photoshop CS2 version 9.0.2 (Adobe Systems, San Joes, CA). Colocalization of fluorescent proteins on vesicles was quantified using the Autoscore co-localization macro for ImageJ as previously described (Murray, et al., 2002;Mukhopadhyay, et al., 2011). Time-lapsed movies of motility experiments were taken at 1 frame per second for 90 seconds starting before addition of ATP. Movies were scored manually using ImageJ 1.39u. Statistical analysis was performed using Chi-square or Student's *t*-test as appropriate.

Results

Motility of oatp1a1-associated Vesicles on Microtubules

Initial experiments were performed to examine whether oatp1a1-containing vesicles bind to and move along microtubules. Figure 1a (also see supplemental movie 1) shows a representative time series of images of an oatp1a1-associated vesicle, prepared from a PDZK1 knockout mouse, moving on a polarity-marked microtubule. The plus and minus microtubule ends are indicated by long and short regions of bright fluorescence respectively. In this example, the vesicle moved towards the minus end. In the 34 sec shown in this figure, it moved approximately 20 µm. Similar analysis of multiple vesicles prepared from wild type and PDZK1 knockout mice was performed. As seen in Figure 1b, approximately 45% of microtubule-bound

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oatp1a1-associated vesicles obtained from wild type or PDZK1 knockout mice moved upon addition of 50 μM ATP. However, quantification of direction of movement shows substantially different behavior between these two populations of vesicles. As seen in Figure 1c, oatp1a1-associated vesicles prepared from wild type mouse liver moved equally towards the plus and minus ends of polarity marked microtubules. In contrast, vesicles prepared from PDZK1 knockout mouse liver had a significant bias towards the microtubule minus end with approximately 70% of motile vesicles moving in the minus-end direction.

Immunolocalization of Microtubule-based Motors on oatp1a1-associated Vesicles

The preceding studies indicate that oatp1a1-containing vesicles are associated with microtubule-based motors that can mediate both plus- and minus-end directed motility. Identification of candidate motors that are associated with these vesicles was determined by immunofluorescence microscopy. Representative studies are shown in Figure 2A, and quantitation of multiple studies is shown in Figure 2B. When association of oatp1a1-containing vesicles with PDZK1 was examined, close to 60% of oatp1a1-containing vesicles prepared from wild type mouse liver were also associated with PDZK1. As expected, there was no colocalization of PDZK1 with vesicles prepared from PDZK1 knockout mice. As seen in Figure 2B there were several substantial differences in motor protein distribution between vesicles from wild type and PDZK1 knockout mice. Kinesin-1 (Kif5B), a plus-end directed kinesin motor, and KifC1, a minus-end directed kinesin, were present in approximately 60% of vesicles from wild type mice, but fewer than 20% of vesicles from PDZK1 knockout mice. In contrast, dynein, a minus-end directed motor, was present in approximately 50% of the vesicles from PDZK1

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knockout mice, but only 25% of vesicles from wild type mice. Further studies were performed to determine whether these motors mediate motility of these vesicles.

Activity of Vesicle-associated Motors

Analysis of total motility

Studies were performed to examine the effect of inclusion of 5 μM vanadate or 1 mM AMP-PNP on motility. Based upon previous observations, at the concentrations used, these compounds inhibit dynein and kinesins, respectively (Murray, et al., 2000). As seen in Figure 3, inclusion of vanadate reduced the number of motile vesicles, more so in vesicles prepared from wild type as compared to PDZK1 knockout mice. Addition of AMP-PNP resulted in almost complete inhibition of motility of vesicles from both wild type and PDZK1 knockdown mice. Of the motor antibodies tested, only antibody to dynein reduced the number of motile vesicles prepared from wild type and PDZK1 knockout mice. Studies quantifying changes in total motility do not take into account potential complex interactions of motors and colocalization of motors of opposite direction on a single vesicle as we and others have observed previously (Nath, et al., 2007;Soppina, et al., 2009;Hendricks, et al., 2010;Schuster, et al., 2011). Subsequent motility studies were performed on polarity marked microtubules to quantify directional motility.

Analysis of directional motility on polarity marked microtubules

Effects of chemical inhibitors

As seen in Figure 4A, there was no effect of addition of vanadate on the proportion of oatp1a1-containing vesicles prepared from wild type mice moving in either direction although

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the total number of motile vesicles was reduced (Figure 3A). In contrast, as seen in Figure 4B, there was a significant reduction in minus-end directed motility of vesicles prepared from PDZK1 knockout mice, and a corresponding increase in plus-end directed motility. As noted above, addition of AMP-PNP resulted in almost complete inhibition of motility of vesicles from both wild type and PDZK1 knockdown mice (Figure 3), and there were not enough motile vesicles to accurately assess directional movement.

Roles of kinesin-1 and dynein

To better assess the role of specific motors in mediating motility of these vesicles, effects of motor antibodies on directional movement of oatp1a1-containing vesicles were quantified. As seen in Figure 5A, there was no effect of dynein antibody on directional motility of oatp1a1-containing vesicles prepared from wild type mice. Similar to results with vanadate, dynein antibody preincubation significantly reduced minus-end motility of vesicles prepared from PDZK1 knockout mice, with proportionately more plus-end motility remaining (Figure 5B). In contrast to results for dynein inhibition, preincubation with antibody to the plus-end kinesin Kif5B (kinesin-1) resulted in reduced plus end directed motility of wild type vesicles and a corresponding proportional increase of movement towards the minus end (Figure 5C). There was no effect of Kif5B antibody on directional motility of oatp1a1-containing vesicles prepared from PDZK1 knockout mice (Figure 5D).

Roles of the minus-end kinesins KifC1 and KifC3

Preincubation of wild type vesicles with antibody to the minus end kinesin KifC1 had no significant effect on their total or directional motility (Figures 3A and 6A). Incubation with this antibody resulted in a significant reduction in the proportion of minus end motility in vesicles

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from PDZK1 knockout mice (Figure 6B). This suggests that KifC1 may mediate some of the minus-end directed motility in these vesicles, and that when its function is inhibited by antibody, compensatory plus-end directed motor activity becomes apparent. Similarly, there was no effect of antibody to the minus end kinesin KifC3 on total or directional motility of wild type vesicles (Figures 3A and 5C), but a significant reduction in minus-end motility of vesicles from knockout mice (Figure 5D), although their total motility was unchanged (Figure 3B).

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Discussion

Organic anion transporting polypeptides (OATPs) are a super family of transport proteins responsible for sodium independent uptake of compounds that include hormones, bile salts, xenobiotics, and drugs (Hagenbuch and Meier, 2003;Hagenbuch and Meier, 2004;Hagenbuch and Stieger, 2013;Iusuf, et al., 2012). The superfamily is composed of six families based on 40% amino acid sequence identity divided into subfamilies that have 60% amino acid homology (Hagenbuch and Stieger, 2013;Iusuf, et al., 2012). OATP family members are expressed in multiple organs including brain, heart, kidney, intestine, and liver, and they share structural similarities that include predicted 12 transmembrane domains (Wang, et al., 2008;Hagenbuch and Stieger, 2013) and 3-4 N-linked glycosylation sites (Wang, et al., 2008;Yao, et al., 2012). Reduced transport activity of OATPs, accompanied by adverse drug reactions, have been described with a number of genetic polymorphisms (Link, et al., 2008;Nakanishi and Tamai, 2012;Clarke and Cherrington, 2012). It is probable that any condition in which plasma membrane localization of oatps is reduced could have a similar effect.

Oatp1a1, first identified in rat (Jacquemin, et al., 1994), is a prototypical member of the OATP family. Our previous studies indicated that oatp1a1 can traffic from the cell surface

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following phosphorylation at serines 634 and 635 (Xiao, et al., 2006) resulting in down regulation of transport function (Glavy, et al., 2000;Choi, et al., 2011). In addition, several members of the OATP family, including oatp1a1, contain a PDZ consensus binding motif (KTKL) at the C-terminus (Choi, et al., 2009). We also showed that this consensus motif mediated binding of oatp1a1 to PDZK1 (Wang, et al., 2005). Mice in which the gene for PDZK1 was knocked out had reduced plasma disappearance of the oatp1a1 ligand sulfobromophthalein (Wang, et al., 2005). Oatp1a1 in these mice accumulated in intracellular vesicles rather than at the cell surface (Wang, et al., 2005). Further studies were performed in cell lines transfected with oatp1a1 with or without cotransfection of PDZK1. In the presence of PDZK1, oatp1a1 was predominantly on the cell surface as determined morphologically and by cell surface biotinylation (Choi, et al., 2011). In the absence of PDZK1 expression, oatp1a1 was predominantly intracellular (Wang, et al., 2005;Choi, et al., 2011). The present study examined whether changes in microtubule-based motility of oatp1a1-containing vesicles could represent the mechanism behind these observations.

Trafficking of a number of proteins between the plasma membrane and an intracellular pool has been shown to require intact microtubules (Sarkar, et al., 2006;Oda, et al., 1995;Soldati and Schliwa, 2006). Based on this, we hypothesized that oatp1a1-containing vesicles traffic through the cell along microtubules and that their direction of movement is determined by specific vesicle associated motors. In the present study, we tested this hypothesis and examined the role of PDZK1 on motor recruitment and directional motility of oatp1a1-containing vesicles. These studies utilized a system that we developed to reconstitute microtubule-based vesicle motility *in vitro* (Murray, et al., 2000;Bananis, et al., 2000;Murray and Wolkoff, 2005). The present studies were performed using oatp1a1-containing vesicles that were prepared from livers

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of wild type or PDZK1 knockout mice. These studies quantified the directional motility of vesicles on microtubules in the absence of cytosol or other subcellular components and provide a platform on which to examine vesicle-associated proteins, including specific microtubule-based motors and regulatory factors (Murray, et al., 2002). It is notable that, as we have described previously, native vesicles can be studied by antibody labeling of cell surface transporter without inhibiting their ability to move on microtubules (Bananis, et al., 2000;Sarkar, et al., 2006). This obviates concerns that could arise with overexpression of a fluorescently labeled protein such as oatp1a1 that could alter stoichiometry of association with other proteins. This could be important for studies of PDZK1 function. PDZK1 is a 70kDa scaffolding protein that can assemble protein complexes and plays an important role in regulating expression of the HDL Scavenger Receptor class B type 1 (SR-B1) (Silver, 2002;Kocher, et al., 2003) as well as cell surface expression of oatp1a1 in the liver (Wang, et al., 2005).

Our studies indicate that PDZK1 is not required for binding of oatp1a1-containing vesicles to microtubules (Figure 1B), but that it regulates the direction in which these vesicles move (Figure 1C). The plus end of microtubules in most cells, including hepatocytes (Novikoff, et al., 1996), is near the cell surface, while the minus end is within the cell at the microtubule organizing center. In the absence of PDZK1, oatp1a1-containing vesicles move predominantly towards the minus end of microtubules which is consistent with their localization within the cell interior. Several microtubule-based motors that are associated with these vesicles were identified. As seen in Figure 2, their distribution on vesicles varies in the presence or absence of PDZK1. Interestingly, oatp1a1-containing vesicles from wild type mice are highly associated with the plus-end motor kinesin-1 (Kif5B), while oatp1a1-containing vesicles from PDZK1 knockout mice have little kinesin-1. Oatp1a1-containing vesicles from both wild type and

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PDZK1 knockout mice were associated with dynein, a minus-end directed motor, but dynein was significantly more abundant on vesicles from knockout mice (Figure 2B). In both groups, the number of motile vesicles was reduced by inhibition of dynein activity either with vanadate or with antibody (Figure 2B). However, inhibition of the knock out vesicles was less effective. Both vanadate and dynein antibody-treated motile vesicles from wild type mice continued to move approximately equally towards the plus and minus directions while those from knockout mice lost their minus-end bias and had motility similar to that of wild type vesicles (Figures 4 and 5). Previous studies showed colocalization and coordination of motor activities of dynein and kinesin-1 (Ligon, et al., 2004;Uchida, et al., 2009;Gross, et al., 2002;Deng, et al., 2010). We hypothesize that a population of vesicles from wild type mice is associated with both motors, and that inhibition of dynein on these vesicles results in dysfunction of this regulatory mechanism producing loss of both plus and minus end motility. In contrast, there is little kinesin-1 associated with knockout vesicles. Consequently, activity of dynein is not co-regulated with that of kinesin-1 so that inhibition of dynein results in reduced minus end motility (Figure 5). In further studies, we found that KifC1 and KifC3, two minus-end directed kinesins are also associated with oatp1a1-containing vesicles from wild type and PDZK1 knockout mice (Figure 2B). Inclusion of antibodies to these motors during motility assays resulted in a decrease in minus-end directed motility of vesicles from PDZK1 knockout but not wild type mice (Figures 3 and 6A,C). This suggests that these minus-end kinesins are active in the absence of PDZK1, consistent with the relatively large amount of residual minus-end directed motility in these vesicles when dynein is inhibited with vanadate or antibody. The reason for their lack of activity in vesicles from wild type mice is not known at this time but could be related to interaction with kinesin-1 as we described in a previous study (Nath, et al., 2007). Vesicles from PDZK1

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knockout mice exhibit a different profile of motor proteins, with increased KifC3 and dynein but decreased KifC1 and Kif5B. These results again suggest that PDZK1 is responsible for motor protein recruitment and in the absence of PDZK1, the profile of motor proteins and their regulation are altered.

That members of the kinesin family are associated with all motile oatp1a1-containing vesicles is suggested by experiments in which AMP-PNP was included in the motility assay. AMP-PNP is known to associate with kinesins resulting in strong binding to microtubules and complete inhibition of motility at high AMP-PNP to ATP ratios (Uemura, et al., 2002;Subramanian and Gelles, 2007). As seen in Figure 3, this compound inhibited virtually all motility for both wild type and PDZK1 knockout vesicles. This suggests that AMP-PNP functionally anchors these vesicles to microtubules via their kinesins, making them unable to move, even if they are also associated with dynein.

In summary, the present study presents a novel mechanism by which PDZK1 regulates intracellular trafficking of oatp1a1 by recruiting specific motors to intracellular oatp1a1-containing vesicles. Our previous studies showed that oatp1a1 binds to only two of the four independent PDZ binding domains on PDZK1 (Wang, et al., 2005). As shown in the cartoon in Figure 7, we suggest that this enables PDZK1 to form complexes with proteins that recruit kinesin-1 to the vesicle. In the absence of PDZK1, oatp1a1-containing vesicles have reduced ability to recruit kinesin-1 and instead associate with dynein as a predominant minus-end directed motor. We know that the terminal four amino acids on the cytoplasmic domain of oatp1a1 are required to recruit PDZK1. Whether this cytoplasmic domain contains sequences that facilitate recruitment of motors and regulatory factors in the absence of PDZK1 is the subject of ongoing investigation.

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Authorship Contributions

Participated in research design: Wang, Murray, Wolkoff

Conducted experiments: Wang

Contributed new reagents or analytic tools: Murray

Performed data analysis: Wang, Wolkoff

Wrote or contributed to the writing of the manuscript: Wang, Murray, Wolkoff

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Footnotes

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Figure Legends

Figure 1. Microtubule-based motility of oatp1a1-associated endocytic vesicles. Oatp1a1-containing endocytic vesicles were prepared from livers of wild type and PDZK1 knockout mice and flowed into microchambers that had been coated with polarity-marked fluorescent microtubules. After binding of vesicles to microtubules, motility was initiated with the addition of 50 μM ATP. (A) Representative images demonstrating minus-end directed movement of an oatp1a1-containing vesicle prepared from PDZK1 knockout mouse liver. A red microtubule with attached green oatp1a1 labeled vesicles runs horizontally and contains markings for microtubule polarity. The polarity marks were generated by polymerizing brightly fluorescent tubulin from short, dimly fluorescent microtubule seeds, allowing the growth of long microtubule plus ends. Visible from left to right is the microtubule minus end (-), a dimly fluorescent seed, and the microtubule plus end (+) to which a green, motile vesicle is bound. The white arrow follows this vesicle as it moves towards the minus end of the microtubule. The yellow arrowhead indicates the starting point for the vesicle. Time in seconds after addition of ATP is indicated at the top left of each panel. In the 34 seconds of this study, the vesicle moved approximately 18 μm (approximately 0.5 μm/sec). Scale bar = 10μm. (B) The percentage of microtubule-bound vesicles that moved following ATP addition is indicated by the bars for wild type (open bars) and PDZK1 knockout (solid bars) derived vesicles. (C) The percentage of motile vesicles from the studies in panel B moving towards the plus (closed bars) or minus ends (open bars) of microtubules is indicated. Numbers in parentheses represent the number of motile vesicles that were examined. Error bars represent mean ± SEM. *$p<0.0001$ as compared to

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plus-end motility of wild type vesicles. **p<0.0001 as compared to minus-end motility of wild type vesicles.

Figure 2: Co-localization of motor proteins and PDZK1 with oatp1a1-associated vesicles. Endocytic vesicles isolated from wild type and PDZK1 knockout mouse livers were attached to the glass surface of microchambers and immunostained for oatp1a1 and motor proteins or PDZK1 as described in Materials and Methods. (A) Representative images are shown in which oatp1a1 is in red and PDZK1 or motor proteins are in green. Vesicles in yellow represent colocalization of the two. Scale bar = 10μm. (B) Quantification of protein colocalization with oatp1a1-containing vesicles. The percentage of oatp1a1-containing vesicles that colocalized with each of the proteins indicated in the figure is represented by filled (wild type) or open (PDZK1 knockout) bars. The number of oatp1a1-associated vesicles examined is in parentheses. Error bars represent mean ± SEM. *p<0.0001 as compared to colocalization in wild type vesicles.

Figure 3: Effects of chemical and motor protein antibody inhibitors on the fraction of oatp1a1-containing vesicles that move. The proportion of oatp1a1-associated vesicles derived from wild type (panel A) and PDZK1 knockout (panel B) mouse liver that move on microtubules in the presence of chemical or motor protein antibody inhibitors are represented by the bars. Buffer addition serves as the control for vanadate and AMP-PNP studies, and non-immune IgG addition serves as the control for antibody studies. The total number of oatp1a1 associated vesicles counted is in parentheses. *p<0.0001 for total motility vs buffer control. **p<0.0001 for total motility vs IgG control.

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Figure 4: Effect of vanadate on directional motility of oatp1a1-associated vesicles. 1 μM vanadate, an inhibitor of dynein, was included in the motility assay performed on polarity marked microtubules. The proportion of motile vesicles moving towards the plus or minus ends of microtubules was quantified for wild type vesicles (panel A) and PDZK1 knockout vesicles (panel B). The total number of motile vesicles observed is shown in parentheses. Error bars represent mean ± SEM. *$p<0.0001$ as compared to plus-end motility. **$p<0.0001$ as compared to minus-end motility.

Figure 5: Effect of antibodies to dynein or kinesin-1 (Kif5B) on directional motility of oatp1a1-associated vesicles. Vesicles prepared from wild type (panels A and C) or PDZK1 knockout (panels B and D) mouse livers were bound to polarity marked microtubules in a microscopy chamber and preincubated for 5 min with non-immune IgG or antibodies to dynein (panels A and B) or kinesin-1 (panels C and D). The proportion of moving vesicles going towards the plus or minus ends of the microtubules was quantified following addition of 50 μM ATP. The total number of motile vesicles observed is shown in parentheses. Error bars represent mean ± SEM. *$p<0.0001$ as compared to plus-end motility. **$p<0.0001$ as compared to minus-end motility.

Figure 6: Effect of antibodies to the minus end kinesins KifC1 and KifC3 on directional motility of oatp1a1-associated vesicles. Vesicles prepared from wild type (panels A and C) or PDZK1 knockout (panels B and D) mouse livers were bound to polarity marked microtubules in a microscopy chamber and preincubated for 5 min with non-immune IgG or antibodies to KifC1 (panels A and B) or KifC3 (panels C and D). The proportion of vesicles moving towards the

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plus or minus ends of the microtubules was quantified following addition of 50 μM ATP. The total number of motile vesicles observed is shown in parentheses. Error bars represent mean ± SEM. *$p<0.0001$ as compared to plus-end motility. **$p<0.0001$ as compared to minus-end motility.

Figure 7: Proposed role of PDZK1 in selective recruitment of motors to oatp1a1-associated vesicles. As seen in panel A, we propose that one or more of the cytosolic domains of vesicle-associated oatp1a1 bind a protein complex that contains dynein, independent of the presence of PDZK1. As seen in panel B, PDZK1 bound to the C-terminal PDZ binding consensus sequence (KTKL) of vesicle-associated oatp1a1 recruits a complex of proteins that includes kinesin-1 (Kif5B). In this situation, activity of these motors is coordinately regulated, such that inhibition of one type of motor will affect activity of the other.

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Figure 1

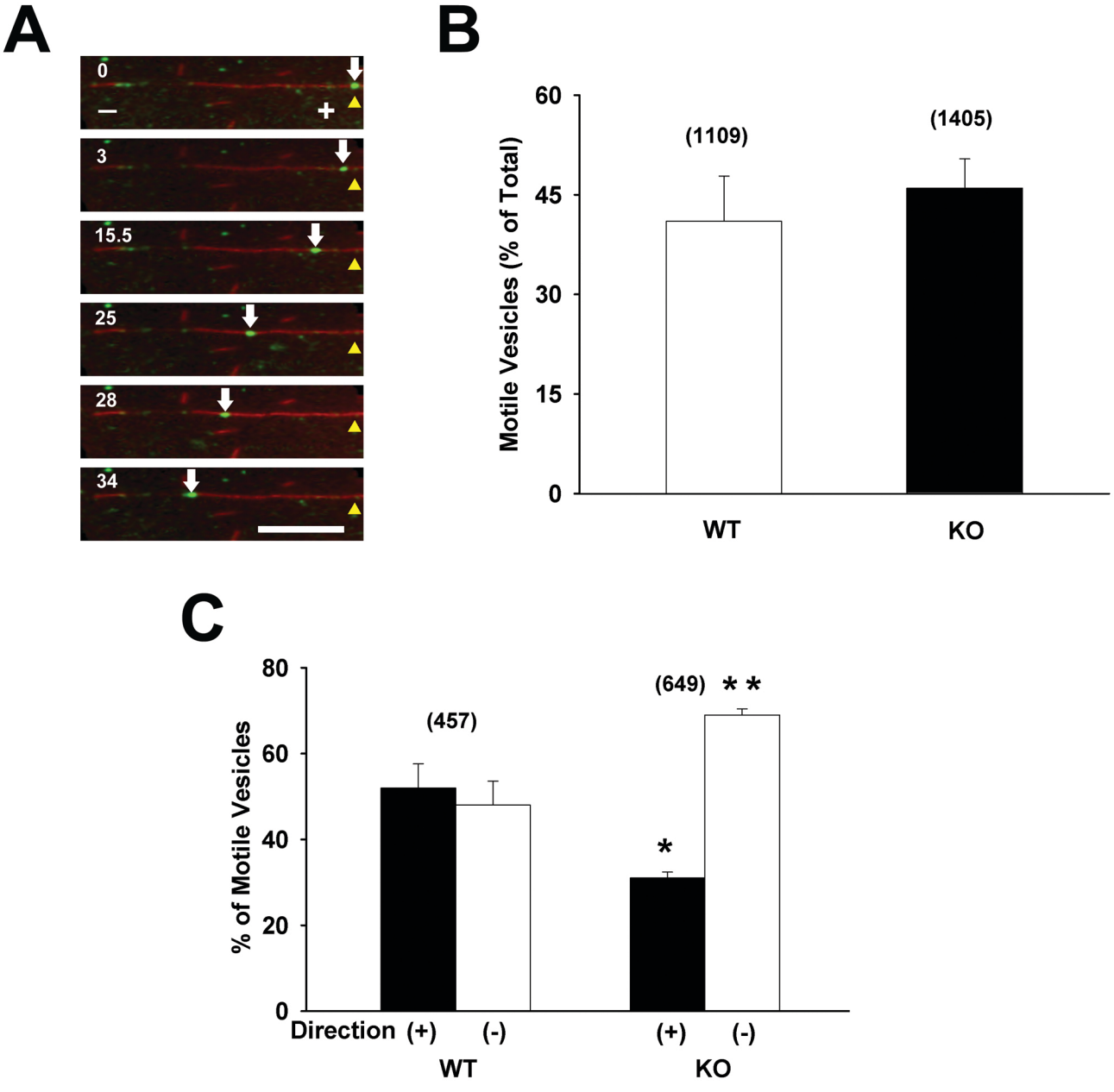

A
0
3
15.5
25
28
34
B
Motile Vesicles (% of Total)
60
45
30
15
0
(1109)
(1405)
WT
KO
C
% of Motile Vesicles
80
60
40
20
0
(457)
(649)
* *
*
Direction (+) (-) (+) (-)
WT
KO

Figure 2

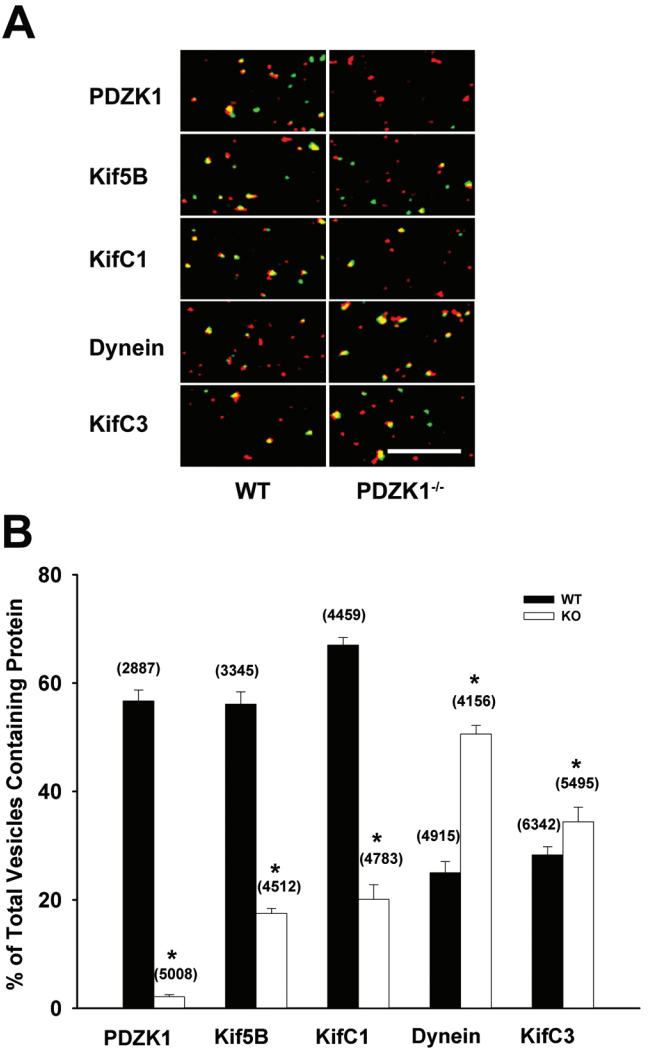

A
PDZK1
Kif5B
KifC1
Dynein
KifC3
WT
PDZK1-/-
B
% of Total Vesicles Containing Protein
80
60
40
20
0
WT
KO
(2887)
(5008)
(3345)
(4512)
(4459)
(4783)
(4915)
(4156)
(6342)
(5495)
*
PDZK1
Kif5B
KifC1
Dynein
KifC3

Figure 3

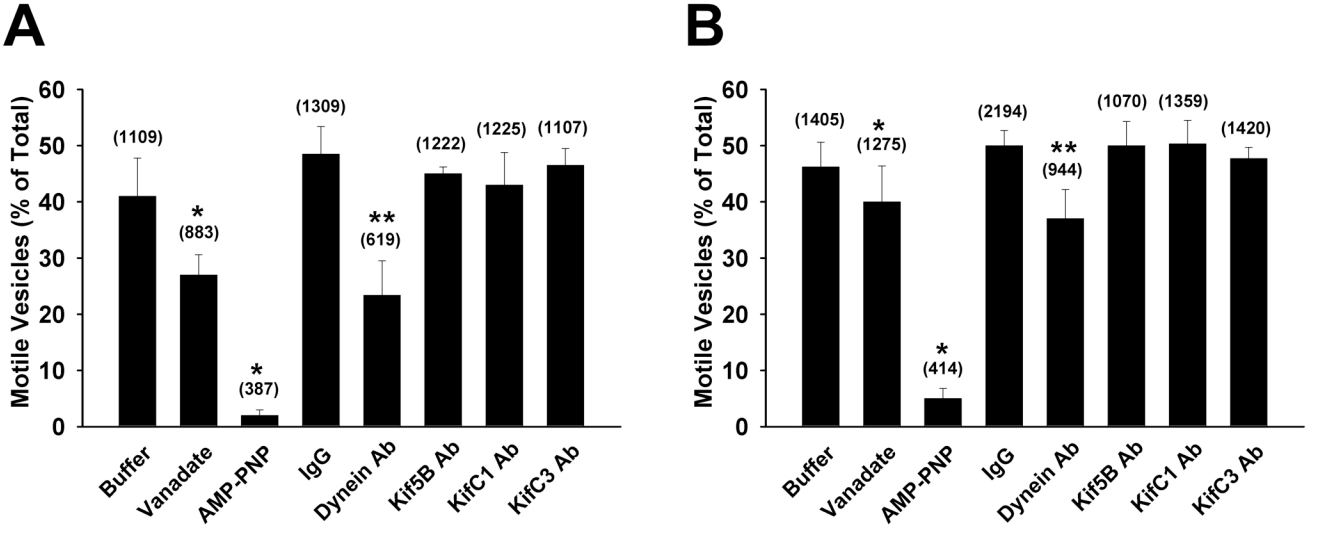

A
Motile Vesicles (% of Total)
60
50
40
30
20
10
0
(1109)
*
(883)
*
(387)
(1309)
**
(619)
(1222)
(1225)
(1107)
Buffer
Vanadate
AMP-PNP
IgG
Dynein Ab
Kif5B Ab
KifC1 Ab
KifC3 Ab
B
Motile Vesicles (% of Total)
60
50
40
30
20
10
0
(1405)
*
(1275)
*
(414)
(2194)
**
(944)
(1070)
(1359)
(1420)
Buffer
Vanadate
AMP-PNP
IgG
Dynein Ab
Kif5B Ab
KifC1 Ab
KifC3 Ab

Figure 4

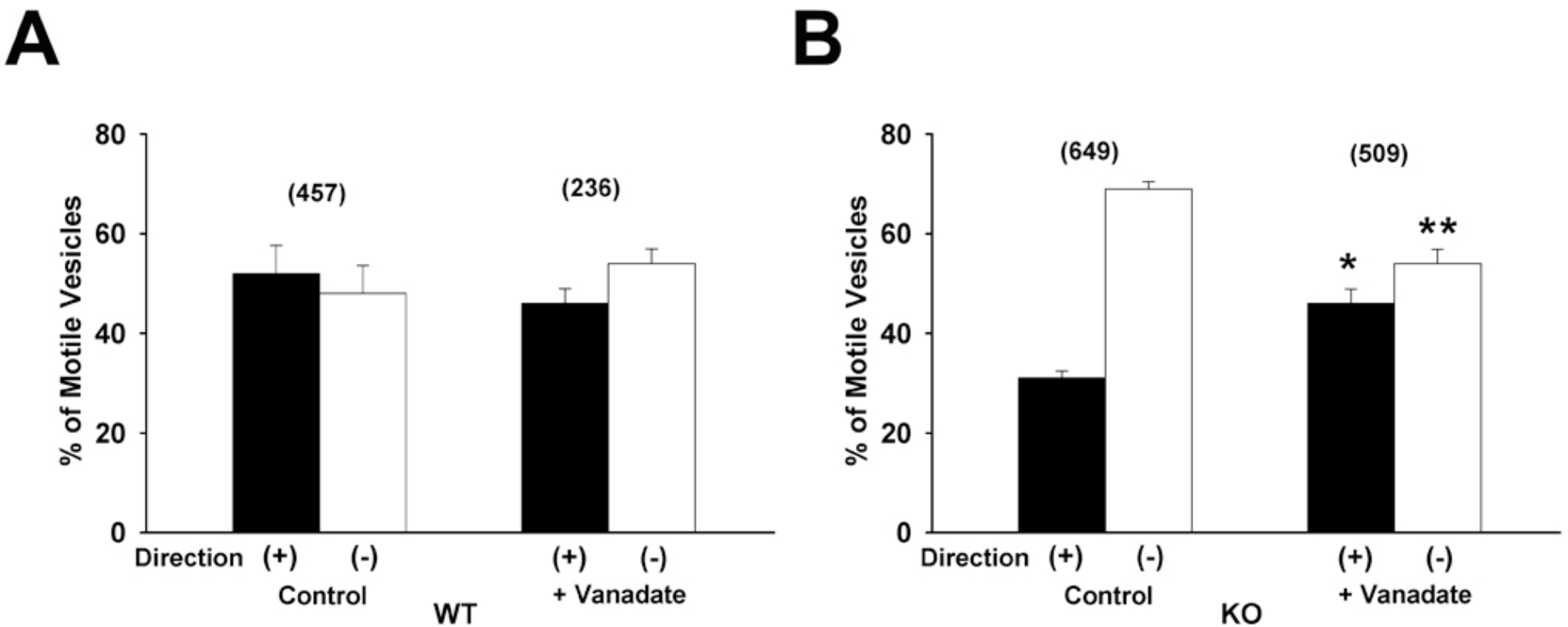

A
B
80
60
40
20
0
% of Motile Vesicles
(457)
(236)
(649)
(509)
*
**
Direction
(+)
(-)
(+)
(-)
Control
+ Vanadate
WT
KO

Figure 5

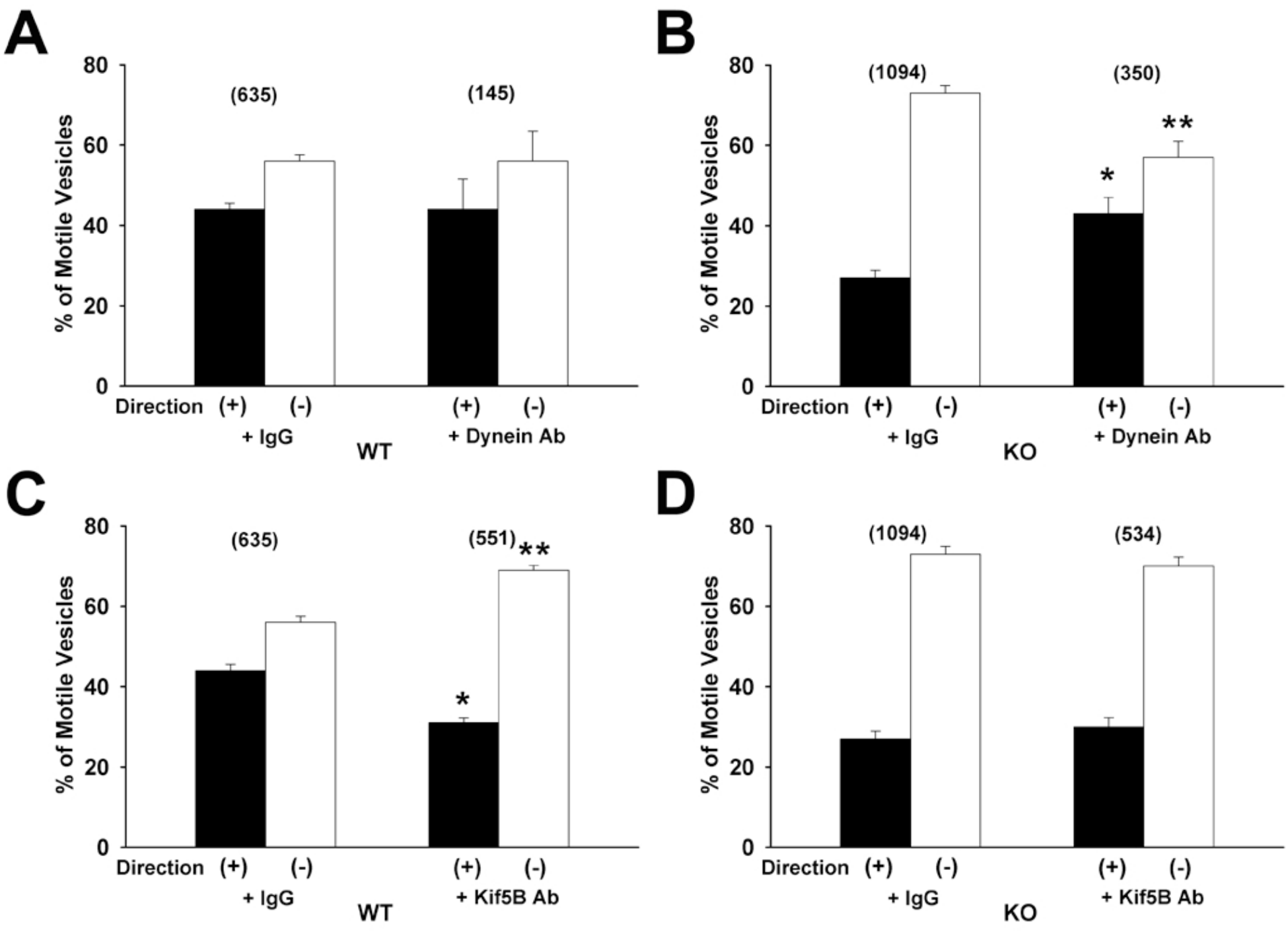

A
80
60
40
20
0
% of Motile Vesicles
(635)
(145)
Direction (+) (-) (+) (-)
+ IgG
+ Dynein Ab
WT
B
(1094)
(350)
*
**
Direction (+) (-) (+) (-)
+ IgG
+ Dynein Ab
KO
C
(635)
(551)
*
**
Direction (+) (-) (+) (-)
+ IgG
+ Kif5B Ab
WT
D
(1094)
(534)
Direction (+) (-) (+) (-)
+ IgG
+ Kif5B Ab
KO

Figure 6

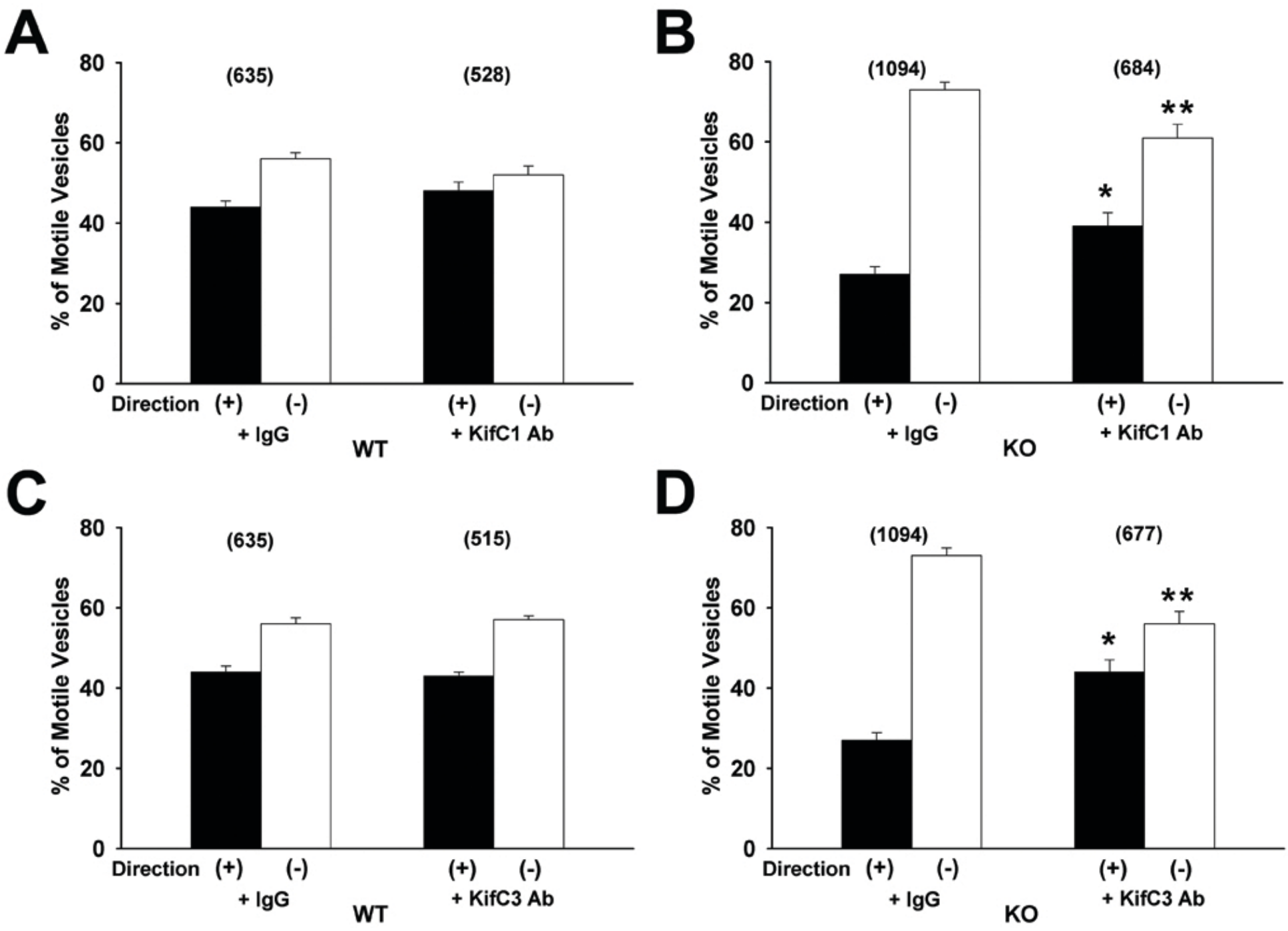
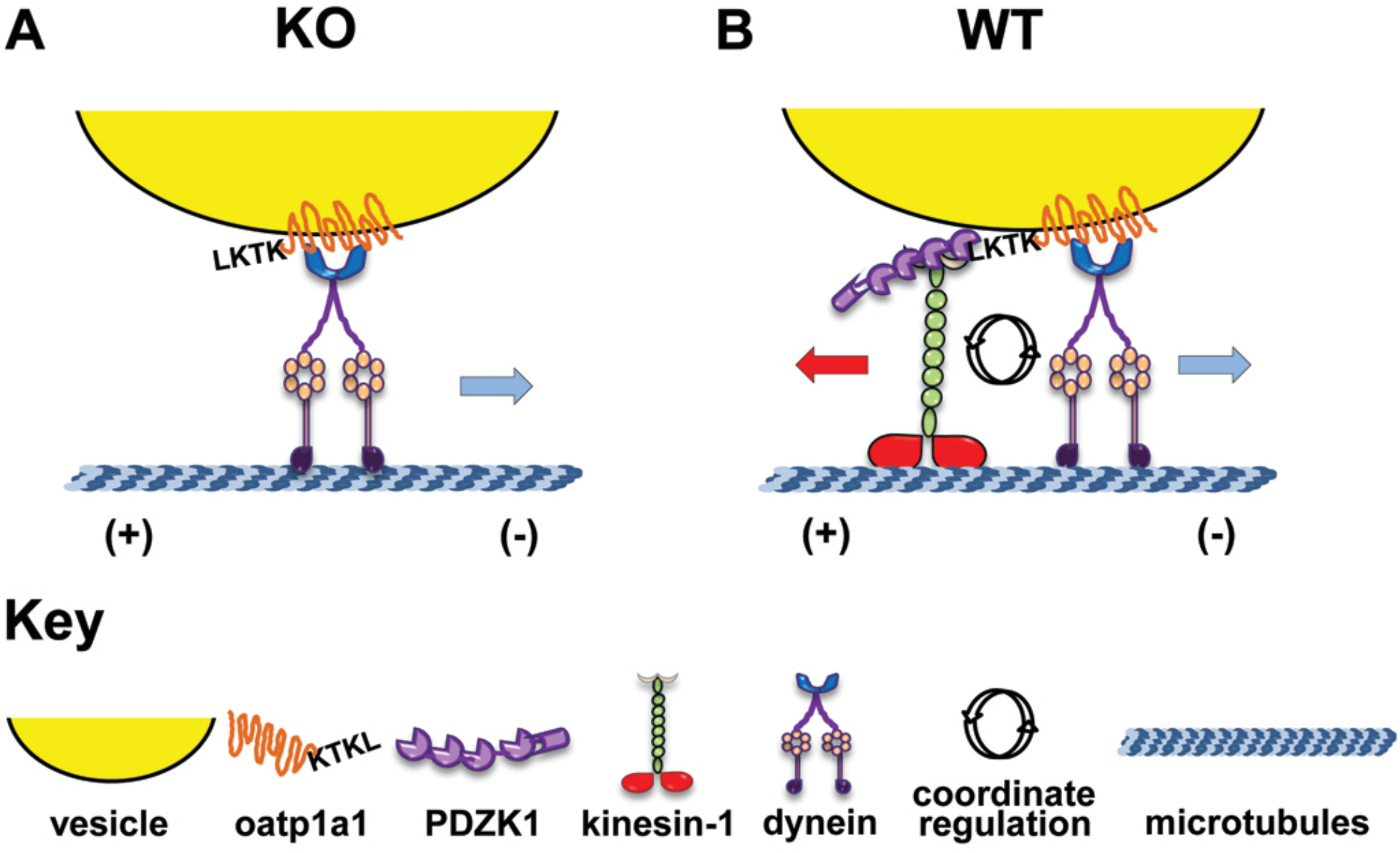

A
80
60
40
20
0
% of Motile Vesicles
(635)
(528)
Direction (+) (-) (+) (-)
+ IgG
+ KifC1 Ab
WT
B
80
60
40
20
0
% of Motile Vesicles
(1094)
(684)
*
**
Direction (+) (-) (+) (-)
+ IgG
+ KifC1 Ab
KO
C
80
60
40
20
0
% of Motile Vesicles
(635)
(515)
Direction (+) (-) (+) (-)
+ IgG
+ KifC3 Ab
WT
D
80
60
40
20
0
% of Motile Vesicles
(1094)
(677)
*
**
Direction (+) (-) (+) (-)
+ IgG
+ KifC3 Ab
KO

Figure 7


A
KO
LKTK
(+)
(-)
B
WT
LKTK
(+)
(-)
Key
KTKL
vesicle
oatp1a1
PDZK1
kinesin-1
dynein
coordinate regulation
microtubules