Sunday Driver/JIP3 binds kinesin heavy chain directly and enhances its motility

Faneng Sun1, Chuanmei Zhu2, Ram Dixit2 and Valeria Cavalli1,*

1Department of Anatomy and Neurobiology, Washington University in St Louis, School of Medicine, St Louis, MO, USA and 2Department of Biology, Washington University in St Louis, St Louis, MO, USA

Neuronal development, function and repair critically depend on axonal transport of vesicles and protein complexes, which is mediated in part by the molecular motor kinesin-1. Adapter proteins recruit kinesin-1 to vesicles via direct association with kinesin heavy chain (KHC), the force-generating component, or via the accessory light chain (KLC). Binding of adaptors to the motor is believed to engage the motor for microtubule-based transport. We report that the adaptor protein Sunday Driver (syd, also known as JIP3 or JSAP1) interacts directly with KHC, in addition to and independently of its known interaction with KLC. Using an in vitro motility assay, we show that syd activates KHC for transport and enhances its motility, increasing both KHC velocity and run length. Syd binding to KHC is functional in neurons, as syd mutants that bind KHC but not KLC are transported to axons and dendrites similarly to wild-type syd. This transport does not rely on syd oligomerization with itself or other JIP family members. These results establish syd as a positive regulator of kinesin activity and motility.

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Introduction

Neurons are extremely polarized cells with specialized domains including a long axon and multiple dendrites. Most proteins needed to function in axons and dendrites are synthesized in the cell body and transported to their final destination. This intracellular transport is essential for neuronal development, maintenance, and function. The kinesin family of motor proteins primarily drives the anterograde transport of cargoes towards distal areas of the neuron. Kinesin uses the energy of ATP hydrolysis to generate the motile force that moves cargo towards the plus-end of microtubules. The neuronal kinesin-1, known as conventional kinesin, drives the transport of a variety of molecules including protein complexes, vesicles, RNA granules, and cytoskeletal components (reviewed in Guzik and Goldstein, 2004; Goldstein et al, 2008 and Hirokawa et al, 2009).

Kinesin-1 conventionally refers to a tetramer of two heavy chains (KHC or KIF5), which harbour the motor domain, and two accessory light chains (KLC), which do not possess any motor activity (Verhey and Hammond, 2009). The association of cargoes to kinesin-1 is mediated by adaptor proteins that directly bind either KHC or KLC (Hirokawa et al, 2009). Over 20 adaptor proteins have now been identified. Adaptor proteins that interact with KHC directly to mediate the transport of diverse organelles and protein complexes include Milton (Glater et al, 2006), mRNP complex (Kanai et al, 2004), synaptobulin (Su et al, 2004; Cai et al, 2005), SNAP25 (Diefenbach et al, 2002), DISC1 (Taya et al, 2007), GRIP1 (Setou et al, 2002), Fez1/unc76 (Gindhart et al, 2003; Blasius et al, 2007), RanPB2 (Cho et al, 2007), mNUDC (Yamada et al, 2010), and bicaudal D1/D2 (Grigoriev et al, 2007). Adaptor proteins also interact with KLC to mediate transport and include the JIP family of proteins (Bowman et al, 2000; Verhey et al, 2001; Kelkar et al, 2005; Nguyen et al, 2005), APP (Kamal et al, 2000), kidins/ARMS (Bracale et al, 2007), alcadein (Araki et al, 2007), CRMP2 (Kimura et al, 2005), cytaxin (Aoyama et al, 2009), calyxtenin (Konecna et al, 2006), nesprins (Roux et al, 2009), and Hsc70 (Terada et al, 2010). The Fragile X mental retardation protein (FMRP) interacts with KLC to mediate the transport of mRNA granules in mouse neurons but does not require KLC in Drosophila S2 cells (Ling et al, 2004; Dictenberg et al, 2008). The growing number of identified KLC and KHC binding partners is thought to reflect the complexity of the molecular machinery controlling kinesin-1’s cargo selectivity.

The binding of adaptors to kinesin-1 is also believed to promote activation of the motor for microtubule-binding and motility. When not transporting cargo, kinesin-1 is thought to be inactive due to a folded conformation positioning the KHC tail domain near the enzymatically active motor domain, thereby preventing ATP hydrolysis (reviewed in Verhey and Hammond, 2009). The KHC tail has also been shown to contain an ATP-independent microtubule-binding domain, which was suggested to ‘park’ kinesin on microtubules when not transporting cargo (Dietrich et al, 2008; Seeger and Rice, 2010; Watanabe et al, 2010). Additionally, in the folded state, KLC is thought to contribute to the autoinhibition of kinesin-1 by pushing the KHC motor domains apart (Verhey et al, 1998; Cai et al, 2007). Binding to both KHC and KLC appears to be required to release the inhibition and to activate microtubule-dependent transport of kinesin-1 (Blasius et al, 2007; Verhey and Hammond, 2009). These findings, together with studies in KLC-deficient animals (Gindhart et al, 1998; Rahman et al, 1999), suggest that KLC may have a role in regulating kinesin-1 activity. However, several lines of evidence indicate that KLC is not essential for kinesin-1 activity and has a role in only some kinesin-1 transport events. Indeed, the tetrameric configuration is not obligatory and multiple lines of evidence suggest
that kinesin-1 exists and functions as a dimer of two heavy chains lacking the light chains: (i) KHC dimers can bind to membrane organelles in the absence of KLC (Skoufias et al., 1994); (ii) A small pool of KHC not associated with KLC has been found in various cultured cells and shown to possess motor properties of conventional tetrameric kinesin-1 (DeLuca et al., 2001; Gyoeva et al., 2004); (iii) In the retina, KLC does not fully colocalize with KHC and even appears to be absent from photoreceptors (Mavlyutov et al., 2002); and (iv) Cellular cargoes can be transported by direct interaction with KHC, without requiring KLC. For example, the adaptor protein syndapin was shown to interact directly with KHC but not with KLC to promote the transport of syntaxin-containing vesicles (Su et al., 2004) and mitochondria (Cai et al., 2005). In Dro sophila, the adaptor protein Milton attaches KHC to mitochondria in a KLC-independent manner (Cai et al., 2005; Glater et al., 2006) and mRNA transport is also KLC independent (Palacios and St Johnston, 2002; Loiseau et al., 2010).

In the absence of KLC, KHC activation relies on relieving the autoinhibition mediated by the KHC tail on the motor domain (Friedman and Vale, 1999; Coy et al., 1999a; Hackney and Stock, 2000, 2008; Cai et al., 2007; Cho et al., 2009; Wong et al., 2009). Recently, native cellular cargoes that are able to activate KHC directly have been identified. The Ran binding protein 2 (RanBP2) interacts with KHC (Cai et al., 2001; Cho et al., 2007) and activates the ATPase activity of KHC (Cho et al., 2009). The Drosophila Pat1 protein interacts with KHC and functions as a positive regulator of KHC motility for the transport of oskar mRNA and dynein in Drosophila oocytes (Loiseau et al., 2010).

To further understand KHC function within neurons, we examined the interaction of KHC with the adaptor protein Sunday Driver (syd). syd was identified in Drosophila in a genetic screen for axonal transport mutants and was shown to interact directly with KLC (Bowman et al., 2000). syd is a member of the JIP family of proteins, which interact with the c-Jun N-terminal kinase (JNK), and is thus also known as JIP3 (Kelkar et al., 2000) or JSAP1 (ito et al., 1999). The C. elegans homologue of syd/JIP3 (unc-16) also integrates JNK signalling and kinesin-1-dependent transport (Byrd et al., 2001; Sakamoto et al., 2005). All known JIP family members interact with KLC (Bowman et al., 2000; Verhey et al., 2001; Kelkar et al., 2005; Nguyen et al., 2005; Sakamoto et al., 2005). The interaction between syd and KLC relies on syd’s Leucine-Zipper (LZ) domain and on the KLC tetratricopeptide repeats (TPR domains) (Kelkar et al., 2005; Nguyen et al., 2005; Hammond et al., 2008). Given its interaction with kinesin, syd was proposed to mediate the axonal transport of at least one class of vesicles (Bowman et al., 2000). In C. elegans, syd/unc16 is involved in synaptic vesicle localization (Byrd et al., 2001; Sakamoto et al., 2005) and in synaptic membrane trafficking (Brown et al., 2009). Recently, we identified two different vesicle populations—endosomes and small antero-gradally moving organelles—as syd cargoes in mouse axons (Abe et al., 2009). Here, we show that syd interacts directly with the tail domain of KHC in addition to and independently of its interaction with KLC. Using an in vitro motility assay, we show that syd activates KHC for microtubule-based transport and promotes efficient motility of KHC along microtubules, increasing both processive run length and velocity. Importantly, syd binding to KHC is functional in neurons, as syd mutants that bind KHC but not KLC are transported to axons and dendrites similarly to wild-type syd. syd’s KHC-dependent transport does not rely on oligomerization with endogenous JIP family members. This work establishes syd as an adaptor for both kinesin-1 chains and as a positive regulator of kinesin-1 motility.

## Results

### syd interacts with KHC independently of KLC

To determine if syd’s interaction with kinesin-1 is entirely dependent on KLC, we performed GST-pulldown assays combined with syd deletion analyses. N-terminal syd (N-syd), but not C-terminal syd (C-syd) was able to pull down both KLC and the neuron-specific kinesin heavy chain KIF5C, hereafter referred to as KHC (Figure 1A and B). VAMP3, an endosomal protein that does not bind to kinesin-1, is used as a negative control. Deletion of the LZ domain (N-sydlAZ) resulted in loss of interaction with KLC1 and KLC2 (Figure 1C), consistent with previous observations (Verhey et al., 2001; Kelkar et al., 2005; Nguyen et al., 2005). However, N-sydlAZ retained the ability to interact with KHC (Figure 1C). Deletion of the JNK binding domain or coiled-coil domains 2 and 3 did not prevent syd’s interaction with either KLC or KHC (Figure 1C). Since KLC1 and KLC2 have almost identical TPR regions (Rahman et al., 1998) and interact with wild-type and mutant syd in a similar manner (Bowman et al., 2000), we focused our study on KLC1. To define the region interacting with KHC, we tested three N-syd fragments for their interaction with KHC and KLC, syd240–540, which contains the LZ domain, interacted with both KLC and KHC (Figure 1D). syd541–772, which lacks the LZ domain, did not interact with KHC or KLC (Figure 1D). However, syd3–239, which lacks the LZ domain, was not associated with KLC, as expected, but retained the ability to interact with KHC (Figure 1D), indicating that this syd fragment interacts with a KHC dimer lacking KLC.

To determine if a pool of KHC lacking the light chain exists in brain extract, we performed sucrose density centrifugation analysis. Similarly to what has been reported previously in cultured mammalian cells (DeLuca et al., 2001), we detected a population of KHC lacking the light chain in brain extracts (Figure 1E). The light chain was not degraded in these extracts and was detected in fractions of higher density along with a subset of KHC (Figure 1E). Quantification of KHC in the two distinct pools indicates that about 14% of KHC lacks the light chain. These results suggest that syd can associate with the pool of KHC that lacks KLC.

To determine the residues required for syd interaction with KHC, we generated a series of truncation mutants. This analysis revealed that aa50–80 was required to promote syd’s interaction with KHC (Figure 2A and B). To determine if aa50–80 (hereafter referred to as Kinesin-1 Heavy Chain Binding Domain, KBD) represents the minimal domain promoting syd–KHC interaction, we performed a deletion analysis. Deletion of the LZ domain abolished syd’s interaction with KLC but not with KHC (Figures 1C and 2C). Deletion of the KBD did not prevent syd’s interaction with tetrameric kinesin-1, consistent with the LZ domain interacting with KLC (Figure 2C). Since GFP–sydlAZ binds to KHC dimer and GFP–sydAKBD interacts with tetrameric kinesin-1 via KLC, deletion of both the KBD and LZ domains was required to...
syd interacts with KHC independently of KLC. (A) Schematic illustration of full-length or truncated syd constructs used. Domains of known function located within the amino-terminal portion of syd (N-syd) are indicated. cc: coiled-coil domains; JBD: JNK binding domain; LZ: leucine-zipper domain (KLC binding domain). (B) Mouse brain lysate was used in pulldown experiments using recombinant GST, GST N-syd, or GST C-syd. Western blot analysis was performed with the indicated antibodies. VAMP3 was used as a negative control. Both KHC and KLC are pulled down with GST N-syd, but not with GST C-syd. (C) As in (B), but the indicated N-syd deletion mutants were used. N-syd lacking the KLC binding site (N-sydΔLZ) interacts with KHC but not KLC. The asterisk points to non-specific reaction with molecular marker loaded in this lane. (D) As in (B), but the indicated syd fragments were used. The syd fragment aa3–239, which does not contain the LZ domain is sufficient to bind KHC. Densitometry analysis of ponceau staining for (B) quantifies the amount of the indicated GST fusion proteins pulled down by each N-syd fragment. Relative GST protein amount (% of input) is calculated by normalizing the signal to the amount of total recombinant GST. (E) Active microtubule-bound kinesin-1 was released by ATP. The soluble kinesin-1 fraction was collected and separated by sucrose density gradient. The fractions were collected and analysed by western blot on two separate gels. The black separating line marks where separate gels have been spliced together. The asterisk points to non-specific crossreacting bands. A population of KHC is detected in lighter fractions at the top of the gradient, which does not contain KLC.

syd’s direct interaction with KHC is functional

To determine whether syd’s interaction with KHC is functional, we analysed the dynamic properties of GFP-tagged syd by total internal reflection fluorescence microscopy (TIRF), as previously described (Blasius et al., 2007). COS cells

Figure 1 syd interacts with KHC independently of KLC. (A) Schematic illustration of full-length or truncated syd constructs used. Domains of known function located within the amino-terminal portion of syd (N-syd) are indicated. cc: coiled-coil domains; JBD: JNK binding domain; LZ: leucine-zipper domain (KLC binding domain). (B) Mouse brain lysate was used in pulldown experiments using recombinant GST, GST N-syd, or GST C-syd. Western blot analysis was performed with the indicated antibodies. VAMP3 was used as a negative control. Both KHC and KLC are pulled down with GST N-syd, but not with GST C-syd. (C) As in (B), but the indicated N-syd deletion mutants were used. N-syd lacking the KLC binding site (N-sydΔLZ) interacts with KHC but not KLC. The asterisk points to non-specific reaction with molecular marker loaded in this lane. (D) As in (B), but the indicated syd fragments were used. The syd fragment aa3–239, which does not contain the LZ domain is sufficient to bind KHC. Densitometry analysis of ponceau staining for (C) and (D) shows the relative amount of recombinant GST proteins used in each condition. Input for (B–D) is 1% of total starting material. (E) Active microtubule-bound kinesin-1 was released by ATP. The soluble kinesin-1 fraction was collected and separated by sucrose density gradient. The fractions were collected and analysed by western blot on two separate gels. The black separating line marks where separate gels have been spliced together. The asterisk points to non-specific crossreacting bands. A population of KHC is detected in lighter fractions at the top of the gradient, which does not contain KLC.

prevent syd’s interaction with kinesin-1 (Figure 2C), indicating that the KBD is necessary for binding to KHC.

The C-terminal tail domain of KHC (aa807–956) mediates its interaction with several cargoes (reviewed in Hirokawa et al., 2009). To test whether syd binds the KHC tail domain, we conducted a GST-pulldown assay using a GST-tagged KHC tail (aa807–956). syd interacted with the GST–KHC tail, but not with GST alone (Figure 2D). To test whether syd directly binds to the KHC tail domain, we performed an in vitro binding assay using GST-tagged KHC tail (aa807–956), and His-tagged syd fragments. All three syd fragments containing the KBD interacted directly with the GST–KHC tail (Figure 2E). To determine whether the affinity between syd and KHC is similar to what has been previously measured between syd and KLC (Bowman et al., 2000), we performed a binding assay. Similarly to what has been reported (Bowman et al., 2000), we measured a Kd of 0.33 ± 0.08 μM between syd and KLC1-TPR (Figure 2F). The syd–KHC tail interaction displayed a lower affinity, with a Kd of 1.83 ± 0.43 μM (Figure 2G). Given the lower affinity of syd for KHC tail compared with KLC-TPR, syd may bind to a kinesin-1 tetramer containing both KHC and KLC, and syd binding to KHC may relieve or alter its autoinhibitory activity. However, kinesin-1 does not always exist as a tetramer, suggesting that syd can bind directly to a population of KHC that lacks KLC. Together, these results reveal an alternative and KLC-independent interaction between syd and kinesin-1.
were chosen for their low amounts of endogenous kinesin-1 (Cai et al., 2007) and were used to express the desired GFP–syd constructs or Flag–KHC. Cell lysates containing either Flag–KHC or GFP–syd were mixed before the motility assay, thereby ensuring identical amounts of KHC in each condition (Figure 3A). When lysate expressing GFP–syd wild-type (wt) was mixed with Flag–KHC lysate, motile events were observed (Figure 3B–D; see Supplementary Movie S1). However, when non-transfected cell extract was used in place of the Flag–KHC lysate, the absence of Flag–KHC dramatically reduced the number of motile events (Figure 3C and D; see Supplementary Movie S2), indicating that the low level of endogenous kinesin-1 in COS cells does not have a significant role under these conditions.

Figure 2 Mapping the syd domain necessary for KHC interaction. (A) Scheme of the constructs used in (B), (C), and (E). (B) GST-pulldown analysis was conducted as described in Figure 1B. Residues 50–80 are required for syd interaction with KHC. VAMP3 is used as a negative control. (C) The indicated N-syd deletion mutants were used in a GST-pulldown assay and analysed by western blot with the indicated antibodies. Deletion of both LZ (KLC binding domain) and KBD (KHC binding domain) was required to prevent the syd–kinesin-1 interaction. (D) Recombinant GST–KHC tail (aa 807–956) was used in a GST-pulldown assay. The GST–KHC tail, but not GST, interacts with syd. Densitometry analysis of ponceau staining for (B–D) shows the relative amount of recombinant GST proteins used in each condition. Input is 10% of total starting material. (E) The indicated purified His-tagged syd fragments were incubated with purified GST or the GST–KHC tail. Direct binding of syd to the KHC tail domain was assessed using an anti-polyhistidine antibody. (F, G) Fixed amounts of recombinant GST N-syd (50 nM) were incubated with His–KLC1-TPR (F) or with His–KHC tail (G) at the indicated concentrations. The amount of bound His–KLC1-TPR or His–KHC tail was analysed by western blot with an anti-polyhistidine antibody and quantified using ImageJ. To estimate the dissociation constant, we plotted the band intensity for each concentration and used the first order binding equation: fraction of bound kinesin = [kinesin]/(Kd + [kinesin]), to fit the data generated.
The movement of GFP–syd observed in these conditions is thus mainly mediated by its interaction with Flag–KHC. We measured a velocity of 0.62 $\pm$ 0.02 $\mu$m/s for GFP–syd wt (Figure 3E), which is similar to the velocity reported for kinesin-1 under comparable conditions (Blasius et al., 2007).

The average run length we measured (3.21 $\pm$ 0.24 $\mu$m; Figure 3F) was greater than previously reported for kinesin-1 under comparable conditions (Blasius et al., 2007), but similar to purified recombinant kinesin-1 (Dixit et al., 2008).

We then tested whether GFP–syd3–239 binding to KHC was sufficient for motility. GFP–syd3–239 displayed motile events, albeit with reduced frequency compared with GFP–syd wt (Figure 3D; Supplementary Movie S3). Surprisingly, we observed that in addition to a reduced frequency of motile events, the velocity and run length decreased significantly for GFP–syd3–239, to 0.39 $\pm$ 0.03 $\mu$m/s and 2.11 $\pm$ 0.17 $\mu$m/run, respectively (Figure 3E and F). The reduced run length could be attributed to GFP–syd3–239 detaching from KHC sooner as
compared with GFP–syd wt. However, the reduced velocity observed for GFP–syd3–239 compared with GFP–syd wt suggests that other syd structural elements may participate in KHC regulation.

syd enhances KHC motility along microtubules
To directly determine whether syd regulates KHC motility, we next observed the movement of KHC–mCit in the presence or absence of Flag-tagged syd wt (Flag–syd wt; Figure 4A).

![Diagram of experimental procedures](image)

**Figure 4** syd regulates kinesin-1 activity and motility. (A) Scheme of the experimental procedures. COS-7 cells were transfected with the indicated Flag–syd constructs or fluorescently tagged KHC at the C-terminus (KHC–mCit). (B) Kymographs were generated from movies recorded for KHC–mCit in the presence or absence of Flag–syd wt or in the presence of Flag–sydΔKBD or Flag–syd3–239. Vertical bar = 20 s, horizontal bar = 5 μm. (C) The proportion of motile versus non-motile events observed for each condition is shown. The difference in the probability for being motile or non-motile in each condition was analysed with χ² analysis. (D, E) Velocity (D) and run length (E) were calculated from the kymographs generated. Velocity and run length values represent mean ± s.e.m. of n = 52 for KHC–mCit alone, n = 358 for KHC–mCit with Flag–syd wt, n = 76 for KHC–mCit with Flag–sydΔKBD, n = 191 for KHC–mCit with Flag–syd3–239. Data were collected from 3 to 4 independent experiments. Asterisks (*): compared with non-transfected condition (**P<0.01, ***P<0.001). Pound (#), compared with each other (###P<0.001). (F, G) Raw distribution data are shown for velocity (F) and run length (G).
When mixed with non-transfected extracts, KHC–mCit displayed few motile events (Figure 4B and C), probably reflecting activation by endogenous adaptors in COS cells. We, however, collected sufficient motile events to analyse KHC–mCit motile properties. We measured a velocity of 0.33 ± 0.03 μm/s and a run length of 1.94 ± 0.39 μm/run (Figure 4D and E; see Figure 4F and G for the raw distribution; and Supplementary Movie S4), which is comparable to what has been reported previously for full-length kinesin-1 motility in similar assays (Sung et al., 2008; Loiseau et al., 2010). In the presence of Flag–syd wt, we observed a dramatic increase in the number of motile events (Figure 4B and C). Remarkably, we also observed a significant increase in velocity (1.01 ± 0.03 μm/s) and run length (5.54 ± 0.28 μm/run) (Figure 4D and E; see Figure 4F and G for the raw distribution data and Supplementary Movie S5). The velocity and run length of Flag–KHC in the presence of GFP–syd wt (Figure 3E and F) were found to be lower than that for KHC–mCit in the presence of Flag–syd wt (Figure 4D and E). This difference may result from the position of the tag, rendering Flag–KHC not as efficient as KHC–mCit. However, importantly, in both cases the presence of syd wt increased KHC motility. This increase in motility is likely due to syd binding to the KHC tail, since Flag–sydΔKBD only minimally affected KHC motility (Figure 4D and E; see Figure 4F and G for the raw distribution data; Supplementary Movie S6). This velocity is comparable to what has been measured for purified tail-less kinesin-1 (Hackney et al., 2003; Yildiz et al., 2008; Shastry and Hancock, 2010), indicating that syd’s binding to the KHC tail is effective in relieving the inhibitory effect of the motor domain by the KHC tail. However, the enhanced run length in the presence of syd is significantly greater than the run length reported for purified tail-less kinesin-1 (Dixit et al., 2008; Yildiz et al., 2008; Shastry and Hancock, 2010). Similarly to what we observed with GFP–syd3–239–mCit motility (Figure 3E and F), we observed that Flag–syd3–239 was able to activate KHC motility, but with reduced velocity and run length compared with Flag–syd wt (Figure 4D and E; see Figure 4F and G for the raw distribution data; Supplementary Movie S7). Together, these results indicate that binding of syd to KHC activates kinesin-1 for microtubule-dependent motion, enhancing both KHC velocity and run length, and that syd structural elements beyond the KHC binding site likely participate in KHC regulation. It is also possible that a small proportion of the observed motile events is mediated by a KHC–KLC tetramer. Indeed, we found that a small fraction of expressed KHC–mCit in COS cells can interact with endogenous KLC (Supplementary Figure S1). The relatively small increase in KHC velocity provided by Flag–sydΔKBD (Figure 4D) may thus represent an effect of Flag–sydΔKBD on a kinesin-1 tetramer via its interaction with KLC.

**syd direct interaction with KHC promotes transport in neurons**

To determine whether syd binding to KHC is sufficient for transport in neurons, we examined the localization of GFP–syd wt and mutants in cultured hippocampal neurons. Previous evidence suggests that syd localizes predominantly to axons during development and mostly to the cell bodies and dendrites in adult brain (Akechi et al., 2001; Setou et al., 2002). In agreement with these previous observations, we found that in cultured embroyonic hippocampal neurons, endogenous syd is highly enriched at the tips of developing axons (Figure 5A). Similarly, expressed GFP–syd wt accumulated mostly at the tip of axons (Figure 5B, upper panel and 5E). GFP–sydΔLZ, which binds KHC, or GFP–sydΔKBD, which interacts with tetrameric kinesin-1 via KLC was transported equally to the axon tip (Figure 5B middle two panels and Figure 5E). GFP–syd wt, GFP–sydΔLZ, and GFP–sydΔKBD all displayed a similar low level distribution along dendrites (Figure 5C and E). In contrast, GFP–sydΔΔ failed to exit the cell body (Figure 5B and C bottom panel and Figure 5E) and was confined to the perinuclear region in proximity of the Golgi apparatus (Figure 5D). Lack of transport due to GFP–sydΔΔ misfolding is unlikely since GFP–sydΔΔ retains the ability to oligomerize with JIP family members in cells (Figure 6). Therefore, syd’s interaction with KHC does not appear to specify axonal versus dendritic transport. Together, these results suggest that syd’s interaction with KHC can mediate transport in neurons in the absence of the KLC binding site, as deletion of both KHC and KLC binding sites is required to prevent syd transport to axon tips.

**syd/JIP family members are known to homo- or hetero-oligomerize** (Yasuda et al., 1999; Kelkar et al., 2000, 2005; Kristensen et al., 2006; Hammond et al., 2008). To determine whether oligomerization with endogenous JIP family members bound to KLC mediates the transport of syd mutants, we first tested the ability of GFP-tagged syd mutants to oligomerize with Flag–syd wt. Flag–syd wt was co-transfected with GFP, GFP–syd3–239, GFP–syd wt, or GFP–sydΔΔ in N2A cells and immunoprecipitation was performed with anti-GFP antibodies. Flag–syd wt co-immunoprecipitated with GFP–syd3–239, GFP–sydΔΔ and GFP–syd wt but not with GFP alone, revealing that both GFP–syd3–239 and GFP–sydΔΔ can oligomerize, similarly to syd wt (Figure 6A). However, despite its ability to oligomerize with Flag–syd wt, GFP–sydΔΔ failed to exit the cell body (Figure 5C and D). Therefore, oligomerization of GFP–sydΔΔ with endogenous syd does not appear to be sufficient for its transport in neurons. We next examined the localization of mCherry–syd3–239 in cortical neurons prepared from mice lacking syd (Kelkar et al., 2003). Immunofluorescence of cultured cortical neurons (Figure 6B) and western blot analysis of brain lysates (Figure 6C) confirmed the lack of syd expression in syd-/- animals. We observed an enrichment of the syd mutants that bind KHC but not KLC, mCherry–syd3–239 and GFP–sydΔLZ, at the axonal tips of neurons lacking syd (Figure 6D–F). These results further exclude that oligomerization with endogenous syd mediates transport of syd mutants that bind KHC but not KLC.

syd can form hetero-oligomers with some JIP family members. In particular, syd can oligomerize with JIP2 and JIP1 (Kelkar et al., 2000; Hammond et al., 2008), but it does not oligomerize with JIP4 (Kelkar et al., 2005). While GFP–syd wt and GFP–sydΔΔ retained the ability to oligomerize with Flag–JIP2, GFP–syd3–239 failed to do so (Figure 6G). In contrast, all three constructs, GFP–syd wt, GFP–syd3–239 and GFP–sydΔΔ displayed the ability to oligomerize with myc–JIP1. Yet, despite its ability to oligomerize with myc–JIP1, GFP–sydΔΔ failed to exit the cell body (Figure 5C and D). This is in agreement with the results from Hammond et al. (2008), showing that the formation of a JIP1/syd/KLC complex is necessary for efficient JIP1 or syd transport in neurons.
neuronal cells. Thus, GFP–sydDD and GFP–syd3–239 may be unable to be stably incorporated in a complex with JIP1 and KLC. Oligomerization with JIP1 may thus not fully account for the transport of syd lacking the KLC binding domain (GFP–syd3–239, Figure 6E). Although we cannot exclude that other yet unknown kinesin-1 binding proteins may be involved in syd transport in neurons, these results, together with our motility experiment, suggest that syd’s interaction with KHC promotes transport in neurons.

**Discussion**

We report here that syd, a previously known KLC adaptor (Bowman et al., 2000), is also capable of interacting directly with KHC independently of KLC. Binding of syd to KHC not only activates kinesin-1 for microtubule-dependent transport, but also enhances KHC velocity and run length. Binding of syd to KHC is functional in neurons, since mutant syd that interacts with KHC only is targeted to axons and dendrites similarly to wt syd. Thus, syd–KHC interaction promotes transport but does not appear to determine transport specificity. Together, these data establish syd as a novel KHC binding partner capable of positively regulating kinesin-1 motility.

**Regulation of kinesin-1 activity**

Kinesin-1 is a processive motor, which takes multiple steps along microtubules before dissociating. How kinesin-1 activation for microtubule transport is controlled in live cells is not well understood, but recent studies couple kinesin-1 activation for microtubule transport with the binding of cellular partners (Blasius et al., 2007; Cho et al., 2009; Loiseau et al., 2010). A proposed regulatory mechanism for kinesin-1 activation is the transition from a ‘folded’ inactive state to an ‘open’ active state. In the inactive folded conformation, the KHC tail domain interacts with both the motor domain and the microtubules to prevent kinesin motion (Friedman and Vale, 1999; Hackney and Stock, 2000;
Cai et al., 2007; Dietrich et al., 2008; Watanabe et al., 2010). Activation of kinesin-1 for transport requires a conformational change in which motor and tail domains are separated and the motor domains come closer together (Cai et al., 2007). Our in vitro motility results indicate that syd, but not sydΔKBD, which lacks the KHC binding domain, increased the number of motile events (Figure 4), suggesting that syd binding to the KHC tail domain efficiently relieves the inhibition by the KHC tail domain, activating or opening KHC to bind microtubules for long-range motility. These results place syd alongside Pat1 (Loiseau et al., 2010) and RanBP2 (Cho et al., 2009) as cellular regulators of kinesin-1 activity.

In the case of tetrameric kinesin-1, it has been proposed that binding of both KLC and KHC is required for motor activation (Blasius et al., 2007). It will be interesting to determine in future studies whether syd may fulfill activation of a KHC/KLC complex via its ability to interact with both KHC and KLC. Alternatively, similar to the JIP1–Fez1

**Figure 6** KHC-dependent syd transport does not depend on oligomerization with endogenous JIP family members. (A) Cell lysates were prepared from N2A cells transfected with Flag–syd together with GFP, GFP–syd3–239, GFP–syd wt, or GFP–sydΔΔ. Immunoprecipitation was performed with GFP antibodies or rabbit IgG as a negative control; western blots were probed with Flag and GFP antibodies. GFP–syd3–239 and GFP–sydΔΔ retain the ability to homo-oligomerize. (B) E18 cortical neurons from syd+/+ or syd−/− pups were stained with syd and tau, confirming the lack of expression in syd−/− neurons. (C) E18 brain lysates from wt (syd+/+) or syd knockout (syd−/−) were analysed by western blot. β-Tubulin is used as a loading control. (D) The ratio of fluorescence intensities between axon tips and the somatic region was measured for endogenous syd (stained with anti-syd antibody in syd+/+ neurons) and for the indicated GFP-constructs (GFP intensity in syd−/− neurons). (E, F) syd−/− cortical neurons were transfected with mCherry–syd3–239 (E) or GFP–sydΔLZ (F) and stained with the axon marker tau. Both constructs were transported to the axon tip. Both constructs were transported to the axon tip. (G) Cell lysates were prepared from N2A cells transfected with Flag-tagged JIP2 together with GFP, GFP–syd3–239, GFP–syd wt, or GFP–sydΔΔ. Immunoprecipitation was performed with GFP antibodies or rabbit IgG as a negative control; western blots were probed with Flag and GFP antibodies. GFP–syd3–239 does not hetero-oligomerize with JIP2. (H) Cell lysates were prepared from N2A cells transfected with myc-tagged JIP1 together with Flag–syd wt, Flag–syd3–239, or Flag–sydΔΔ. Immunoprecipitation was performed with Flag antibodies or rabbit IgG as a negative control; western blots were probed with Flag and myc antibodies. All syd constructs oligomerize with JIP1. Arrows: axon tips; arrowheads: cell body. (B–E) Bar = 50 μm, except that bar = 10 μm for high magnification (high mag.).
complex (Blasius et al., 2007), syd may require additional interacting partners for the activation of tetrameric kinesin-1.

Regulation of kinesin-1 motility
Kinesin-1 stepping along microtubules is believed to involve concerted conformational change and diffusive movement of the tethered head to the next binding site. The precise mechanisms regulating the speed and the distance that kinesin-1 can achieve are fairly well understood for purified kinesin-1. Yet less is known about how kinesin-1 motility is regulated in a cellular environment. The observation that different kinesin-1 cargoes move at different rates in a cellular environment illustrates the complexity of in vivo motor regulation (Araki et al., 2007). The increase in KHC velocity and run length in the presence of full-length syd and syd3–239 suggests that syd binding to the KHC tail relieve the ‘brake’ provided by the tail binding to the microtubule (Dietrich et al., 2008; Seeger and Rice, 2010; Watanabe et al., 2010), thus allowing efficient forward movement. A recent study indicates that the *Drosophila* Pat1 protein interacts with KHC and functions as a positive regulator of KHC motility for the transport of *oskar* mRNA and dynein in *Drosophila* oocytes (Loiseau et al., 2010). In the absence of Pat1, both kinesin-1 velocity and run length are reduced (Loiseau et al., 2010). The Ran binding protein 2 (RanBP2) activates the ATPase activity of KHC (Cho et al., 2009), suggesting that RanBP2 may also regulate kinesin-1 velocity and processivity. These observations support the notion that adaptors contribute to regulate kinesin-1 motile properties, in addition to their roles in recruiting cargoes.

KLC-independent functions of KHC
Our results indicate that syd is capable of associating with both tetrameric and dimeric kinesin-1. This result is consistent with studies indicating that kinesin-1 exists and functions as a dimer of two heavy chains lacking the light chains, in addition to its conventional tetrameric conformation. KLC-independent transport has been reported for mitochondria (Cai et al., 2005; Glater et al., 2006), syntaxin-containing vesicles (Su et al., 2004), and RNA particles (Palacios and St Johnston, 2002; Kanai et al., 2004; Loiseau et al., 2010), in agreement with earlier studies reporting that KHC dimers can bind membrane organelles in the absence of KLC (Skoufias et al., 1994). Furthermore, a small pool of KHC not associated with KLC has been found in cultured HeLa cells and bovine brain (Hackney et al., 1991; DeLuca et al., 2001; Gyoeva et al., 2004) and we obtained similar results from mouse brain (Figure 1E). Degradation of KLC during kinesin-1 isolation can be excluded, since we detected KLC in our experiment (Figure 1E). Furthermore, significant molar excess of KHC over KLC has been reported in CV-1 cells (Gyoeva et al., 2004). It is thus conceivable that spatially and temporally, KHC and KLC do not always fully colocalize. Indeed, it has been shown that KLC is absent in photoreceptor cells and that throughout the retina, KLC does not fully colocalize with KHC (Mavlyutov et al., 2002), suggesting that at the cellular and subcellular levels KHC localization does not fully overlap with KLC. In addition, during brain development, KHC and KLC decline after the first week of postnatal life, but the decline in KLC appears to be more pronounced (Morfini et al., 2001). Therefore, it is possible that syd interacts with tetrameric kinesin-1 via KLC, while in cells or subcellular regions where KLC is absent syd instead interacts and regulates the activity of a kinesin-1 dimer. Our observation that syd interacts with KHC in addition to and independently from its known interaction with KLC is not inconsistent with the data published so far. In *C. elegans*, the localization of UNC-16 (syd) depends on both UNC-116 (KHC) and KLC (Sakamoto et al., 2005). Furthermore, syd transport to neurite tips in differentiated CAD cells is mostly, but not completely dependent on KLC (Verhey et al., 2001; Hammond et al., 2008). Similarly to syd, the FMRP was found to bind KLC (Dictenberg et al., 2008), and also KHC in a KLC-independent manner (Ling et al., 2004).

Syd role in axonal transport
Kinesin-1 drives different sets of cargoes to axons or dendrites, but how kinesin-1 distinguishes between axonal or dendritic cargoes for directional sorting remains poorly understood. Previous studies suggested that there was a dendritic preference for KHC cargoes and an axonal preference for KLC linkage (Setou et al., 2002; Hirokawa and Takemura, 2005). Our results showed that regardless of its mode of association with kinesin-1, syd is predominantly targeted to axon tips (Figure 5). It is thus more likely that the uploaded cargoes, and not the adaptor–motor complexes, determine the destination of kinesin-1 and its adaptor. We have recently shown that syd mediates the transport of at least two distinct types of vesicles in axons: endosomes and small anterogradely moving vesicles (Abe et al., 2009). It will be interesting to define in future studies the nature of the syd cargoes transported by KLC-dependent or KLC-independent interaction. Indeed, KLC isoforms have been proposed to mediate targeting of KHC to proper cargo (Stenoien and Brady, 1997; Khodjakov et al., 1998; Gyoeva et al., 2000; Wozniak and Allan, 2006), but KHC can also associate with membranous cargo in the absence of KLC (Skoufias et al., 1994).

The propensity of JIP family members to form homo or hetero-oligomers (Yasuda et al., 1999; Kelkar et al., 2000, 2005; Kristensen et al., 2006; Hammond et al., 2008; Koushika, 2008) suggests that syd may be transported via oligomerization. Indeed, Hammond et al. (2008) reported that syd and JIP1 require each other for efficient transport of JIP1 or syd in non-neuronal cells. Such cooperative transport is due to an interaction between JIP1 and syd as well as distinct binding sites on the KLC-TPR domain. We found that despite its ability to oligomerize with myc–JIP1, GFP–sydΔΔ failed to exit the cell body (Figure 5C and D). Thus, GFP–sydΔΔ may be unable to be stably incorporated in a complex with JIP1 and KLC. Oligomerization with JIP1 may thus not fully account for the transport of the syd mutant GFP–sydΔ–239 lacking the KLC binding domain. Although we cannot exclude that other yet unknown kinesin-1 binding proteins may be involved in syd transport in neurons, our results suggest that syd’s interaction with KHC may promote transport in neurons and that oligomerization may provide additional layers of regulation of syd-dependent transport.

In summary, we identified syd as an adaptor for kinesin-1 heavy chain and we determined that syd enhances KHC motility along microtubules. Future studies are needed to determine the precise mechanisms by which syd regulates kinesin-1 activation and processivity, and examine whether the distinct modes of interaction with kinesin-1 provide...
specificity for cargo selection and delivery to particular subcellular destinations.

Materials and methods

Antibodies and reagents

syd antibody was previously described (Bowman et al., 2000). The antibodies are as follows: anti-GFP (Invitrogen), anti-VAMP3 (Synaptic System), anti-kinesin heavy chain (KIF5C; Xi et al., 2003), anti-KLC1 (Santa Cruz), anti-KLC1–KLC2 (63–90) (gift from Dr Scott Brady), anti-His6 (Qiagen), anti-Flag (Sigma), anti-β-tubulin (Sigma), anti-myrc (Cell Signaling), anti-β-actin (Chemicon), anti-giantin (Abcam), anti-MAP2 (Chemicon), and anti-tau (Millipore). syd knockdown mice were previously described (Keikar et al., 2003). For wild-type animals, C57Bl6 mice were used.

Plasmid construction

KHC–mCit and myc–JIP1 were obtained from Dr Kristen Verhey and were previously described (Cai et al., 2007; Hammond et al., 2008). Full-length mouse syd cDNA was purchased from Open Biosystems (Huntsville, AL). Constructs for GST fusion proteins (N-terminal syd (aa 3–722), C-terminal syd (aa 773–1337), syd3–239, syd240–540, syd541–772, syd3–80, syd3–160, syd50–239, syd101–239, syd150–239, 203–239, syd429–459, syd50–82, KIF5C 807–956) were generated by inserting their coding sequence in-frame downstream of sequences encoding a Flag tag in the pcDNA3 vector. A PCR-generated by inserting their coding sequence in-frame downstream of sequences encoding a Flag tag in the pcDNA3 vector. To generate polyhistidine-tagged KLC1, KLC1–TPR (KLC1 177–413) and Flag-tagged proteins were purified using PrepEase His-tag resin (USB Corporation, Cleveland, OH). The clear lysates were then mixed with Protein A Magnetic Beads (Invitrogen) were coated with anti-GFP antibody or rabbit IgG as a negative control, or with anti-myc with mouse IgG as a negative control. N2A cells were lysed in ice-cold lysis buffer 24 h after transfection and the lysate was incubated with coated magnetic beads for 2 h at 4°C on a rotating wheel. Beads were then washed three times with ice-cold PBS. The bound material was eluted by boiling beads in sample buffer for 5 min and analysed by western blot.

Protein binding assay. GST-tagged proteins were incubated with Glutathione Sepharose 4B beads for 30 min at room temperature, washed with PBS, and incubated with His6-tagged proteins at 4°C on a rotating wheel for 30 min. Beads were washed three times with ice-cold PBS and bound material was eluted by boiling beads in sample buffer for 5 min and analysed by western blot. This assay was used to determine the dissociation constant between syd and KLC1–TPR and between syd and KHC tail, as previously described (Bowman et al., 2000). We used a fixed concentration of recombinant GST–syd N-terminal (50 nM) and various concentrations of the ligands His–KLC-TPR or His–KHC tail over a 3000-fold range. The relative amount of bound KLC1–TPR or KHC tail was analysed by western blot and quantified using ImageJ. The dissociation constants were measured by plotting the band intensity of bound kinesin for each concentration and using the first order binding equation: fraction of bound = [kinesin]/(Kd + [kinesin]) to fit the data.

Sucrose density centrifugation

Mouse brains were homogenized in PIPES buffer (50 mM PIPES, 1 mM EGTA, 1 mM MgSO₄, and protease inhibitors, pH 6.9). The homogenate was then diluted at 100,000 g for 30 min at 4°C to obtain a clear brain lysate. Glycerol (20%), and GTP (1 mM), taxol (20 μM) and AMP-PNP (2.5 mM) were added to brain lysates and incubated for 30 min at 37°C. The lysates were centrifuged at 100,000 g for 30 min at 22°C to pellet down microtubules and the associated kinesin motor. The resulting pellet was rinsed once with PIPES buffer with AMP-PNP (0.1 mM) and Taxol (20 μM). To release the motor from microtubules, the microtubule pellets were resuspended in ATP-containing buffer (100 mM PIPES, 1 mM EGTA, 1 mM MgSO₄, and ATP 20 mM, pH 6.9), and incubated for 1 h on ice. The re-suspension was then centrifuged from above to collect the supernatant. The supernatant containing released motor proteins was loaded onto a sucrose gradient (5–50% in PIPES buffer) and centrifuged with a SW41 swinging bucket rotor for 14 h at 200,000 g at 4°C. Fractions were collected for western blot analysis with KHC and KLC antibodies.

In vitro motility assay

Twenty-four hours after transfection with the indicated constructs, C6 cells were lysed N2A buffer with the addition of 1 mM EGTA and 0.1% Triton X-100. The supernatant was collected at 16,000 g for 30 min at 4°C. The clear lysates were then mixed with Glutathione Sepharose 4B beads (GE Healthcare) for 30 min at room temperature. The beads were washed with PBS three times, and GST fusion proteins were eluted with 20 μM reduced glutathione in Tis buffer (50 mM, pH 8.0). His6-tagged proteins were purified using PrepEase His-tag resin (USB Corporation, Cleveland, OH). Briefly, cells were resuspended in LEW buffer (NaH₂PO₄, 50 mM, NaCl 300 mM, AEBSF 1.0 mM, pH 8.0), sonicated and incubated with Triton X-100 (0.2%) for 40 min at 4°C. The lysates were clarified by centrifugation at 16,000 g for 30 min at 4°C and then mixed with resin at room temperature for 30 min. After the washings with LEW buffer, the bound His6-tagged proteins were eluted with 250 mM imidazole in LEW buffer. The eluted recombinant proteins were dialysed in PBS or ultrafiltered for buffer exchange before their application.

GST pulldown and immunoprecipitation

GST pulldown. Mouse brains were homogenized in ice-cold lysis buffer (Tris–HCl 20 mM, NaCl, 200 mM, EDTA 1 mM, 0.5% Nonidet P-40, pH 8.0), and microtubule homogenate were overlaid on long distance, images were captured with a back-thinned electron multiplier-CCD camera (ImageEM, Hamamatsu) at 1 s intervals. Non-transfected cell lysates were used for control experiments in parallel on the same day. The motion of GFP–syd or
KHc–mCit was analysed using kymographs generated with Slidebook (Intelligent Imaging Innovations). Vertical lines in kymographs were defined as non-motile events, while diagonal lines were defined as motile events. Only lines spanning more than two frames were counted as events. For non-motile events, only vertical lines with a fluorescence intensity similar to motile, diagonal lines were counted, to avoid inclusion of aggregates bound to microtubules.

**Cell culture, transfection, and image analysis**

N2A and COS-7 cells were grown in DMEM medium supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamate, 0.1 mM non-essential amino acids and 1% Pen/Strep. Cells were grown on T25 flasks overnight before transfection using Lipofectamine 2000 (Invitrogen). Primary hippocampal and cortical cultures were prepared from mouse embryos at embryonic day 18 (E18). Hippocampal or cortical were treated with papain and DNase for 30 min, and triturated in neurobasal medium with 0.1% FBS. Dissociated neurons were cultured on coverslips coated with poly-d-lysine in Neurobasal medium containing 2% B27, 0.5 mM L-Glutamax and 1% Pen/Strep. About 5–6 × 10^6 neurons were plated per well in 24-well plates. Amaxa Nucleofection® was used to transfect neurons before plating. After 5–6 days in culture, neurons were fixed with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamate, 0.1 mM sucrose for 10 min. Neurons were permeabilized and blocked with 10% goat serum, 0.1% Triton X-100 in PBS and incubated with the indicated primary antibodies and Alexa Fluor-conjugated secondary antibodies. Nuclei were stained by DAPI in the Prolong anti-fade mounting medium (Invitrogen). The images were acquired with fluorescence microscopy (Nikon, Eclipse TE 2000-E) and analysed with Nis-Element software.

**Statistical analysis**

Statistical analyses were performed using Student's t-test, except that χ2 analysis was used to determine the statistical differences in motile frequency. For the motility experiments, the Kolmogorov–Smirnov test was used to test for normality of distribution. If the distribution was not normal, then statistical analysis was performed with the Kolmogorov–Smirnov test.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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**Author contributions**

FS and VC designed the experiments. FS performed all biochemical and cellular experiments and data analysis. FS and CZ performed and analysed the in vitro motility assay. RD guided and supervised the in vitro motility experiments. FS, RD, and VC wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

**References**


required for axonal transport in the Drosophila nervous system. 

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