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Keeping Neuronal Cargoes on the Right Track: New Insights into Regulators of Axonal Transport

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Abstract
In neurons, a single motor (dynein) transports large organelles as well as synaptic and dense core vesicles toward microtubule minus ends; however, it is unclear why dynein appears more active on organelles, which are generally excluded from mature axons, than on synaptic and dense core vesicles, which are maintained at high levels. Recent studies in Zebrafish and *C. elegans* have shown that JIP3 promotes dynein-mediated retrograde transport to clear some organelles (lysosomes, early endosomes, and Golgi) from axons and prevent their potentially harmful accumulation in presynaptic regions. A JIP3 mutant suppressor screen in *C. elegans* revealed that JIP3 promotes the clearance of organelles from axons by blocking the action of the CSS system (Cdk5, SAD Kinase, SYD-2/ Liprin). A synthesis of results in vertebrates with the new findings suggests that JIP3 blocks the CSS system from disrupting the connection between dynein and organelles. Most components of the CSS system are enriched at presynaptic active zones where they normally contribute to maintaining optimal levels of captured synaptic and dense core vesicles, in part by inhibiting dynein transport. The JIP3-CSS system model explains how neurons selectively regulate a single minus-end motor to exclude specific classes of organelles from axons, while at the same time ensuring optimal levels of synaptic and dense core vesicles.

Keywords
***C. elegans*, Zebrafish, axonal transport, active zone, dynein, JIP3 (JSAP1), SYD-2 (Liprin-α), Cdk5, SAD Kinase, BRSK1**

Introduction
The unique architecture and functions of neurons demand sophisticated regulation of their organelle transport system. For example, synaptic vesicle precursors and dense core vesicles (DCVs) must travel long distances into axons where they accumulate, while cell soma organelles, such as Golgi, lysosomes, and some classes of endosomes, are selectively excluded from the presynaptic region of mature axons in adult animals in both invertebrates (Edwards and others 2013) and mammals (Block and others 2015). However, under special conditions of growth or repair, or in different kinds of neurons, there may be a need for cell soma organelles in axons, so the organelle transport system must include both positive and negative regulators.

The association of mutations in the axonal transport machinery with neurodegenerative disorders in mice and humans underscores the importance of a properly functioning transport system for the long-term viability of neurons (De Vos and others 2008; Maday and others 2014; Millecamps and Julien 2013; Schiavo and others 2013). A recent study highlighted the importance of the axonal localization of early/ recycling endosomes for axon outgrowth (van Bergeijk and others 2015). The regulation of axonal retrograde transport (movement from the axon to the cell soma) is also especially important for axon-soma communication after axon injury and thus has relevance for axon regeneration (Abe and Cavalli 2008; Rishal and Fainzilber 2014). Now, with the

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recent discoveries highlighted in this review, there is new potential to understand how neurons regulate the transport of endosomal organelles, as well as synaptic and dense core vesicles, to develop and maintain a functional axon.

The neuron's axonal transport system consists of microtubule tracks and motor proteins that carry cargos along the tracks, as well as proteins that regulate the system. Microtubules have an intrinsic plus- and minus-end polarity, and axonal microtubules are oriented with their plus-ends pointing outward toward the synaptic region (Baas and Lin 2011; Burton and Paige 1981; Heidemann and others 1981). Plus-end directed motors from the large family of kinesins carry synaptic vesicles (SVs) and organelles outward, while the minus-end directed motor dynein moves them in the opposite direction (Hirokawa and others 2010; Hirokawa and others 2009; Holzbaur 2004; Vale 2003). The transport of SVs, DCVs, and at least some organelles is bidirectional (Edwards and others 2013; Hendricks and others 2010; Hoover and others 2014; Kumar and others 2010; Ou and others 2010; Wong and others 2012), meaning that organelles reverse direction repeatedly *en route* to their final destination. This suggests that regulated directional biases, as well as capture mechanisms (Bulgari and others 2014; Shakiryanova and others 2006; Wong and others 2012), determine their steady state distributions.

Despite the importance of motor regulation in determining the organelle composition of axons and dendrites, little is known about how mature neurons regulate organelle transport *in vivo*. One exception is mitochondrial transport, for which the relevant motors and adaptors have been identified, and a mechanistic understanding of regulatory mechanisms is emerging (See Box 1 after references). In addition, recent studies have made important progress in understanding the roles of microtubule plus-end binding proteins (CLIP-170, p150^{Glued}, and EB1/EB3) and tubulin tyrosination in the initiation of dynein-mediated organelle transport at nerve process terminals in cultured mouse dorsal root ganglion (DRG) neurons (Lazarus and others 2013; Moughamian and Holzbaur 2012; Moughamian and others 2013; Nirschl and others 2016) and *Drosophila* motor neurons (Lloyd and others 2012).

Recent genetic studies from *C. elegans* and Zebrafish have provided another entry point for understanding how neurons regulate the transport of organelles such as early endosomes, lysosomes, and Golgi stacks. Mutants lacking the conserved protein JIP3 accumulate lysosomes, early endosomes, and Golgi in their axons (Brown and others 2009; Drerup and Nechiporuk 2013; Edwards and others 2013). Thus, the normal function of JIP3, in this context, is to clear organelles from axons. A recent *C. elegans* study used a JIP3 genetic suppressor screen as a starting point to discover a previously unknown organelle transport regulatory system, the CSS system (Cdk5, SAD-A Kinase, SYD-2/ Liprin-α) (Edwards and others 2015a). Most components of the CSS system are enriched at presynaptic active zones, where they normally contribute to maintaining optimal levels of captured synaptic and dense core vesicles, in part by inhibiting dynein (Edwards and others 2015b; Goodwin and Juo 2013; Goodwin and others 2012; Ou and others 2010; Wu and others 2013). Although this is a good thing, the CSS system can also inhibit the clearance of potentially harmful organelles from the presynaptic region unless those organelles are protected from the CSS system by JIP3 (Edwards and others 2015a). JIP3 thus acts on specific classes of organelles to block the dynein-inhibiting action of the CSS system and ensures that those organelles are transported back to the cell soma by dynein.

Possibly tempering the enthusiasm for these new findings is the problem of reconciling them with other studies showing that JIP3 can function as an adaptor that stimulates Kinesin-1 transport of specific cargos into axons (Huang and others 2011; Sun and others 2011; Watt and others 2015). These two functions of JIP3 seem completely opposite. In one function, the "adaptor" function, JIP3 directly interacts with Kinesin-1 and Kinesin-1 cargos to promote the *plus-end* movement of those cargos *into* axons (Figure 1A, B). In its other function, the "organelle clearance" function, JIP3 blocks the action of the CSS system, which promotes the *minus-end* movement of cargos *out of* axons (Figure 1C, D). This review begins by highlighting the evidence for both JIP3 functions and proposing an appealing explanation based on a synthesis of past and new results. The review then shows how genetic, biochemical, and live animal quantitative imaging results are pointing to a new JIP3-CSS system model for organelle clearance and the maintenance of optimal SV and DCV levels at synapses.

The JIP3 and JIP1 regulators of axonal transport

JIP3 can function as a "scaffold" protein that brings together components of a JNK signaling cascade (a subgroup of MAP Kinases) (Ito and others 1999; Kelkar and others 2000). However, JIP3's ability to bind JNK components does not appear to be critical for its transport-related functions *in vivo* (Edwards and others, 2013; Drerup and Nechiporuk, 2013). In mammals, JIP3 is also known as JSAP1 (Ito and others 1999). Vertebrates have a second, closely related JIP3 gene variously known as JIP4, JLP, or SPAG 9

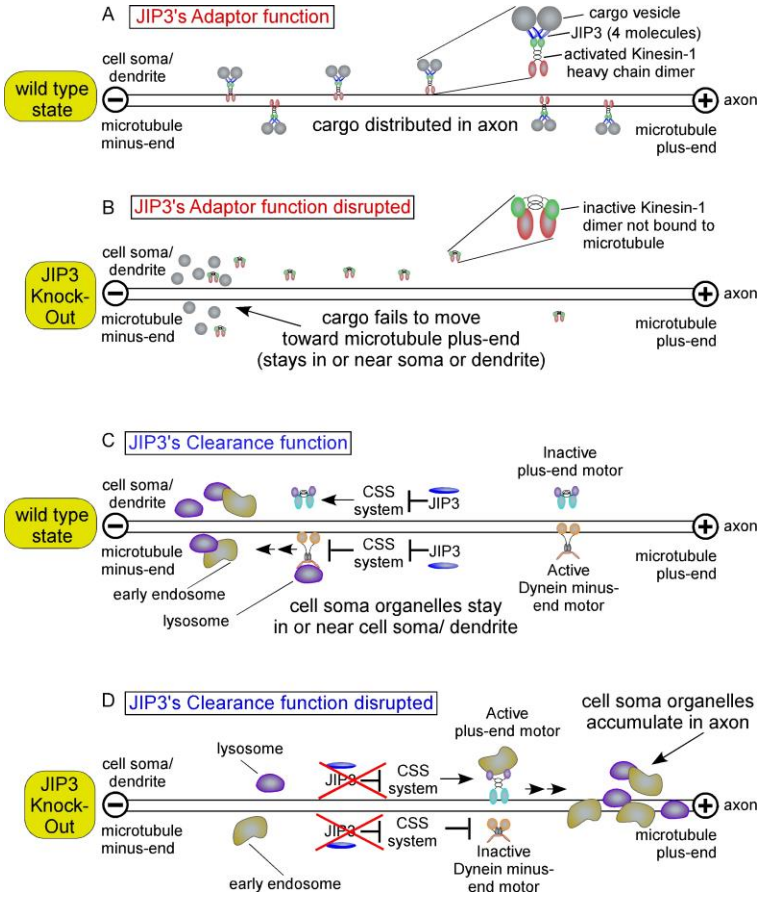


Figure 1. JIP3's Adaptor and Clearance Functions.

(A) and **(B)** In JIP3's Adaptor function, JIP3 directly interacts with the tail regions of the Kinesin-1 dimer, relieving autoinhibition, and promotes binding of Kinesin-1 to microtubules followed by processive plus-end directed movement. JIP3 connects activated Kinesin-1 to TrkB or other unidentified cargos that regulate axon elongation and, in some cases, axon branching. When this function is disrupted the cargos fail to move into the axon **(B)**. Based on the biochemical studies of (Sun and others 2011) and (Watt and others 2015) and the genetic studies in Table 1.

(C) and **(D)** In JIP3's Clearance function, JIP3 inhibits the CSS system (vertical bars indicate inhibition), which in turn prevents the CSS system from performing its normal function of activating a plus-end motor (that is not Kinesin-1) and/ or inhibiting the Dynein minus-end motor. When this function is disrupted, cell soma organelles, including early endosomes, Golgi fragments, and lysosomes, accumulate in axons due to plus-end dominated movements **(D)**. Based on the genetic studies in Table 2.

(Jagadish and others 2005; Kelkar and others 2005; Lee and others 2002). Invertebrates have one JIP3 ortholog known as Sunday Driver (*Drosophila*) or UNC-16 (*C. elegans*). For simplicity, this review refers to any JIP3 ortholog as JIP3, and uses the name JIP4 for the second related gene in vertebrates.

Two other proteins, known as JIP1 and JIP2, share an unfortunate common naming scheme with JIP3 based on their isolation as JNK interacting proteins in yeast two hybrid screens (Dickens and others 1997; Kelkar and others 2000). JIP1 has been shown to regulate the axonal transport of several cargoes, including APP (Amyloid Precursor Protein) vesicles, autophagosomes, and Rab10-postive vesicles (Deng and others 2014; Fu and Holzbaur 2013; Fu and Holzbaur 2014b). JIP1 can function as an adaptor that is capable of binding complexes containing either Kinesin-1 or Dynein and connecting those motors with their cargoes (Fu and Holzbaur 2013; Fu and others 2014). The motor complex with which JIP1 interacts can be regulated by phosphorylation of JIP1 at a specific site (Fu and Holzbaur 2013). Thus, JIP1 has been described as a bidirectional scaffolding protein or bidirectional adaptor (Fu and Holzbaur 2014a). A single JIP1/ JIP2 ortholog is conserved in lower animals such as *C. elegans* and *Drosophila.* JIP1 and JIP2 are related to each other, but both are completely unrelated to JIP3, and there is no sequence homology, even in the domains of each protein that interact with JNK and molecular motors. This suggests that JIP1/JIP2 and JIP3 have distinct functions in organelle transport. The remainder of this review focuses on functions related to JIP3.

JIP3's Kinesin-1 Adaptor function

JIP3 was first proposed to be an adaptor when a biochemical study of *Drosophila* Sunday Driver (JIP3) showed that it directly interacts with Kinesin-1 light chain, and a genetic analysis of Sunday Driver mutants found altered organelle transport (Bowman and others 2000). However, in this case, the organelle transport was not altered in the manner predicted for loss of Kinesin-1 - mediated transport. Instead of having fewer organelles in axons, as would be predicted for loss of an adaptor/ plus-end motor interaction (Figure 1A, B), the fly Sunday Driver mutants had more organelles in axons (Bowman and others 2000). Thus, despite being the first study to propose an adaptor function for JIP3, this does not fit the definition of a Kinesin-1 adaptor function, in which less, not more, of the Kinesin-1 cargo should accumulate in axons when the adaptor is disrupted (e.g., as is the case with mitochondria; See Box 1 after references).

Still, several independent studies have verified the JIP3-Kinesin-1 light chain interaction in both vertebrates and invertebrates (Nguyen and others 2005; Sakamoto and others 2005; Verhey and others 2001). Moreover, recent strong biochemical studies

Table 1. Genetic studies of JIP3's Adaptor function

Study	Organism	Cell type	Cargo	Organelle or compartment	Adaptor phenotype caused by loss of JIP3 function
(Huang and others 2011)	rat	PC12 cells and primary cell cultured hippocampal neurons	Various tagged versions of TrkB receptors that bind Brain-Derived Neurotrophic Factor (BDNF)	unidentified	less TrkB in neurite tips of PC12 cells; less anterograde movement in hippocampal neuron axons
(Drerup and Nechiporuk 2013)	Zebrafish	pLL sensory neuron axons in intact animals	native TrkB receptors	unidentified	decreased levels of TrkB in axon terminals
(Sato and others 2015)	Mouse	primary cultured hippocampal neurons (Cre-mediated double knockouts of JIP3 and JIP4)	Mito-GFP	mitochondria[1]	decreased anterograde (plus-end-directed) movement[2]
(Sato and others 2015)	Mouse	primary cultured hippocampal neurons (Cre-mediated double knockouts of JIP3 and JIP4)	APP-GFP (Amyloid Precursor protein)	unidentified	decreased anterograde (plus-end-directed) movement[2]
(Sun and others 2013)	Rat	siRNA and overexpression studies using primary cultured hippocampal neurons and *in utero* electroporation of cortical progenitor cells	unidentified; suggests JIP3 itself is the only relevant Kinesin-1 cargo for axon elongation in these neurons	unidentified or not applicable	decreased axonal elongation, dependent on Kinesin-1 and JNK activated locally at the axon tip
(Watt and others 2015)	Rat and mouse	cultured hippocampal neurons from JIP3 KO mouse; DN experiments with rat hippocampal neurons and mouse dorsal root ganglia neurons	unidentified; possibly JIP3 itself and/ or a JIP3 interacting protein	unidentified or not applicable	decreased axonal elongation and regeneration, dependent on JIP3's interaction with Kinesin-1 heavy chain

Shown are a list of studies in which loss of JIP3 led to decreased levels of a cargo in axons, decreased anterograde transport of a cargo, or defective axonal growth caused by a loss of JIP3's interaction with Kinesin-1. These phenotypes are indicative of JIP3's Adaptor function.

[1]Mitochondria are unique in that they are the only organelle that has so far been shown to be absolutely dependent on Kinesin-1 for anterograde transport in axons, and they are much less affected by JIP3's clearance function compared to endosomal organelles (Edwards and others 2013). Mitochondria also appear to use a unique adaptor system not involving JIP3 (See Box 1 after references), so it is unclear if this is an example of JIP3 acting directly as an adaptor.

[2]Sato and others (2015) also reported accumulations of Mito-GFP and APP-GFP in axons, which seems inconsistent with decreased anterograde movement.

support a Kinesin-1 adaptor function for JIP3. In addition to interacting with Kinesin-1 light chain, JIP3 directly interacts with Kinesin-1 heavy chain at a specific site near its N-terminus (Sun and others 2011). In *in vitro* motility assays, JIP3 relieves the autoinhibition of Kinesin-1 and enhances its motility (Sun and others 2011). JIP3 binding to Kinesin-1 light chain allows Kinesin-1 to bind microtubules, while JIP3

binding to Kinesin-1 heavy chain starts Kinesin-1 moving on microtubules (Watt and others 2015).

Recent genetic studies have found that JIP3 can indeed function as an adaptor for Kinesin-1 *in vivo*. Table 1 summarizes these studies. In this analysis of the literature, JIP3 is considered to have an adaptor function if loss of JIP3 leads to decreased levels of a cargo in axons, decreased anterograde transport of a cargo, or defective axonal growth that is caused by a loss of JIP3's interaction with Kinesin-1.

The cargo compartments relevant to JIP3's Adaptor function

Although the biochemistry of JIP3's interaction with Kinesin-1 is well-studied, one area requiring further investigation is the identity of the cargo – bearing compartments that are relevant to the Kinesin-1 adaptor function. In fact, the only JIP3 adaptor cargo that has been clearly defined is the TrkB receptor (Huang and others 2011; Drerup and Nechiporuk 2013). This transmembrane cargo must be carried on a membrane compartment. The anterograde route by which TrkB receptors reach synaptic sites in axons is poorly understood, but may involve "transcytosis", in which mature receptors on the surface of the neuronal soma are endocytosed and initially mobilized onto Rab11-positive recycling endosomes (Ascano and others 2009). JIP3 directly or indirectly binds TrkB receptors in this adaptor function (Huang and others 2011). However, it is unknown whether the TrkB cargo compartment that is transported into axons is endosomal (organelle size) or vesicular (i.e. smaller transport vesicles that bud from the cell soma recycling endosomes).

In the cases involving the JIP3-Kinesin-1 function that is important for axonal growth (Sun and others 2013; Watt and others 2015), it is not clear if JIP3 is functioning as an adaptor in (i.e. connecting Kinesin-1 to a third component) because no cargo compartment or molecule has been identified. One study provided evidence that JIP3 itself may be a relevant Kinesin-1 cargo for stimulating axon elongation in rat hippocampal primary neurons in cell culture, and that JIP3 exerts its stimulatory effects at growing axon tips by locally activating JNK signaling (Sun and others 2013).

Other studies have also found that JIP3 regulates axon elongation, branching, and regeneration (Bilimoria and others 2010; Drerup and Nechiporuk 2013; Nix and others 2014; Suzuki and others 2010). However, the effects (i.e. promoting versus inhibiting these processes) differ between different cell types, perhaps dependent on how the cell is set up to respond to the cargos or signals with which JIP3 interacts, and no specific cargos were identified in those studies.

JIP3's Organelle Clearance function

An attempt to explain the massive organelle accumulation in fly Sunday Driver (JIP3) mutant axons in terms of a defective JIP3 - Kinesin-1 adaptor function led to the "stalled transport" explanation. This hypothesis was based on the fact that fly JIP3 mutants die as larvae and thus must be derived from heterozygous mothers. The basic idea is that a small amount of wild type JIP3 from the maternal contribution of mRNA allows organelles to enter axons using Kinesin-1 as the motor, but that transport stalls as the maternal JIP3 supply is depleted and leaves the organelles stranded (Bowman and others 2000).

While this was a reasonable hypothesis with the data available at the time, the first hints that it might not explain the organelle accumulation in JIP3 mutant axons came from biochemical studies showing that JIP3 can also interact (directly or indirectly) with dynactin (Cavalli and others 2005), which is a large regulatory complex that associates with the dynein motor (Kardon and Vale 2009). Moreover, JIP3 physically associates with two distinct populations of membranous compartments in mouse axons and mouse cortical synaptosomes (Abe and others 2009; Cavalli and others 2005). One is a class of small transport vesicles, distinct from synaptic vesicles, and the other is a class of organelle size endosomes, including early/ recycling endosomes. Sciatic nerve ligation experiments showed that the small transport vesicles that are associated with JIP3 primarily move anterogradely, consistent with a JIP3-Kinesin-1 adaptor function, while the organelle size compartments move bidirectionally, consistent with movements mediated by dynein and at least one plus-end directed motor (Abe and others 2009; Cavalli and others 2005).

Since then, genetic studies in *C. elegans* and Zebrafish have produced strong evidence that JIP3 has a dynein-dependent organelle clearance function. Table 2 summarizes these studies. In this analysis of the literature, JIP3 is considered to have a clearance function if loss of JIP3 leads to *increased* levels of a cargo in axons (Figure 1 C, D).

JIP3's clearance function removes some organelle cargos from axons

The cargos associated with JIP3's clearance function are mainly organelles, including Golgi stacks, early/ recycling endosomes, and lysosomes (Brown and others 2009; Drerup and Nechiporuk 2013; Edwards

Table 2. Genetic studies of JIP3's Clearance function

Study	Organism	Neuron type	Cargo	Organelle(s) or compartment(s)	Clearance Phenotype caused by loss of JIP3 function
(Bowman and others 2000)	*Drosophila*	larval segmental nerves	unspecified "various axonal membrane bound cargos"	unidentified	"massive accumulation" of cargos in axons
(Brown and others 2009)	*C. elegans*	GABAergic motor neurons	YFP-RAB-5	early endosomes and unidentified membranous cisternae	accumulation in axons
(Arimoto and others 2011)	*C. elegans*	sublateral cholinergic motor neurons	APL-1-GFP (Amyloid Precursor Protein)	unidentified	decreased retrograde (minus-end directed) transport
(Drerup and Nechiporuk 2013)	Zebrafish	pLL sensory neuron axons	Lamp1 and Lysotracker Red	lysosomes	accumulation in axon terminals
(Drerup and Nechiporuk 2013)	Zebrafish	pLL sensory neuron axons	mTangerine-DLIC	unidentified	accumulation in axon terminals (other dynein and dynactin components showed normal localization)
(Drerup and Nechiporuk 2013)	Zebrafish	pLL sensory neuron axons	activated JNK	Not applicable; no compartment necessary since JIP3 directly interacts with JNK	decreased retrograde transport of activated JNK and accumulation of activated JNK in axon terminals
(Edwards and others 2015a; Edwards and others 2013)	*C. elegans*	DA and DB type cholinergic motor neurons	AMAN-2-Venus, PST-2-GFP, YFP-RAB-5, RFP-SYN-13, CTNS-1-RFP, LMP-1-GFP	Golgi (2 markers), early endosomes (2 markers), lysosomes (2 markers)	5-10 – fold accumulations of these organelles in the synaptic region of axons; not dependent on Kinesin-1
(Edwards and others 2013)	*C. elegans*	ventral and dorsal cholinergic motor neuron axons	N/A	endosomal compartments identified by electron microscopy	6-9 – fold accumulations in axons

Shown are a list of studies in which loss of JIP3 led to increased levels of a cargo in axons or decreased retrograde transport of a cargo. These phenotypes are indicative of JIP3's clearance function.

and others 2013).

In JIP3 mutants, these organelles accumulate in axons at levels that are ~5-10 – fold higher than wild type (Edwards and others 2013). However, other organelles, such as ER membranes (Edwards and others 2013), late endosomes (Rab7 positive) (Drerup and Nechiporuk 2013), and autophagosomes (Drerup and Nechiporuk 2013), do not accumulate in JIP3 mutant axons. Mitochondria are affected to a lesser extent (~2.5-fold increased) (Edwards and others 2013). Thus, in contrast to its Kinesin-1 adaptor

function, which promotes cargo movement *into* axons, JIP3 has a second clearance function that promotes the movement of some organelles *out of* axons.

JIP3 can exert its clearance function at the axon initial segment to keep organelles out of the axon

Mature wild type *C. elegans* cholinergic motor neuron axons normally contain no lysosomes in their presynaptic regions and only very low levels of endosomes (Edwards and others 2013), and this is also the case in mouse brain dopaminergic neurons (Block and others 2015). Although it is not clear why these organelles are excluded from the synaptic region, it is easy to imagine that they may interfere with synaptic vesicle trafficking at synapses and thus impair synaptic transmission.

In blocking organelle accumulation in cholinergic motor neuron axons, JIP3 exerts its strongest effects at the axon initial segment. That is, even in wild type, lysosomes frequently move out of the cell soma into the axon initial segment where they exhibit bidirectional movements that only rarely take them beyond this region and almost never to the presynaptic region (Edwards and others 2013). Lysosomes in JIP3 null mutants also exhibit bidirectional movements, but the movements are biased toward microtubule plus ends (Edwards and others 2015a), leading to lysosomes that often escape the initial segment and move deeper into the axon (Edwards and others 2013). These observations led one study to apply the metaphor "organelle gatekeeper" to JIP3 (Edwards and others 2013). Although this appears apt for JIP3 in this context, it is likely that JIP3 also functions throughout the axon, since immunostaining experiments in intact animals showed that native JIP3 has slightly higher concentrations in the cell soma and initial segment, but is also distributed throughout neuronal processes with local areas of concentration even in the synaptic region of axons (Edwards and others 2013).

A JIP3 isoform also regulates endosome movements in some non-neuronal cells

The highly similar JIP4 protein in humans has a core function in regulating endosome movements. A fraction of JIP4 has been shown to localize to early endosomes (Rab5-positive) in HeLa cells (Montagnac and others 2009), and JIP4 speeds up the microtubule-dependent recycling of endosomes (Montagnac and others 2011). JIP4 also regulates the speed and movement of a specific class of endosomes in and out of the intercellular bridge in HeLa cells prior to abscission during cell division (Montagnac and others 2009). In this case, JIP4 regulates both Kinesin-1-dependent plus-end movements and Dynein-dependent minus end movements. Biochemical experiments suggest that the small G protein ARF6 may determine which motor is regulated by JIP4 (Montagnac and others 2009).

JIP3's kinesin-1 adaptor and organelle clearance functions are two independent functions of the same protein

The biochemical and genetic data summarized above suggest that, in neurons, JIP3 has evolved two distinct functions that are distinguished by different cargos and different motors. JIP3's adaptor function appears to be specific to Kinesin-1 and Kinesin-1 cargos that are carried on or within the small transport vesicles that can be immunoisolated with JIP3 and that, consistent with this idea, move primarily toward microtubule plus-ends (Abe and others 2009; Cavalli and others 2005). JIP3 itself, without serving as an adaptor for any cargo, may also "piggy-back" on Kinesin-1 to move into axons (Sun and others 2011; Watt and others 2015).

JIP3's clearance function appears to be specific for the large endosomal classes of organelles that were also immunoisolated with JIP3 and that, consistent with being relevant to the clearance function, move bidirectionally (Abe and others 2009; Cavalli and others 2005), but are generally biased toward microtubule minus ends (Edwards and others 2015a; Edwards and others 2013). These organelles include early/ recycling endosomes, Golgi, and lysosomes, but not all classes of cell soma organelles (Drerup and Nechiporuk 2013; Edwards and others 2013). JIP3's clearance function also acts on a non-organelle cargo: the JNK cargo that binds directly to JIP3 (Drerup and Nechiporuk 2013).

Another model for JIP3 proposes that the Kinesin-1 and dynein functions of JIP3 are not separable and that JIP3 functions as an adaptor for both motors in transporting the same cargoes. This "dual adaptor" model was proposed in a study from *C. elegans* (Arimoto and others 2011). However, that study did not assay organelle transport (only transport of Kinesin-1, dynein, and APL-1-GFP transport vesicles), and its main hypothesis: that Kinsesin-1, dynein, and JIP3 move into axons as a complex, was not supported by biochemical data and has been called into question by biochemical data in separate studies showing that Kinesin-1 and dynein can't stably bind JIP3 simultaneously (Cavalli and others 2005; Montagnac and others 2009), by imaging data showing that levels of dynein subunits are unaffected in JIP3

mutant axons (Drerup and Nechiporuk 2013), and by new *C. elegans* studies demonstrating that JIP3 does not function as a dynein adaptor during organelle clearance (discussed in detail below). Other proteins, such as milton (mitochondrial transport) and JIP1 (autophagosome transport) have been shown to be dual adaptors (Fu and Holzbaur 2014a; Schwarz 2013; Zinsmaier and others 2009). However, despite sharing an unfortunate common "JIP" naming scheme with JIP1, JIP3 is a completely unrelated protein that has absolutely no sequence homology to JIP1.

Which kinesin motor carries organelles into axons in JIP3 mutants?

Since organelles accumulate in JIP3 mutant axons, and since axonal microtubules, including those in JIP3 mutant axons (Arimoto and others 2011; Edwards and others 2015a), are oriented with their plus ends out, there must be a plus-end motor that carries them into axons. Indeed, time lapse studies demonstrated that lysosomes move anterogradely at fast axonal transport speeds in JIP3 null mutant axons (Drerup and Nechiporuk 2013; Edwards and others 2013). The motor that carries organelles into axons in JIP3 mutants is likely to be the same motor that is normally responsible for axonal transport of these organelles; however, with the exception of mitochondria (See Box 1 after references), the plus-end motor that is normally responsible for the transport of endosomal classes of organelles into axons has not been identified.

One attractive candidate for the relevant plus-end motor is Kinesin-1, because JIP3 directly interacts with Kinesin-1 in its adaptor function, and because Kinesin-1 appears to be universally required for the plus-end directed transport of mitochondria in axons, including in worms, flies, and Zebrafish (Campbell and others 2014; Rawson and others 2014; Stowers and others 2002) (See Box 1 after references). However, a genetic analysis *in C. elegans* showed that reducing the function of Kinesin-1 has no effect on the endosomal/ lysosomal organelle accumulation that occurs in JIP3 null mutants (Edwards and others 2013). In contrast, impairing Kinesin-1 blocks the transport of mitochondria into *C. elegans* motor neuron axons (Rawson and others 2014). Surprisingly, axonal accumulation of early endosomes and lysosomes also occurs in *C. elegans* Kinesin-1 mutants, although to a lesser extent than in JIP3 mutants (Edwards and others 2013). Does this mean that Kinesin-1, the very paradigm of plus-end motors, promotes minus-end organelle movement? That is extremely unlikely. The most likely explanation is that Kinesin-1 is required for the plus-end transport of JIP3 into axons, as is known to be the case in mouse neurons (Sun and others 2013; Watt and others 2015). Then, without JIP3 in axons (or lower levels), JIP3 can't exert its clearance function, and organelles accumulate via the unidentified plus-end motor(s). The finding that axonal organelle accumulation in mutants lacking both JIP3 and Kinesin-1 is no worse than JIP3 single mutants is consistent with this explanation (Edwards and others 2013).

At first glance, it seems surprising that Kinesin-1 is not the motor that is relevant to JIP3's clearance function, and thus probably not the motor responsible for the plus-end axonal transport of these classes of organelles (Golgi, early/ recycling endosomes, and lysosomes). However, an analysis of the literature reveals no data that conflicts with this idea. With the exception of mitochondria (See Box 1 after references), there is no data showing that Kinesin-1, or any specific kinesin, is required in neurons for the axonal transport of any of these classes of organelles (i.e. the organelles relevant to JIP3's Clearance function). Studies have shown that Kinesin-1 is required for moving/ "dispersing" some classes of endosomes as well as lysosomes toward the outer boundaries (microtubule plus ends) of non-neuronal cells (Hollenbeck and Swanson 1990; Loubery and others 2008; Montagnac and others 2009; Nakata and Hirokawa 1995; Rosa-Ferreira and Munro 2011; Tanaka and others 1998). However, in neurons, with the exception of mitochondria, Kinesin-1's cargos in axons seem to be smaller transport vesicles carrying specific proteins, such as GAP-43 and Synapsin I (Ferreira and others 1992), APP (Kamal and others 2000), the sodium channel Nav1.8 (Su and others 2013), the potassium channel Kv1 (Rivera and others 2007), and TrkB receptors (Arimura and others 2009; Huang and others 2011). Indeed, there are few or no examples of Kinesin-1 axonal transport cargos beyond these specific proteins and mitochondria.

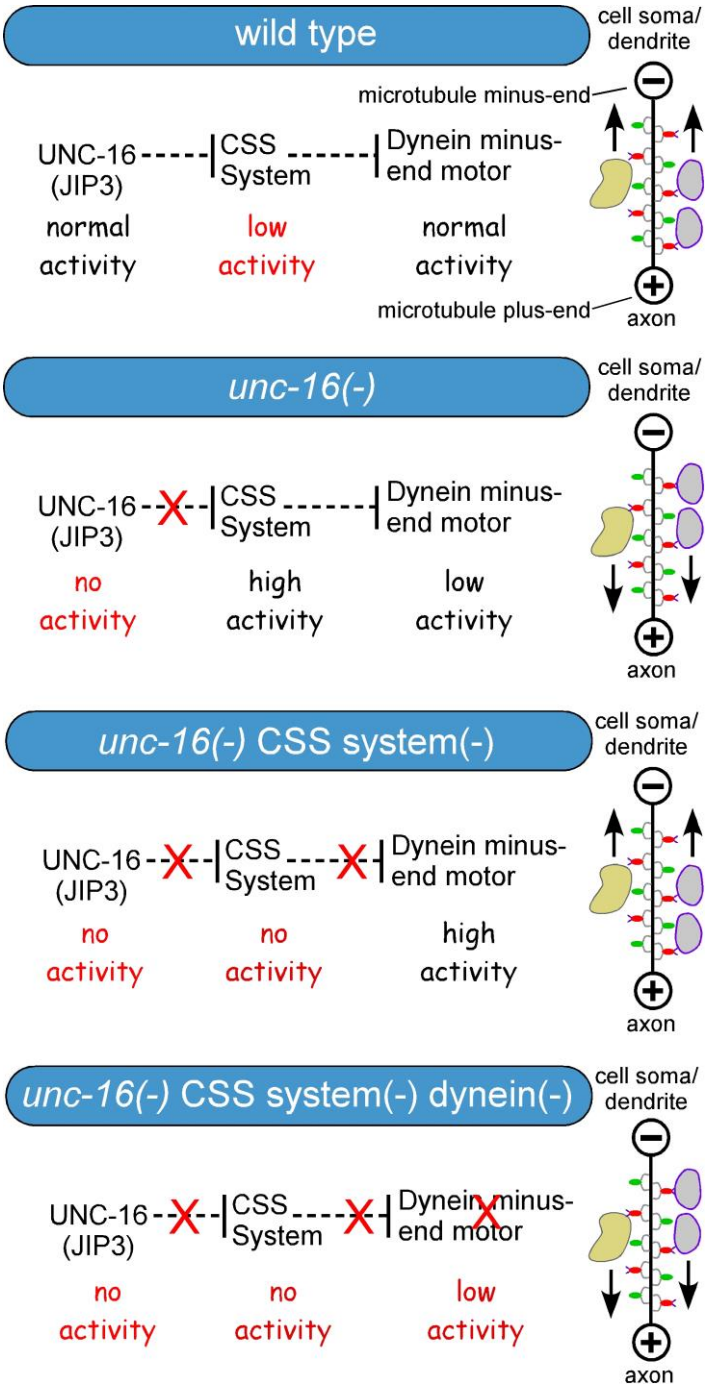
***C. elegans* Kinesins**		
Kinesin family	***C. elegans* protein(s)**	**Good candidate for plus-end motor for JIP3's clearance function?**
Kinesin-1	UNC-116	No. Not required for axonal organelle accumulation in *unc-16* (JIP3) mutants.
Kinesin-2	OSM-3, KLP-11, KLP-20	Yes. KLP-11 and KLP-20 are expressed in ventral cord motor neurons.
Kinesin-3	UNC-104, KLP-4, KLP-6	Yes, but UNC-104 single mutants have no effect on axonal organelle accumulation in *unc-16* (JIP3) mutants.
Kinesin-4	KLP-12, KLP-19	No. Only expressed in germ cells and embryos and involved in mitosis.
Kinesin-5	BMK-1	No. Acts as a brake during anaphase spindle elongation in mitosis.
Kinesin-6	ZEN-4	No. Only expressed during early and mid-embryogenesis.
Kinesin-12	KLP-10, KLP-18	No. KLP-10 lacks Switch I region for ATP interaction. KLP-18 is only expressed in germline and early embryos.
Kinesin-13	KLP-7	No. Undirected motor that diffuses to microtubule ends where it promotes microtubule disassembly in mitosis.
Kinesin-14	KLP-3, KLP-15, KLP-16, KLP-17	No. These are all predicted to be minus-end directed motors.
Yeast Kip3	KLP-13	No. Atypical kinesin that lacks certain amino acids that would make it a functional kinesin.

Figure 2. The ten families of *C. elegans* Kinesins. The two yellow highlighted families are the best candidates for the motor or motors related to JIP3's clearance function. Data for this figure come from Wormbase version WS240 and the following references: (Edwards and others 2013; Hall and Hedgecock 1991; Huckaba and others 2011; Monteiro and others 2012; Morsci and Barr 2011; Ou and others 2005; Pan and others 2006; Robin and others 2005; Siddiqui 2002; Snow and others 2004).

Although the kinesin motor that is relevant to the transport of endosomal classes of organelles in axons hasn't been identified, a recent study using rat embryonic fibroblast cells revealed a strong pattern of endosome binding by Kinesin-3 family members (Bentley and others 2015). *C. elegans* has 19 Kinesin-related proteins in 10 families (Figure 2). Some can be ruled out because they are predicted to be minus-end motors, undirected motors, lack key residues necessary for being a functional kinesin, or are expressed only in embryos (Figure 2). All three *C. elegans* Kinesin-3 family members are expressed in neurons, as well as the three Kinesin-2 family members. Impairing the function of one of the Kinesin-3 family members, the UNC-104 (KIF1A) motor that transports SV precursors, was recently found to have no effect on lysosome transport in *unc-16* mutant neurons (Edwards and others 2015a). However, among the remaining five members of the Kinesin-2 and -3 families, there is likely to be redundancy, especially given the finding that a single endosomal cargo compartment can bind two different Kinesin-3 family members (Bentley and others 2015). *C. elegans* mutants are available in all of these genes, and although testing these motors in various combinations requires some challenging genetics, the identity of the plus-end motor(s) relevant to JIP3's organelle clearance function should be forthcoming.

The CSS system: an emerging view of a new axonal transport regulatory system

With the goal of identifying the relevant plus-end motor and/ or other proteins important for JIP3's organelle clearance function, a recent *C. elegans* study performed a forward genetic screen to look for mutations that suppress the accumulation of lysosomes in *unc-16* (JIP3) mutant axons (Edwards and others 2015a). The *unc-16* suppressor screen revealed that JIP3 inhibits, or blocks the actions of, CDK-5 (Cdk5) and two conserved active zone proteins: SAD-1 (SAD-A Kinase), and SYD-2 (Liprin-α). Genetic analysis of all combinations of double and triple mutants in JIP3(+) and JIP3(-) backgrounds showed that the three proteins are all part of the same organelle transport regulatory system, which was



named the CSS system based on its founder proteins (CDK-5, SAD-1, and SYD-2) (Edwards and others 2015a). Further genetic analysis revealed roles for SYD-1 (another active zone protein) and STRADα (a SAD-1 – interacting protein) in the CSS system (Edwards and others 2015a). In a JIP3 null background, loss of the CSS system improves the sluggish locomotion of JIP3 null mutants, inhibits axonal lysosome accumulation, and leads to the accumulation of lysosomes in dendrites (Edwards and others 2015a). Microtubules in *C. elegans* motor neuron dendrites are almost uniformly minus-end out (Goodwin and others 2012; Yan and others 2013). Thus, when organelles accumulate in dendrites, it is a good indication of over-active minus-end motor activity (or underactive plus-end motor activity). Consistent with over-active dynein transport in animals lacking both JIP3 and the CSS system, additionally impairing dynein activity (i.e. making the triple mutants) reverts the lysosome distribution to the JIP3 single mutant state of high axonal levels and low dendritic levels (Edwards and others 2015a).

Figure 3. Genetic pathway representation of Edwards et al., 2015 results.
Shown is one possible model consistent with the genetic results of (Edwards and others 2015a). In wild type animals JIP3 directly or indirectly inhibits the CSS system (vertical bars indicate inhibition). When CSS system activity is low, minus- and plus-end motor activity is properly balanced and cell soma organelles (lysosomes and endosomes; tan and purple blobs) remain in the soma and/ or enter the dendrite at optimal rates. When CSS system activity is high, plus-end motor activity dominates due to inhibition of dynein and organelles accumulate in axons. In other equally plausible models the CSS system could positively regulate a plus-end motor or regulate both motors (as shown in Figure 1), and the effect on organelle transport would be the same.

Figure 3 summarizes these genetic results graphically using a formal genetic pathway representation. In this representation, JIP3 promotes the movement of organelles in a minus-end direction (i.e. keeps them in or near the cell soma and dendrite) by directly or indirectly inhibiting the action of the CSS system, which prevents the CSS system from inhibiting the dynein minus-end motor and/ or promoting the activity of a plus-end motor. In other words, the data show that JIP3 mutants accumulate organelles in their axons because, in the absence of JIP3, CSS system activity is too high, thus inhibiting dynein and/ or promoting a plus-end motor. Time lapse imaging of lysosomes in the dendrites of CSS system mutants in JIP3(+) and JIP3(-) backgrounds revealed active transport defects (i.e. biases in one direction or another) consistent with this model (Edwards and others 2015a). The CSS system was also shown to regulate the transport of early/ recycling endosomes and, to a lesser extent, Golgi (Edwards and others 2015a).

Possible mechanistic actions of the CSS system

Although the pathway in Figure 3 depicts the CSS system inhibiting dynein action, the genetics and organelle distribution results are equally consistent

with the CSS system promoting the action of the unidentified plus-end motor, or regulating both motor systems. In other words, the genetics (and imaging of mutants) only informs us that it is the *balance* of plus and minus-end motor activity that is disrupted in JIP3 mutants. However, when considering the various models, the models need to take into account all of the available data from all systems where these proteins have been studied, including genetic and imaging studies in Zebrafish and *C. elegans*, biochemical data from mice, and new transport studies in mouse cultured DRG neurons. When this is done, the data point most strongly to a model in which the CSS system inhibits the action of dynein in organelle transport; however, additional regulation of the plus-end motor cannot and should not be ruled out.

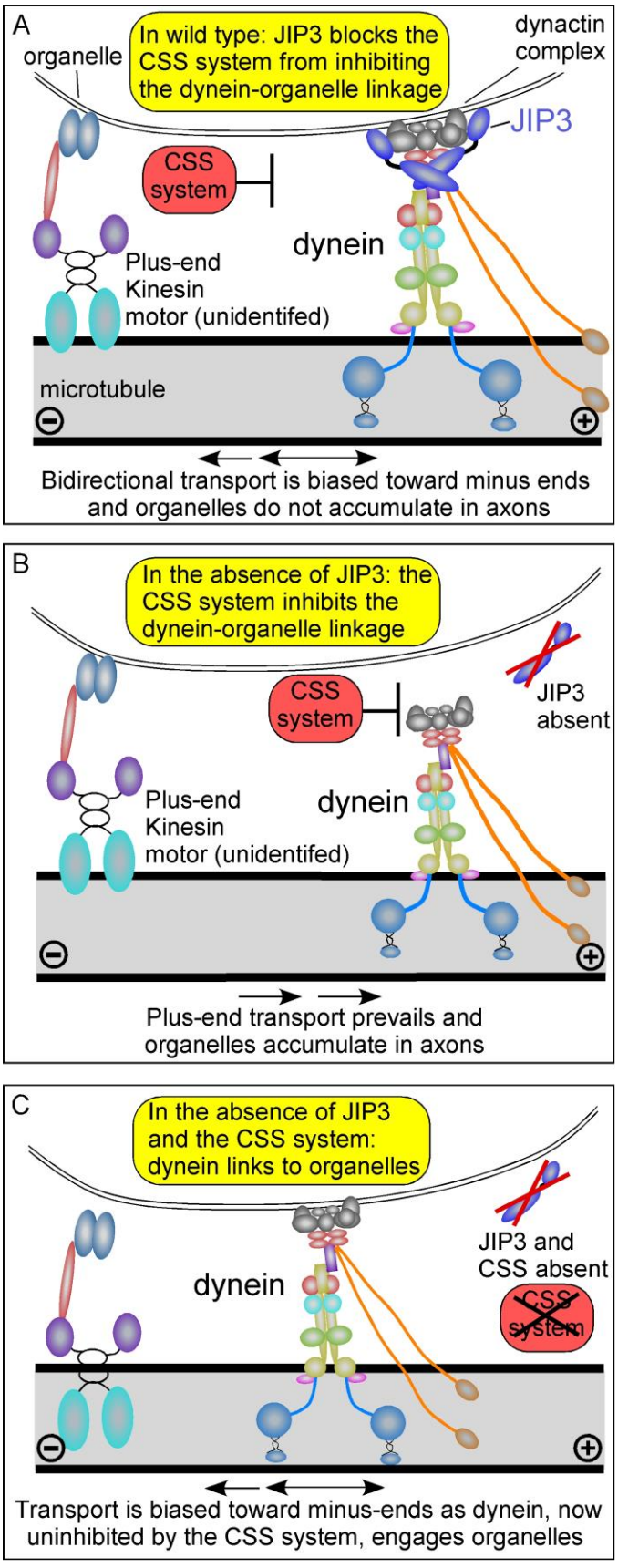
Model 1: the CSS system inhibits the dynein – based transport of organelles

A time lapse imaging study in Zebrafish neurons contributed a key observation by showing that Jip3 mutants have decreased association of dynein (specifically the dynein light intermediate chain) with lysosomes during transport (Drerup and Nechiporuk 2013). On the basis of this, as well as biochemical data showing that JIP3 physically associates with bi-directionally moving organelles, and that JIP3 can be found in a complex with the dynactin components $p150^{glued}$ and p50 (Abe and others 2009; Cavalli and others 2005), JIP3 was proposed to function as an adaptor that links dynein to lysosomes and thus stimulates the minus-end transport of lysosomes (Drerup and Nechiporuk 2013). This was a reasonable hypothesis with the data available at the time; however, the new *C. elegans* data suggest that JIP3 is not a dynein adaptor, because mutants lacking both JIP3 and the CSS system have overactive dynein minus-end transport of lysosomes (Edwards and others 2015a). Thus, according to the new data, as long as the CSS system is not interfering with dynein transport, the dynein motor transports lysosomes similar to wild type in the complete absence of JIP3.

The new *C. elegans* data instead suggest a different interpretation that is consistent with the Zebrafish co-transport data (Drerup and Nechiporuk 2013) and the mouse biochemical data of Cavalli and others (2005) and Abe and others (2009) (Figure 4). According to this model JIP3 interacts directly or indirectly with dynactin (Cavalli and others 2005; Montagnac and others 2009) and also physically associates with large endosomal organelles (Abe and others 2009). JIP3 then promotes the minus-end directed transport of those organelles (Drerup and Nechiporuk 2013; Edwards and others 2015a; Edwards and others 2013) by blocking the action of the CSS system (Edwards and others 2015a). When JIP3 is present, organelle transport is bidirectional but biased toward minus-ends, with plus-end movement mediated by one or more unidentified kinesin motors. In the absence of JIP3, there is decreased association of dynein with organelles during minus-end transport (Drerup and Nechiporuk 2013), likely caused by the CSS system, which is normally inhibited by JIP3 (Edwards and others 2015a). JIP3 may inhibit the CSS system by physically blocking/ interfering with the actions of the CSS system on the dynactin complex and/or other dynein regulators, although other mechanisms of inhibition, such as microtubule regulation, are also possible. Impaired minus-end transport in JIP3 mutants allows plus-end transport to dominate, thus causing organelle accumulation in axons. Additional elimination of the CSS system and dynein affects organelle transport in predictable ways consistent with this model (Edwards and others 2015a) (see Figure 3), but not consistent with JIP3 having a dynein adaptor function. Although these data seem to rule out a dynein adaptor function for JIP3 in organelle transport, further studies will be needed to identify the protein that links organelles to the dynein motor. One candidate is the bidirectional adaptor JIP1, which, as noted earlier, is unrelated to JIP3.

Although this mechanistic model is consistent with the genetic data, the formal genetic pathway places JIP3 *upstream* of the CSS system (i.e. inhibiting its activity). While this is still a formal possibility, the mechanistic interpretation in Figure 4, which is more consistent with data from other systems (Abe and others 2009; Drerup and Nechiporuk 2013), shows how JIP3 can "inhibit" the actions of the CSS system by preventing CSS system proteins from accessing dynein, dynein modulators, and/ or the dynactin complex (see See Box 2 after references: "Interpreting JIP3 genetic pathway results"). Essentially, in this model, JIP3 protects specific classes of organelles from the dynein-inhibiting activity of the CSS system (blocks the CSS system), thus allowing JIP3-protected organelles to be cleared from axons by dynein.

Because the mutations in individual CSS system components do not have additive effects on organelle transport, the proteins of the CSS system may ultimately affect the same target(s), or possibly different targets that converge on the same end effect, to inhibit dynein minus-end transport. However, the specific targets through which inhibits dynein in the context of JIP3's clearance function are unknown. Clues may come from a recent study showing that Cdk5 hyperactivation inhibits the dynein-mediated transport of late endosomes, lysosomes,



autophagosomes, and mitochondria in cultured mouse DRG neurons, and that this inhibition is dependent on Cdk5 phosphorylation sites on the

Figure 4. Model for JIP3's Clearance function. Shown is one possible model consistent with both the biochemical data (Abe and others 2009; Cavalli and others 2005) and the genetic and live animal imaging data (Drerup and Nechiporuk 2013; Edwards and others 2015a; Edwards and others 2013). **(A)** In wild type animals JIP3 interacts directly or indirectly with dynactin (Cavalli and others 2005; Montagnac and others 2009) and large endosomal organelles (Abe and others 2009) and allows minus-end transport of those organelles (Drerup and Nechiporuk 2013; Edwards and others 2013) by blocking the action of the CSS system (Edwards and others 2015a). The transport is bidirectional but biased toward minus ends, with plus-end movement mediated by one or more unidentified kinesin motors. **(B)** In the absence of JIP3, there is decreased association of dynein with organelles during minus-end transport (Drerup and Nechiporuk 2013) possibly caused by the CSS system inhibiting the dynein-organelle linkage (Edwards and others 2015a). Impaired minus-end transport allows plus-end transport to dominate. Plus end transport may be promoted directly by the CSS system or indirectly by increasing the availability of plus-end motor connections to the organelle after dynein connections are lost. **(C)** In the absence of both JIP3 and the CSS system, transport is again biased toward minus-ends as dynein, now no longer inhibited by the CSS system, engages with organelles (Edwards and others 2015a). See also See Box 2 after references.

dynein regulator Ndel1 (Klinman and Holzbaur 2015). In both DRG neurons and *C. elegans* motor neurons, eliminating Cdk5 alone has no, or subtle, effects on axonal organelle transport (Edwards and others 2015a; Klinman and Holzbaur 2015). According to the model in Figure 4, this is because, under normal conditions, JIP3 blocks, or otherwise interferes with, Cdk5's ability to inhibit dynein-based organelle motility, so knocking out Cdk5 has very little effect unless JIP3 is also removed. Cdk5 hyperactivation in the cultured DRG neurons may partially overcome this blockade by mass action. However, further experiments will be needed to determine if Cdk5 is functioning in the context JIP3's clearance function in mouse cultured DRG neurons.

Model 2: the CSS system regulates both plus- and minus-end motor systems

Although the data suggest that the CSS system inhibits dynein-mediated transport in the absence of JIP3 protection, there is no evidence against the hypothesis that the CSS system also regulates one or more plus-end motors. Indeed, one of the CSS system proteins, SYD-2 (Liprin-α) has been found to directly interact with Kinesins, including the Kinesin-3 family member UNC-104 (Wagner and others 2009) and the Kinesin-4

family member Kif7 (Liu and others 2014). However, SYD-2 belongs to a class of "intrinsically unstructured proteins" that can bind several partners in a "structurally adaptive process" (Wagner and others 2009), so past studies of its binding partners are not necessarily relevant to its function in the JIP3 clearance system. Further studies will be needed to determine the extent to which the CSS system regulates plus-end motors in axons.

The Significance of the Connection between Active Zone/ Synapse Assembly Proteins and Organelle Clearance

The genetic data show that neurons wouldn't need JIP3 to ensure organelle clearance from axons if they didn't have a CSS system. So why do neurons have a CSS system that interferes with organelle clearance? It turns out that most components of the CSS system, including SYD-2, SYD-1, and SAD-1, are enriched at the presynaptic density that lies at the center of the active zone where neurotransmitters are released (Inoue and others 2006; Owald and others 2012; Weimer and others 2006). These CSS system proteins function to cluster SVs near active zones at sites of neurotransmitter release (Crump and others 2001; Dai and others 2006; Kittelmann and others 2013; Patel and others 2006; Stigloher and others 2011; Zhen and Jin 1999). In the absence of SYD-2, SVs appear more prone to dissociate from mature synapses (Kittelmann and others 2013; Stigloher and others 2011; Wu and others 2013). Interestingly, in this region of the neuron, components of the CSS system appear to inhibit both minus- and plus-end directed transport to keep SVs captured at synapses and out of the flanking asynaptic regions and the cell soma/ dendrite region (Edwards and others 2015b; Liu and others, 2014; Wu and others 2013).

The concept that synapse assembly/ active zone proteins can regulate axonal transport of SVs is not new. SYD-2 has been shown to regulate SV transport in *Drosophila* (Miller and others 2005) and both SV and DCV transport in *C. elegans* (Edwards and others 2015b; Goodwin and Juo 2013; Wagner and others 2009; Zheng and others 2014), along with CDK-5 and the related Pctaire Kinase (Goodwin and others 2012; Ou and others 2010). A recent *C. elegans* study provided evidence that the accumulation of SVs in *sad-1* and *syd-1* mutant dendrites (microtubule minus ends) also results from a defect in the regulation of SV transport (Edwards and others 2015b). However, the involvement of synapse assembly/ active zone proteins in the regulation of *organelle* transport (i.e. Golgi, early/ recycling endosomes, and lysosomes) in neurons is a completely novel and unprecedented concept and begs the question "What is the significance of the connection between active zone proteins and organelle clearance?"

A recent study provided two key insights into this question. First, *syd-2* null mutants were shown to have a dynein-dependent shift of SV density from the synaptic region to cell somas and dendrites (Edwards and others 2015b). Second, the same study used a temperature sensitive mutation in the SV motor protein UNC-104 (KIF1A) to stop plus-end directed transport of SV precursors in adult animals and observe what happens to mature SV clusters in a single cholinergic motor neuron over time when only dynein-mediated transport is allowed (Edwards and others 2015b). The data showed an abrupt loss of ~50% of the SVs from the clusters, but then no further loss over time. When the same experiment was performed using locomotion rate as the assay parameter, there was a corresponding abrupt 50% decrease in locomotion rate, but then no further decrease over time. This indicates that the SVs that remained in the clusters after impairing the UNC-104 motor were not simply trapped or permanently tethered in the cytomatrix, but were instead capable of moving through the stages of fusion, endocytosis, and recycling at synapses and thus were motile at some points and potentially vulnerable to removal by motors. Under these conditions of low UNC-104 motor activity, the presence of SVs at synaptic sites was completely dependent on the CSS protein SYD-2 (Edwards and others 2015b). Although tethering could also be an important component of SV capture/ clustering at synapses, these data suggest that one function of CSS proteins is to make about half of the SVs at synaptic sites resistant to minus-end transport by dynein, thus maintaining stable recycling populations of SVs at synaptic sites. It is important to note that the CSS proteins SYD-2, SYD-1, and SAD-1 also function to inhibit the plus end-directed movement of SVs into the asynaptic region that lies beyond the synaptic region in some neurons (Liu and others, 2014; Edwards and

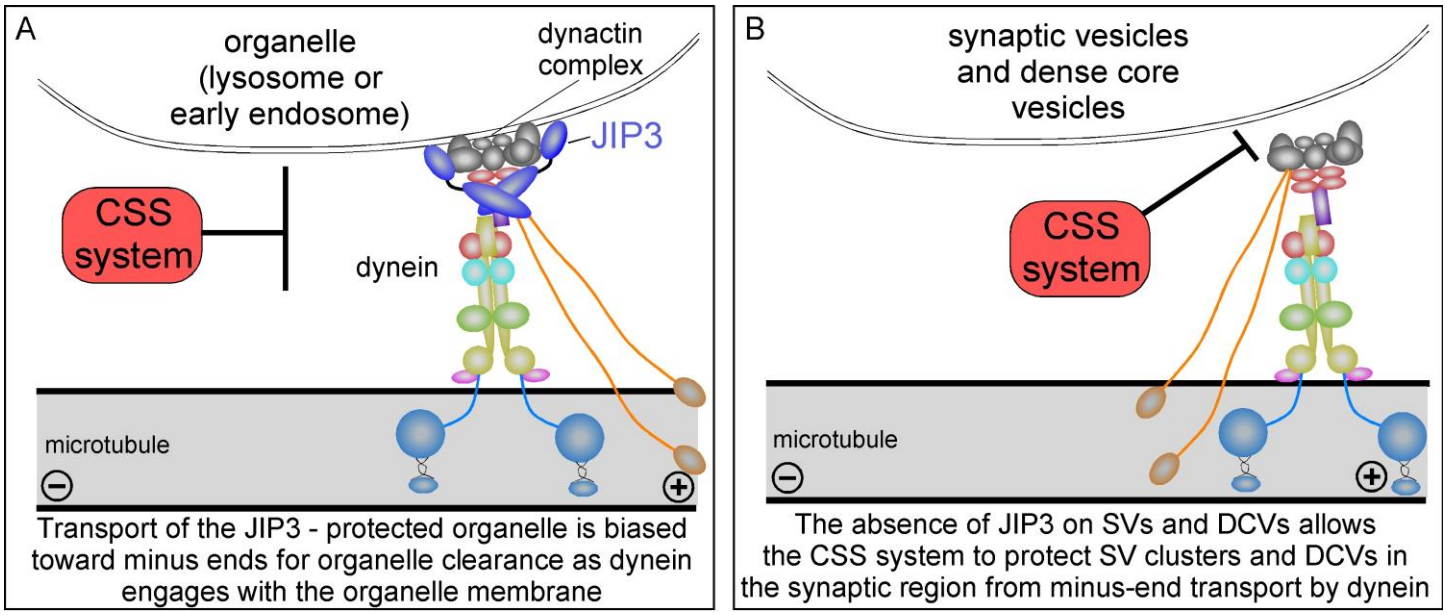


Figure 5. Comparison of CSS system actions on organelles versus synaptic vesicles and dense core vesicles.

(A) In its organelle clearance function, JIP3 protects organelles from the CSS system and allows them to be cleared from the axon by dynein. Based on findings from Edwards and others (2015a) and the finding that JIP3 interacts with dynactin and endosomal organelles (Abe and others 2009; Cavalli and others 2005; Montagnac and others 2009) **(B)** The absence of JIP3 on SVs and DCVs allows the CSS system to protect SV clusters near active zones and DCVs in the synaptic region. Most CSS system proteins are enriched at the presynaptic density at the center of the presynaptic active zone.

others, 2015b; Wu and others, 2013), so at least in this region of the axon, CSS system proteins appear to regulate both motor systems and/ or the microtubules in this region, to facilitate the capture of SVs at synapses.

The dynein-related function of the CSS system at synaptic active zones fits with JIP3's organelle clearance function: CSS system proteins may inhibit dynein by a similar mechanism in organelle transport and SV cluster stability. In organelle transport this inhibition is normally blocked by JIP3 to prevent the accumulation of potentially harmful organelles in the all-important synaptic region where they could interfere with synaptic transmission. However, in SV cluster stability, the CSS system is not blocked by JIP3. Synaptic vesicle interactions with CSS system proteins, perhaps at or near the active zone/ presynaptic density itself (where most CSS system proteins are enriched), may inhibit the ability of dynein to remove SVs from the synaptic region (Figure 5) and contribute to their stable capture and accumulation in this region. However, the CSS system may also regulate SV transport in regions of the axon that are more proximal to the cell soma, including the axon initial segment, where the CSS system appears to exert its strongest effects in preventing endosomes and lysosomes from returning to the cell soma in the absence of JIP3 protection (Edwards and others, 2013). Regardless of the sites of action of the CSS system, the new findings reveal how neurons can selectively regulate a single minus-end directed motor to exclude specific classes of organelles from axons, while at the same time ensuring optimal levels of SVs at synapses.

The CSS system proteins also regulate dense core vesicles (DCVs) levels in the synaptic region. However, unlike SVs, DCVs are only occasionally seen associated with tethers at synapses (Stigloher and others 2011), and are not clustered around the active zone (Hammarlund and others 2008; Hoover and others 2014; Stigloher and others 2011). This suggests that tethering is not essential for CSS system regulation of vesicle transport, at least for DCVs. Studies in *Drosophila* have shown that DCVs, like SVs, are captured at boutons in the synaptic region during long range bidirectional circulation (Wong and others 2012). Another study demonstrated that synaptic

activity stimulates the capture of DCVs at synapses by reducing the number of DCVs moving toward microtubule minus ends (Shakiryanova and others 2006). This represents dynein inhibition, since dynein is the only motor that carries DCVs toward microtubule minus ends in these neurons (Cavolo and others 2015). The finding that DCVs are also depleted from the synaptic region and accumulate at microtubule minus ends in a dynein-dependent manner when the CSS system proteins *cdk-5* and *syd-2* are knocked out (Edwards and others 2015b; Goodwin and Juo 2013; Goodwin and others 2012) suggests that the CSS system also contributes to DCV capture via dynein inhibition in the synaptic region, although this remains to be directly tested. Differences in the concentrations or activity levels of CSS system proteins may explain the different efficiencies of DCV capture at different kinds of synapses (Bulgari and others 2014).

Concluding remarks

The discoveries of Kinesin-1 and Dynein set the stage for arguably one of the most interesting and impactful fields in cell biology: trying to understand how cells use motors to move various cargos from one location to another to establish and maintain their internal structure. Understanding motor regulation has especially heightened relevance when studying neurons. One important concept the studies in this review have underscored is that neurons have developed highly specialized mechanisms to meet their highly specialized transport needs.

As evidenced by milton, JIP3, the CSS system, and the new SV transport regulator SAM-4 (Zheng and others 2014), genetic studies, especially forward genetic screens in model organisms, can play important roles by producing entry point discoveries that reveal axonal transport regulators. However, a mechanistic understanding requires not only genetics, but strong biochemistry as well as functional studies in mouse mutants. As highlighted in this review, JIP3 is a great example of this. Studies focusing on its interactions with Kinesin-1 revealed its adaptor function that is important for regulating axonal growth and for transporting specific transmembrane protein cargos into axons. Genetic studies focusing on the axonal organelle accumulation that occurs in the absence of JIP3 revealed a distinct organelle clearance function that promotes organelle movement out of axons. However, making sense of these two distinct functions required viewing the genetic data in light of biochemical and cell biological studies in mice (and vice versa).

Many questions remain about JIP3's adaptor and clearance functions. The intricacies and complexities of mitochondrial transport (See Box 1 after references) hint at the path ahead for understanding the axonal transport of other organelles. However, many of the remaining questions are experimentally addressable: What determines whether JIP3 functions as a Kinesin-1 adaptor or an organelle clearance regulator? Which kinesin motor(s) promote organelle transport into axons when JIP3's clearance function is compromised? What are the specific mechanisms by which JIP3 blocks the CSS system and the mechanism by which the CSS system inhibits dynein transport? Are there signals, such as axonal injury or cellular stress, that regulate JIP3 to control organelle content and/ or that facilitate information exchange between cell somas and axons? Answering these questions will require the strengths of model organisms as well as functional and biochemical studies in mammals, with clues flowing in both directions to maximally benefit the field.

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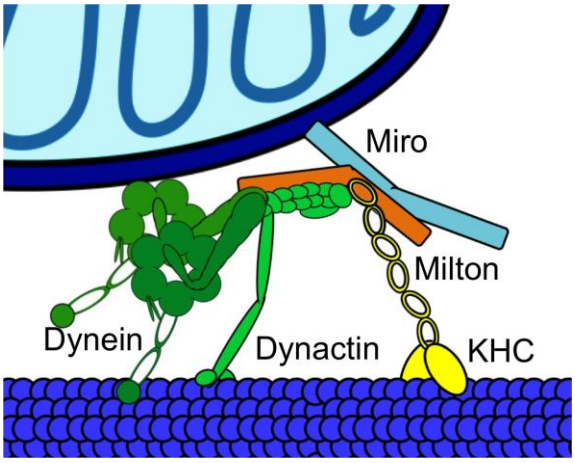
Box 1. Regulation of mitochondrial transport

Mitochondria are currently the only large non-vesicular organelles that have been shown to require Kinesin-1 for their transport into axons. A series of elegant genetic and biochemical studies have revealed the motor/ adaptor complex by which this occurs. The discovery began when a forward genetic screen for blind flies identified mutants whose axons and dendrites were completely devoid of mitochondria (Stowers and others 2002). The defective protein, named milton, directly interacts with the cargo-binding domain of Kinesin-1 and with mitochondria through a direct interaction with Miro, which was also discovered in a "blind flies" genetic screen (Fransson and others 2006; Glater and others 2006; Guo and others 2005; Smith and others 2006; Stowers and others 2002). The milton-Miro system also serves as an adaptor that connects mitochondria to minus-end directed dynein transport (Guo and others 2005; Russo and others 2009; Stowers and others 2002; van Spronsen and others 2013). The milton-Miro system is a fundamental mechanism for mitochondrial axonal transport that is conserved in mammals (Schwarz 2013; Zinsmaier and others 2009).

Miro is a transmembrane protein with two GTPase domains and two Ca^{2+}-binding EF hands that is attached to the mitochondrial outer membrane. Elevated Ca^{2+} levels are known to arrest the transport of mitochondria in axons, possibly to allow them to assist in Ca^{2+} buffering, to provide ATP for the active transport of Ca^{2+} from the axons, and/ or to provide energy to synaptic regions that have recently been energetically active. Blocking the Ca^{2+}-binding capacity of Miro prevents mitochondria from stopping at activated synapses and increases the susceptibility of neurons to excitotoxic death (Macaskill and others 2009; Wang and Schwarz 2009; Zinsmaier and others 2009).

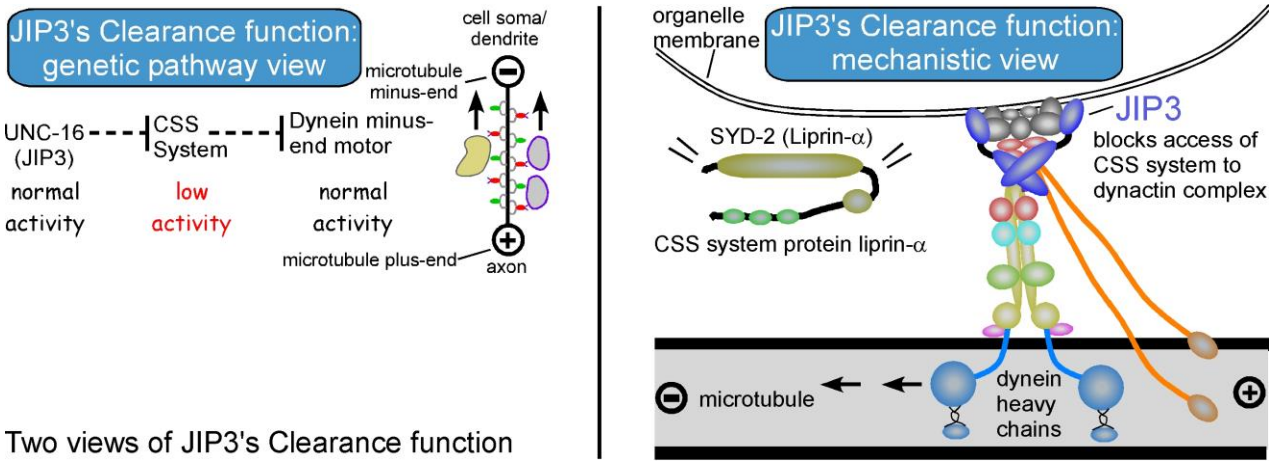
The milton-Miro system is also regulated in other ways to control the ratio of stationary to moving mitochondria (Schwarz 2013; Zinsmaier and others 2009). In one mechanism, mitochondrial damage activates the PINK1/ Parkin pathway to cause the proteasome-dependent degradation of Miro, which severs the connection to kinesin and milton and stops mitochondrial transport (Chan and others 2011; Wang and others 2011). This has been proposed to reduce the mixing of damaged and healthy mitochondria by fusion (Schwarz 2013).



The milton-Miro adaptor system (Figure courtesy of Jarom Chung).

Box 2. Interpreting JIP3 genetic pathway results

In *C. elegans*, mutations that eliminate any or all of the CSS system proteins CDK-5 (Cyclin-Dependent Kinase 5), SAD-1 (SAD-A kinase), and SYD-2 (Liprin-α) suppress the axonal organelle accumulation in UNC-16 (JIP3) null mutants. The additional elimination of dynein function (i.e. triple mutants) in turn epistatically restores axonal organelles to the UNC-16 null mutant state (Edwards and others 2015a). Thus, UNC-16 null mutants accumulate axonal organelles because they have too much CSS system activity and mutants lacking both UNC-16 and the CSS system lack axonal organelles because they have too much dynein activity. In the language of genetic pathway analysis, this means that UNC-16 normally inhibits the CSS system proteins, which in turn inhibits dynein motor activity (see genetic pathway view). This makes it look like UNC-16 is "upstream" of the CSS system proteins and inhibits their activity. However, biochemical data indicate that JIP3 interacts directly with the dynactin complex on endosomal organelles (Cavalli and others 2005). The genetic data suggest that JIP3 is not a dynein adaptor because mutants lacking both UNC-16 and the CSS system have overactive minus-end activity that can be suppressed by additional elimination of dynein. Thus, one mechanistic model that is consistent with both the biochemical and genetic data is that JIP3 physically blocks CSS system proteins from accessing dynein/ dynactin on organelles and thus prevents the CSS proteins from inhibiting minus-end transport. In this mechanistic model, CSS system proteins could already be fully activated (even in the presence of JIP3), but just unable to access the dynactin complex due to JIP3 binding that complex (see mechanistic view).



Two views of JIP3's Clearance function