Presynaptic Mechanisms for Cargo Recruitment

The Harvard community has made this article openly available. **Please share** how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:42013129">http://nrs.harvard.edu/urn-3:HUL.InstRepos:42013129</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Presynaptic Mechanisms for Cargo Recruitment

A dissertation presented

by

Hajnalka Nyitrai

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

Harvard University

Cambridge, Massachusetts

August 2019
© 2019 Hajnalka Nyitrai

All rights reserved.
Presynaptic Mechanisms for Cargo Recruitment

Abstract

Delivery and assembly of presynaptic material over long axonal distances is a daunting challenge for neurons. Many presynaptic proteins are thought to be delivered from the Golgi apparatus via precursor vesicles, the recruitment of which at nerve terminals is crucial for the maintenance of presynaptic function. But how do these transport vesicles know where to stop along the axon? In most cellular secretory pathways, Golgi-derived cargos are labeled with Rab GTPases that are recognized by tethering complexes at their fusion sites. Surprisingly, we know very little about what presynaptic tethers and cargo labels facilitate presynaptic cargo capture.

I here address how transport vesicles are captured at nerve terminals. I find that Rab6 resides in the neuronal Golgi apparatus, and its active form also localizes to nerve terminals. Rab6 associates with highly mobile vesicles in axons and it binds to ELKS in the vesicle-associated form via a short sequence near the C-terminus of ELKS. Genetic ablation of Rab6B leads to a loss of synaptic vesicles and active zone material from nerve terminals together with an accumulation of uncaptured vesicles throughout the axon. ELKS1 is a large coiled coil protein that is localized to nerve terminals and is broadly distributed within them.
Strikingly, conditional neuronal knockout of ELKS1 phenocopies Rab6B ablation with a similar loss of presynaptic material in the bouton and cargo accumulation in the axon, accompanied by loss of synaptic Rab6. Reversing these phenotypes by ELKS1 rescue requires Rab6-binding of ELKS1. Finally, mistargeting of ELKS1 or Rab6 onto mitochondria leads to aberrant cargo capture, indicating that ELKS1 and Rab6 are sufficient to mediate capture.

I propose the new model that ELKS1 acts as a presynaptic cargo tether, capturing Rab6-labeled precursor vesicles transported from the Golgi apparatus. This mechanism is important for regulating the assembly and maintenance of presynaptic nerve terminals.
ACKNOWLEDGEMENTS

I am dedicating my doctoral dissertation to my little niece, Fanni, my mini-me, who at a very early age already shows signs of a strong-willed, persistent and inquisitive nature with a love for comedy. I am also dedicating it to my nieces, Eszter and Sarolta, and to my nephew, Janos, who have been sources of so much joy and happiness in my life.

I would like to thank Hobbes, my best friend, my partner in crime, my source of imagination. You have been endlessly patient, loving and supportive, often the voice of reason, and source of my sanity and comic relief. I could not have done this without you.

I would like to thank my physicist brother, Gábor, the smart one in the family, with whom I can have the most mind-bending philosophical conversations; my logician brother, Mózes, who I cannot ever beat in five-square; my humanist sister, Móni, who keeps our family connected to its roots; my ecclesiastic father, whose intelligence is an inspiration; and my loving mother, whose common sense has pulled the family through a lot of difficult times.

I would like to thank my friends and colleagues, who have been an incredible support both mentally and practically. Bea, Sophie, Peti, Clare, Jonah, Nat, Tanne, Marsha, Betty, Stephanie: you make my life richer and happier! People of the Kaeser lab, both past and present members: you made and make this lab a fun, collaborative, engaging and productive place to be! It has been a true privilege getting to know you. I have grown into a better scientist and person because of you. I will cherish both the good and difficult times we have had. From the bottom of my heart: thank you!

I would like to give a special word of gratitude to my mentor: Dr. Pascal Kaeser, who has been an inspiration, a scientific force and a challenge in the best ways. Thank you for taking me on and pulling and pushing and supporting me through all these years!

Lastly, but certainly not the least, I would like to thank the members of my dissertation advisory committee, Dr. Thomas Schwarz (chairing both my DAC and the thesis examination), Dr. Matthew Pecot, Dr. Samara Reck-Peterson and Dr. John Flanagan, whose scientific guidance throughout my graduate years was an invaluable contribution to developing an exciting project. I also would like to thank my thesis examiners, Dr. David Van Vactor, Dr. Wade Regehr, and Dr. Angela Ho, who took the time to read and provide insightful feedback on my dissertation.
TABLE OF CONTENTS

Abstract ............................................................................................................................... iii

Acknowledgments .................................................................................................................. v

Table of Contents .................................................................................................................. vi

CHAPTER ONE: Introduction ................................................................................................. 1

CHAPTER TWO: Small GTPase Rab6 accompanies trafficking organelles in the axon and
localizes to presynaptic terminals ....................................................................................... 15

CHAPTER THREE: The presynaptic coiled coil protein ELKS captures Rab6-marked synaptic
transport cargos .................................................................................................................... 38

CHAPTER FOUR: Are there broader cell biological roles of ELKS beyond the synapse? ........ 69

CHAPTER FIVE: Discussion .................................................................................................. 85

CHAPTER SIX: Materials and Methods .................................................................................. 101

APPENDIX ONE: Reagent Tables .......................................................................................... 122

APPENDIX TWO: Supplemental Figures ............................................................................... 126

References ............................................................................................................................ 134
CHAPTER ONE:

INTRODUCTION
Rapid and precise information processing in the brain is mediated through chemical transmission between neurons at their synaptic sites. Most presynaptic terminals are formed along axonal branches that are carefully aligned with an equally specialized density on the postsynaptic membrane of another neuron, containing neurotransmitter receptors and signaling apparatus (Südhof, 2017; Van Vactor and Sigrist, 2017; Yogev and Shen, 2014). A typical neuron maintains thousands of synaptic connections while retaining the flexibility to adjust synapse size or form new ones on demand. The maintenance of such an enormous and plastic synaptic network is a daunting challenge for neurons, requiring fast, efficient and tightly controlled intracellular transport. My thesis focuses on the regulation of presynaptic transport to axonal nerve terminals.

To maintain synaptic connections, neurons have to efficiently deliver presynaptic components from the soma to specific presynaptic regions over very long axonal distances (Kurshan and Shen, 2019). The majority of presynaptic proteins are thought to be delivered from the Golgi apparatus on precursor transport vesicles, the recruitment of which is crucial for the maintenance of presynaptic terminals and ultimately for healthy signal transduction (Ziv, 2018). But how do transport vesicles know where to stop along the axon? Dysfunctional axonal transport is a source of a multitude of neurological disorders (Franker and Hoogenraad, 2013; Goldstein and Das, 2018), yet we still know very little about its regulation.

Material generated in the soma are sorted onto vesicular cargo at the Golgi apparatus, transported by motor complexes along cytoskeletal tracks, captured at and integrated into the appropriate target site, then cycled back to the Golgi or to degradation sites via retrograde trafficking (Bonifacino and Glick, 2004; Feyder et al., 2015; Makaraci and Kim, 2018). Both the contents and the destinations of cargos vary greatly, thus to ensure efficient cargo delivery, cells utilize sophisticated systems to match cargo with their appropriate target compartments. Most types of transport cargos are packaged at the Golgi with unique sets of small GTPases, SNAREs, adaptors proteins and motors that both facilitate the movement of cargo and serve as
recognition signals for target sites. Various cargo-tethering complexes are stationed at acceptor compartments that recognize and capture transport cargo (Cai et al., 2007; Witkos and Lowe, 2016; Yu and Hughson, 2010). Cargo-capture is thought to occur in multiple stages, where the longest proteins from the tethering complex make the initial contact with the motor-attached cargo. These tethers frequently have long coiled coils domains forming rod-like shapes that could reach up to 100 nm to facilitate easy cargo capturing. The initial contact between cargo and tether is thought to be highly transient, after which the cargo is passed through a series of further tethering steps, pulling it closer to the target site (Gillingham and Munro, 2019). Surprisingly, such tethering mechanisms have remained largely elusive for presynaptic protein transport.

**Organization of the presynaptic terminal**

Presynaptic terminals are highly specialized cellular compartments along axonal branches with the sole purpose of establishing and maintaining sites of communication with neighboring cells (Mochida, 2015). The major constituents of presynaptic terminals are synaptic vesicles that are continuously cycled between exo- and endocytosis. These neurotransmitter-filled vesicles are at the core of neuronal communication. The primary function of the presynaptic bouton is to release the contents of these vesicles into the synaptic cleft. The released neurotransmitters stimulate the postsynaptic neuron by binding and activating receptors on the postsynaptic membrane. After vesicle exocytosis, endocytotic complexes retrieved vesicle components from the plasma membrane for either reuse or degradation, thus completing the vesicle cycle (Kokotos and Cousin, 2015). The presynaptic boutons contain a heterogenous mixture of vesicles in various states, including maturated, primed or retrieved, thus a continuous exchange of places is required to move vesicles from one stage to the next (Jahn, 2003; Rizzoli, 2014). Presynaptic boutons are unusual in that they define their boundaries not by a membrane but by the agglutination of vesicles and scaffolding proteins into
a liquid phase, which enables flexible fluid-like motion within an otherwise cramped presynaptic bouton (Milovanovic et al., 2018).

To ensure the proper cycling of synaptic vesicles, presynaptic boutons are densely populated with a multitude of protein complexes each with their designated function: synaptic vesicle proteins, active zone complexes, ion channels, fusion machineries, endocytotic complexes, adhesion molecules, cytoskeletal proteins and many others (Wilhelm et al., 2014). Adhesion molecules and cytoskeletal proteins, for example, are essential for matching and coupling the appropriate pre- and postsynaptic membranes (Südhof, 2017; Van Vactor and Sigrist, 2017; Yogev and Shen, 2014), while ion channels inserted in the plasma membrane determine membrane potentials during resting state and stimulation (Kaeser and Regehr, 2014).

The active zone is a special interest in my thesis, since multiple members of this complex have been implicated in synapse assembly and trafficking. The active zone is a highly specialized proteinaceous region near the plasma membrane that is positioned between the synaptic vesicles and the vesicle fusion sites on the membrane (Mochida, 2015; Petzoldt et al., 2016; Schoch and Gundelfinger, 2006; Südhof, 2012; Ziv, 2018). Its primary function is the spatial and temporal organization of vesicle fusion, facilitating fast and synchronous coupling between excitation and release, and ultimately modulating synaptic strength and presynaptic plasticity (Kaeser and Regehr, 2014). It is composed of seven protein families, including Bassoon, Piccolo, Munc-13, RIM, RIM-BP, ELKS and liprin-α, all of which are large scaffolding proteins. Components of the active zone interact extensively among themselves forming a main platform near the plasma membrane of the presynaptic bouton, to which they recruit the vesicle release machinery.

Prior to exocytosis, synaptic vesicles are stored in two major states: a resting pool that requires some maturation before exocytosis and a readily releasable pool that fuses easily with the plasma membrane when prompted (Crawford and Kavalali, 2015). The active zone maintains the readily releasable vesicle pools, docks a fraction of them onto the plasma
membrane and recruits SNAREs, voltage gated calcium channels and other calcium-sensors near the docked vesicle (Kaeser and Regehr, 2017). When an electric current of a stimulated neuron reaches the membrane of a presynaptic bouton, calcium channels open and flood the vicinity of the docked vesicle with calcium. Calcium-sensors on and near the vesicle subsequently trigger a cascade of events that result in the fusion of the vesicle with the plasma membrane and the release of its contents into the synaptic cleft (Jahn, 2003; Rizzoli, 2014).

**Axonal trafficking of precursor vesicles for presynaptic maintenance**

All proteins have a half-life and eventually require recycling and replenishment. Synaptic proteins are no exception (Ziv, 2018). There are multiple reports suggesting that there is local protein synthesis in synapses (Alvarez-Castelao and Schuman, 2015; Hafner et al., 2019; Jung et al., 2012; Li et al., 2015; Scarnati et al., 2018), but this likely serves to replenish proteins with high turnover rate that are easier to synthesize. Many proteins in the presynaptic bouton are large molecules with transmembrane regions or membrane associations and would pose a challenge to synthesize without sophisticated sorting compartments like the endoplasmic reticulum (ER) or the Golgi apparatus. The molecular composition of the presynaptic terminal has been investigated in great detail, and while parts of translational machineries have been identified, currently there is no evidence that the ER or the Golgi exist in their full capacity at presynaptic terminals (Wilhelm et al., 2014).

Most presynaptic proteins are thought to be trafficked from the Golgi apparatus on vesicle cargo attached to a motor complex that walks on microtubule cables over long axonal distances (Bury and Sabo, 2016; Goldstein et al., 2008; Maeder et al., 2014a; Schwarz, 2013). Most cell types have a morphology that allows newly synthesized material to be accessible relatively quickly via cytoskeletal transport. However, the enormous distances, at which synapses are frequently positioned on axons away from the soma, tests the limits of cellular
transport. The diameter of the neuronal soma is between 10-40 microns on average, while axons often reach thousands of microns in length. Thus, efficient axonal transport is crucial for the assembly and maintenance of synapses (Franker and Hoogenraad, 2013; Goldstein and Das, 2018).

Multiple types of transport packets have been identified in axonal transport. It is known, for example that mitochondria, lysosomes, autophagosomes, mRNA or cytoskeletal segments form their own cargo with unique sets of molecular motors, Rab GTPases, adaptors and other proteins to ensure targeting and movement of the cargo (Maday et al., 2014). Despite the fact that a multitude of protein complexes exist in the presynaptic terminal, it is still poorly understood how they get there. We currently know very little about the exact compositions of presynaptic protein cargos, which proteins are trafficked together, how they are targeted to the presynaptic terminal, or how they are incorporated into the terminal.

Current transport models generally group cargos that carry presynaptic proteins into two major categories: synaptic vesicle proteins (SVPs) or Piccolo-Bassoon transport vesicles (PTVs), both of which are thought to play a critical role in synaptogenesis and presynaptic maintenance (Bury and Sabo, 2016; Chia et al., 2013; Goldstein et al., 2008; Gondré-Lewis et al., 2012; Gundelfinger et al., 2015; Schlager and Hoogenraad, 2009; Ziv, 2018; Ziv and Garner, 2004). The main distinction between these two is thought to be their content, where PTVs are large dense-core vesicles (DCVs) that carry components of the presynaptic active zone (Ahmari et al., 2000; Dresbach et al., 2006; Zhai et al., 2001), while SVPs are smaller clear vesicles that carry vesicle proteins (Hall and Hedgecock, 1991; Okada et al., 1995; Pack-Chung et al., 2007). However, there are conflicting data on how clear the boundaries are between these two cargo types. Piccolo and Bassoon, two of the largest active zone proteins were initially shown to traffic together from the Golgi apparatus travelling on 80-nm dense-core vesicles, which were subsequently shown to contain additional active zone proteins (Shapira et al., 2003). These PTV packets were thought to contain a heterogeneous mixture of active zone components, and
given the relatively low copy number of active zone proteins per synapse, a couple of these packets would be enough for replenishment. Maas et al (2012) later showed that active zone proteins do not all occupy a common transport cargo. While Bassoon, Piccolo and ELKS2 co-localized with trans-Golgi markers and exited the trans-Golgi network on a common vesicle cargo, Munc13-1 trafficked separately (Maas et al., 2012). On the other hand, synaptic vesicle proteins, regulated by the anterograde kinesin-3 motor protein Unc-104/KIF1A and KIF1B (Hall and Hedgecock, 1991; Okada et al., 1995; Pack-Chung et al., 2007) were suggested to be trafficked separately from PTVs. These reports, however, were challenged by showing that active zone and synaptic vesicle proteins can be transported together on the same cargo (Bury and Sabo, 2011; Lipton et al., 2018; Wu et al., 2013). The regulators of the post-Golgi trafficking of PTVs and SVPs are poorly understood.

Interestingly, some of the active zone proteins themselves have been implicated in regulating synaptic assembly and axonal trafficking in addition to their function at the active zone. Of these, two of the active zone proteins stand out as potential organizers of synaptic trafficking, the protein families of liprin-α and ELKS. Genetic studies in C. elegans have revealed that SYD-2/liprin-α is one of the first synaptic organizers to arrive to the presynaptic site and is a key regulator of active zone assembly (Edwards et al., 2015; Zhen and Jin, 1999), the removal of which leads to smaller synaptic clusters (Kittelmann et al., 2013; Stigloher et al., 2011). SYD-2/liprin-α subsequently recruits additional scaffolding proteins (Dai et al., 2006; Patel et al., 2006). Furthermore, both the Drosophila (Miller et al., 2005) and the mammalian (Shin et al., 2003) liprin-α have been shown to regulate KIF1A activity, a kinesin motor that is important for the trafficking of synaptic vesicle proteins (Hall and Hedgecock, 1991; Okada et al., 1995).

Genetic analysis of Drosophila synaptogenesis showed similar results, where SYD-2/liprin-α together with SYD-1 was found to act as a nucleation site for synapses (Fouquet et al., 2009; Owald et al., 2010), followed by the recruitment of Unc13, the fly homolog of Munc-13 (Böhme et al., 2016) and Bruchpilot, the fly homolog of ELKS (Fouquet et al., 2009).
ELKS and liprin-α protein families share interesting structural and functional homologies. Both ELKS (Monier et al., 2002) and liprin-α (Schoch et al., 2002) contain large coiled coil domains and both interact with a small GTPase. Mammalian ELKS was originally discovered as a Rab6 interacting protein (Monier et al., 2002), while the C. elegans SYD-2/liprin-α is thought to interact with Arl-8 small GTPase (Klassen et al., 2010; Maeder et al., 2014b; Wu et al., 2013).

The large coiled coil protein ELKS as a multifunctional presynaptic vesicle tether

Mammals express two ELKS genes (also called Rab6IP2, ERC, and CAST), ERC1 and ERC2 that give rise to eight isoforms. ELKS-α and ELKS-β variants are generated via alternative start sites, while ELKS-A and ELKS-B variants are generated via alternative splicing. Protein products of ERC1 gene are ELKS1αA, ELKS1αB, ELKS1βA, ELKS1βB; protein products of ERC2 gene are ELKS2αA, ELKS2αB, ELKS2βA, ELKS2βB. The main isoforms in the mammalian brain are ELKS1αB and ELKS2αB, both highly expressed in the central nervous system and are enriched in presynaptic terminals in neurons (Deguchi-Tawarada et al., 2004; Kaeser and Held, 2018; Kaeser et al., 2009; Liu et al., 2014; Monier et al., 2002)

The significance of ELKS in presynaptic function was demonstrated by genetic studies, where removal of the major isoforms, ELKS1α or ELKS2α disrupted synaptic transmission without altering presynaptic ultrastructures (Held et al., 2016; Kaeser et al., 2009; Kiyonaka et al., 2012; Liu et al., 2014). The simultaneous removal of ELKS1α and ELKS2α resulted in a 50% decrease in neurotransmission together with a 30% decrease in presynaptic calcium-influx and with a reduced release probability in inhibitory synapses (Liu et al., 2014). In excitatory neurons, the simultaneous of removal ELKS1α and ELKS1α 2α decreased the size of the readily releasable vesicle pools, while the release probability and the calcium-influx remained unaffected (Held et al., 2016). The difference in the effects in excitatory and inhibitory synapses suggests a specialization in ELKS function across different synapse (Kaeser and Held, 2018).
The C. elegans ELKS, which has a high homology to mammalian ELKS, showed a similar disruption of the worm synaptic transmission (Deken, 2005).

In Drosophila synapses, Bruchpilot, the fly homolog of the mammalian ELKS, has been shown to play a more central role in both structural assembly and neurotransmission than the vertebrate ELKS. Removal of Bruchpilot from fly neurons resulted in a dramatic loss of T-bars, the fly equivalent of active zones, and a major inhibition in synaptic transmission (Kittel et al., 2006; Matkovic et al., 2013). It is important to note, however, that only a short N-terminal segment of the Bruchpilot has homology with the vertebrate ELKS (Wagh et al., 2006).

The importance of ELKS in tethering and docking vesicles to vesicle fusion sites in presynaptic terminals has been demonstrated (Wang et al., 2016). Removal of ELKS1/2α together with another active zone protein family, RIM1/2, led to a complete loss of docked vesicles in presynaptic boutons (Wang et al., 2016). The removal of either of these protein families alone could not reproduce the magnitude of this effect, suggesting that vesicle docking depends on the collaborative action of these two protein families (Wang et al., 2016). While the exact mechanism of its action is not yet clear, these data suggest the role of ELKS as a vesicle tether in presynaptic terminals.

To date, functions of ELKS were almost exclusively evaluated in context of presynaptic vesicle exocytosis. ELKS, however, was originally discovered as a Rab6-binding protein from a yeast two-hybrid screen of a mouse embryo cDNA library, which was later confirmed in brain pulldown assays (Monier et al., 2002). Rab6, a mainly Golgi-resident small GTPase, is an important regulator in cellular trafficking, yet the physiological relevance of its interaction to ELKS have not been explored in neuronal context. Interestingly, ELKS shares striking structural homologies with the Golgi-resident vesicle tethers, the Golgins (Monier et al., 2002) that also bind Rab6 (Munro, 2011). Despite structurally looking similar to Golgins and binding to Golgi-resident Rab6, ELKS has little expression in the Golgi both in neurons (Kaeser et al., 2009; Liu et al., 2014) and in nonneuronal cells (Grigoriev et al., 2007; Lansbergen et al., 2006).
While there is little known about the function of ELKS in nonneuronal systems, there are reports suggesting that ELKS1 is part of an adhesion complex for microtubule plus-ends and dock Golgi-derived secretory vesicles at the cell edge (Astro et al., 2014; Fourriere et al., 2019; Grigoriev et al., 2007, 2011; Lansbergen et al., 2006; Patwardhan et al., 2017). In HeLa cells and fibroblasts, it accumulates at the growing cell edge that is not yet in contact with other cells (Astro et al., 2014; Lansbergen et al., 2006). ELKS was shown to directly interact with microtubule associating LL5β (Lansbergen et al., 2006) and MICAL3 (Grigoriev et al., 2011), forming a complex that also includes CLASPs (Grigoriev et al., 2007), liprin-α1 (van der Vaart et al., 2013) and other tethers (Astro et al., 2014).

Based on all the previously published data on ELKS and data from our lab, I hypothesized that ELKS acts as a multifunctional vesicle tether in the presynaptic terminal that includes tethering Golgi-derived synaptic transport cargos arriving to the terminal.

**Presynaptic small GTPases as cargo labels in synaptic trafficking**

Identifying molecules that are responsible for the accurate targeting of vesicle cargos to appropriate acceptor compartments has been a long-standing interest in the field of cellular transport. The superfamily of small GTPases, monomeric small nucleotide binding proteins are thought of as the most likely candidates to act as cargo labels given their enormous variety with approximately 150 family members. This superfamily is divided into additional subgroups: Ras, Rab, Arf, Rho and Ran, of which the Rab subfamily is the largest with approximately 70 RAB genes identified in humans (Wandinger-Ness and Zerial, 2014).

Small GTPases serve as molecular switches in a wide range of cellular functions, from which the Rab and Arf subfamilies are tasked with regulating vesicle dynamics in transport, fusion and endocytosis. Rabs and Arfs exist in two states: a GDP-bound inactive form and a GTP-bound active form. In the active form, Rabs and Arfs associate with membranes of various
organelles, including transport vesicles. As the GTP is hydrolyzed to GDP, the small GTPase becomes inactive and dissociates from the membrane. Their cycling between active and inactive states are assisted by other proteins, namely GDIs, GAPs, and GEFs. GDIs are guanine nucleotide dissociation inhibitors that prevent GTP from dissociating from the GTPase. GAPs are guanine nucleotide activating proteins that speed up the GTP-hydrolysis process, since the intrinsic hydrolysis activity of GTPases is otherwise slow. GEFs, guanine nucleotide exchange factors are proteins that assist the final step in the GTPase cycle after hydrolysis by facilitating the exchange of GDP for a new GTP molecule on the GTPase (Stenmark, 2009).

Besides acting as molecular switches for cargo motility, Rabs and Arfs are also thought to serve as recognition signals for appropriate tethering complexes on target compartments (Hutagalung and Novick, 2011; Mima, 2018; Pfeffer, 2013; Pylypenko et al., 2018; Stenmark, 2009; Zerial and McBride, 2001). Both Rab and Arf protein families have been implicated in axonal transport and were shown to be an underlying cause for many neurological disorders (D’Adamo et al., 2014; Kiral et al., 2018; Veleri et al., 2018; Zhen and Stenmark, 2015), yet their exact function in axonal transport is poorly understood.

The most abundant Rab protein in synapses is Rab3 (Wilhelm et al., 2014) that is exclusively expressed in neurons. The Rab3 primarily localizes to presynaptic terminals, where the majority of synaptic vesicles contain multiple copies of Rab3 (Takamori et al., 2006). Given the relative abundance of Rab3 on synaptic vesicles, its removal would be expected to lead to a massive decrease in bouton size if it was responsible for presynaptic transport. While Rab3 was shown to play an important role in vesicle biogenesis in chromaffin cells (Schonn et al., 2010), the simultaneous genetic removal of all four mammalian Rab3 isoforms (Rab3A, B, C, D) from neurons did not lead to any obvious transport defects or structural alterations of the presynaptic bouton (Schluter et al., 2004). In Drosophila, loss of the singular Rab3 led to defects in active zone organization and mild functional defects (Graf et al., 2009). Furthermore, there is no significant Rab3 expression in the Golgi apparatus (Fischer von Mollard et al., 1990; Matteoli at
al., 1991), which makes it an unlikely candidate for Golgi-derived cargo targeting. The most likely function of Rab3 is the regulation of vesicle dynamics locally at the synapse (Graf et al., 2009). The active zone protein RIM is a major interactor of the active GTP-bound Rab3 (the vesicle associated state). In fact, RIM is thought to mediate synaptic vesicle priming partly via a direct interaction with the active Rab3 (Dulubova et al., 2005; Kaeser et al., 2011).

Another small GTPase implicated in presynaptic function is the Arf GTPase Arl-8. In C. elegans neurons, Arl-8 was suggested to act as a marker in presynaptic trafficking (Maeder et al., 2014a; Wu et al., 2013) and was proposed to be recruited to presynaptic terminals via interaction with SYD-2/liprin-α, the worm homolog of the mammalian liprin-α. Interestingly, the loss of Arl-8 also caused abnormal vesicle distribution in C. elegans axon (Wu et al., 2013).

**Rab6 GTPase as a marker for secretory vesicles**

Proteomic screens identified a number of other potentially synaptic Rabs (Morciano et al., 2009; Takamori et al., 2006; Wilhelm et al., 2014), from which Rab6 stands out as the most promising candidate to mediate Golgi-derived synaptic transport. RAB6 is one of only five RAB genes that is evolutionarily conserved from yeast to humans (Echard et al., 2000; Heffernan and Simpson, 2014; Opdam et al., 2000). Mammals have two major isoforms, Rab6A and B, and the primate specific Rab6C. Rab6A is thought to be ubiquitous (Schlager et al., 2010), while Rab6B is brain specific. Rab6 is highly enriched in the central nervous system (Opdam et al., 2000; Schlager et al., 2010; Short et al., 2002; Zahraoui et al., 1989).

The majority of published data on Rab6 comes from work in nonneuronal systems, consequently information on neuronal Rab6 is very limited. Rab6 is thought to serve as a major membrane anchor for Golgins and other vesicle tethers, like p150glued and Bicaudal-D, thus mediating the retrograde flow of cargo between endosomes to trans-Golgi and from cis-Golgi to ER. At the trans-Golgi, the large coiled coil Golgin vesicle tethers are recruited to the Golgi
membrane via their C-terminal GRIP domain binding to Rab6 or Arl-1, while their N-terminus extends far into the cytosol to capture vesicles. The capturing happens via the Golgin tethers directly binding cargo-resident Rabs, coat proteins, or lipids on the surface of the cargo (Cheung and Pfeffer, 2016; Witkos and Lowe, 2016).

While most studies suggest that Rab6 primarily resides and functions in the Golgi apparatus, a few studies showed that Rab6 also accompanies post-Golgi secretory vesicles in nonneuronal cells (Coutelis and Ephrussi, 2007; Grigoriev et al., 2007; Matanis et al., 2002; Miserey-Lenkei et al., 2017; Schlager et al., 2010, 2014; Seifert et al., 2015; Wanschers et al., 2007; White et al., 1999). In HeLa cells, both overexpressed (Seifert et al., 2015; Wanschers et al., 2007; White et al., 1999) and endogenous (Grigoriev et al., 2007; Matanis et al., 2002) Rab6 was shown to be in the cell periphery beside localizing to the Golgi. In live HeLa cells (White et al., 1999) and MRC5-SV cells (Schlager et al., 2014), fluorescently tagged Rab6 was observed to be highly mobile trafficking to the cell periphery. In Drosophila oocytes, Rab6 was observed on secretory vesicles, and its genetic removal led to a loss of Syntaxin-1 from the plasma membrane and defects in vesicle exocytosis (Coutelis and Ephrussi, 2007).

In respect to localization in neurons, data lacking appropriate Rab6 knockout controls are either indirect or inconclusive; however, they do suggest that Rab6 may not be restricted to the Golgi apparatus in neuronal cell types (Chan et al., 2011; Deretic and Papermaster, 1993; Gumy et al., 2017; Iwanami et al., 2016; Jasmin et al., 1992; Kobayashi et al., 2016; Shetty et al., 1998; Tixier-Vidal et al., 1993; Tortosa et al., 2017). In Drosophila retinal rod photoreceptors, Rab6 was shown to localize on post-Golgi vesicles and regulate anterograde transport of rhodopsin (Deretic et al., 1993, Iwanami et al., 2016; Shetty et al., 1998). Furthermore, in a systematic analysis of Drosophila Rab GTPases, Rab6 was identified as one of many Rabs with possible synaptic function (Chan et al., 2011). In a high-throughput proteomic screen to identify a comprehensive list of presynaptic proteins, Rab6 was listed as the second most abundant Rab after Rab3 (Wilhelm et al., 2014). Rab6 was observed on trafficking organelles in mouse
inner retinas, where its motility increased with increased cell activity after exposing the retina to light (Huang et al., 2009). In primary cultured hippocampal neurons, overexpressed Rab6 exhibited a punctate pattern in axonal segments (Schlager et al., 2014) and was highly mobile in both anterograde and retrograde directions (Gumy et al., 2017; Tortosa et al., 2017). Most interestingly, Rab6 was suggested to bind ELKS in both neuronal (Monier et al., 2002) and nonneuronal cells (Kobayashi et al., 2016); furthermore, in multiple nonneuronal mammalian cells, Rab6-positive cargos were observed to traffic to ELKS-enriched regions of the cell cortex (Fourriere et al., 2019; Grigoriev et al., 2007). Taken these together, I hypothesized that Rab6 could be a marker for presynaptic protein cargos.

My thesis is built on one overarching question: What mechanisms mediate the capturing of precursor transport vesicles at the presynaptic bouton? I approached this from two angles simultaneously by asking (1) what vesicle-associated molecules may serve as cargo labels in presynaptic transport, and (2) which presynaptic proteins act as tethering complexes to facilitate cargo capture.
CHAPTER TWO:
Small GTPase Rab6 accompanies trafficking organelles in the axon

Manuscript in preparation

to publish data from chapter two in combination with data in chapter three

Concepts and experimental designs are by H.N. and P.K. Experimental procedures, analyses, and presentations were carried out by H.N. I would like to thank L. Kershberg for providing the cortical synaptosome samples (Figure 1), L. Bickford for cloning some of the constructs (Figure 5), and the Harvard EM Facility and the Harvard Neuro Imaging Core Facility for their assistance.
While the majority of previous reports suggest Rab6 to primarily function as a Golgi-resident protein, where it regulates retrograde vesicle trafficking and Golgi membrane integrity, a number of studies have suggested its presence on post-Golgi anterograde secretory vesicles (Schlager et al., 2010, 2014; Seifert et al., 2015). Rab6 has been proposed to bind to the presynaptic coiled coil protein ELKS (Monier et al., 2002), and was implicated in rhodopsin trafficking in retinal cells (Iwanami et al., 2016), yet we know very little about its neuronal localization and function. In this chapter, I aimed to characterize neuronal Rab6 and its interaction with ELKS, using various biochemical and molecular tools. I hypothesized that Rab6 accompanies post-Golgi transport vesicles in neurons.

Rab6 is highly expressed in neurons and associates with presynaptic secretory vesicles

Based on previous reports that Rab6 is enriched in the central nervous system and that it binds to ELKS (Monier et al., 2002), I hypothesized that Rab6 may in part be present in nerve terminals. I first used Rab6 antibodies to assess Rab6 expression in homogenates of various mouse tissues harvested at various developmental time points (postnatal days P1-P90). Rab6 was enriched in mouse brain relative to other tissues, and its expression increased from P1 to P90 (Fig. 1A). In collaboration with a colleague, cortical brain lysates from adult mice were fractionated to prepare synaptosome samples (Fig. 1B). I visualized proteins in synaptosome fractions using Western blotting and found that Rab6B was highly enriched in synaptosomes (Fig. 1C), similar to other synaptic proteins, while the Golgi marker GM130 failed to enrich.

Since Rab GTPases are known to associate with membranes of either the target compartment or the membrane of a vesicular cargo (Fig. 1D), I wanted know if Rab6 can associate with secretory vesicles in neurons. This was especially important, since the majority of publications report Rab6 to primarily associate with the Golgi membrane. I subjected adult
mouse brain tissue to a vesicle fractionation protocol that primarily fractionated small vesicles from neuronal branches, including synaptic vesicles from synapse and small transport vesicle from axonal regions. The membranous compartments and vesicles from the soma were largely excluded (Fig. 1E). I found that Rab6B enriched in the same fractionates as synaptic vesicle proteins, while the Golgi marker (GM130) or cytoskeletal markers did not fractionate (Fig. 1F).

Next, I examined where exactly the vesicle-bound Rab6 localizes in neuronal branches. Rab6, like other Rab GTPases, regulate their association with vesicles and other membranes by cycling between active (GTP-bound) and inactive (GDP-bound) states (Fig. 1E). Rabs are attached to membranes when they are bound to a GTP molecule. Once the GTP is hydrolyzed to GDP, the Rab falls off the membrane (Stenmark, 2009). These GTP- and GDP-bound states can be reliably mimicked with specific point mutations for Rab6. I cultured hippocampal neurons from wild type mice and expressed Cerulean-tagged Rab6B active (Q72L, Rab6B_{QL}) or inactive (T27N, Rab6B_{TN}) point mutants using lentiviruses. Protein expression levels were comparable across conditions (Fig. 1I). Neuronal cultures were fixed at DIV 14, stained with anti-GFP antibodies (which recognize the Cerulean tag), co-stained for the active zone protein Bassoon to mark synapses, and acquired images using confocal microscopy. Constitutively active Rab6B_{QL} appeared punctate away from the neuronal soma and showed colocalization with the synaptic marker Bassoon (Figs. 1G and 1H). In contrast, the inactive Rab6B_{TN} was diffusely localized throughout neuronal branches, but not enriched in synapses. Together, these data suggest that Rab6B may at least be partially synaptic in addition to its known localization in the Golgi apparatus.
Chapter 2: Figure 1

**A**

- Brain tissue: P1, P10, P20, lung, kidney, liver, heart
- Rab6B, Rab6A/B, Synapsin-1, β-actin

**B**

- Brain homogenate
  - P1: S1
  - P2: S2
- 0.9k x g
- 12k x g
- Sucrose gradient, 140k x g
- Synaptosome

**C**

- Brain homogenate
  - P1, S1
- 0.8 M sucrose
  - P2, S2
- 1.2 M sucrose
- Synaptosome

**D**

- Rab6B-GTP: active, vesicle-bound form
  - Rab6B-Q72L = Rab6B<sub>QL</sub>
  - Constitutive active point mutant that mimicks active state

- Rab6 cycle
  - GTP
  - GAP
  - GDP
  - GEF

- Rab6B-GDP: inactive, vesicle-dissociated form
  - Rab6B-T27N = Rab6B<sub>N</sub>
  - Constitutive inactive point mutant that mimicks inactive state

**E**

- Brain homogenate
  - P1: S1
  - P2: S2
  - P2': S2'<sup>*</sup>
  - Osmotic lysis, 25k x g
  - LP4, LS4, LS5, vesicles

**F**

- Vesicle-enriched
  - S1, P2<sup>+</sup>, vesicles
  - Rab6B, Rab6A/B, Rab3A, Rabphilin, Synapsin-1, Synaptotagmin-1, Synaptophysin-1, Synaptobrevin-2

- Presynaptic
  - S1, P2<sup>+</sup>, vesicles
  - Syntaxin-1, SNAP-25, Munc-18, ELKS1, ELKS2, Liprin-α3, Synaptobrevin-2, Munc13-1, RIM1

- Other
  - Complexin-1/2, Neurofilament, β-actin, PSD-95, GluR1, VCP, GM130

**G**

- Cerulean-Rab6B<sub>QL</sub>, Bassoon, Map2, merge

**H**

- Normalized Rab6B intensity
  - Control, Rab6B<sub>QL</sub>, Rab6B<sub>N</sub>
  - **p < 0.01**

**I**

- Control, Rab6B<sub>QL</sub>, endogenous Rab6
  - β-actin
Chapter 2: Figure 1

The vesicle-bound active form of Rab6 fractionates with synaptic material

(A) Example Western blot images of various tissue homogenates harvested from developing or adult mice. P1-P90 indicates postnatal age of mice at the time of tissue harvest.

(B and C) Schematic representation (B) and example Western blot images (C) of the cortical synaptosome fractions harvested from adult (P90) mouse brains.

(D) Schematic representation of the Rab6 cycle between GTP-bound active and the GDP-bound inactive states, and the active and inactive point mutants of Rab6.

(E and F) Schematic representation (E) and example Western blot images (F) of the vesicle fractions harvested from adult (P90) mouse brains.

(G and H) Confocal images (G) and quantification (H) of wild type hippocampal neurons overexpressed with cerulean tagged Rab6B-Q72L (Rab6BQL, active mutant) or Rab6B-T27N (Rab6BTN, inactive mutant). Cerulean was visualized by immunostaining with GFP antibodies. Quantification (H) shows fluorescent intensity levels within regions of interest defined by Bassoon objects and normalized to Rab6BQL intensity levels. Rab6BQL, n = 8 fields of view / 3 independent cultures (fields of view = 211.2 μm x 211.2 μm, typically containing 1500-4500 Bassoon objects per field of view); Rab6BTN, 7/3.

(I) Western blot analysis of overexpressed Cerulean-Rab6BQL or Rab6BTN levels in hippocampal cultures.

Experiments in (Fig. 1G-I) were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test. For specifications on the primary antibodies used, see Table S2 in Appendix 1.
Presynaptic terminals have significant endogenous Rab6 expression

To assess the localization of endogenous Rab6B, I used antibody stainings and generated constitutive Rab6B knockout mice for negative controls. Rab6B knockout mice were generated by CRISPR-mediated deletion of exon2 of the Rab6B gene in embryonic stem cells (Fig. 2A). The offspring ratio of Rab6B heterozygotes showed normal Mendelian distributions (Fig. 2B), different from constitutive Rab6A knockout, which leads to embryonic lethality (Bardin et al., 2015). Deletion of exon2 removed Rab6B protein in brain homogenates as assessed by Western blotting with Rab6B specific antibodies (Fig. 2D) and its mRNA as measured by qRT-PCR (Fig. 2C). Remarkably, Rab6B knockout also removed most antibody signal for an antibody that recognizes Rab6A and Rab6B, suggesting that Rab6B is the dominant Rab6 isoform in brain (Fig. 2D).

To visualize endogenous nanoscale distribution of Rab6B, first I used confocal microscopy on fixed hippocampal cultures of Rab6B wild type (+/) or Rab6B knockout (-/-). The cultures were immunostained against a pan-Rab6 antibody that recognizes both Rab6A and Rab6B isoforms, and against Synaptophysin-1 synaptic marker, then were co-stained with the cis-Golgi marker GM130 (Fig 2E) or co-stained with neuronal branch marker Map2 (Fig. 2F). The majority of the Rab6 signal appeared in the Golgi apparatus (Fig. 2E) as was predicted from previous publications. The Golgi signal of Rab6 was accompanied by significant levels of localization in neuronal branches (Fig. 2F).

Since Rab6 is predicted to express in glial cells as well, I used STED superresolution imaging to exclude the possibility that the synaptic Rab6 signal in confocal microscopy is a bleed-through from closely positioned glial layers underneath. Primary neuronal cultures were prepared same as above, and were co-stained with Rab6B and Synaptophysin-1 antibodies.
Rab6B signal was acquired by STED superresolution microscopy, while Synaptophysin-1 signal was acquired by confocal.

I found that Rab6B antibodies labeled small punctate structures that overlapped with synaptic vesicle marker Synaptophysin-1. The superresolution imaging confirmed that the Rab6 signal indeed came from synaptic regions (Figs. 2G and 2H). Finally, the synaptic Rab6B intensities were positively correlated with levels of Synaptophysin-1 (Figs. 2I and 2J), further supporting that the Rab6B signal arises from its synaptic localization. Together, these data establish that Rab6B is localized partially to nerve terminals and associates with vesicles in addition to its known Golgi localization.
Chapter 2: Figure 2

A

Rab6B wild type (+)  E1  #  Rab6B knockout (-)  E2  #  + Cas9

CRISPR guide RNAs  0.5 kb

B

number of offspring

D

Rab6B
Rab6A/B
Synapsin-1
β-actin

C

normalized Rab6A mRNA levels
normalized Rab6B mRNA levels

E

Rab6A/B
GM130
Synaptophysin-1

F

Map2 + Synapsin-1

G

Synaptophysin-1 intensity

H

normalized Rab6B area (μm²)

I

synapses from only Rab6B^{+/+}

J

normalized Rab6B intensity vs. Synaptophysin-1 intensity

R^2 = 0.88
Chapter 2: Figure 2

Rab6 is present both in the Golgi apparatus and in nerve terminals
(A) Rab6B gene targeting strategy constitutively removing exon 2 from mouse genome.
(B) Mendelian survival analysis of the offspring of Rab6+/− mouse breeding pairs using Chi-square test. Total number of pups = 207 over 24 litters.
(C) Real-time q-PCR analysis of Rab6A and Rab6B mRNA levels harvested from Rab6B+/− and Rab6B+/+ mouse hippocampal cultures. For both genotypes, n = 3 independent cultures.
(D) Example images of fluorescent Western blots of Rab6B+/+ or Rab6B−/− brains harvested from adult (P90) littermate mice.
(E and F) Example confocal images of Rab6B+/+ and Rab6B−/− hippocampal cultures showing Rab6 localization in the neuronal soma (E) and branches (F). Hippocampal cultures were immunostained against a pan-Rab6 antibody that recognizes both Rab6A and Rab6B isoforms, and Synaptophysin-1 synaptic marker. Then cultures were co-stained with the cis-Golgi marker GM130 and imaged the neuronal soma (E) or were co-stained neuronal branch marker Map2 and imaged neuronal branches. All images were acquired using confocal microscopy.
(G-I) Example superresolution images and quantification of Rab6B+/+ and Rab6B−/− of individual hippocampal synapses. Neurons were immunostained with Rab6B or Synaptophysin-1 antibodies. Rab6B signals were acquired by STED microscopy, while Synaptophysin-1 signals were acquired by confocal microscopy. (G) Representative images of Rab6B+/+ and Rab6B−/− conditions. (H) Analysis shows normalized Rab6B intensity levels per Synaptophysin-1 objects and the average Synaptophysin-1 object sizes. Rab6B+/+, n = 93 fields of view / 3 independent cultures (fields of view = 1.5 μm x 1.5 μm, always containing exactly one Synaptophysin object per field of view); Rab6B−/−, 97/3. (I) Example images of intensity Rab6B+/+ only for showing correlation between Rab6B and Synaptophysin-1 signal intensities per synapses. Rab6B+/+ synapses, n = 401 over 3 independent cultures (average synapses sizes were between 0.2 - 2.0 μm²).

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test (Figs. 2C and 2H), Pearson’s correlation (Fig. 2J), or Chi square test (Fig 2B). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
Removal of Rab6B from neurons decreases levels of active zone materials

I next assessed the protein composition of Rab6B−/− neurons relative to controls using quantitative fluorescent Western blotting. Ablation of Rab6B was accompanied by decreases in the levels of several active zone proteins, but other presynaptic or postsynaptic proteins were unaffected (Figs. 3A and 3B). Since there was a decrease in total levels of active zone proteins, I next asked whether Rab6B removal impairs delivery and incorporation of these proteins into the active zone. I used STED superresolution microscopy of synapses as was described before (de Jong et al., 2018; Wong et al., 2018). In brief, I labeled synapses with antibodies against synaptic vesicles, the postsynaptic density marker PSD-95, and the active zone protein RIM1. I then selected synapses in which PSD-95 forms a bar aligned to one edge of the vesicle cloud, and positioned a rectangular region of interest perpendicular to the axis of the PSD-95 bar across the synapse. Within this region, I assessed the localization and the peak intensity of RIM1 signals. I found that RIM1 peak intensity was reduced in Rab6−/− synapses relative to Rab6+/+ neurons, but the remaining RIM1 signal was localized at the correct position (Figs. 3C and 3D). This finding suggests that ablation of Rab6 impairs the delivery of presynaptic material.
Chapter 2: Figure 3

A

B

C

D

(normalized protein levels)

(normalized RIM1 intensity)

(normalized PSD-95 intensity)

(distance from PSD-95 peak (µm))
Chapter 2: Figure 3

Decreased active zone levels in Rab6B knockouts

(A and B) Example images (A) and quantification (B) of fluorescent Western blots from cultured Rab6B+/+ or Rab6B−/− hippocampal neurons of littermate mice. For both genotypes, n = 3 independent cultures.

(C and D) Example images (C) and quantification (D) of Rab6B+/+ and Rab6B−/− hippocampal neurons from STED microscopy. (C) RIM1 and PSD95 signals were acquired by STED microscopy, while Synaptophysin1 signals were acquired by confocal microscopy. (D) Quantification shows normalized intensity profiles of RIM1 signals of side-view synapses. The area of line-scan was 1 μm x 0.2 μm. The distance = 0 μm was set to the PSD95 peak position, shown as a dotted line on the graph. The positive μm values indicate distances towards the Synaptophysin-1 signal. Data are shown as mean (solid line) ± SEM (shaded area). Rab6B+/+, n = 76 fields of view / 3 independent cultures (fields of view = 1.5 μm x 1.5 μm, always containing exactly one Synaptophysin object per field of view); Rab6B−/−, 77/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by one-sample Student’s t-test (Fig. 3B). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
Rab6B knockout leads to enhanced axonal cargo deposition away from synapses

I hypothesized that Rab6B is involved in the delivery of material to the presynaptic nerve terminal. I employed high-pressure freezing and chemical fixation of hippocampal neurons followed by transmission electron microscopy to assess neuronal ultrastructure. I found that the number of vesicles per bouton and the overall bouton size were somewhat decreased in Rab6B knockouts relative to controls, but vesicle docking and the length of the postsynaptic density were unaffected (Figs. 4A and 4B).

Previous studies reported that axonal delivery defects can result in aberrant accumulation of cargo in axons away from synapses (Barber et al., 2017; Miller et al., 2005). I next analyzed electron micrographs of axonal segments in Rab6B−/− neurons compared to Rab6B+/+ neurons. Remarkably, there was a ~3-fold increase in the number of small vesicles (diameters < 50 nm) accumulating outside of presynaptic boutons in neurons fixed by high pressure freezing (Figs. 4C and 4D) or classical glutaraldehyde fixation (4E and 4F). I also quantified larger vesicles that were grouped into two categories, large dense core vesicles (LDCVs, diameters between 50-100 nm) and endosomes that were not LDCVs (diameters between 50-100 nm) and found similar results (Supplemental Fig. 2A). This suggests that uncaptured vesicles are more frequently present in axons of Rab6B−/− neurons and that Rab6B influences the trafficking and capturing of different types of vesicles.

The data from Western blot and superresolution imaging (Fig. 3) together with the data from transmission electron microscopy (Fig. 4) of Rab6B−/− neurons suggest that ablation of Rab6 impairs the delivery of presynaptic material, namely the genesis, transport, and/or capture of synaptic cargo.
Chapter 2: Figure 4
Chapter 2: Figure 4

Increased number of extra-synaptic vesicles in Rab6B knockouts

(A and B) Electron micrograph images (A) and quantification (B) of boutons from high pressure frozen Rab6B^{+/+} or Rab6B^{-/-} hippocampal cultures. (B) Quantifications show the average number of all vesicles and docked vesicles per bouton and PSD length. Rab6B^{+/+}, n = 144 boutons / 2 independent cultures; Rab6B^{-/-}, 158/2.

(C and D) Electron micrograph images (C) and quantification (D) of axons from high pressure frozen Rab6B^{+/+} or Rab6B^{-/-} hippocampal cultures. (D) Quantifications show the average number of vesicles per 5 μm axonal length and shows the average axonal width. Rab6B^{+/+}, n = 207 axons / 2 independent cultures; Rab6B^{-/-}, 167/2.

(E and F) Electron micrograph images (E) and quantification (F) of axons from Rab6B^{+/+} or Rab6B^{-/-} hippocampal cultures acquired by chemical fixation. Quantification (F) shows the average number of vesicles per 5 μm axonal length and the average axonal width. Rab6B^{+/+}, n = 56 axons / 1 culture; Rab6B^{-/-}, 54/1.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test; except for the vesicle distribution analysis at 0-1000 nm distances in (Fig. 4B), which was analyzed by extra sum of squares F-test.
Rab6 binds both isoforms of presynaptic ELKS via a short C-terminal segment

I argued that if Rab6 acts as a Golgi-derived synaptic cargo marker, it would follow to look for Rab6 binding partners that reside in the presynaptic terminal. ELKS was originally discovered when searching for Rab6 interactors (Monier et al., 2002); however, the physiological relevance of their interaction has remained largely elusive.

To examine whether Rab6 binds endogenous ELKS, I used purified brain lysates from adult wild type mice and assayed them with GST-tagged Rab proteins immobilized on glutathione-Sepharose beads. I found that the active form of Rab6 (Rab6\textsubscript{AQT} and Rab6B\textsubscript{QT}) specifically interacted with both endogenous ELKS isoforms (ELKS1 and ELKS2), but not with any other presynaptic proteins (Fig. 5A). However, RIM1, another large active zone protein is a major binding partner of ELKS, so to exclude the involvement of RIM1 in the ELKS-Rab6 interaction, I repeated the above affinity assay using brain lysates from RIM1\textsuperscript{−/−} mice. I found that the removal of RIM1 did not negatively affect the interaction between ELKS and Rab6 (Fig. 5B).

To determine isoform specificity of this interaction, I used the above procedure with HEK cell lysates that expressed various ELKS isoforms (via calcium-phosphate transfection) and observed similar results (Fig. 5C), confirming that both isoforms of Rab6 bind all ELKS isoforms.

To map the Rab6-binding site on the ELKS protein sequence, I used recombinant His-tagged Rab6A (Rab6\textsubscript{AQT} or Rab6\textsubscript{ATN}) protein solutions and assayed them with GST-tagged ELKS1αB fragments immobilized on glutathione-Sepharose beads (Figs. 5D-5F). First, I identified which of the four major coiled coil domains of ELKS1 interacts with Rab6. I found that only the fourth coiled coil domain (CC\textsubscript{D}) of ELKS1αB interacted with Rab6 and the binding was GTP-specific (Fig. 5D). Using a similar approach, I further narrowed the binding site by creating
shorter fragments of ELKS1αB-CC_D and found that a 17-amino-acid region near the C-terminus of ELKS1 is responsible for Rab6 binding (Fig. 5E). This 17-amino-acid region is also found on the ELKS2 sequence.

To test if the same region is responsible for the binding of ELKS2 to Rab6, I generated ELKS1 and ELKS2 fragments (ELKS1αB$_{808-971}$ and ELKS2αB$_{765-884}$, respectively) that were homologous to each other and both contained the Rab6 binding sequence. ELKS2 binding to Rab6 was facilitated by the same sequence region in ELKS2 as in ELKS1 (Fig. 5F). Furthermore, the interactions were replicable using the native Rab6 constructs (Rab6$^{WT}$ and Rab6B$_{WT}$) in combination with non-hydrolyzable GTP or GDP analogs (Fig. 5F).
Chapter 2: Figure 5

A

<table>
<thead>
<tr>
<th>GST-Rab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
</tr>
<tr>
<td>GST</td>
</tr>
<tr>
<td>3A</td>
</tr>
<tr>
<td>6A</td>
</tr>
<tr>
<td>6B</td>
</tr>
<tr>
<td>CCa</td>
</tr>
<tr>
<td>CCb</td>
</tr>
<tr>
<td>CCo</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>GST-Rab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
</tr>
<tr>
<td>GST</td>
</tr>
<tr>
<td>3A</td>
</tr>
<tr>
<td>6A</td>
</tr>
<tr>
<td>6B</td>
</tr>
<tr>
<td>CCa</td>
</tr>
<tr>
<td>CCb</td>
</tr>
<tr>
<td>CCo</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>GST-Rab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input</td>
</tr>
<tr>
<td>GST</td>
</tr>
<tr>
<td>3A</td>
</tr>
<tr>
<td>6A</td>
</tr>
<tr>
<td>6B</td>
</tr>
<tr>
<td>CCa</td>
</tr>
<tr>
<td>CCb</td>
</tr>
<tr>
<td>CCo</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>GST-ELKS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>input</td>
</tr>
<tr>
<td>GST</td>
</tr>
<tr>
<td>6A</td>
</tr>
<tr>
<td>6Atn</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GST input</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>His-Rab</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
</tr>
<tr>
<td>6Atn</td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>GST-ELKS1αB</th>
</tr>
</thead>
<tbody>
<tr>
<td>input</td>
</tr>
<tr>
<td>GST</td>
</tr>
<tr>
<td>6A</td>
</tr>
<tr>
<td>6Atn</td>
</tr>
</tbody>
</table>

F

<table>
<thead>
<tr>
<th>GST-ELKSαB</th>
</tr>
</thead>
<tbody>
<tr>
<td>input</td>
</tr>
<tr>
<td>GST</td>
</tr>
<tr>
<td>ELKS1 808-971</td>
</tr>
<tr>
<td>ELKS2 765-884</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>His-Rab6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A wt</td>
</tr>
<tr>
<td>B wt</td>
</tr>
<tr>
<td>+GTP</td>
</tr>
<tr>
<td>+GDP</td>
</tr>
</tbody>
</table>

32
Chapter 2: Figure 5

Rab6 binds presynaptic ELKS in a GTP-dependent manner

(A and B) Example Western blot images from affinity assays using purified brain lysates as prey from adult RIM1\textsuperscript{+/+} (A) or RIM1\textsuperscript{-/-} (B) mice. GST-tagged recombinant Rab proteins were used as baits. Endogenous proteins from brain lysates trapped by GST-Rab baits were visualized by staining with various primary antibodies. For GST-Rab6A and GST-Rab3A baits, n = 3 independent repeats; for GST-Rab6B, n = 1.

(C) Western blot images and Coomassie stain of affinity purifications using HEK cell homogenates expressing HA-tagged ELKS isoforms. HA-tagged ELKS proteins (ELKS\textsubscript{1αA}, ELKS\textsubscript{1αB}, ELKS\textsubscript{1βB} and ELKS\textsubscript{2βB}) were overexpressed in HEK cells using Ca\textsuperscript{2+}-phosphate transfection. Purified HEK cell lysates were used as soluble prey and GST-tagged Rab proteins were used as baits immobilized on beads. Constitutive active and inactive forms of Rab proteins were acquired by point-mutations. Active forms: Rab3A-Q81L (3A\textsubscript{QL}), Rab6A-Q72L (6A\textsubscript{QL}), and Rab6B-Q72L (6B\textsubscript{QL}); inactive forms: Rab3A-T36N (3A\textsubscript{TN}), Rab6A-T27N (6A\textsubscript{TN}), and Rab6B-T27N (6B\textsubscript{TN}). HA-ELKS trapped by GST-Rab baits were visualized by using an HA antibody. For condition Ha-ELKS\textsubscript{1βB} + GST-Rab6A, n = 3; for all other conditions, n = 1.

(D and E) Schematic representation and Western blot images of the recombinant affinity assays to map the Rab6-binding site on ELKS1. Purified recombinant His-tagged Rab6A proteins were used as soluble prey. His-Rab6A inputs trapped by GST-ELKS1 were visualized by a T7 antibody. (D) Recombinant GST-tagged ELKS\textsubscript{1αB} fragments across the entire ELKS sequence were used as immobilized baits. The experiment was replicated in 2 independent trials. (E) Schematic representation and Western blot images of the recombinant affinity assays to narrow the Rab6-binding site on ELKS1. The fourth coiled coil domain (CC\textsubscript{D}) of ELKS\textsubscript{1αB} was further fragmented and used as immobilized baits. The experiment was replicated in 3 independent trials.

(F) Western blot images of recombinant affinity assays showing that ELKS2 also binds Rab6 with a sequence region (ELKS\textsubscript{2765-884}) that is homologous to the Rab6-binding region of ELKS1 (ELKS\textsubscript{1αB\textsubscript{808-971}}). GST-tagged ELKS\textsubscript{1808-971} or ELKS\textsubscript{2765-884} protein fragments were used as immobilized baits. For prey, purified recombinant His tagged Rab6A or Rab6B point-mutant proteins (QL = active point mutant, TN = inactive point mutant) were used as described in Fig 3A, or recombinant His-Rab6\textsubscript{WT} proteins were used in combination with GMP-PNP or GDP. His-Rab6 inputs trapped by GST-ELKS1 were visualized by a T7 antibody. For specifications on the primary antibodies used, see Table S2 in Appendix 1.
The direct interaction between ELKS and Rab6 determines Rab6 localization in HEK cells

To test the specificity of the binding site and exclude the possibility that other regions of ELKS may contribute to Rab6-binding, I generated an HA-tagged ELKS1αB construct that lacked only the 17-amino-acid segment identified previously. The HA-tagged ELKS1 constructs were expressed in HEK cells and assayed with GST-tagged Rab6 baits as described before (Fig. 6A and 6B). The mutant ELKS1αBΔ955-971 construct was unable to bind either Rab6 isoform, confirming the specificity of the binding site (Fig. 6B).

To confirm that ELKS1 affects Rab6 localization and not the other way around, I simultaneously expressed HA-ELKS1 (wild type or mutant) and Cerulean-Rab6B (active or inactive) in HEK cells in various combinations (Fig. 6C and 6D). There is very little endogenous ELKS1 in HEK cells, and overexpressed ELKS1 frequently localizes to the cell periphery. On the other hand, based on previous findings Rab6 was predicted to remain largely in the Golgi. HEK cells were transfected with either Cerulean tagged Rab6BQL (6BQL) or Rab6BTN (6BTN), and simultaneously co-transfected with HA-tagged ELKS1αB or ELKS1αBΔ955-971. To visualize overexpressed Cerulean-Rab6B, HEK cells were immunostained against an anti-GFP antibody. To visualize overexpressed HA-ELKS1, HEK cells were immunostained against an anti-HA antibody. All signals were acquired by confocal microscopy. The levels of protein overexpressions were monitored by Western blotting (Fig. 6E).

I found that both forms of overexpressed HA-ELKS1 (ELKS1αB or ELKS1αBΔ955-971) localized to the cell periphery independent of conditions (Fig. 6C). Meanwhile Rab6B localization was heavily influenced by which form of ELKS1 was present. When the active Rab6BQL was co-transfected with the full-length HA-ELKS1αB (ELKS1αB + 6BQL), Rab6 accumulated into large aggregates near the cell periphery. This Rab6-aggregation was almost entirely absent in the other two conditions, with either the inactive Rab6BTN or with the mutant
ELKS1αB^{495-971} (Figs. 6C and 6D). This finding confirms several previous observations that ELKS1 only interacts with active Rab6 via its C-terminal segment and their interaction determines the subcellular localization of Rab6, indicating that ELKS1 actively recruits Rab6 cargo.

**Conclusion**

I found that while Rab6 largely resides at the Golgi in neurons, it also has significant localization in axonal branches and in presynaptic terminals. It associates with small vesicles in neuronal branches and its synaptic enrichment is driven by its active vesicle-associated state. Furthermore, only the active vesicle-associated form of Rab6 binds to ELKS near its C-terminal segment, similarly to Rab6 binding Golgin tethers. The removal of Rab6B from mouse hippocampal neurons caused vesicle tethering defects, with increased extra-synaptic vesicle deposition in axons and decreased number of vesicles per bouton. Additionally, there was a decreased level of active zone material upon Rab6 removal. Overall, my data strongly suggests the involvement of Rab6 in post-Golgi transport in neurons, possibly acting as a marker for transport vesicles targeted to presynaptic terminals.
Chapter 2: Figure 6

A

ELKS1\textsubscript{\alpha B}  
955 R M K L M A D N Y E D D H F R S S 971  

<table>
<thead>
<tr>
<th></th>
<th>HA</th>
<th>CC\textsubscript{\alpha}</th>
<th>CC\textsubscript{\beta}</th>
<th>CC\textsubscript{\gamma}</th>
<th>CC\textsubscript{\delta}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELKS1\textsubscript{\Delta 955-971}</td>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

ELKS1\textsubscript{\alpha B}  

|  |  |  |  |  |  |
|---|---|---|---|---|
| ELKS1\textsubscript{\Delta 955-971} |  |  |  |  |

C

ELKS1\textsubscript{\alpha B} + Rab6\textsubscript{B\textsubscript{QL}}  

ELKS1\textsubscript{\Delta 955-971} + Rab6\textsubscript{B\textsubscript{QL}}  

ELKS1\textsubscript{\alpha B} + Rab6\textsubscript{B\textsubscript{TN}}  

Cerulean-Rab6  

HA-ELKS1  

merge  

D

<table>
<thead>
<tr>
<th>Experiments</th>
<th>number of Rab6 clusters</th>
<th>Rab6 intensity (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ + + + HA-ELKS1\textsubscript{\alpha B}</td>
<td>1.00±0.15***</td>
<td>1.8±0.2***</td>
</tr>
<tr>
<td>+ - - - HA-ELKS1\textsubscript{\Delta 955-971}</td>
<td>0.8±0.1**</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>+ + - - Cerulean-Rab6\textsubscript{B\textsubscript{QL}}</td>
<td>0.6±0.1*</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>- - + + Cerulean-Rab6\textsubscript{B\textsubscript{TN}}</td>
<td>0.4±0.1</td>
<td>2.0±0.2</td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>Experiments</th>
<th>HA</th>
<th>GFP</th>
<th>(\beta)-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ + + + HA-ELKS1\textsubscript{\alpha B}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ - - - HA-ELKS1\textsubscript{\Delta 955-971}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ + - - Cerulean-Rab6\textsubscript{B\textsubscript{QL}}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- - + + Cerulean-Rab6\textsubscript{B\textsubscript{TN}}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2: Figure 6

The direct interaction between Rab6 and ELKS determines Rab6 localization in HEK cells (A and B) Schematic representation (A) and example Western blot images (B) of affinity assays using HA-tagged ELKS1αB proteins overexpressed in HEK cells. GST-Rab6 proteins were used as immobilized baits, while purified HEK cell lysates were used as prey that were overexpressed with HA-tagged control (ELKS1αB) or a mutant ELKS1αBΔ955-971 that was missing the Rab6-binding segment. HA-ELKS1αB trapped by GST-Rab6A baits was visualized by using an anti-HA antibody. For Rab6A baits, n = 1 independent trials; for Rab6B baits, n = 3.

(C and D) Example images (C) and quantification (D) of the localization of co-expressed ELKS1 and Rab6B proteins in HEK cells. HEK cells were transfected either with either Cerulean tagged Rab6BQL or Rab6B1N, and simultaneously co-transfected with HA-tagged ELKS1αB or ELKS1αBΔ955-971. To visualize overexpressed Cerleuan-Rab6B, HEK cells were immunostained against an anti-GFP antibody. To visualize overexpressed HA-ELKS1, HEK cells were immunostained against an anti-HA antibody. All signals were acquired by confocal microscopy. (D) Quantifications show the average number of large Cerulean-Rab6B aggregates per cell and the average total Cerulean-Rab6 intensity per cell. ELKS1αB + Rab6BQL, n = 23 fields of view / 3 independent cultures (fields of view = 184.88 μm x 184.88 μm, typically containing 50-100 cells per field of view); ELKS1αBΔ955-971 + Rab6BQL, 27/3; ELKS1αB + Rab6B1N, 22/3. (E) Example Western blot images of HEK cells showing protein expression levels.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by one-way ANOVA, followed by Holm-Sidak post hoc comparison to condition (ELKS1αB + Rab6BQL). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
CHAPTER THREE:

ELKS captures trafficking cargo via a direct interaction with Rab6

Manuscript in preparation
to publish data from chapter three in combination with data in chapter two

Concepts and experimental designs are by H.N. and P.K. Experimental procedures, analyses, and illustrations were prepared by H.N. I would like to thank R. Held for the initial breeding of the ELKS1α/β mouse line (Figure 2), A. de Jong for sharing EM images of RIM cDKO for axonal segment analysis (Figure 4), S.S. Wang for cutting EM sections in the ELKS1α/β rescue experiment (Figure 5), Dr. T. Schwarz for the mitoDsRed and Tom20 constructs, and the Harvard EM Facility and the Harvard Neuro Imaging Core Facility for their assistance.
ELKS is a large scaffolding protein that is highly expressed in the central nervous system and localizes primarily to presynaptic terminals in neurons (Kaeser et al., 2009). Together with other large scaffolding proteins, ELKS forms the active zone complex, a highly specialized protein matrix near the plasma membrane that regulates the precision of synaptic vesicle release (Südhof, 2012). The genetic removal of ELKS leads to disruption of synaptic transmission (Held et al., 2016; Kaeser et al., 2009; Kiyonaka et al., 2012; Liu et al., 2014). Recent data from our lab demonstrated the importance of ELKS as a vesicle tether at the active zone. Removal of ELKS together with another active zone protein, RIM, leads to a complete loss of docked vesicles at the presynaptic release site (Wang et al., 2016).

Functions of ELKS were almost exclusively evaluated in context of vesicle exocytosis at the presynaptic terminal; however, there is evidence from the literature that ELKS might be involved in transport-associated pathways. ELKS is a large coiled coil protein (Monier et al., 2002) with striking sequence homologies to the Golgi-resident vesicle tethers, the Golgins that bind Rab6 (Munro, 2011). In nonneuronal cells, ELKS primarily localizes to the cell edge and is thought to form an adhesion complex for microtubules and/or mark docking sites for Rab6-positive secretory vesicles (Astro et al., 2014; Fourriere et al., 2019; Grigoriev et al., 2007, 2011; Lansbergen et al., 2006; Patwardhan et al., 2017). My data on neuronal Rab6 indicated that Rab6 has synaptic expression in a vesicle-associated GTP-bound state, at which state it also binds ELKS in presence of ELKS1. Furthermore, the localization of Rab6 was dramatically altered in HEK cells (chapter 2). In my second aim I addressed the physiological relevance of the Rab6 binding to ELKS. Based on my data on Rab6 taken together with data from the literature, I hypothesized that there is a functional interaction between Rab6 and ELKS at presynaptic terminals.
Presynaptic ELKS1 has a wide distribution within presynaptic terminals

In neurons, ELKS is known to primarily localize to presynaptic terminals and was shown to be important for neurotransmitter release as part of the active zone complex. While both ELKS1 and ELKS2 isoforms bound Rab6 in biochemical assays, I needed to address their subsynaptic distribution. In order to consider them as possible interactor of a Rab6-marked transport cargo, some fraction of ELKS would need to be localized away from the active zone. I used a previously established mouse line, ELKS1/2α conditional double knockouts. Hippocampal neurons were cultured from P0 pups of ELKS1/2α homozygous floxed mice. ELKS protein removal was achieved by lentiviral delivery of Cre recombinase to neuronal hippocampal cultures at day-in-vitro (DIV) 5. Cultures were harvested at DIV 14-16 and used in subsequent experiments.

Analysis of confocal images confirmed that both ELKS1 and ELKS2 isoforms are primarily synaptic in control conditions, and signal for both ELKS are entirely lost in the knockout condition (Figs. 1C). For analysis of the subsynaptic localization, line-scan intensity profiles of side view synapses were obtained for the synaptic vesicle cluster marker (either Synapsin-1 or Synaptophysin-1, imaged with confocal microscopy), the active zone marker (Bassoon, imaged by STED), and ELKS1 or ELKS2 proteins (imaged by STED). Side-view synapses were selected manually, and a rectangular 0.2 μm x 1.0 μm bar was placed perpendicular to the signal from the active zone marker. The intensity values of the target proteins were aligned such that the peak intensity of their corresponding active zone markers was always at zero.
Line-scan profiles of superresolution images revealed that there is a major difference in the subsynaptic localization of the two ELKS proteins. Only ELKS2 was confined to the active zone, while ELKS1 appeared widely distributed and largely away from the active zone (Figs. 1D-1J). Measuring the total ELKS signal at or away from the active zone within synaptic clusters showed similar results, where the majority of the ELKS1 signal fell outside of the active zone marker Bassoon (Fig. 1J). This suggests that not ELKS2, but ELKS1 has a subsynaptic distribution that could facilitate capture of arriving vesicle cargos.

When transmission electron micrographs of chemically fixed neurons were examined, I found an increased number of extra-synaptic vesicles in axonal segments (Figs. 1A and 1B), which resembled the phenotype seen upon Rab6B removal from neurons.
Chapter 3: Figure 1
Chapter 3: Figure 1

ELKS1 has a wide-spread subsynaptic distribution

(A and B) Electron micrograph images (A) and quantification (B) of axons of ELKS1/2α control and cDKO cultures acquired by chemical fixation. ELKS1/2α control, n = 54 axons / 1 culture; ELKS1/2α cDKO, 58/1.

(C) Example confocal images of neuronal branches for ELKS1 (D) and ELKS2 (E) proteins from ELKS1/2α control and cDKO cultures. Hippocampal cultures were were immunostained with anti-ELKS1 or anti-ELKS2 antibodies and were co-stained with an anti-Bassoon antibody to mark synapses.

(D-J) Analysis of subsynaptic localization of ELKS1 and ELKS2 in STED microscopy using ELKS1/2α conditional double knockout (cDKO) mouse line. ELKS1/2α cDKO hippocampal cultures were acquired by infecting ELKS1/2α floxed neurons with a lentivirus to express Cre recombinase, while ELKS1/2α control cultures were acquired by infecting with a truncated inactive form of Cre recombinase. Bassoon was used to mark active zone regions. Synapsin-1 was used to mark synapses. ELKS1, ELKS2 and Bassoon signals were acquired by STED microscopy, while Synapsin-1 signals were acquired by confocal.

(D-F) Example images (D) and quantification (E and F) of ELKS1 subsynaptic localization. (E) Quantification shows total ELKS1 signals within Synapsin-1 objects. ELKS1/2α control, n = 567 synapses / 3 independent cultures; ELKS1/2α cDKO, 380/3. (F) Line scan analysis of side-view synapses showing normalized intensity profiles of ELKS1 and Bassoon signals. The dotted line indicates distance = 0 μm from Bassoon peak. Data are shown as mean (solid line) ± SEM (shaded area). ELKS1/2α control, n = 73 fields of view / 3 independent cultures; (fields of view = 1.5 μm x 1.5 μm, always containing exactly one Synapsin-1 object per field of view); ELKS1/2α cDKO, 71/3.

(G-I) Example images (G) and quantification (H and I) of ELKS2 subsynaptic localization. (H) Quantification shows total ELKS2 signals within Synapsin-1 objects. ELKS1/2α control, n = 407 synapses / 3 independent cultures; ELKS1/2α cDKO, 286/3. (I) Line scan analysis of side-view synapses showing normalized intensity profiles of ELKS2. ELKS1/2α control, n = 42 fields of view /3 (fields of view = 1.5 μm x 1.5 μm, always containing exactly one Synapsin-1 object per field of view); ELKS1/2α cDKO, 34/3.

(continued on next page)
Quantification shows the fraction of total ELKS1 or ELKS2 signal away from the active zone within Synapsin-1 objects. ELKS1 or ELKS2 signal that did not colocalize with Bassoon signal a pixel-to-pixel signal analysis was counted as outside the active zone.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test (Fig 1B, 1E, 1H, and 1J). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
**ELKS1α/β knockout leads to decreased active zone material**

To address the functional significance of the interaction between Rab6 and ELKS, new ELKS1α/β conditional mouse knockout line was established (Fig. 2A). The previously established ELKS1/2α cDKO line did not remove ELKSβ, an isoform that is also able to bind Rab6 (Fig. 3A). Given that only the ELKS1 isoform showed a wide subsynaptic distribution, we considered the ELKS1α/β conditional floxed line appropriate to address the functional relationship between Rab6 and ELKS.

The conditional ELKS1α/β mouse line was generated as KO-first line, the ELKS1α/β knockin (ki) with a reporter-tagged insertion into the exon13 region. The targeting vector inserted a splice acceptor and a neomycin selection sequence flanked by two FRT and followed by a loxP site upstream exon13 and another loxP site downstream exon13 (Fig. 2A). Because of the splice acceptor, alleles carrying this insertion frequently failed to produce viable isoforms of the ELKS1 protein and could be considered as a knockout. I found that the ELKS1α/β^+/ki breeders never produced homozygous ELKS1α/β^ki/ki (Supplemental Fig. 1A) as was predicted based on previous publications where constitutive ELKS1 removal results in embryonic lethality (Liu et al., 2014). To generate the conditional ELKS1α/β floxed line, the FRT-flanked neomycin cassette (including the splice acceptor) was removed by crossing them to a mouse line that carried the Flp gene. The survival ratio of the offspring from the newly generated ELKS1α/β^+/floxed animals were also analyzed, and found normal Mendelian ratios (Supplemental Fig. 1B). To confirm that the mouse line did not experience any spontaneous recombination, I tested ELKS protein levels in crude brain homogenates of ELKS1α/β^+/+ and ELKS1α/β floxed littermates, and found no disturbance in protein levels (Supplemental Fig. 1D).
Since our previous antibodies were not sensitive enough to all ELKS isoforms, specifically to the ELKS-β isoforms, I generated a new ELKS antibody (ELKS1/2 α/β, also called HM1083) that is able to detect all ELKS isoforms (Supplemental Fig. 1C). This antibody was used to confirm that ELKS1β was also removed along with the major ELKS1α isoforms (Figs. 2B and 2C). To generate ELKS1α/β conditional knockout (cKO) neurons, primary hippocampal cultures harvested from newborn pups of homozygous ELKS1α/β floxed animals were infected at day in vitro 5 (DIV 5) with a lentiviral solution expressing an eGFP-fused Cre recombinase with a nuclear localization signal. ELKS1 removal was achieved by Cre recombinase inducing the recombination of the two loxP sites flanking exon13. Expression of the Cre recombinase was monitored by the nuclear GFP signal in neurons and the cultures were harvested at DIV 14-16 for subsequent experiments.

Screening a panel of synaptic proteins in fluorescent Western blotting, I found that removal of ELKS1 decreased the total levels of active zone proteins specifically (Figs. 2B and 2C). To see whether levels of synaptic Rab6 was affected upon ablation of ELKS1, I immunostained ELKS1α/β control and cKO cultures against anti-ELKS1 and anti-Rab6B, and with anti-Synaptophysin-1 marking synaptic vesicle clusters. I then examined signal localizations and intensities via superresolution (ELKS1 and Rab6) and confocal (vesicle marker) imaging. I found that the ELKS1 signals were indeed removed from synaptic regions observed both in larger views of neuronal branches (Fig. 2D) and in individual synapses (Figs. 2E and 2H). Remarkably, I observed a significant decrease in synaptic Rab6B levels in ELKS1α/β cKO neurons relative to controls (Figs. 2E and 2F). I also examined Rab6 signal intensities in the ELKS1α/β control synapses that were expressing different levels of ELKS1 and found a positive correlation between ELKS1 and Rab6B signal intensities in synaptic boutons (Figs. 2G and 2H). The fact that total Rab6 protein levels did not change (Fig 2B and 2C) is in accordance with the observation that the majority of Rab6 resides in the Golgi where ELKS has no known function in neurons.
Chapter 3: Figure 2

A

ELKS1α/β wild type (+) targeting construct
ELKS1α/β knockin (ki)
ELKS1α/β floxed (f)
ELKS1α/β cKO

B

ELKS1α/β
ELKS1α/β (HM1083)
ELKS1/2β
ELKS2β
Rab6A
Rab6B
Liprin-α3
Liprin-α4
Rab3A
Rab3B
Synaptophysin-1
Synaptophysin-2
Synaptoctobrevin-2
Synaptoctobrevin-3
PSD-95
β-actin

C

D

ELKS1α/β control
ELKS1α/β cKO

E

ELKS1α/β
Rab6B
Synaptophysin-1
+ merge

F

G

synapses from only ELKS1α/β control

H

normalized ELKS1 intensity
normalized Rab6B intensity

R2 = 0.52

normalized ELKS1 intensity
**Chapter 3: Figure 2**

**Synaptic ELKS1 levels coordinate synaptic Rab6 localization**

**(A)** Schematic representation of the Erc1 gene targeting strategy.  
**(B and C)** Example images (B) and quantification (C) of fluorescent Western blots from cultured ELKS1α/β control or ELKS1α/β conditional knockout (cKO) hippocampal neurons. Knockout cultures were acquired by infecting ELKS1α/β^floxed/floxed^ neurons with a lentivirus to express Cre recombinase, while control cultures were acquired by infecting ELKS1α/β^floxed/floxed^ neurons with a lentivirus to express a truncated inactive form of Cre recombinase. Protein levels were normalized to corresponding Synapsin-1 protein levels. For both genotypes, n = 3 cultures.  
**(D)** Example confocal images of ELKS1α/β control and cKO hippocampal cultures.  
**(E and F)** Example images (E) and quantification (F) of ELKS1α/β control or cKO hippocampal neurons acquired by STED microscopy. ELKS1 and Rab6B signals were acquired by STED microscopy, while Synaptophysin-1 signals were acquired by confocal microscopy. (F) Quantifications show normalized intensities of ELKS1 or Rab6B within Synaptophysin-1 objects, and the average area of the Synaptophysin-1 objects. ELKS1α/β control, n = 25 fields of view / 3 independent cultures (fields of view = 15 μm x 4 μm, each field of view typically containing approximately 20-40 Synaptophysin objects); ELKS1α/β cKO, n = 20/3.  
**(G and H)** Example images (G) and quantification (H) of Rab6B and ELKS1 signal correlation in wild type hippocampal neurons. ELKS1 and Rab6B signals were acquired by STED microscopy, while Synaptophysin-1 signals were acquired by confocal microscopy. (H) Quantification shows correlation analysis of normalized ELKS1 and Rab6B signals per Synaptophysin-1 object. The area of Synaptophysin-1 objects fell within a narrow range of 0.5-0.8 μm². Synaptophysin objects, n = 162 synapses / 3 independent cultures.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test (Figs. 2C and 2F) and Pearson's correlation (Fig 2H). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
ELKS1α/β knockout leads to enhanced axonal cargo deposition away from synapses

When transmission electron micrographs of both high-pressure frozen and chemically fixed neurons from ELKS1α/β control and cKO neurons were examined, I found a decreased number of vesicles in presynaptic boutons (Figs. 3A-3C) and an increased number of extra-synaptic vesicles (with diameters ≤ 50 nm) in axonal segments (Figs. 3D-3H). I also quantified larger vesicles (with diameters between 50 to 100 nm). Large vesicles were grouped into two categories, large dense core vesicles (LDCVs) and endosomes that were not LDCVs (clear center). I found that these two groups were affected by ELKS1α/β removal only in certain conditions (Supplemental Figs. 2B and 2C); however, the effect on small vesicles was consistent across all electron microscopic preparation. The effect on extra-synaptic axonal vesicle numbers did not differ between various EM preparations (Figs. 3D-3H); furthermore, earlier removal of ELKS1 (DIV 1 infection) also did not change the outcome (Figs. 3F and 3G). I also analyzed transmission electron micrographs of axons from RIM control and cDKO neurons and found no change in axonal vesicle deposition (Fig. 3I). The effects of ELKS1α/β removal differed from the removal of Rab6B in that ELKS1α/β cKO neurons showed a decreased number of docked vesicles at the active zone (Figs. 3A and 3B).

The striking similarities between ELKS1 and Rab6B removal on axonal vesicle accumulation, total bouton size, and active zone levels indicate that these two proteins share a functional pathway.
Chapter 3: Figure 3

A

ELKS1α/β control  ELKS1α/β cKO

B

**B**

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>cKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>docked vesicles</td>
<td>21</td>
<td>90</td>
</tr>
<tr>
<td>PS length (µm)</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>cKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>bouton area (µm²)</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>number of endosomes</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>number of vesicles</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

D

ELKS1α/β control  ELKS1α/β cKO

E

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>cKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>vesicles per 5 µm axon</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>axon width (µm)</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

F

DIV1 cre, high pressure freezing

G

DIV1 cre, chemical fixation

H

DIV5 cre, chemical fixation

I

DIV5 cre, high pressure freezing

RIM control  RIM cKO
Chapter 3: Figure 3

ELKS1α/β knockout leads to vesicle tethering defects

(A-C) Electron micrograph analysis of boutons from high pressure frozen ELKS1α/β control or cKO hippocampal cultures. (B and C) Quantifications show the average number of total vesicles and docked vesicles per bouton, and the average PSD length. ELKS1α/β control, n = 134 boutons / 2 independent cultures; ELKS1α/β cKO, 148/2.

(D and E) Electron micrograph analysis of axons from high pressure frozen ELKS1α/β control or cKO hippocampal cultures. (E) Quantifications show the average number of vesicles per 5 μm axonal length and the average axonal width. ELKS1α/β control, n = 209 axons / 2 independent cultures; ELKS1α/β cKO, 221/2.

(F-H) Electron micrograph analysis of axons of ELKS1α/β control and cKO cultures acquired by three different protocols. Example images and quantification of cultures infected with Cre or inactive-Cre lentivirus (F) at day-in-vitro (DIV) 5 and processed by chemical fixation, (G) infected at DIV 1 and processed by chemical fixation, (H) infected at DIV 1 and processed by high pressure freezing. Quantifications show number of vesicles per 5 μm axonal segment. For all three protocols, n = 1 culture, approximately 50-100 axons per genotype.

(I) Example electron micrographs and quantification of axons from RIM1/2+/+ and RIM1/2−/− hippocampal cultures. RIM1/2 control, n = 141 axons / 2 independent cultures; RIM1/2 cDKO, 143/2.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test; except for the vesicle distribution analysis at 0-1000 nm distances in (Fig. 3C), which was analyzed by extra sum of squares F-test.
**Binding of Rab6 to ELKS1 is necessary for cargo delivery**

To further address the functional significance of a direct interaction between ELKS1 and Rab6 at the presynaptic terminal, I used HA-tagged ELKS1αB constructs in a series of rescue experiments to examine, which phenotypes can be rescued upon ELKS1α/β removal. The ELKS1αB constructs were generated in chapter 2 and were tested were Rab6-binding (chapter 2 Fig 6). HA-ELKS1αB rescue protein is able to bind Rab6, while ELKS1αBΔ955-971 lacks binding. Localization of the rescue constructs was determined, showing normal synaptic localization, determined by confocal microscopy (Fig. 4A). I hypothesized that if the decrease in synaptic Rab6 recruitment and any additional phenotypes observed in the ELKS1α/β cKO neurons is due to a direct ELKS1-Rab6 interaction, rescue should only be possible with the ELKS1αB protein where the Rab6-binding is uninterrupted.

Homozygous ELKS1α/β floxed hippocampal neurons were cultured as described previously. For non-rescue conditions, I used ELKS1α/β floxed neurons infected at DIV 5 only with one lentivirus expressing either Cre (for cKO condition) or with the inactive Cre (for control condition). To generate rescue conditions, separate ELKS1α/β floxed neurons from the same culture preparations were first infected with a lentivirus at DIV 3 expressing one of the HA-tagged ELKS1αB rescue proteins (ELKS1αB or ELKS1αBΔ955-971), then were infected again with a second independent lentiviral solution at DIV 5 expressing the Cre recombinase. Levels of endogenous and rescue ELKS1 expressions were monitored with Western blotting (Fig. 4B). Cultured neurons were then subjected to either high-pressure freezing for electron microscopy or immunostaining for superresolution imaging. In the superresolution imaging, I found that both the synaptic Rab6 (Figs. 4C and 4D) and RIM1 levels (Figs. 4E and 4G) were rescued with only the full-length but not with the mutant ELKS1 protein. Furthermore, Bassoon levels were not affected in the ELKS1αB cKO neurons (Figs. 4F and 4G).
Chapter 3: Figure 4

A) HA-ELKS1αβ and HA-ELKS1αβΔ665-971

B) ELKS1, Synapsin-1, β-actin

C) ELKS1α/β control vs ELKS1α/β cKO + ELKS1αβΔ665-971

D) ELKS1 intensity comparisons

E) RIM1 and Synaptophysin-1 + merge

F) Bassoon and PSD-95 + merge

G) PSD-95 peak intensity comparisons
Chapter 3: Figure 4

**Binding of Rab6 to ELKS1 is necessary to rescue active zone levels in ELKS1α/β cKO**

**(B)** Example Western blot images showing protein expression levels in the ELKS1α/β control, ELKS1α/β cKO, ELKS1α/β cKO + ELKS1αB and ELKS1α/β cKO + ELKS1αBΔ955-971 conditions.

**(C and D)** Example images (C) and quantification (D) of the ELKS1 and Rab6 levels of ELKS1α/β rescue cultures in STED microscopy. (C) Example images show cultures immunostained against anti-Rab6B, anti-ELKS1 and anti-Synaptophysin-1 antibodies. Synaptophysin-1 signals were acquired by confocal microscopy; all other signals were acquired by STED microscopy. (D) Quantifications show normalized intensities of ELKS1 and Rab6 signals within Synaptophysin-1 fields of views (1.5 μm x 1.5 μm, always containing exactly one Synaptophysin object per field of view). ELKS1α/β control, 78/3; ELKS1α/β cKO, 73/3; ELKS1α/β cKO + ELKS1αB, 75/3; and ELKS1α/β cKO + ELKS1αBΔ955-971, 76/3.

**(E-G)** Example images (E and F) and quantification (G) of the RIM1 and Bassoon subsynaptic localization relative to PSD-95 in ELKS1α/β rescue cultures acquired by STED microscopy. Example images show cultures immunostained against anti-RIM1 (E) or anti-Bassoon (F), and co-stained with anti-PSD-95 and anti-Synaptophysin-1 antibodies. RIM1, Bassoon and PSD-95 signals were acquired by STED microscopy, while Synaptophysin-1 signals were acquired by confocal microscopy. (G) Quantification shows line-scan profiles of normalized RIM1 and Bassoon intensities of side-view synapses relative to the PSD-95 peak position (0 μm). ELKS1α/β control, 63/3; ELKS1α/β cKO, 61/3; ELKS1α/β cKO + ELKS1αB, 53/3; and ELKS1α/β cKO + ELKS1αBΔ955-971, 60/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by one-way ANOVA, followed by Holm-Sidak post hoc comparison of conditions to ELKS1α/β cKO (Fig. 4D). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
When the transmission electron images of boutons and axons were analyzed in all four conditions, I found that much of the initially observed ultrastructural phenotypes, namely the decrease in bouton size (Figs. 5A and 5B) and the increase in extra-synaptic vesicle deposition (Figs. 5C and 5D) were rescued only with the full-length ELKS1 protein but not with the mutant ELKS1 that lacked the Rab6-binding region (Figs. 5A-5D). On the other hand, the decrease in the number of docked vesicles observed in the ELKS1α/β cKO was rescued with both ELKS1 proteins (Figs. 5A and 5B).

Data from these rescue experiments suggest that the direct Rab6-ELKS1 interaction is responsible for the synaptic recruitment of Rab6 (Figs. 4C and 4D), and for some of presynaptic RIM levels (Figs 4E and 4F). The decreased number of synaptic vesicles in boutons (Figs. 5A and 5B), accompanied by an increased number of uncaptured vesicles in axonal segments (Figs. 5C and 5D) are also due to lacking the direct association between ELKS and Rab6, while vesicle docking is not a result of their interaction. These data suggest that the extra-synaptic vesicles may be Rab6-positive cargo that carry active zone material to the presynaptic terminal, and these cargos are recruited to the terminal by ELKS1 directly binding to Rab6. Abolishing their direct interaction leads to delivery deficits of a subtype of vesicle cargos.

In subsequent experiments, I addressed the existence of an ELKS1-Rab6 cargo recruitment mechanism at the presynaptic terminal.
Chapter 3: Figure 5

A

ELKS1α/β control

ELKS1α/β cKO

ELKS1α/β cKO + ELKS1αB

ELKS1α/β cKO + ELKS1αβ

500 nm

B

docked vesicles

vesicles per button

PSD length (μm)

C

ELKS1α/β control

ELKS1α/β cKO

ELKS1α/β cKO + ELKS1αB

ELKS1α/β cKO + ELKS1αβ

500 nm

D

vesicles per 5 μm axon

axon width (μm)
Chapter 3: Figure 5

Binding of Rab6 to ELKS1 is necessary to rescue axonal vesicle accumulation, but not for rescuing vesicle docking in ELKS1α/β cKO neurons

(A-D) Electron micrograph analysis of high pressure frozen ELKS1α/β hippocampal cultures in a rescue experiment. ELKS1α/β control and cKO cultures were acquired as described in Fig 4. Rescue conditions were acquired by first driving the expression of the HA-tagged ELKS1αB or ELKS1αBΔ955-971 rescue proteins under a human Synapsin promoter via lentiviral infection at DIV 3, then inducing the ablation of ELKS1 by introducing the Cre recombinase with a second independent lentiviral infection at DIV 5. (A and B) Example images (A) and quantification (B) of boutons. (B) Quantifications show the average number of docked and total vesicles per bouton, and the length of PSD. ELKS1α/β control, n = 82 boutons / 2 independent cultures; ELKS1α/β cKO, 116/2; ELKS1α/β cKO + ELKS1αB, 103/2; ELKS1α/β cKO + ELKS1αBΔ955-971, 105/2.

(C and D) Example images (C) and quantification (D) of axons. (D) Quantifications show the average number of small vesicles per 5 μm axonal length and the average width of analyzed axons. ELKS1α/β control, n = 143 axons / 2 independent cultures; ELKS1α/β cKO, 165/2; ELKS1α/β cKO + ELKS1αB, 133/2; ELKS1α/β cKO + ELKS1αBΔ955-971, 148/2.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by one-way ANOVA, followed by Holm-Sidak post hoc comparison of conditions to ELKS1α/β cKO.
While ELKS1 is stably localized to synapses, axonal Rab6 is mobile

The rescue experiments in ELKS1α/β cKO neurons indicated that there is a physiologically relevant direct interaction between Rab6 and ELKS1 at presynaptic terminals. Furthermore, synaptic Rab6 recruitment was directly mediated by presynaptic ELKS1. Based on previously published data that neuronal ELKS1 is largely synaptic, we hypothesized that synapse-anchored ELKS captures Rab6-labeled synaptic cargo by recognizing and directly interacting with GTP-bound Rab6 on newly delivered vesicle cargos. However, there is a possibility that ELKS1 may be transported along with Rab6 on the cargo. To address this, I asked how the proteins behaved in live cells and examined their mobility. I expressed Cerulean-tagged versions of either ELKS1αB or Rab6B<sub>WT</sub> proteins in wild type neurons using a calcium-phosphate transfection delivery method at DIV 11-12. I simultaneously transfected neurons with a tdTomato-tagged SV2A protein to mark synaptic vesicle clusters and to make sure that only axonal regions that exhibited vesicle trafficking were analyzed. I chose to employ the transfection method because viral infection did not produce high enough Cerulean signal in Cerulean-ELKS1 overexpression to establish any mobility patterns. Neurons were imaged live at DIV 14-15 using time lapse imaging at 35°C in a pH-buffered solution optimized for fluorescent imaging. Each axonal segment was imaged for 2 minutes with 1 frame per minute frequency using an LED light source.

For analysis of time-lapse images, Cerulean puncta (marking ELKS1 or Rab6B) were categorized as either stationary or moving objects. Any Cerulean object that remained completely motionless within the 2-minute imaging period was considered stationary. Interestingly, the total number of stationary objects for ELKS1 and Rab6 were very similar, and both accumulated almost exclusively at SV2-positive regions (Figs. 6A and 6B). On the other
hand, when moving objects of Rab6 and ELKS1 were compared, I found that the two proteins had remarkably different mobility pattern. While Rab6 had a high number of moving events additionally to the stationary ones, ELKS1 signals remained mostly stationary compared (Figs. 6C and 6D). It was necessary to use the wild type form of Rab6B (Rab6B\textsubscript{WT}) for the mobility experiment because in initial trial experiments the active point mutant (Rab6B\textsubscript{QL}) showed significantly less trafficking in axons near synaptic regions. This supported the idea that the active form of Rab6 is readily recruited to synaptic regions.

To confirm that recruitment of mobile Rab6 to synapses was not an overexpression artifact, I examined overexpressed active Cerulean-Rab6A\textsubscript{QL} or Rab6B\textsubscript{QL} proteins in ELKS1\textalpha/\textbeta cKO neurons and compared them to controls. I found that synaptic accumulation of both Rab6A (Figs. 6H-6J) and Rab6B (Figs. 6E-6G) were significantly decreased in ELKS1\textalpha/\textbeta cKO relative to control condition. Furthermore, correlation analysis for both Rab6A and Rab6B overexpression signal with endogenous ELKS1 levels showed a positive correlation (Figs. 6G and 6J), indicating that overexpressed Rab6 favorably accumulated at ELKS1-enriched synaptic regions. These suggest that Rab6 is indeed a highly trafficked protein in axons, the recruitment of which to synapses depends of synaptic ELKS1.
Chapter 3: Figure 6

Rab6 behaves similarly to trafficking proteins in live imaging (A-D) Example images (A), kymographs (C) and quantifications (B and D) of the axonal mobility assay for ELKS1 and Rab6B. Cerulean tagged ELKS1αB or Cerulean-Rab6B\textsubscript{WT} expression was induced in wild type neuronal cultures via a calcium-phosphate transfection method. Cultures were simultaneously transfected with tdTomato-SV2A to mark synaptic regions. Time-lapse images of Cerulean and tdTomato signals from live cells were acquired by fluorescence microscopy. Cells were imaged for 2 minutes with 1 frame / sec imaging speed. (B) Quantification shows the average number of stationary ELKS1 and Rab6B objects per 100 μm axonal distance and shows the fraction of stationary objects that associated with SV2A-marked synaptic regions. An object was considered stationary if it appeared as a straight vertical bar on the kymograph. (C) Example kymographs of axonal segments where the X-axis corresponds to the measured distance in μm and the Y-axis corresponds to the elapsed time in frames (1 frame = 1 second). (D) Quantification shows the average number of moving ELKS1 and Rab6B objects per 100 μm axonal distance per minute and shows the average instant speed of puncta in μm/sec. An object was considered mobile if it showed any displacement during the time period it was within view. An object was considered mobile even if it was mostly stationary but moved at least 5 μm. For both (D and F) ELKS1αB, n = 34 cells / 3 independent cultures; Rab6B\textsubscript{WT}, 34/3. (E-G) Cerulean tagged Rab6B\textsubscript{WT} overexpressed in ELKS1α/β control and cKO neuronal cultures using confocal microscopy. ELKS1α/β control and cKO neurons were acquired as described before. The cultures were infected with a second independent lentivirus expressing Cerulean tagged Rab6B\textsubscript{WT} proteins. (E) Example images show cultures immunostained with anti-GFP (to visualize overexpressed Rab6), anti-ELKS1, and Synaptophysin-1 antibodies. (F) Quantification in shows normalized intensities of Rab6B\textsubscript{WT} within Synaptophysin-1 objects. ELKS1α/β control, 870 synapses / 3 independent cultures; ELKS1α/β cKO, 1196/3. (G) Quantification shows correlation between overexpressed Rab6B\textsubscript{WT} and endogenous ELKS1 signals within Synaptophysin-1 objects in ELKS1α/β control condition only, n = 870/3.

(continued on next page)
(Chapter 3: Figure 6 continued)

(H-J) Cerulean tagged Rab6A<sub>WT</sub> overexpressed in ELKS1α/β control and cKO neuronal cultures using confocal. (H) Example images show cultures immunostained the same as above. (I) Quantification shows normalized intensities of Rab6A<sub>WT</sub> within Synaptophysin-1 objects. ELKS1α/β control, 1108 synapses / 3 independent cultures; ELKS1α/β cKO, 1521/3. (J) Quantification shows correlation between overexpressed Rab6A<sub>WT</sub> and endogenous ELKS1 signals within Synaptophysin-1 objects in ELKS1α/β control condition only, n = 1108/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test (Figs 6B, 6D, 6F and 6I), and Pearson’s correlation (Figs. 6G and 6J). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
Hijacking of Rab6-ELKS1 interactions to mistarget vesicles or mitochondria

The results discussed so far demonstrate that ELKS1 directly interacts with Rab6 at the synapse and recruits Rab6-labeled small vesicle cargos. I asked whether this mechanism could be hijacked and applied to other types of cargo. To test this, I mis-localized either ELKS1 or Rab6 to mitochondria and examined how it effects the behavior of mitochondria in axons. I generated a mito-tag that was composed of the transmembrane region of Tom20 protein (from the outer leaflet of the mitochondria) coupled to Cerulean, which was then fused to the N-terminus of either ELKS1αB or Rab6B (Figs. 7A and 7F).

I asked whether ELKS1 is sufficient to tether vesicles using the mito-tagged ELKS1. I first tested that the mito-tag was able to effectively mis-localize ELKS1 to the mitochondria (Fig. 7B). I expressed mitoDsRed mitochondrial marker in wild type hippocampal cultures and co-expressed it with either the mito-tagged ELKS1αB or a soluble Cerulean-ELKS1αB without mito-tag using lentiviral infections as delivery methods. Cultures were immunostained at DIV 14 against anti-RFP antibody to visualize mitoDsRed, anti-GFP antibody to visualize overexpressed ELKS1, and anti-Bassoon antibody to mark synaptic clusters, then imaged using confocal microscopy. Mito-ELKS1αB but not Cerulean-ELKS1αB showed colocalization with mitoDsRed (Fig. 7B). Then, I expressed either mito-ELKS1αB, mito-ELKS1αB△965-971 or the mito-tag alone in wild type hippocampal cultures using a lentiviral delivery method (Fig. 7A). The levels of protein expressions were monitored by Western blotting (Fig. 7C). Cultures were high-pressure frozen at DIV 14 and imaged with transmission electron microscopy. I employed this approach, since this provided the desired resolution to count individual vesicles. For analysis, I examined mitochondria in axonal segments and counted the number of small vesicles (diameter ≤ 50 nm) that fell within 70 nm from mitochondrial surface. The maximum distance of 70 nm was
determined by measuring the longest visible string or tether between vesicles and mitochondria. (Note that after the initial determination of the maximum distance, a tether did not have to be visible as long as the vesicle fell within 70 nm from the mitochondrial membrane.) I found that mito-tagged ELKS1αB was able to recruit small vesicles to the mitochondrial surface relative to the mito-tag control; furthermore, this vesicle recruitment could be abolished when the Rab6-binding region from ELKS1 was removed (Figs. 7D-7E).

Then I examined what happens to mitochondria when was Rab6 mis-targeted to their surface (Fig. 7F). I fused Rab6BQT and Rab6BTN proteins to the mito-tag (Fig. 7F) and confirmed that they colocalized with mitochondrial markers (Fig. 7G). Then I expressed the mito-tagged Rab6BQT or Rab6BTN proteins in combination with the previously described Ha-tagged ELKS1αB proteins (ELKS1αB or ELKS1αBΔ955-971) in wild type hippocampal cultures. Levels of overexpressed proteins were monitored by Western blotting (Fig. 7H). Neurons were harvested at DIV 15 and immunostained with an anti-GFP antibody to visualize overexpressed mito-Rab6B, an anti-HA antibody to visualize overexpressed HA-ELKS1, and an anti-Bassoon antibody to mark endogenous synaptic regions (Fig. 7I). For analysis, intensity-thresholded objects for both the GFP signals (indicating mito-Rab6) and objects for endogenous Bassoon signals were defined using ImageJ. Then, the association of Bassoon and GFP objects was measured using the BioVoxxel ImageJ plugin. In this method, the area of Bassoon objects that overlapped with a GFP object was measured using three different association thresholding: anything greater than 0 μm² (least stringent), > 0.1 μm², or > 0.25 μm² (most stringent).

I found that both full-length and mutant HA-ELKS1 proteins primarily localized to synapses independent of conditions (Fig. 7I), similarly to results in HEK cells (chapter two Fig 6). Remarkably, synaptic enrichment of mitochondria increased only when the active mito-Rab6BQL was overexpressed with the full-length HA-ELKS1, while in the other two conditions I did not observe this increase (Figs. 7I-7J). The analysis in Fig 7J shows results using the most
stringent (> 0.25 μm²) area thresholding for association between Bassoon and GFP (see Supplemental Fig. 3 for results using the additional two thresholding methods.)

These findings indicate several things. One is that ELKS1 localization to synapses is not dependent on Rab6. Second is that the Rab6-ELKS1 interaction is able to recruit cargo to synapses, even if the cargo is as large as a mitochondrion.

Conclusions

I found that ELKS1 has a wide subsynaptic distribution that would allow its association with newly arriving cargo. Strikingly, the removal of ELKS1α/β from mouse hippocampal neurons phenocopied Rab6B removal, namely increasing extra-synaptic vesicle deposition, decreasing the number of vesicles per bouton, and decreasing levels of active zone material per synapse, suggesting that ELKS1 and Rab6 share functional roles. ELKS1α/β removal also decreased the number of docked vesicles at the presynaptic active zone that was independent from its interaction with Rab6. The synaptic enrichment of Rab6 heavily depended on the presynaptic presence of ELKS1. This is in accordance with my previous data on Rab6 showing that Rab6 is driven to synapses by its active vesicle-bound state, and that only the active form of Rab6 binds ELKS. These together suggest that ELKS1 recruits Rab6 to synaptic terminals only when Rab6 is bound to vesicles.

In live imaging experiments, while both Rab6 and ELKS accumulated at synaptic clusters, they dramatically differed in their mobility. Characteristically to a trafficking protein, Rab6 appeared highly mobile, while ELKS1 remained largely anchored to synapses, indicating that cargo recruitment mediated by the two proteins travelling together. Instead, I propose that that ELKS1 is a synapse-based cargo-tether that captures Golgi-derived transport cargo labeled with Rab6.
Figure 7

A. Mitochondria

B. Cerulean-ELKS1αβ

C. mito-tag

D. mito-ELKS1αβ

E. GFP

F. Synapsin-1

G. HA-ELKS1αβ

H. merge

I. HA-ELKS1Δ955-971

J. HA-ELKS1αβ

Note: The figure shows various experimental setups involving mitochondrial and synaptic proteins, along with corresponding images and graphs.
Chapter 3: Figure 7

Reconstitution of the ELKS-Rab6 vesicle capturing mechanism on the mitochondria

(A) Representative schema of the mito-ELKS1 assay in neuronal cultures. The mito-tag is composed of the trans-membrane domain (TMD) of the mitochondrial Tomm20 protein and the Cerulean fluorescent protein. The mito-tag is fused to the N-terminus of ELKS1αB, which is then attached to the mitochondrial surface via the Tomm20-TMD.

(B) Example confocal images of mito-ELKS1αB (mito-tag = Tomm20-TMD + Cerulean) and Cerulean- ELKS1αB to compare their localization. Wild type neuronal cultures were infected with a lentivirus to express either the mito-tagged or the Cerulean-tagged ELKS1αB. Cultures were infected with a second independent lentivirus to express mitoDsRed, which is an established mitochondria-localizing fusion protein. To visualize overexpressed ELKS1αB, cultures were immunostained against an anti-GFP antibody. To visualize overexpressed mitoDsRed, cultures were immunostained against an anti-RFP antibody. Cultures were co-stained against an anti-Bassoon antibody to mark synaptic regions.

(C) Example Western blot images of neuronal cultures showing the levels of the overexpressed mito-tagged protein across all three conditions. To visualize the overexpressed proteins, membranes were immunoblotted against an anti-GFP antibody.

(D) Wild type mouse hippocampal cultures were infected with a lentivirus to express the mito-tag alone for negative control, the mito-tagged ELKS1αB, or the mito-tagged ELKS1αBΔ955-971. High pressure frozen cultures were imaged with electron microscopy.

(E) Quantification shows the number of small vesicles (diameter ≤ 50 nm) tethered to mitochondrial surface. A vesicle was considered tethered if it was within 70 nm from a mitochondrial surface. The maximum distance of 70 nm was determined by measuring the longest visible string or tether between vesicles and mitochondria; however, a tether did not have to be visible as long as the vesicle fell within the 70-nm-distance from the mitochondrial membrane. Mito-tag, n = 198 mitochondria / 2 independent cultures; mito- ELKS1αB, n = 208/2; mito-ELKS1αBΔ955-971, n = 197/2.

(F) Representative schema of the mito-Rab6B assay in neuronal culture. To localize Rab6B proteins to the mitochondria, Rab6B protein was fused with a mito tag as described above. Wild type cultures were infected with a lentivirus to express either mito-Rab6BQL or mito-Rab6BTN, and co-infected with a second independent lentivirus to express the HA-tagged ELKS1αB or ELKS1αBΔ955-971.

(continued on next page)
(G) Example confocal images of the colocalization of mito-Rab6 with the mitochondrial control mitoDsRed. Mito-tag = Tomm20-TMD + Cerulean. Wild type neuronal cultures were infected with a lentivirus to express either the mito-Rab6B_{QL} or mito-Rab6B_{TN}. Cultures were infected with a second independent lentivirus to express mitoDsRed. To visualize overexpressed Rab6B, cultures were immunostained against an anti-GFP antibody. To visualize overexpressed mitoDsRed, cultures were immunostained against an anti-RFP antibody. Cultures were co-stained against an anti-Synaptophysin-1 antibody to mark synaptic regions.

(H) Example Western blot images showing the levels of the overexpressed proteins in neuronal cultures across all three conditions. To visualize overexpressed mito-Rab6B, cultures were blotted against an anti-GFP antibody. To visualize overexpressed HA-ELKS1, cultures were blotted against an anti-HA antibody.

(I) To visualize overexpressed mito-Rab6B, cultures were immunostained against an anti-GFP antibody. To visualize overexpressed HA-ELKS1, cultures were immunostained against an anti-HA antibody. Cultures were also immunostained against an anti-Bassoon antibody to mark synaptic regions. All signals were acquired by confocal microscopy.

(J) Quantification shows the fraction of Bassoon objects that contained mito-Rab6 signal (the threshold of co-localization was set to > 0.25 μm^2 of the Bassoon area. ELKS1αB + mito-Rab6B_{QL}, n = 19 fields of view / 3 independent cultures (fields of view = 50 μm x 10 μm, each field of view typically containing about 30-60 Bassoon objects). ELKS1αB_{Δ955-971} + mito-Rab6B_{QL}, n = 21/3; ELKS1αB + mito-Rab6B_{TN}, n = 18/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0. analyzed by one-way ANOVA, followed by Holm-Sidak post hoc comparison to mito-ELKS1αB condition (Fig. 7E) or to ‘ELKS1αB + mito-Rab6B_{QL}’ condition (Fig. 7J). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
CHAPTER FOUR:

Are there broader cell biological roles of ELKS beyond the synapse?

Concepts and experimental designs are by H.N. and P.K. Experimental procedures, analyses, and illustrations were carried out and presented by H.N. I would like to thank R. Held for sharing his mass spectrometry data on ELKS1 baits, and Dr. T. Schwarz for the mitoDsRed and EB3 constructs.
Given that the function of ELKS have been studied almost exclusively in context with presynaptic vesicle exocytosis, any possible roles in upstream pathways may have been missed. To address cell biological functions of ELKS upstream of the active zone, I took a systematic approach, addressing four key cellular domains that depend on Golgi and/or microtubule organization, namely cell and Golgi morphology, microtubule dynamics, and axonal transport.

A role for ELKS (Dai et al., 2006; Grigoriev et al., 2007; Ko et al., 2003; Lansbergen et al., 2006; Monier et al., 2002; van der Vaart et al., 2013) in regulating transport-associated pathways has been suggested in the literature, but the supporting data is mostly circumstantial and does not provide mechanistic insight. The fact that ELKS1 knockout results in embryonic lethality in mice also supports the notion that this protein family has crucial role in developmental processes (Liu et al., 2014) and both ELKS1 and ELKS2 interacts with multiple molecular players involved in transport and microtubule stability, including Rab6 (Grigoriev et al., 2007; Monier et al., 2002), liprin-α (Dai et al., 2006; Ko et al., 2003), CLASP2 (Lansbergen et al., 2006), and LL5β (Lansbergen et al., 2006; van der Vaart et al., 2013). CLASP2 is trans-Golgi resident protein aiding microtubule nucleation and stability and LL5β forms microtubule-anchoring complexes on the plasma membrane. It is possible that ELKS' role in axonal transport upstream of synaptic vesicle exocytosis contributes to these effects.

Additionally, in a broad interaction screen, I identified a potential interaction between ELKS and VPS13, a protein with yet unknown function in mammalian cells. Evidence from the literature, however, supports the hypothesis that ELKS and VPS13 might interact with a functional relevance in cellular transport.
ELKS plays a role in axonal trafficking in neurons

I designed various neuron-based trafficking assays. I used primary hippocampal cultures that were transfected with either mitoDsRed mitochondrial marker (Hollenbeck and Saxton, 2005) or with a tdTomato-SV2A protein synaptic vesicle cluster marker, and imaged them in ELKS1/2α conditional double knockout (cDKO) neurons, using live imaging conditions as described in previous experiments. For determining general health of neurons after transfection, I used a soluble marker to visualize the shape of the imaged cell. Imaged neurons showed healthy branch arborization. Axonal mitochondrial transport was significantly compromised (Figs. 1A-C), where the distance of continuous movement was much shorter in ELKS1/2α cDKO relative to control. The flux and velocity of trafficking events; however, remained unchanged. Furthermore, the moving mitochondrial particles did not pause for longer periods of time (Figs. 1A-C).

SV2 is a known synaptic vesicle protein readily trafficked along axons (Lazzell et al., 2004). My initial trial experiments suggested an effect on axonal trafficking of SV2 when a manual method was used to trace SV2 trafficking events on kymographs (Figs. 1D-F). However, as the health of the cultures improved with better transfection methods, speeds of SV2 trafficking events increased such that data became difficult to quantify without photobleaching (Figs. 1G-I). Nevertheless, the data suggests that trafficking of SV2-containing packages are compromised in ELKS1/2α cDKO neurons.
Chapter 4: Figure 1
Chapter 4: Figure 1

Removal of ELKS1/2α leads to shorter distances travelled between pauses (called processivity) by transport packets in axons

*Processivity* = distance travelled by puncta between pauses; *instant speed* = speed of continuous movement between pauses; *flux* = number of events per 10 μm during the imaging time; *manual tracing method* = manual tracing of events on kymographs generated by ImageJ; *automated method* = quantification of movements using a custom ImageJ tracing program written by the Harvard Neuro Imaging Facility (L. Ding).

Primary hippocampal cultures were acquired as described previously, were transfected with mitoDsRed via calcium-phosphate transfection at DIV 11 and imaged live on a fluorescent microscope with an LED light source. Cells were imaged live on 35 °C in Hibernate-A (BrainBits) solution for 5 min with 0.5 sec/frame frequency.

(A-C) Representative mitoDsRed images (A), kymographs (B), and analysis using an automated method (C) of the movements of mitoDsRed events in live hippocampal neurons of ELKS1/2α control and cDKO cultures. ELKS1/2α control, n = 40 cells / 3 independent cultures; ELKS1/2α cDKO, 45/3.

(D-F) Representative images (D), kymographs (E), and quantification manual tracing (F) of the movements of tdTomato-SV2A events in live hippocampal neurons of ELKS1/2α control and cDKO cultures. Cultures were acquired as described previously in mitoDsRed experiment. Image quantification was done manual with ImageJ. ELKS1/2α control, n = 20 cells / 3 independent cultures; ELKS1/2α cDKO, 20/3.

(G-I) Representative images (G), kymographs (H), and analysis using an automated method (I) of the movements of tdTomato-SV2A events in live hippocampal neurons of ELKS1/2α control and cDKO cultures. Cultures were acquired as described previously in mitoDsRed experiment. Image quantification was done using a custom ImageJ program written by the Harvard Neuro-Imaging Core Facility. ELKS1/2α control, n = 19 cells / 3 independent cultures; ELKS1/2α cDKO, 21/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test.
Removal of ELKS only mildly affects Golgi integrity and axonal growth

There are several lines of evidence from the literature pointing to ELKS being involved in Golgi-derived pathways. ELKS was originally discovered as a Rab6-binding protein in a yeast-two-hybrid assay (Monier et al., 2002), and was found to interact with the LL5β/CLASP2 complex that aids microtubule nucleation from the Golgi (Astro et al., 2014; Dai et al., 2006; Drabek et al., 2006; Grigoriev et al., 2007, 2011; Ko et al., 2003), thus I examined whether ELKS contributes to Golgi integrity.

I immunostained ELKS1/2α control or cDKO hippocampal cultures with an anti-GM130 antibody to mark the Golgi apparatus and costained them with an anti-Map2 antibody to mark dendrites ELKS1/2α cDKO cultures exhibited diminished Golgi area, along with higher number of fragmented Golgi (Figs 2A and 2B), which is a sign of compromised Golgi structures (Nakagomi et al., 2008). The mild effect of ELKS removal on the Golgi structure suggest that ELKS may not be a direct effector on Golgi; however, it does suggest its involvement in Golgi-related pathways (Figs 2A and 2B).

To analyze branch complexities, I performed Sholl analysis (Schmitz et al., 2011) on ELKS1/2α cDKO hippocampal cultures. To better isolate processes of a single neuron, I transfected hippocampal cultures with a soluble Cerulean fluorescent protein, labeling only a small fraction of cells (Fig. 2C). I then employed Sholl analysis to quantify arbor complexity. I found that axonal branch development in ELKS1/2α cDKO neurons was only mildly affected relative to controls (Fig. 2D). These data suggested that ELKS does not have a major role in neuronal arborization, although mild effects on axonal branching are present. It does, however, suggest that ELKS may be regulating a cellular process, the disruption of which could negatively impact neurite extension, for example axonal trafficking or microtubule stability.
Chapter 4: Figure 2

A. ELKS1/2α control and ELKS1/2α cDKO stained with GM130 and Map2. Scale bar: 10 μm.

B. Bar graphs showing GM130 area, number of fragments, and GM130 intensity for control, cDKO, ELKS1/2α, and ELKS1/2α cDKO conditions.

C. Soluble Cerulean signal in live wild type neurons. Scale bar: 50 μm.

D. Graphs showing the number of intersections versus distances from cell soma for dendritic and axonal regions of control and ELKS1/2α cDKO neurons. Step size = 10 μm.

E. Bar graphs showing number of axons and axon length for control and cDKO conditions.
Chapter 4: Figure 2

ELKS removal has mild effects on Golgi and branch development

(A and B) Representative confocal images (A) and quantification (B) of ELKS1/2α control and cDKO hippocampal neurons. Cultures were acquired as described before, infecting ELKS1/2α floxed neurons with a Cre-recombinase expressing virus at DIV 5, and fixing at DIV 14. Cultures were immunostained with an anti-GM130 to visualize Golgi apparatus and with an anti-Map2 to visualize dendritic branches. ELKS1/2α control, n = 150 cells / 2 independent cultures; ELKS1/2α cDKO, 132/2.

(C-E) Representative confocal images (C) and quantifications (D and E) of ELKS1/2α control and cDKO hippocampal neurons in Sholl analysis. Cultures were acquired as described before, and were transfected at DIV 7 via a calcium-phosphate transfection method with soluble Cerulean under a human Synapsin promoter. Cultures were fixed at DIV 8-10 and Cerulean signal was imaged on a confocal microscope. Quantifications show either Sholl analysis (D) and counting total axonal branches and lengths (E). Black = control, grey = ELKS1/2α cDKO. ELKS1/2α control, n = 50 cell / 3 independent experiments; ELKS1/2α cDKO, 45/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test (Figs. 2B and 2E). For specifications on the primary antibodies used, see Table S2 in Appendix 1.
ELKS might form a complex with microtubule associated proteins, but it does not contribute to microtubule dynamics.

In an effort to identify additional interaction partners for ELKS, in collaboration with a colleague, a broad screening approach was employed. GST-tagged ELKS1 and ELKS2 fragments were used from their N- and C-terminal regions as immobilized baits in a pulldown assay, where purified adult mouse brains were used as inputs. The interaction assay was then run on an SDS-PAGE gel, and bands at different sizes were submitted for mass spectrometric analysis. We identified many microtubule associating proteins as potential interactors for ELKS (Supplemental Figure 5), including many MAP proteins, kinesins (KIF1 and KIF2), and dynein and dynamin subunits. Thus, I hypothesized that ELKS may play a role in microtubule related pathways in the neuron.

Previous studies implicated ELKS as a protein forming an adhesion complex for microtubules near the cell edge in nonneuronal systems via interactions with Bicaudal-D, LL5β, CLASP2 and MICAL3 (Grigoriev et al., 2007; Lansbergen et al., 2006; Stehbens et al., 2014). To test if ELKS plays a role in microtubule function in the axon, I measured axonal microtubule polymerization using eGFP-tagged EB3 as a reporter. EB3 is a microtubule plus-end capping protein, where it moves together with the plus-tip of the microtubule as it grows (polymerizes) and collapses (depolymerizes). The growing phase is much slower than the collapse phase, thus the direction in which the microtubule cable grows can be easily identified. Since microtubule plus-ends always face towards the cell edge (anterograde direction) in axons, a branch can be identified whether it is an axon or dendrite based on the directionality of the EB3 comets. In an axon, EB3 comets always grow in one direction, while in the dendritic branches EB3 grows in both directions.
Chapter 4: Figure 3

A. Example eGFP-EB3 in live wild type neuron

B. ELKS1/2α control vs. ELKS1/2α cDKO

C. Comparison of various parameters:
- Distance polymerization (μm)
- Fraction of time spent polymerizing
- Flux (number per 10 μm)
- Instant speed of comets (μm/s)

Control vs. cDKO for ELKS1/2α
ELKS does not contribute to microtubule dynamics

(A-C) Example image (A), kymograph (B), and quantification (C) of eGFP-EB3 movements in ELKS1α conditional knockout cultures. ELKS1/2α homozygous floxed hippocampal cultures were infected with a Cre-recombinase (active for cDKO or truncated for control) lentivirus at DIV 5. Cultures were transfected with eGFP-tagged EB3 at DIV 11, followed by live imaging at DIV 13 using a fluorescent scope. Individual axonal segments were identified by the directionality of EB3 comets. eGFP-EB3 comets were analyzed to determine microtubule polymerization using kymographs generated in ImageJ. (C) Quantification shows growth-distance, speed, time, and flux of EB3 comets. ELKS1/2α control, n = 224 axons / 16 cells / 3 independent cultures; ELKS1/2α cDKO, 268/19/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test.
ELKS1/2α conditional knockout cultures were acquired as described before, where ELKS1/2α homozygous floxed hippocampal cultures were infected with a Cre-recombinase (active for cDKO or truncated for control) lentivirus at DIV 5. Then, cultures were transfected with eGFP-tagged EB3 at DIV 11, followed by live imaging at DIV 13 using a fluorescent scope. Individual axonal segments were identified by the direction of EB3 comets. The direction, speed, and number of eGFP-EB3 comets were analyzed to determine microtubule polymerization using kymographs generated in ImageJ. Microtubule polarization appeared unaffected in ELKS1/2α cDKO neurons relative to controls (Fig. 3). Thus, I concluded that ELKS does not contribute to plus-end microtubule stabilization.

**VPS13 proteins as possible presynaptic proteins and ELKS interactors**

In the same broad interaction screen mentioned in the previous section, I also identified VPS13, a protein with yet unknown function in mammalian cells, as a potential interaction partner for presynaptic ELKS. In yeast, VPS13p gene interacts with multiple other genes involved in vesicle formation and transport, including Ypt6 the yeast homolog of mammalian Rab6 and it functions in protein sorting and transport between the trans-Golgi (Bankaitis et al., 1986; Brickner and Fuller, 1997; Park and Neiman, 2012). Both ELKS (Monier et al., 2002) and VPS13 (Seifert et al., 2015) have been independently suggested to functionally interact in Rab6-mediated transport pathways, and both protein families have been implicated in neurological disorders, specifically autism (Abdelmoity et al., 2011; Yu et al., 2013). These led me to hypothesize that ELKS and VPS13 interact with one another to control transport.

VPS13 (SOI1) gene was first identified in mutant yeast cells defective in the delivery of CPY to the vacuole (Bankaitis et al., 1986), and in the cycling of Kex2 between the Golgi and
the vacuole (Brickner and Fuller, 1997), indicating a role in trafficking. It is also thought to be one of the essential genes required for proper growth of the pro-spore membranes that are membrane-bound cytosolic compartments used to sort and package cellular material prior to sporulation (Rabitsch et al., 2001).

VPS13 gene family is highly conserved across eukaryotic organisms. In humans there are four VPS13 orthologs, VPS13A, B, C and D (Velayos-Baeza et al., 2004). Genetic mutations in VPS13A (or CHAC) and VPS13B (or COH1) genes give rise to hereditary neurological disorders in humans, chorea acanthocytosis (Rampoldi et al., 2001) and Cohen syndrome (Kolehmainen et al., 2003; Cohen et al., 2005), respectively, indicating that at least these two genes play important roles in the nervous system function. Chorea-acanthocytosis is an autosomal recessive neurodegenerative movement disorder, the symptoms of which closely resemble Huntington’s chorea (Dobson-Stone et al., 2004; Kurano et al., 2006; Rampoldi et al., 2001; Tomemori et al., 2005). Cohen syndrome is a debilitating neurodevelopmental disorder causing mental retardation and facial dysmorphism (Kolehmainen et al., 2003; Seifert et al., 2009; Velayos-Baeza et al., 2004). Clear parallels have been drawn between Cohen syndrome and autism spectrum disorder (ASD) showing significant overlap and comorbidity; furthermore, VPS13B gene was identified as one of the loci associated with ASD (Cohen et al., 2005).

There are multiple publications characterizing the human VPS13A and B genes and their predicted protein sequences (Kolehmainen et al., 2003; Mizuno et al., 2007; Rampoldi et al., 2001; Velayos-Baeza et al., 2004) but almost no data are available on VPS13C and D, thus I conducted sequence and transcript analysis comparing all four genes simultaneously (Fig. 4A). I aligned genetic sequences (SwissProt) and found that VPS13A and C are the most homologous with ~60% homology. In the mass spectrometric screen, I identified VPS13C as a potential ELKS interactor.
### Chapter 4: Figure 4

#### A

<table>
<thead>
<tr>
<th></th>
<th>Vps13A CHAC</th>
<th>Vps13B C0H1</th>
<th>Vps13C</th>
<th>Vps13D</th>
<th>Vps13p/S011 (yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mw (kDa)</strong></td>
<td>360</td>
<td>444</td>
<td>420</td>
<td>490</td>
<td>358</td>
</tr>
<tr>
<td><strong>evolutionarily conserved</strong></td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>domains</strong></td>
<td>chorein DUF1162</td>
<td>chorein DUF1162 ATG-C</td>
<td>chorein DUF1162</td>
<td>chorein DUF1162 UBA</td>
<td>chorein DUF1162</td>
</tr>
<tr>
<td><strong>localization</strong></td>
<td><em>cis</em>-Golgi (neurons)</td>
<td>Golgi (HeLa, HEK)</td>
<td>n/a</td>
<td>n/a</td>
<td>trans-Golgi, endosomes</td>
</tr>
<tr>
<td><strong>function</strong></td>
<td>n/a</td>
<td>protein sorting</td>
<td>n/a</td>
<td>n/a</td>
<td>protein sorting, transport, membrane formation</td>
</tr>
<tr>
<td><strong>disease (in humans)</strong></td>
<td>Chorein synd</td>
<td>Autism Cohen synd</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

#### B

![Normalized mRNA expression levels](image)
Chapter 4: Figure 4

The vacuolar protein sorting VPS13 protein family expresses in the brain and is important for healthy neuronal functions

(A) The table shows gathered knowledge base on VPS13 protein family. Green columns indicate mammalian proteins, purple column indicates yeast protein.

(B) mRNA expression levels of genes Vps13A-D. Expression levels of genes VPS13A-D were compared to expression levels of established neuronal active zone proteins Synapsin1 (Syn1), ELKS (ERC1 and ERC2) and Munc13 (Unc13a and Unc13b) levels were used as controls across six different mouse-tissues (brain, lung, spleen, heart, kidney, liver); n = 3 mice assayed with 2 repeats in a probe-based assay, mRNA levels of Cq values were normalized to GAPDH from appropriate samples.
Currently we know very little about their localization and function in mammalian cells. VPS13A has been suggested to have transmembrane regions (Kolehmainen et al., 2003), but I did not find transmembrane domains, which is in agreement with another group’s suggestion that these are not transmembrane proteins (Seifert et al., 2009). VPS13B (Cohen syndrome protein) has been reported to localize to the cis-Golgi where it interacts with Rab6, and possibly plays a role in protein sorting and trafficking (Seifert et al., 2009, 2011, 2015). In PC12 cells, VPS13A (Chorein syndrome protein) was shown to localizes to the trans-Golgi (Hayashi et al., 2012).

I conducted expression profiling of VPS13 genes by RT-qPCR for all four isoforms using mRNAs isolated from adult mouse tissues. The results showed that the VPS13 isoforms are ubiquitously expressed, including the brain, but only VPS13A (Chorein syndrome protein) showed mRNA levels comparable to other synaptic proteins, like ERC1 (protein product: ELKS1) or Unc13a (protein product: Munc13-1) (Fig. 4B).

Conclusions

I explored the function of ELKS outside the active zone, given that its role in upstream pathways have not been examined. I found that Golgi integrity and neuronal branch development were only mildly affected, while microtubule dynamics remained unchanged. Processivity of cargo transport was significantly disturbed; however, it could be explained by secondary effects following disruptions within the synapse. Transport motor integrity remained unaffected as I found no change in instant cargo speed. While not excluding the possibility that ELKS may have a role in pathways yet unexamined, my data together with previous publications from our lab (Held et al., 2016; Kaeser et al., 2009; Liu et al., 2014, Wang et al., 2016) support the hypothesis that in neurons ELKS primarily functions at presynaptic regions.
CHAPTER FIVE:

DISCUSSION
I addressed two questions in this study: (1) Is there a Rab-dependent cargo delivery to synaptic boutons? (2) Is there a mechanism at the synapse that actively tethers transport cargo? I found that while Rab6 largely resides at the Golgi in neurons, it also has significant localization in axonal branches. This peripheral Rab6 fraction is highly mobile and accumulates at synaptic regions, similarly to trafficking proteins. The synaptic enrichment of Rab6 is driven by its active vesicle-associated state and the presence of presynaptic ELKS1. On the other hand, ELKS1 remains anchored at synapses with a wide subsynaptic distribution that allows association with newly arriving cargo. ELKS binds primarily the active vesicle-associated form of Rab6 via a short segment near its C-terminus, similarly to the Rab6-binding Golgin vesicle tethers. The separate removal of Rab6B and ELKS1α/β from mouse hippocampal neurons phenocopy each other, both increasing extra-synaptic vesicle deposition, decreasing the number of vesicles per bouton, and decreasing levels of active zone material per synapse. Cargos artificially tagged with Rab6 are recruited to ELKS-enriched synapses via a direct interaction between ELKS and Rab6.

Both ELKS and Rab6 protein families have been implicated in neurological disorders. Disruption in ELKS genes were found in cases of autism spectrum disorder (Silva et al., 2014), language disorders (Chen et al., 2017), and other developmental delays (Abdelmoity et al., 2011). Rab6 has been implicated in the neurodevelopmental Cohen syndrome (Seifert et al., 2015) and in Alzheimer’s diseases (Elfrink et al., 2012; Scheper et al., 2007). Their involvement in neurological disorders suggests their importance in healthy neuronal function.
Interaction between the cargo label Rab6 and the coiled coil vesicle tether ELKS1 mediates the final steps in presynaptic cargo delivery

There is an active cargo recruitment mechanism at presynaptic terminals that depends on the direct interaction between Rab6 and ELKS1. A subtype of presynaptic cargo is labeled with Rab6 at the Golgi apparatus and transported along microtubule tracks of axonal branches towards presynaptic boutons. When a Rab6-labeled cargo arrives at presynaptic boutons, the C-terminal region of ELKS1 engages the cargo via directly binding GTP-Rab6, while the rest of the long coiled-coil ELKS1 region acts as a recruiting lasso.
Is ELKS at the active zone or not?

ELKS, a large coiled coil scaffolding protein in the presynaptic terminals, was originally discovered as a Rab6-binding protein in a yeast two-hybrid screen of a mouse embryo cDNA library, which was later confirmed in brain pulldown assays (Monier et al., 2002). However, the physiological relevance of their interaction in neurons remained unexamined. Soon after its initial discovery, ELKS was suggested to be part of the active zone in neurons showing associations with RIM1 and Munc13-1, two integral active zone proteins (Ohtsuka et al., 2002). A quick succession of studies followed confirming the interaction of ELKS with these and additional active zone members (Ko et al., 2003; Takao-Rikitsu et al., 2004; Lu et al., 2005; Dai et al., 2006; Inoue et al., 2006; Kittel et al., 2006; Ko et al., 2006), narrowing the focus entirely on the function of ELKS at the active zone in presynaptic vesicle exocytosis. By the time one of the first nonneuronal studies came along (Lansbergen et al., 2006), the field had settled on the working model that ELKS is primarily an active zone protein. A series of follow-up studies confirmed that ELKS expresses and functions in nonneuronal cells and was suggested to tether microtubules and Golgi-derived cargos near the cell edge (Grigoriev et al., 2007, Astro et al., 2014; Fourriere et al., 2019; Grigoriev et al., 2007, 2011; Lansbergen et al., 2006; Patwardhan et al., 2017). Despite the numerous publications from nonneuronal systems, the role of ELKS in neurons upstream of the active zone remained unaddressed.

The significance of ELKS in mediating synaptic vesicle exocytosis was demonstrated by our lab using genetic studies, where removal of the major isoforms ELKS1α or ELKS2α disrupted synaptic transmission (Held et al., 2016; Kaeser et al., 2009; Kiyonaka et al., 2012; Liu et al., 2014). Yet we could not explain an important discrepancy. The significant decrease in transmission that was observed in the ELKS knockouts was never accompanied by any structural/tethering defects at the active zone (Kaeser et al., 2009; Liu et al., 2014). A recent publication from our lab demonstrated the importance of ELKS in tethering and docking
vesicles to the vesicle release site when ELKS1/2α were removed together with RIM1/2 (Wang et al., 2016), but ELKS1/2α removal alone had no effects on presynaptic ultrastructure. This suggested that ELKS has yet unexplored functions upstream of the active zone. In light of this, I set out to systematically test the neuronal localization of ELKS and to assess potential defects in upstream pathways upon ablation of the main ELKS1a and ELKS2a isoforms. I examined (in chapter four) branch development, Golgi integrity, microtubule dynamics and axonal trafficking dynamics; however, I did not find any dramatic effect on these cellular functions, with the exception of the processivity of axonal trafficking. The frequent pausing of trafficking organelles; however, did not conclusively point to a role of ELKS outside of the presynaptic terminal. In fact, it could be explained as a secondary effect from a dysfunctional presynaptic terminal.

Given the primarily presynaptic localization of ELKS in neurons and the lack of any significant role outside of the terminal, I narrowed the possible site of additional function inside the presynaptic terminal, but upstream the active zone. The fact that Rab6 was suggested to interact with ELKS in neurons, I followed up on Rab6. Rab6 was reported to mainly reside in the Golgi in many cell types where it recruits and anchors large coiled coil Golgin vesicle tethers to the trans-Golgi surface. This led me to draw structural parallels between ELKS and Golgin tethers (Figure 2B), and address whether ELKS plays a role as a cargo tethering protein at the presynaptic terminals similar to Golgins tethering cargos at the Golgi surface.
ELKS is the largest known coiled coil protein in the presynaptic terminal

Comparing coiled coil predictions of various protein sequences. Human protein sequences were acquired from the UNIPROT protein sequence database in FASTA format and were submitted for secondary sequence structure analysis using the ExPasy COIL program from EMBL. The black line indicates the length of the protein; the blue boxes indicate predicted coiled coil regions. The coiled coil region on ELKS1 is represented with a darker blue bar for emphasis.

(A) Comparisons of coiled coil regions of presynaptic proteins. Scales of sequences are comparable within Fig 2A. (B) Comparisons of coiled coil regions of ELKS1 with some of the Golgin protein sequences. Scales of sequences are comparable within Fig 2B.
Several issues needed to be addressed before examining the role of ELKS in presynaptic terminals in context with Rab6: (1) First was its subsynaptic localization, (2) second was the remaining beta isoforms that were left behind in the original ELKS1/2a knockouts (Liu et al., 2014), and (3) the third was examining neuronal Rab6.

**Issue (1): subsynaptic localization.** It has been established that presynaptic transport is mediated by microtubule-based mechanisms, where cargos are carried by various motor complexes, attached directly to microtubule cables (Goldstein et al., 2008; Kapitein and Hoogenraad, 2015). When a microtubule cable runs through a synaptic bouton, a dense cloud of vesicles surrounds the transport track. The active zone, on the other hand is situated adjacent to the plasma membrane without much access to other synaptic regions. In STED microscopy, side-view synapses show an active zone sitting at the plasma membrane with a range no more than 100-200 nm towards the center of the presynaptic cloud (Wong et al., 2018). When observed under electron microscopy, microtubule cables are rarely seen within this limited range of the active zone (Schrod et al., 2018). This would pose a serious obstacle for ELKS1 to capture vesicle cargos if it was only sitting in the active zone. While the subsynaptic localization of the *Drosophila* Bruchpilot (homolog to the mammalian ELKS) has been characterized, the subsynaptic distribution of ELKS in vertebrate presynaptic terminals was simply assumed to be at the active zone based on functional data, but was never directly demonstrated. Employing superresolution imaging of primary hippocampal cultures, I determined that ELKS1 and ELKS2 exhibit strikingly different subsynaptic distributions (chapter three Fig. 1). While ELKS2 is confined to the active zone, ELKS1 is distributed throughout the synapse with the majority away from the active zone (chapter three Fig. 1J).

**Issue (2): beta isoforms of ELKS.** In a series of biochemical experiments, I confirmed that all isoforms of ELKS was able to bind Rab6 (chapter one Fig. 5), which confirmed that the beta isoforms are also able to bind Rab6 with equal efficiency. Thus, to have a better genetically controlled study, we generated a new ELKS1 knockout line that removed all isoforms of ELKS.
Issue (3): Rab6 localization and function in neurons. There was a lack of evidence for Rab6 existing in presynaptic terminals and having synaptic functions. In chapter three, I addressed this shortcoming by systematically characterizing neuronal Rab6, and determined that Rab6 has significant presynaptic levels that are vesicle-bound and directly interacts with ELKS.

Addressing these three points led to the findings that ELKS1 is not an active zone protein and that vesicle-bound Rab6 is recruited to presynaptic terminals by ELKS1, which opened the path to propose and test a new model for ELKS function in neurons. In this new model, I propose that ELKS1 acts as a presynaptic cargo tether, recruiting Rab6-labeled precursor vesicles transported from the Golgi to the presynaptic terminal.

Maintaining the presynaptic terminal

Synaptic assembly and maintenance are a persistently daunting challenge for neurons during both development and mature state. Neurons are required to continuously recycle and replenish presynaptic material. Beside homeostatic maintenance of their synapses, neurons also need to ensure activity dependent synaptic plasticity, the basis for learning and memory. Plasticity is partly achieved by neurons adjusting the strength of their synaptic connections via increasing or decreasing total protein levels of their presynaptic terminals (Alvarez-Castelao and Schuman, 2015). While there is evidence for local protein synthesis at presynaptic terminals (Jung et al., 2012; Li et al., 2015), the building blocks and components of this machinery would still have to be transported from the soma. There is no way around this. Neurons need long-range trafficking.

The currently prevailing model is that presynaptic proteins are generated in the soma, sorted onto vesicular cargo in the Golgi apparatus and transported in by motor proteins along microtubules over long axonal distances (Bury and Sabo, 2016; Maeder et al., 2014a). The
challenge of large distances can be tackled by a continuous conveyor-belt method, where instead of a need-based protein synthesis the soma continuously makes and sends out all types of cargos towards distant synapses, and synapses take what they need (Wong et al., 2012). On a general basis, there is probably more cargo in circulation than what synapses need. Since neurons are constantly sending and receiving stimuli, there is a high chance that the strength of a synapse needs to be adjusted on short notice. There are reports demonstrating the formation of nascent synapses within minutes after stimulation with high concentrations of potassium at the *Drosophila* neuromuscular junctions (Ataman et al., 2008; Vasin et al., 2014). Due to the large distances of axons, material would not get to presynaptic sites fast enough if they had to wait for the soma to respond. Keeping a surplus of cargo in circulation, however, could ensure immediate response, where synapses with increased activity can quickly capture cargo to increase their size, providing the basis for short-term plasticity. The soma might have a mechanism to keep track of the ratio between sent and returned cargo and possibly use it as feedback to arrange more long-term changes if necessary. When a cell is stimulated over longer periods of time and fewer or no cargo returns to the soma, the soma could respond by increasing the number of newly generated cargos, which over time could become the new *status quo*.

Is cargo delivery random along axons or is it targeted? Synaptic changes are site specific, thus neurons adjust the size of their synapses only at the appropriate sites. Most presynaptic boutons are formed *en passant* along axons, where microtubule tracks do not terminate (Kapitein and Hoogenraad, 2015). Cargos frequently pass through synaptic boutons without pausing. Not having to stop at every bouton considerably speeds up transport to reach more distant regions. But this could also make cargo selection more difficult, especially in light of the fact that cargos are not made all equal and their contents can vary greatly. Yet neurons manage to replenish their synapses at the right regions with the right content, which suggest the existence of a precise transport targeting mechanism.
How does a cargo know where to stop along axonal tracks? All other cellular compartments are known to have a cargo-capturing complex that serves to recognize and capture newly arriving transport cargo. Yet, a similar mechanism has remained largely elusive for the capture of presynaptic cargo.

**Mechanisms of cargo targeting via tethering complexes**

The mechanism to target and recruit essential proteins (active zone components, adhesion molecules, ion channels, and many others) to the presynaptic terminal is poorly understood. Since most other cellular compartments have a known cargo targeting mechanism, we can gain inspiration from those models. The general targeting mechanism across compartments appears to operate on a common principle: proteins are sorted onto vesicular cargos, labeled by content and destination, trafficked along cytoskeleton by motors and captured by cargo-tethering complexes at their final destination (Stenmark, 2009; Yu and Hughson, 2010; Zerial and Mcbride, 2001). To achieve appropriate matching of cargo with target sites, cells use a two-part mechanism. In part one, cargos are labeled by content and destination; in part two, tethering complexes are built at target sites with specificity for only certain types of cargo labels. There are two major types of cargo-capturing complexes: long coiled coil tethers, like the Golgins on the trans-Golgi surface, and multisubunit tethering complexes that frequently contain proteins with coiled coil domains (Yu and Hughson, 2010). Likely, evolution did not come up with a different method to replenish synaptic proteins.

ELKS falls into the category of large coiled coil vesicle tethers (Fig. 2). These tethers are frequently above 100 kDa in size, where the majority of the sequence is comprised of multiple long alpha-helical coils, positioned one after another (Cheung and Pfeffer, 2016). When an alpha-helical segment wraps around another, frequently by dimerizing with another alpha-helical protein, it forms a double coil, also called a coiled coil (Truebestein and Leonard, 2016). The
formation of the double coil straightens and stiffens the protein, forcing it into an elongated rod-like shape. Large tethers with multiple coiled coil domains can reach up to 100 nm to facilitate easy tethering (Cheung and Pfeffer, 2016). A tether is not composed of one continuous coil, rather multiple shorter domains with hinges separating each domain. The coiled regions provide tensile strength, while the hinges provide flexibility. ELKS is made up of four coiled coil domains with short hinges between each (Kaeser and Held, 2018; Monier et al., 2002).

Given that ELKS shares strikingly similar sequence features with vesicle tether Golgins (Fig. 2) (Barr, 1999; Cheung and Pfeffer, 2016; Munro, 2011), reviewing how Golgins tether vesicles could be an invaluable resource to understand the mechanism of ELKS. Endosomal cargo continuously arrive to the trans-Golgi surface from other cellular regions that need to be captured and fused with the Golgi membrane, which is achieved by the Golgins, like TMF1, Golgin-245 or GCC185. They frequently have a short (less than 50 amino acids) disordered C-terminal region that contains a GRIP domain. The GRIP domain anchors itself into the trans-Golgi membrane by binding to a small GTPase, either Rab6 or Arl-1. The N-terminal region of the Golgins have a longer disordered region (up to a couple of hundred amino acids in length) filled with Proline residues, ALPS motifs and other hydrophobic regions that are thought to serve as curvature-sensors and lipid-interactors. When Golgins dimerize and form coiled coils, the disordered N-termini of the dimerized proteins form a Y-shape and capture vesicles by binding directly to vesicle lipids, adaptor proteins and small GTPases. Golgins have been shown to have Rab GTPase binding sites on their N-terminus as well, and are thought to capture vesicles via both Rab- and lipid-binding (Cheung and Pfeffer, 2016).

The initial contact between cargo and Golgin tether is thought to be highly transient, which is enough to halt a moving vesicle in its tracks but can quickly disengage to pass the cargo onto the next tethering site. Subsequent tethering can be done by other regions on the same tether or by other proteins, pulling the cargo closer to the target site (Gillingham and Munro, 2019). The highly transient nature of this initial contact between cargo and tethering
complex along with redundancy across multiple proteins has been a major challenge for this field. It is probably one of the reasons why demonstrating the existence of such a mechanism at the synapse has been left largely unexplored.

**ELKS as a multifunctional vesicle tether at the presynaptic terminal**

ELKS is a very large protein (the main alpha isoforms ~ 115 kDa), and 80% of its sequence is composed of long coiled coil domains, with a shorter C-terminal and a longer N-terminal disordered region, similarly to Golgins. In fact, I ran most of the currently known presynaptic proteins through a secondary structure prediction algorithm (COIL by EMBL) and found that ELKS by far had the longest coiled coil domain with Liprin-α coming in as distant second (Figure 2). As mentioned in the introduction, there are a lot of interesting similarities between ELKS and liprin-α, including the coiled coil domain, small GTPase binding, not being restricted to the active zone, and being implicated in synapse assembly and vesicle trafficking.

In addition to having coiled coil domains, ELKS is also thought to form homo- and heterodimers with other ELKS1 and ELKS2 isoforms, but this dimerization proved difficult to see reliably. The N-terminal disordered region is decorated with Proline residues and other motifs that resemble lipid-binding and membrane curvature sensing sequences. All of which makes ELKS a promising candidate to act as a vesicle-capturing protein.

While there are some striking similarities between the presynaptic ELKS and the Golgi-resident Golgins, I am proposing a capturing mechanism seemingly different than what exists at the Golgi apparatus. At the Golgi, Rab6 anchors Golgins to the Golgi membrane, and it is the Golgins’ N-terminus that mediates vesicle capture (Munro and Nichols, 1999). On the other, I propose that in the presynaptic terminal, it is the C-terminus of ELKS that acts as a direct vesicle tether. It is unlikely that Rab6 sits together with ELKS in the presynaptic terminal to provide an anchoring point for ELKS1. My results suggest that Rab6 associates with vesicles
and behaves like a transport protein in axons. Furthermore, in multiple experiments I observed that it was ELKS1 that determined Rab6 localization and not the other way around. These together suggest at the synapse ELKS is the recruiter for Rab6, unlike at the Golgi where Rab6 determines tether localization.

If the Rab6 interaction with the C-terminus of ELKS serves to mediate vesicle capturing, the N-terminus of ELKS needs a way to anchor itself. ELKS1 could also be using the microtubules and microtubule-binding complexes as an anchor, which would have the perfect position to screen and catch passing cargo. Studies have already shown that ELKS1 in nonneuronal cells is part of a microtubule adhesion complex at the cell edge that also contains LL5β and MICAL3. This complex, beside anchoring the plus-end of the microtubules, is also thought to serve as cargo a capturing complex for Golgi-derived Rab6-positive cargos (Grigoriev et al., 2011). It is conceivable that part of this microtubule plus-end binding complex exists in presynaptic terminals.

Microtubules are highly polarized structures with a stable minus-end that is anchored, and a highly dynamic plus-end that cycles between a growing phase (polymerization) and collapse (depolymerization) (Kapitein and Hoogenraad, 2015). In most cell types, the minus-end of microtubules reaches all the way back to the Golgi. In the axon, however, thousands of relatively short overlapping microtubule cables are spaced at regular intervals with their minus-ends anchored along the axons at microtubule organizing centers, while their plus ends are continuously polymerizing and depolymerizing (Kapitein and Hoogenraad, 2015). A recent paper reported that presynaptic terminals are enriched in microtubule plus-ends that may serve as a stop-signal for kinesin-based transport (Guedes-Dias et al., 2019). It is plausible that part of the ELKS1/LL5β/MICAL3 adhesion complex mentioned above coordinates with the microtubule plus-ends in presynaptic terminals to capture and unload synaptic transport cargo.

Another alternative for ELKS1 anchoring itself is by using the membrane-binding motifs on its N-terminus as an anchor. This would be a suitable anchoring method in a highly dynamic
environment of the vesicle cloud. It is possible that ELKS1 dimerizes forming a Y-shaped cup with the N-termini to bind vesicles, similarly to Golgins do. Except in the nerve terminal, ELKS1 might be using its Y-shaped N-terminus not to capture new vesicles, but to anchor itself onto one in absence of a more stable membrane platform. The non-specific nature of membrane-binding could allow ELKS to use any available vesicle from the bouton as an anchor. The Y-shaped membrane association may provide enough stability, while also allowing ELKS to dissociate quickly and change anchoring points to avoid being dragged away if the vesicles within the cloud shift positions.

**Future directions**

*Mechanism of cargo capturing via Rab6–ELKS interaction*

It is yet to be determined what the immediate effect of the interaction Rab6-ELKS1 has on the cargo: Is the interaction responsible for the initial halting and/or dislodging of the moving cargo from the microtubule? Future studies could address this by examining the radius, in which uncaptured vesicle are found in the axon. If the ELKS-Rab6 interaction is responsible for the initial halting of the cargo, in absence of their interaction, cargo should remain attached to motor complexes and stay in motion, thus uncaptured cargo would be distributed throughout the axon. On the other hand, if the ELKS-Rab6 interaction is not responsible for the uncoupling of the cargo from the motor complex, the cargo that already was dislodged from the motor by some other mechanism but remained uncaptured by ELKS should stay in the vicinity of the presynaptic bouton. It is also yet to be determined whether ELKS1 is already anchored prior to its interaction with the vesicle-bound Rab6.

There is of course the possibility that Rab6 acts as a regulator of retrograde trafficking in the synapse as well. However, it is important to note that Rab6 is thought to mediate retrograde traffic at the Golgi because it recruits and anchors the C-terminus of the Golgins to the Golgi surface. In turn the N-terminus of the Golgins capture and tether retrograde transport vesicles.
arriving from the cell periphery. However, Rab6 in that model sits on the Golgi membrane, not on the retrograde vesicle. There are many papers showing that Rab6 either sits on the Golgi membrane or on anterograde secretory vesicles, but never on vesicles travelling back to the Golgi. In our model, Rab6 acts similarly as it would at the Golgi recruiting a tether onto a membrane surface, with the exception that in the presynaptic terminal the membrane surface is the vesicle.

**What happens to the cargo after the initial capturing step?**

Endosomes do exist within presynaptic terminals, but it is not known whether newly arriving cargo fuse with endosomes. The prevailing model for endosome function in the presynaptic nerve terminal is the recycling and regeneration of vesicle material endocytosed from the plasma membrane after exocytosis (Jahne et al., 2015; Kokotos and Cousin, 2015). What happens with newly arriving cargo at presynaptic terminals? Are they fused with endosomes or the plasma membrane? Are they incorporated into the vesicle cloud without fusion? The truth is we do not know what happens with the cargo once it is captured.

**What is the cargo?**

I find it is important to make a clear distinction between general synaptic vesicles that possibly make up the bulk of the synaptic boutons and Golgi-derived presynaptic cargo that carry newly synthesized material from the Golgi and serve to maintain presynaptic protein composition. The fact that in both the Rab6B and in the ELKS1α/β knockout neurons the number of vesicles in boutons only moderately decreases suggests that Rab6-marked vesicles are a subtype of cargos with a specific content. It will be important to identify the exact composition of Rab6-positive cargos.
Outlook

From a general perspective, understanding how neurons solve the issue of axonal transport and ensure accurate material delivery over such incredibly large distances is a necessary step forward in neurobiology. Healthy brain function is based on neurons communicating with one another through an extensive and extremely complex synaptic network, the functional units of which are the properly assembled and maintained individual synapses. Thus, the proper assembly and maintenance of these individual synapses that in part occurs via axonal transport is a crucial starting point in building a healthy brain and is a central issue for neurons. The importance of axonal transport and the challenges that it poses for neurons is well reflected by the multitude of neurodevelopmental and neurodegenerative disorders that arise from disruption to this pathway. Yet, we still know so little about how axonal transport is regulated.

The work presented in this thesis aimed to shed light on one small aspect of this issue, but there are so many questions regarding axonal transport that remain unanswered. How many different cargos are there that carry presynaptic proteins; what are their exact composition; what proteins travel together; in what order do different cargo types arrive during synapse assembly; what are the cargo labels; what other capturing complexes sit at the presynaptic bouton; what is the exact molecular mechanism of capture and cargo integration; how are capturing complexes anchor themselves; are capturing mechanisms regulated in an activity dependent manner; what activates or triggers cargo capturing? And the list can go on.

In order to find more powerful therapeutic targets for many neurobiological disorders, finding answers to many these or similar questions will likely be inevitable.
CHAPTER SIX:

MATERIALS AND METHODS
Overview of Methods

Mouse lines
Real time quantitative PCR
ELKS1/2 antibody generation
Neuronal cell cultures and lentivirus production
Crude sample harvest for protein quantitation
Western blotting
Immunofluorescence staining and confocal microscopy
STED microscopy and analysis
Electron microscopy and analysis of vesicle distribution
Mitochondrial tagging
Live imaging of transfected neuronal cultures
Localization assay in HEK cells
Cortical synaptosome and vesicle fractionation
Protein expression and purification
GST pulldowns from mouse brain and HEK cells
GST pulldowns with His-tagged proteins

Mouse lines

Constitutive Rab6B knockout mice were generated by CRISPR/Cas9 gene editing, targeting Rab6B gene (RRID:MMRRC_049340-UCD, also called CRISPR_JR28993) in embryonic stem cells. Cas9 RNA and 4 guide sequences (GTAAGACCAGCCTATGGCTA, CTAGCCATAGGCTGGTCTTA, GCTCTCCAGACAGGGTCCAC, and AGGGAGCATTGACGCTGAG) were injected into C57BL/6NJ-derived fertilized eggs with well recognized pronuclei. This resulted a deletion exon 2 spanning 178 bp, beginning at Chromosome 9 positive strand position 103,140,328 and ending after position 103,140,505 (GRCm38/mm10). The following primer pair was used for genotyping:

GAGCCAGCCTTTAAGTGCGCGT and CCTGCCTCTTCAAAAGATCC that produce a 466-bp band for a wild type allele and a 288 bp band for knockout allele. The line was kept
heterozygotes, and litters from Rab6B$$^+/-$$ parents were genotype prior to dissection, and only Rab6B$$^+/+$$ and Rab6B$$^-/-$$ littermates were used for experiments.

**Conditional ELKS1α/β mouse lines** was generated as KO-first line (reporter-tagged insertion with conditional potential) targeting the *Erc1* gene (RRID:MMRRC\_041523-UCD). To generate the original ELKS1α/β knockin line (ki), the targeting vector with the L1L2_Bact_P cassette was inserted at position 119,743,809 of Chromosome 6 upstream of the critical exon 13 (Build GRCm38). The cassette contains an FRT site followed by a splice acceptor, a lacZ sequence and a loxP site. This first loxP site is followed by neomycin (under the human β-actin promoter), SV40 polyA, a second FRT site and a second loxP site. A third loxP site is inserted downstream of exon 13 at position 119,743,018. The exon 13 was thus flanked by loxP sites. Because of the splice acceptor inserted before exon 13, alleles carrying this insertion will frequently fail to produce viable isoforms of the ELKS1 protein and can be considered as a knockout. The ELKS1α/β$$^{+/ki}$$ breeders never produced homozygous ELKS1α/β$$^{ki/ki}$$ as was predicted based on previous publications (Kaeser et al., 2009; Liu et al., 2014) that constitutive ELKS1 removal results in embryonic lethality. The knockin line was genotyped using two reactions: the primer pair CCGTTGATTCTGAACAGTGTAGG (forward) and CCGAACATTGGAAGTAGGTAATCC (reverse) produced a 375-bp band in wild type and no band knockin alleles; the primer pair GGGATCTCATGCTGGAGTTCTTCG (forward) and the same reverse primer above produced no band in wild type and a 745-bp band in knockin alleles.

To generate the conditional ELKS1α/β floxed line, the FRT-flanked neomycin cassette (including the splice acceptor) was removed by crossing the line to another mouse line carrying the *Flp* gene under an actin promoter. The *Flp* gene was subsequently outbred, and the new ELKS1α/β floxed line was bred to homozygosity. The floxed line was genotyped with two reactions: primer pair GCCCAAACAGAAGTTGACCGTC (forward) and CTTTGACTCTCTAGAACATAGC (reverse) produces a 360-bp band in wild type and no band
in floxed allele; the primer pair using the same forward oligo with
GAACTGATGGCGAGCTCAGACC (reverse) produces a 390-bp band in floxed and no band in wild type allele.

ELKS1/2α conditional knockout line was previously described (Liu et al., 2014) where loxP sites were inserted into the Erc1 gene ((Liu et al., 2014) RRID:IMSR_JAX:015830) and Erc2 ((Kaeser et al., 2009) RRID:IMSR_JAX:015831). RIM1/2 conditional knockout line was previously described (Kaeser et al., 2011) where loxP sites were inserted into the Rims1 gene ((Kaeser et al., 2008) RRID:IMSR_JAX:015832) and the Rims2 gene (RRID:IMSR_JAX:015833 (Kaeser et al., 2011)). RIM1 constitutive knockout mouse line was previously described ((Kaeser et al., 2008)). All three mouse lines were maintained as homozygote lines.

Survival analysis was performed for Rab6B, ELKS1α/β knockin and ELKS1α/β floxed lines by comparing the observed genotype distribution of offspring of heterozygote matings with the expected distribution based on Mendelian inheritance. The Chi-square test was used to determine whether the obtained offspring ratio was significantly different from the expected ratio. All animal experiments were approved by the Harvard University Animal Care and Use Committee.

**Real time quantitative PCR**

Real-time q-PCR analysis of Rab6A and Rab6B mRNA levels harvested from hippocampal cultures of Rab6B+/− and Rab6B+/+ littermate mice. DIV 14 cultures were washed with PBS and RNA extraction, purification, quantification, and probe-based RT-qPCR was performed by standard methods and quantified by spectrophotometry. One-step RT-qPCR was performed with TaqMan Gene Expression Assays (Life Technologies) and the iScript Reverse Transcriptase (Bio-Rad). The following gene-expression assays were used: Rab6A (assay ID:
Mm00445868_m1, gene: *Rab6A*), Rab6B (assay ID: Mm00620652_m1, gene: *Rab6B*),
Synapsin1 (assay ID: Mm00449772_m1, gene: *Syn1*), and GAPDH (assay ID:
Mm99999915_g1, gene: *Gapdh*). Reactions were performed three times, using 10 ng DNA per
10 μl reaction. Data were analyzed by determining the cycle threshold values (CT) relative to
internal Synapsin1 mRNA levels. Relative expression ratios were expressed as
$2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT_{cDKO} - \Delta CT_{control}$, and ΔCT is the Synapsin1 normalized value.

**ELKS1/2 antibody generation**

We generated a new ELKS1/2α/β antibody (HM1083), raised in rabbits against a GST-fused
ELKS1αB fourth coiled coil domain (CCD) fragment (pLB12025). The immunogen site was
highly similar to antibody the previously described p224 antibody (Kaeser et al., 2009). To
generate the antigen, the GST-fusion protein was purified and eluted from the beads with 10
mM glutathione for 3 h at 4°C. After overnight dialysis in PBS in 4°C, the protein solution was
snap-frozen in ethanol/dry ice and submitted to Cocalico Biologicals for immunization in rabbits
using standard procedures. Rabbits were given booster injections every two weeks, and bleeds
were collected every three weeks. Sera were screened using Western blots against protein
samples harvested from both ELKS1/2α cDKO and ELKS1α/β cKO neuronal cultures. Sera with
the highest immunoreactivity (bleeds 3 to 6) were used at 1:2000 dilution. To test the isoform
reactivity of the new HM1083 antibody, HEK cells were overexpressed with various ELKS
isoforms via a Ca$^{2+}$-phosphate transfection method. The HA-tagged ELKS isoforms were in
pCMV backbone with a CMV promoter to drive high protein expression (pLB12010, pLB12011,
pLB12013, and pLB14065). HEK cell cultures were transfected in T25 flasks with 6.7 μg DNA.
Control HEK cells were not transfected. After 24 h post-transfection, cultures were placed on ice
and washed with ice-cold PBS, then harvested in 1 ml 1X SDS for Western blotting.
Neuronal cell cultures and lentivirus production

Dissociated high-density hippocampal cultures were prepared from newborn mice (P0-P1) as described (Kaeser et al., 2011; Liu et al., 2014) on chemically stripped glass coverslips in 24-well plates. For all experiments, neuronal cultures were harvested at DIV 14-16.

Lentiviruses were produced in HEK293T cells. HEK cells were maintained in DMEM supplemented with 10% bovine serum and 1% penicillin/streptomycin and were split every 2 days to maintain optimal growth rate. For virus production, HEK cells in neuronal culture media were transfected using Ca\textsuperscript{2+}-phosphate transfection protocol with 3\textsuperscript{rd} generation lentiviral packaging plasmids (pVSVG [pHN120108], pRRE [pHN120109], pREV [pHN120110]) and a separate lentiviral plasmid (FSW containing the human Synapsin promoter) encoding the recombinant gene of interest. After 24 h, the culture medium was exchanged to neuronal cell growth medium and allowed viral production for another 24 h. The culture medium from HEK cells was harvested and centrifuged for 5 min at 700 x g, and the supernatant was used immediately for infection.

Generation of knockout cell cultures from floxed mouse lines

Conditional floxed neuronal cultures were infected 5 days in vitro (DIV) with 130 μl of the lentiviral solution expressing GFP-Cre recombinase (pHN131014) or a truncated inactive GFP-Cre (pHN131015) with enhanced nuclear localization (Kaeser et al., 2011; Liu et al., 2014). Infection rates were monitored by the nuclear eGFP expression, and only cultures with nearly 100% infection rates were used in experiments.

Lentiviral expression of rescue proteins in ELKS1α/β cultures

Rescue protein expressions were achieved by first infecting floxed cultures with 200 μl of lentiviral solution expressing the HA-ELKS1αB (pHN161031) or a mutant HA-ELKS1αB\textsuperscript{Δ955-971} (pHN170936) rescue proteins at DIV 3. A second independent lentiviral solution expressing Cre
or inactive Cre recombinase was used to infect the same cultures at DIV 5 as described above. Expression of rescue proteins was monitored by Western blotting.

**Lentiviral expression of Rab6 proteins**

Rab6 proteins in wild type cultures: Wild type neuronal cultures and lentiviral solutions were generated as described above. Cultures were infected at DIV 5 with 100 μl of lentiviral solution expressing Cerulean tagged constitutive active Rab6BQL (pHN160705) or inactive Rab6BTN (pHN160706) mutants.

Rab6 proteins in ELKS1α/β floxed cultures: ELKS1α/β floxed cultures were first introduced to Cre and inactive Cre recombinase by lentiviral infection at DIV 5 as described above. Simultaneously at DIV 5, the same cultures were also infected with 100 μl of lentiviral solution produced in a second independent lentiviral preparation expressing Cerulean tagged active Rab6AQL (pHN160326) or active Rab6BQL (pHN160705). Rab6 expression levels for both experiments was monitored by Western blotting against an anti-GFP antibody.

**Crude sample harvest for protein quantitation**

*Harvesting mouse tissue:* Mice were anesthetized on ice (P0-P5) or placed in an isoflurane chamber (P10-P90). After decapitation, harvested organs were washed in ice-cold PBS, weighed and were homogenized using a glass-Teflon homogenizer in 10% w/v homogenizing buffer (150 mM NaCl, 25 mM HEPES, 4 mM EDTA and 1% Triton-X-100, at pH 7.5). Homogenized tissues were incubated on 4°C for 1 h while slowly rotating, then added SDS to a final 1X concentration and denatured by boiling for 10 min on 95°C.

*Harvesting neuronal cultures:* Neuronal cultures grown on glass coverslips in 24-well culture plates were harvested in 1X SDS solution per coverslip, then denatured by boiling for 10 min on 95°C. Neurons were harvested at DIV 14 in 15 μl 1X SDS.
Western blotting

Western blotting was performed according to standard protocols. After SDS-Page electrophoresis, gels were transferred to nitrocellulose membranes (GE Healthcare) for 6.5 h at 4°C in Tris-glycine buffer containing 20% methanol.

Non-quantitative ECL Western blotting: Nitrocellulose membranes were blocked in 10% (w/v) non-fat milk and 5% (v/v) goat serum in TBST for 1 hr on RT. Membranes were incubated with primary antibodies in 5% (w/v) non-fat milk and 5% (v/v) goat serum in TBST overnight at 4°C. After washing 3 x 5 min with TBST, blots were stained for 1 hr with HRP-conjugated secondary antibodies in the same solution, and washed another round of 3 x 5 min. Protein bands were visualized using enhanced chemiluminescence (ECL) developed onto X-ray films.

Quantitative fluorescent Western blotting: Nitrocellulose membranes were blocked in 5% (w/v) non-fat milk 5% (v/v) goat serum in TBS without Tween-20 for 1 h on RT. Membranes were incubated overnight at 4°C in 5% BSA / TBST solution with primary antibodies against the protein of interest and an anti-Synapsin-1 antibody for loading control. After washing 3 x 5 min with TBST, blots were stained for 1 h in the same solution with secondary antibodies conjugated to fluorescent tags (680CW or 800CW conjugated IR dyes from LICOR), and washed in another round of 3 x 5 min. Blots were scanned on a LICOR Odyssey Fluorescent Scanner with acquisition settings recommended for publication, then the original 16 bit fluorescent images were analyzed in ImageJ software. Each target protein was normalized to its corresponding Synapsin-1 level and protein levels were expressed normalized to the average of the three wild type control samples. For figure representation, the 16 bit images were compressed to 8 bit images. Because some images had very large grey value ranges, data compression caused the appearance of a near white background in these cases in the representative images. Student’s t test was used to determine whether experimental and control conditions were significantly
different. All quantitative analyses were performed in three independent cultures. For a complete list of primary antibodies, see Table S2.

**Immunofluorescence staining and confocal microscopy**

Cultures grown on glass coverslips were washed twice with warm PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at RT. Cells were permeabilized in blocking solution containing 0.1% Triton X-100, 3% BSA in PBS, and incubated with primary antibodies in blocking solution overnight at 4°C. Secondary antibodies conjugated to Alexa 488, 555, or 633 were used for detection (Life Technologies, 1:500) was performed by incubating cultures overnight at 4°C. Coverslips were air-dried at room temperature and mounted on to glass slides in mounting medium. Slides were allowed to dry for two days on RT in the dark before they were imaged or stored in 4°C.

For samples analyzed only in confocal microscopy, images were acquired at an Olympus FV1000 or FV1200 confocal microscope using either 100x or 60x oil immersion objectives with 1.4 numerical aperture, the pinhole was set to one airy unit, and identical settings were applied to all samples within an experiment. Single confocal sections were analyzed in ImageJ software (NIH) as described before (Liu et al., 2014). When necessary, representative images were enhanced for brightness and contrast to facilitate visual inspection; all such changes were made after analysis and were made identically for all experimental conditions.

For quantification of synaptic protein levels in confocal images, fields of views were defined by Bassoon or Synaptophysin-1 objects. The average intensity of the protein of interest was quantified within these synaptic objects. All quantitative data are derived from 3 independent cultures and 5-10 fields of view were quantified per culture per genotype. The experimenter was blind to the experimental condition of the culture.
STED microscopy and analysis

STED microscopy was performed as described in (Wong et al., 2018; de Jong et al., 2018). Neuronal cultures were grown on 0.15-mm-thick chemically stripped glass and harvested as described for confocal microscopy above. Secondary antibodies conjugated to Oregon green 488, Alexa 555, and Alexa 633 were used for detection (Life Technologies, 1:500 for confocal channel, 1:200 for STED channels). Images were acquired with a Leica SP8 Confocal/STED 3X microscope with an oil immersion 100X 1.44 NA objective. Triple-color sequential confocal scans were followed by a dual-color sequential STED scans. Alexa 633, Alexa 555 and Oregon green 488 were excited with 633 nm, 555 nm and 488 nm white light lasers respectively, in this order of sequence, at 2–5% of 1.5 mW laser power. Two-times line accumulation and two-times frame averaging were applied during STED scanning. In all STED experiments, the synapse marker (Synapsin-1 or Synaptophysin-1) with Alexa 633 was acquired in the confocal channel. During STED scanning, Oregon green 488 and Alexa Fluor 555 signals were depleted with 592 nm (75% of max power) and 660 nm (50% of max power) time-gated depletion lasers on the X-Y axis, and with 30% 3D STED depletion on the Z axis. Identical settings were applied to all samples within an experiment.

For analysis of subsynaptic intensity distribution, line-scan intensity profiles of side view synapses were obtained for the synaptic vesicle cluster marker (either Synapsin-1 or Synaptophysin-1, imaged with confocal microscopy), the active zone marker (either Bassoon or PSD-95, imaged by STED), and the test protein (imaged by STED). Side view synapses were selected manually, and a rectangular 0.2 μm x 1.0 μm bar was placed perpendicular to the signal from the active zone marker. Intensity values were extracted using a custom ImageJ program. The intensity values of the target proteins were aligned such that the peak intensity of their corresponding active zone markers was always at zero. All quantitative analyses were
performed on original images without adjustments and were done identically for all experimental conditions. For all image acquisition the experimenter was blind to the experimental condition.

**Analysis of total protein intensity** of the target protein acquired by STED was performed using a similar concept to confocal analysis. The confocal signal of synaptic markers (either Synapsin-1 or Synaptophysin-1) of individual synapses were used to create fields of interests, within which of target protein intensities (acquired by STED) were measured. To measure subsynaptic distribution of the total ELKS signals relative to the active zone marker, first fields of views (objects) were generated in ImageJ based on the confocal intensities of the synaptic clusters (Synapsin-1) as described above. This was followed by the generation of a second object based on only the Bassoon intensities (by STED) that colocalized with Synapsin-1 (Bassoon/Synapsin objects). The total ELKS1 or ELKS2 intensities (by STED) were then measured both within the Synapsin objects and the Bassoon/Synapsin objects. To get the fraction of ELKS signal that fell within the active zone, ELKS intensities that colocalized within synaptic Bassoon objects were divided by the total ELKS intensities within the Synapsin objects.

**Electron microscopy and analysis of vesicle distribution**

*For high-pressure freezing* was performed as previously described (Wang et al., 2016). Neurons were cultured on 6 mm carbon-coated sapphire coverslips were frozen using an HPM 100 high-pressure freezer in extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM Ca\(^{2+}\), 2 mM Mg\(^{2+}\), 10 mM HEPES pH 7.4, 10 mM glucose with ~310 mOsm, 50 µM picrotoxin, 50 µM AP5, and 20 µM CNQX) was added to block synaptic transmission. Samples were stored in liquid nitrogen and processed for sectioning. Frozen samples were freeze-substituted (in 1% glutaraldehyde, 1% osmium tetroxide, 1% water and anhydrous acetone), Epon infiltrated, and polymerized by baking at 60 °C for 2-3 days, then at 100 °C overnight immediately before sectioning at 50 nm. To enhance contrast, mounted sections were stained for 10 sec with lead citrate.
For glutaraldehyde fixation was performed as previously described (Wang et al., 2016). Neurons were cultured on glass coverslips and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 37°C for 10 minutes. The Electron Microscopy Facility at Harvard Medical School carried out the following procedure: fixed samples were stained with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 h at RT, washed in water, washed in maleate buffer (pH 5.15) 3 times, stained with 1% uranyl acetate for 1 h, dehydrated in of EtOH and propylene oxide, resin infiltrated, and finally baked for 24 h at 60°C before sectioning at 50 nm.

Images of both high-pressure frozen and glutaraldehyde fixed samples were taken with a transmission electron microscope (JEOL 1200 EX at 80 kV accelerating voltage) and processed with ImageJ. Bouton analysis: The total number of vesicles, the number of docked vesicles, the length of the PSD, the area of the presynaptic bouton, and the distance of each vesicle from the active zone were analyzed with SynapseEM MATLAB. Bouton size was calculated from the perimeter of each synapse. Docked vesicles were defined as vesicles touching the presynaptic plasma membrane opposed to the PSD. Axon analysis: Axon segments were chosen for analysis based on the following: plasma membranes are parallel and non-tapering, at least 1 μm segment is in view, the width is between 0.2 μm and 0.75 μm, and microtubules are visible throughout the whole segment. The diameter of vesicles was measured and only vesicle with diameters of 50 nm or less were counted. Vesicles were not counted in the vicinity of obvious bouton regions. Student’s t test was used to determine whether all experimental and control conditions were significantly different. All experiments and analyses were performed by an experimenter blind to the genotype.
Mitochondrial tagging experiments

To localize proteins to the mitochondrial membrane, a mito tag was made composed of the trans-membrane domain (TMD) of the mitochondrial Tom20 protein and the Cerulean fluorescent protein. The mito-tag was fused to the N-terminus of target proteins.

For the mito-tagged ELKS1 in, wild type hippocampal cultures were infected at DIV 3 with 200 μl lentiviral solution expressing mito-tagged ELKS1αB (pHN161033) or ELKS1αBΔ955-971 (pHN190429). For negative control, the small mito-tag alone (pHN161037) was expressed by infecting neurons with 50 μl lentiviral at DIV 5 (the mito-tag alone expressed more efficiently than the ELKS constructs). Expression levels were monitored via Western blotting by staining against an anti-GFP antibody. High-pressure frozen cultures were processed and imaged with electron microscopy as described above. For analysis, the number of small vesicles (diameter ≤ 50 nm) tethered to mitochondrial surface were counted. A vesicle was considered tethered if it was within 70 nm from a mitochondrial surface. The maximum distance of 70 nm was determined by measuring the longest visible string or tether between vesicles and mitochondria; however, a tether did not have to be visible as long as the vesicle fell within the 70-nm-distance from the mitochondrial membrane.

For the mito-tagged Rab6 in, wild type hippocampal cultures were infected at DIV 3 with 150 μl lentivirus to express the HA-tagged ELKS1αB (pHN161031) or ELKS1αBΔ955-971 (pHN170936). Subsequently, the same cultures were infected at DIV 5 with 100 μl mito-Rab6BQL (pHN181203) or mito-Rab6BTN (pHN181203). Cultures were harvested for confocal imaging and Western blotting as described before. To visualize overexpressed mito-Rab6B, cultures were immunostained against an anti-GFP antibody. To visualize overexpressed HA-ELKS1, cultures were immunostained against an anti-HA antibody. Cultures were also immunostained against an anti-Bassoon antibody to mark synaptic regions. All signals were acquired by confocal
microscopy. Quantification shows the fraction of Bassoon objects that contained mito-Rab6 signal (the threshold of co-localization was set to 50% of the Bassoon area, i.e.: at least half the area of a Bassoon object had to contain mito-Rab6). Images were analyzed in ImageJ. For co-localization of Bassoon synaptic marker with the mito-tagged Rab6, the ImageJ plugin BioVoxxel was used in the object to object comparison (The BioVoxxel Image Processing and Analysis Toolbox. Brocher, 2015, EuBIAS Conference). Student's t test was used to determine whether experimental and control conditions were significantly different. All experiments and analyses were performed by an experimenter blind to the genotype.

To test how the efficiency of the mito-tag localized proteins to the mitochondrial, mito-tagged ELKS1αB localization was compared with Cerulean-tagged ELKS1αB in cultures that simultaneously expressed the mitoDsRed mitochondrial marker (pHN161038). Wild type neuronal cultures were infected with mito-tagged ELKS constructs as described above, and were subsequently with 50 μl mitoDsRed lentiviral solution at DIV 7. Cultures were harvested for confocal imaging as described before. To visualize overexpressed Rab6B, cultures were immunostained against an anti-GFP antibody. To visualize overexpressed mitoDsRed, cultures were immunostained against an anti-RFP antibody. Cultures were co-stained against an anti-Synaptophysin-1 antibody to mark synaptic regions.

Live imaging of transfected neuronal cultures

For live imaging, wild type neuronal cultures overexpressed with Cerulean tagged ELKS1αB (pMYW12018) or with Rab6BWT (pHN160704) using a Ca²⁺-phosphate transfection method, and were simultaneously transfected with tdTomato-SV2A (pHN141024) to mark synaptic regions. For transfection, the original culture media was gently removed from DIV 12 neuronal cultures washed 3 x 10 min in warm MEM (the original culture media was stored on 37°C for the duration of the procedure). Cultures were added 0.5 ml warm MEM per well supplemented with 50 μM AP5 to reduce excitotoxicity and were incubated for 10 min before transfection. Cultures were
transfected with a total of 4 μg DNA per well: 3 μg ELKS1αB + 1 μg SV2A; 2 μg ELKS1αB + 2 μg SV2A. (Note that proteins express with different levels of efficiency, especially when transfected in combination of other proteins. I optimized transfection amounts such that protein expressions were comparable across conditions. Initial DNA precipitation in the DNA/Ca\(^{2+}\)/HEPES solution was allowed for 5 min before 150 μl solution was added onto cells. Precipitate formation continued in cultures, which was carefully monitored under a light microscope where precipitates appeared as small even pebbles. Once the pebbles started aggregating (after approximately 10 min on RT), the transfection solution was immediately removed and cells were washed twice in 1 ml AP5-supplemented MEM to stop any further precipitate formation. Cells were then washed 2 x 10 min in AP5/MEM and 3 x 10 min in MEM alone. Between each wash, cells were placed back in their incubator. After the final wash, the original cell culture medium was added back onto cells. To maintain optimal culture health, it was crucial to leave ~200 μl media on cells at each step. Cultures were monitored for overall health and were imaged 2-3 days post-transfection.

Live imaging was carried out on an Olympus light microscope (U-TV1X-2) with a pE-Universal LED light source (pE-4000), and images were recorded on a Hamamatsu digital camera (ORCA-Flash4.0). Live cultures were imaged at 35°C in Hybernate-A (BrainBits) solution optimized to fluorescent microscopy. Cerulean signals were excited at 435 nm and tdTomato signals excited at 550 nm. Time-lapse images were recorded for 2 min with 1 frame / sec imaging speed. Images were analyzed in ImageJ. Kymographs were generated from line-scan profiles of Cerulean signals along an axonal track, where X-axis shows distance and Y-axis shows elapsed time in frames (1 frame = 1 second). Moving objects appear as diagonal lines, while stationary objects appear as straight vertical bars on a kymograph. An object was considered stationary if it appeared as a straight vertical bar on the kymograph. Student’s t test was used to determine whether all experimental and control conditions were significantly
different. All experiments and analyses were performed by an experimenter blind to the genotype.

Localization assay in HEK cells

For the localization assay in HEK cells, tagged ELKS1aB and Rab6B proteins were co-expressed and analyzed in confocal microscopy as described above. For both ELKS1 and Rab6B, previously described constructs were used: HA-ELKS1αB (pHN161031), HA-ELKS1Δ955-971 (pHN170936), Cerulean-Rab6BQL (pHN160705), and Cerulean-Rab6BTN (pHN160706). To express these proteins in HEK cells, we used a standard Ca²⁺-phosphate transfection method. HEK cell were grown in HEK cell media described above on glass coverslips in 24-well plates for 24 h. Cultures were washed in PBS, then transfected in fresh HEK medium with a total of 1.5 μg DNA that contained 1.2 μg ELKS1 and 0.3 μg Rab6B DNA. Transfected HEK cells were grown for an additional 24 h before harvest. Cells were harvested for either immunostaining for confocal microscopy or for Western blotting as described above. To visualize overexpressed Cerleuan-Rab6B, HEK cells were immunostained against an anti-GFP antibody. To visualize overexpressed HA-ELKS1, HEK cells were immunostained against an anti-HA antibody

Cortical synaptosome and vesicle fractionation

Mouse cortices were homogenized by 3 x 10 strokes on ice with a glass-Teflon homogenizer in 10% w/v homogenizing buffer (320 mM sucrose, 4mM HEPES pH 7.4, and 1X protease inhibitor cocktail) and centrifuged at 1,000 g for 10 min at 4°C. The pellet (P1) was discarded and the supernatant (S1) was centrifuged at 12,500 x g for 15 min at 4°C. The supernatant (S2) was discarded and the pellet (P2) was used in subsequent fractionations.
Synaptosome preparation was carried as described before (Liu et al., 2018). The pellet P2 was resuspended in 1 ml homogenizing buffer, was added to the top of a sucrose gradient (5 ml of 1.2M sucrose and 5 ml of 0.8M sucrose), and was centrifuged at 69,150 x g for 70 min at 4°C. The synaptosome layer (1-1.5 ml) was collected from the interface of the two sucrose layers. The cortical homogenate, S1, S2, and P2 fractions were diluted with homogenizing buffer to equal the final dilution of the sucrose gradient and the synaptosome fractions. The samples analyzed by a standard Western blotting protocol described above.

Vesicle fractionation: For vesicle fractionation, the pellet P2 was resuspended in 10 ml homogenizing buffer and was again centrifuged at 12,500 x g for 15 min in 4°C. The pellet (P2") was resuspended in 10 ml lysis buffer (4 mM HEPES pH 7.4 and protease inhibitors) and incubated for 30 min on ice for osmotic lysis. The lysate was centrifuged at 25,000 x g for 20 min at 4°C. To get the vesicle fraction, the supernatant (LS4) was ultracentrifuged at 245,000 x g for 2 h in 4°C (Beckman-Coulter, SW41 swing bucket rotor). The pellet (LP5, the vesicle fraction) was resuspended in 100 µl homogenizing buffer. Fractions were processed for Western blotting in 1X SDS as described before. Total protein concentration of samples (S1, P2", and LP5) used for Western blotting were determined using Coomassie staining as described above, and concentrations were adjusted accordingly so that the total protein concentrations across all samples were comparable. For the list of primary antibodies used, see Table S2.

Protein expression and purification

GST- and His-tagged fusion proteins were expressed and purified according to standard procedures. In brief, fusion proteins were expressed at 20°C in electrocompetent E. coli BL21 for 20 h with 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and pelleted by centrifugation.
**GST-tagged fusion proteins:** Bacterial pellets were resuspended in GST-lysis buffer (1X PBS, 0.5 mg/mL lysozyme, 0.5 mM EDTA, and bacterial protease inhibitors, pH 8.0), allowed to incubate for 30 min on ice, then briefly sonicated. Bacterial homogenates were pelleted by centrifugation, and cleared bacterial supernatant was purified using glutathione-Sepharose resin in PBS and used as immobilized baits in affinity assays. The concentration of proteins trapped on beads were determined by comparing against them to known BSA concentrations using SDS-gel electrophoresis and Coomassie staining. The following GST-tagged proteins were produced from pGEX-KG2 constructs: GST alone (pAJ13017), Rab3A Q81L (pHN150605), Rab3A T36N (pHN150606), Rab6A Q72L (pHN150809), Rab6A T27N (pHN150808), Rab6B Q72L (pHN160708), Rab6B T27N (pHN160709), ELKS1αB 2-208 (pLB12022), ELKS1αB 209-358 (pLB12023), ELKS1αB 359-696 (pLB12024), ELKS1αB 697-992 (pLB12025), ELKS1αB 654-955 (pHN160636), ELKS1αB 654-971 (pHN160637), ELKS1αB 654-992 (pHN160638), ELKS1αB 769-992 (pHN160615), ELKS1αB 808-992 (pHN160618), ELKS1αB 850-992 (pHN160619), ELKS1αB 808-971 (pHN160617), ELKS2αB 765-884 (pHN160912).

**His-tagged fusion proteins:** Bacterial pellets were resuspended in His-lysis buffer (300 mM NaCl, 10 mM imidazole, 50 mM NaH2PO4, 0.5 mg/mL lysozyme, and bacterial protease inhibitors, pH 8.0), allowed to incubate for 30 min on ice, then briefly sonicated. Bacterial homogenates were pelleted by centrifugation, cleared bacterial supernatant was purified using Ni-NTA agarose and were eluted from the resin in the same buffer containing 100-300 mM imidazole. After overnight dialysis into 150 mM NaCl, 25 mM HEPES (pH 8.0), protein solution concentrations were determined by comparing against them to known BSA concentrations using SDS-gel electrophoresis and Coomassie staining. Protein aliquots were stored at -80C until use as soluble recombinant protein input for affinity assays. The following His-tagged proteins were produced from pET28a constructs: Rab6A WT (pHN160210), Rab6A Q72L (pHN160211),
Rab6A T27N (pHN160212), Rab6B WT (pHN160701), RAB6B Q72L (pHN160702), RAB6B T27N (pHN160703).

**GST pulldowns from mouse brain and HEK cells**

*Mouse brains lysates:* Brains from 6-10 weeks old mice were homogenized (one brain / 10 ml) in ice cold homogenizing buffer containing 100 mM NaCl, 4 mM EGTA, 25 mM HEPES (pH 7.4), 1 mM DTT, 1X protease inhibitor cocktail (Sigma Mammalian Protease Inhibitor Cocktail) and 1% w/v Triton-X 100. Tissue was homogenized with 3 x 10 strokes on ice using a glass-Teflon homogenizer, then incubated on 4°C for 1 h while gently rotating. The insoluble fraction was removed by ultracentrifugation at 40,000 rpm for 1 h in a Beckman-Coulter Ultracentrifuge using an 70Ti rotor. Supernatant was precleared from glutathione-binding proteins by incubating lysates with 200 μl of 50% glutathione-Sepharose beads for 30 min on 4°C. Subsequently, 0.4 mM GST-fusion Rab proteins (described in previous section) were used in 0.5 ml cleared brain lysate, supplemented with 6 mM Mg²⁺ and incubated for 1.5 h at 4°C with gentle agitation. Beads were washed 6 times with 1.5 ml wash buffer (contained everything except for the protease inhibitors) on 4°C, and proteins were eluted from the beads with 75 μl 1X SDS sample buffer and processed for Western blotting as described in previous sections.

*HEK cell lysates:* HA-tagged ELKS1αA (pLB12010), ELKS1αB (pLB12011), ELKS1βB (pLB12013) and ELKS2βB (pLB14074) were expressed in HEK cells under a CMV promoter; or HA-tagged ELKS1αB (pHN161031) and a mutant HA-ELKS1αBΔ955-971 (pHN170936) were expressed in HEK cells under a human Synapsin promoter. T75 HEK cell flasks were transfected with 20 ug ELKS DNA using standard Ca²⁺-phosphate transfection protocol, and were harvested after 24 hours. HEK cells were placed on ice and washed in ice-cold PBS, then were homogenized, assayed and processed using the same procedure as described above for brain lysates. HA-ELKS trapped by GST-Rab baits were visualized by using an HA antibody.
GST pulldowns with His-tagged proteins

To map and narrow the Rab6-binding region on the ELKS sequence, recombinant affinity assays were set up using GST-tagged ELKS fragments as baits immobilized on glutathione-Sepharose beads and soluble His-tagged Rab6 proteins as input. Concentrated His-tagged Rab protein stocks (described above) were diluted to 0.5 μM in a recombinant buffer containing 150 mM NaCl, 25 mM HEPES (pH 7.4), 4 mM EGTA, 5 mM Mg\(^{2+}\), 1 mM DTT, 1% w/v Triton-X 100, 0.1 mg/mL BSA. His protein solutions were then precleaned with empty glutathione beads and centrifuged with 25,000 x g for 30 min on 4°C to remove any protein precipitates. For the assay, GST-tagged protein beads (described above) was added to 0.5 ml His-Rab input and were incubated for 1 h on 4°C with gentle agitation. The molar ratio of GST-ELKS and His-Rab proteins was 1:1 (both at a 0.5 μM final concentration) in assays where the His-Rab point mutants were used as input. After the assay, glutathione beads were washed 6 times with 1.5 mL in the same recombinant buffer on 4°C, and proteins were eluted from the beads with 75 ul 1X SDS sample buffer and processed for Western blotting as described in previous sections. His-Rab6A inputs trapped by GST-ELKS1 were visualized by a T7 antibody.

For recombinant affinity assays with GTP analogs, the assay was carried out with the addition of the non-hydrolysable forms of GMP-PNP and GDP when the wild type forms of the His-Rab proteins were used as inputs (His-Rab6\(_{\text{AWT}}\) and His-Rab6\(_{\text{BWT}}\)). His-tagged Rab protein solutions were first coupled with the non-hydrolysable GMP-PNP or GDP by diluting the protein stocks to 1.2 μM final concentration in a coupling buffer without Mg\(^{2+}\) (150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM DTT, 1% w/v Triton-X 100, 0.1 mg/mL BSA, 10 mM EDTA, 0.1 mM GMP-PNP or GDP). The coupling was quenched after 1 h on 4°C by supplementing the protein solution with 20 mM Mg\(^{2+}\). Coupled Rab protein solutions were precleaned as described above. The molar ratio of GST-ELKS and His-Rab proteins was 1:3 (0.4 μM and 1.2 μM respectively) in assays where the wild type His-Rab proteins were used as input. His-Rab6 inputs trapped by a T7 antibody.
Statistical analysis methods

Unless otherwise specified, all data are means ± SEM and p-values are set as * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test. The following experiments were analyzed by Pearson’s correlation to determine correlation between fluorescent signal intensities acquired either by confocal or STED superresolution imaging: Ch.2 Fig.2J (p.22); Ch.3 Fig.2H (p.47); Ch.3 Fig.6G and 6J (p.60). The Mendelian survival ratios were analyzed by Chi square test in the following experiments: Ch.2 Fig.2B (p.22); Supplemental Fig.1A and 1B (p.126). Vesicle distribution analysis at 0-1000 nm distances in electron micrographs of presynaptic boutons were analyzed by comparing Gaussian fits, where the extra sum of squares F-test was used to determine significance: Ch.2 Fig.4B (p.28); Ch.3 Fig.3C (p.50). Statistical comparisons between more than two conditions were done using one-way ANOVA, followed by Holm-Sidak multiple comparisons test: Ch.2 Fig.6D (p.36); Ch.3 Fig.4D (p.53); Ch.3 Fig.5B and 5D (p.56); Ch.3 Fig.7E and 7J (p.66); and Supplemental Fig.3. (p.129). All experiments were done using a minimum three independent cultures and, in each culture, multiple cells (typically 5-10 per culture and genotype) or multiple images (typically 10 images per culture and genotype) were analyzed. The experimenter was blind to conditions in all experiments that were subjected to quantitative analysis.
APPENDIX ONE:

REAGENT TABLES
<table>
<thead>
<tr>
<th>TABLE 1: PLASMIDS</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHN120108 pVSVG</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td>pHN120109 pRE</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td>pHN120110 pREV</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td>pHN131014 pFSW eGFP ΔCRE</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td>pHN131015 pFSW eGFP CRE</td>
<td>Liu et al., 2014</td>
</tr>
<tr>
<td>pAJ13017 pGEX-KG2</td>
<td>de Jong 2018</td>
</tr>
<tr>
<td>pHN150605 pGEX Rab3A Q81L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN150606 pGEX Rab3A T36N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160210 pET Rab6A WT</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160211 pET Rab6A Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160212 pET Rab6A T27N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN150809 pGEX Rab6A Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN150808 pGEX Rab6A T27N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160326 pFSW Cerulean Rab6A Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160701 pET Rab6B WT</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160702 pET Rab6B Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160703 pET Rab6B T27N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160708 pGEX RAB6B Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160709 pGEX RAB6B T27N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160704 pFSW Cerulean Rab6B WT</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160705 pFSW Cerulean Rab6B Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160706 pFSW Cerulean Rab6B T27N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN181203 Tom20TMD Cerulean Rab6B Q72L</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN181204 Tom20TMD Cerulean Rab6B T27N</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN181033 Tom20TMD Cerulean ELKS1αB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN190429 Tom20TMD Cerulean ELKS1αB Δ955-971</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN161037 Tom20TMD Cerulean</td>
<td>this thesis</td>
</tr>
<tr>
<td>pMYW12018 pFSW Cerulean ELKS1αB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN161031 pFSW HA ELKS1αB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN170936 pFSW HA ELKS1αB Δ955-971</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12010 pCMV HA ELKS1αA</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12011 pCMV HA ELKS1αB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12013 pCMV HA ELKS1βB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB14065 pCMV HA ELKS2αB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB14074 pCMV HA ELKS2βB</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12022 pGEX ELKS1αB 2-208</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12023 pGEX ELKS1αB 209-358</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12024 pGEX ELKS1αB 359-696</td>
<td>this thesis</td>
</tr>
<tr>
<td>pLB12025 pGEX ELKS1αB 697-992</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160636 pGEX ELKS1αB 654-955</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160637 pGEX ELKS1αB 654-971</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160638 pGEX ELKS1αB 654-992</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160615 pGEX ELKS1αB 769-992</td>
<td>this thesis</td>
</tr>
<tr>
<td>Vector ID</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>pHN160618 pGEX ELKS1αB 808-992</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160619 pGEX ELKS1αB 850-992</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160617 pGEX ELKS1αB 808-971</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN160912 pGEX ELKS2αB 765-884</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN12107 pGEX ELKS2αB 657-921</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN150802 pFSW Cerulean</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN141024 pFSW tdTomato SV2A</td>
<td>this thesis</td>
</tr>
<tr>
<td>pHN161038 pFSW mitoDsRed</td>
<td>this thesis</td>
</tr>
<tr>
<td>pCMV mitoDsRed</td>
<td>Schwarz Lab</td>
</tr>
<tr>
<td>pCMV eGFP-EB3</td>
<td>Schwarz Lab</td>
</tr>
<tr>
<td>Antibodies</td>
<td>Source</td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
</tr>
<tr>
<td>mouse anti-β-actin</td>
<td>Sigma</td>
</tr>
<tr>
<td>guinea pig anti-Bassoon</td>
<td>Sysy</td>
</tr>
<tr>
<td>mouse anti-Bassoon</td>
<td>Enzo Life Sciences</td>
</tr>
<tr>
<td>rabbit anti-Complexin-1/2</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>rabbit anti-ELKS1/2α/β (1083)</td>
<td>Kaeaser laboratory</td>
</tr>
<tr>
<td>rabbit anti-ELKS1/2α/β (4790)</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>mouse anti-ELKS1/2α (E30)</td>
<td>Abcam</td>
</tr>
<tr>
<td>mouse anti-ELKS1α (E1)</td>
<td>SCBT</td>
</tr>
<tr>
<td>mouse anti-ELKS2α (U5004)</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>mouse anti-ELKS2α (1029)</td>
<td>Kaeaser laboratory</td>
</tr>
<tr>
<td>mouse anti-ELKS2α (abErc2)</td>
<td>Abcam</td>
</tr>
<tr>
<td>mouse anti-GFP (JL8)</td>
<td>CloneTech</td>
</tr>
<tr>
<td>rabbit anti-GFP</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>mouse anti-GluA1 (GluR1)</td>
<td>Synaptic Systems</td>
</tr>
<tr>
<td>mouse anti-GM130</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>mouse anti-HA</td>
<td>Biolegend</td>
</tr>
<tr>
<td>rabbit anti-Liprin-α3</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>rabbit anti-Munc23</td>
<td>Sigma</td>
</tr>
<tr>
<td>rabbit anti-Munc13-1</td>
<td>Brose laboratory</td>
</tr>
<tr>
<td>rabbit anti-Munc18-1</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>mouse anti-Neurofilament</td>
<td>Covance</td>
</tr>
<tr>
<td>mouse anti-PSD-95</td>
<td>NeuroMab</td>
</tr>
<tr>
<td>rabbit anti-Rab3A</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>rabbit anti-Rab6A/B</td>
<td>LifeSpan</td>
</tr>
<tr>
<td>rabbit anti-Rab6B</td>
<td>LifeSpan</td>
</tr>
<tr>
<td>rabbit anti-Rabphilin</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>rabbit anti-RFP</td>
<td>Rockland</td>
</tr>
<tr>
<td>rabbit anti-RIM-1</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>rabbit anti-SNAP-25</td>
<td>Sysy</td>
</tr>
<tr>
<td>mouse anti-Synapsin-1</td>
<td>Sysy</td>
</tr>
<tr>
<td>rabbit anti-Synapsin-1</td>
<td>Abcam</td>
</tr>
<tr>
<td>rabbit anti-Synaptobrevin-2</td>
<td>Sysy</td>
</tr>
<tr>
<td>rabbit anti-Synaptotagmin-1</td>
<td>DSHB</td>
</tr>
<tr>
<td>guinea pig anti-Synaptophysin-1</td>
<td>DSHB</td>
</tr>
<tr>
<td>rabbit anti-Synaptophysin-1</td>
<td>DSHB</td>
</tr>
<tr>
<td>rabbit anti-Syntaxin-1</td>
<td>DSHB</td>
</tr>
<tr>
<td>mouse anti-T7</td>
<td>Novagen</td>
</tr>
<tr>
<td>rabbit anti-VCP</td>
<td>Südofh laboratory</td>
</tr>
<tr>
<td>IRDye 680CW anti-mouse IgG</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye 680CW anti-rabbit IgG</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye 800CW anti-mouse IgG</td>
<td>LI-COR</td>
</tr>
<tr>
<td>IRDye 800CW anti-rabbit IgG</td>
<td>LI-COR</td>
</tr>
</tbody>
</table>
APPENDIX TWO:

Supplemental Figures
(A and B) Mendelian survival ratio using Chi square test analysis of ELKS1α/β knockin (ki) (A) and floxed (B) mouse line.

(C) Representative Western blot images of testing the new HM1283 (ELKS1/2α/β) antibody. HEK cells were overexpressed with various ELKS isoforms.

(D) Representative Western blot images of crude brain lysates harvested from ELKS1α/β homozygous wild type (+/+ ) and floxed (f/f) littermate mice.
Supplemental Figure 2

A

vesicles per 5μm axon

Rab6B^{+/+}  

Rab6B^{-/-}

SVs  endosomes  LDCVs

B

DIV5 cre  high pressure freezing  DIV5 cre  chemical fixation

vesicles per 5μm axon

ELKS1α/β

control  cKO

SVs  endosomes  LDCVs

C

DIV1 cre  high pressure freezing  DIV1 cre  chemical fixation

vesicles per 5μm axon

ELKS1α/β

control  cKO

SVs  endosomes  LDCVs
Supplemental Figure 2:

Quantifying different vesicles types in axons
Analysis of electron micrograph images of axons from either Rab6B or ELKS1α/β cultures. Three types of vesicles were distinguished based on size: synaptic vesicles (SVs, < 50 nm), large dense core vesicles (LDCVs, between 50 - 100 nm), and endosomes that were not LDCVs (between 50 – 100 nm). Quantifications show the average number of vesicles per 5 μm axonal length.

(A) Electron micrograph analysis of axons from high pressure frozen Rab6B+/− or Rab6B−/− hippocampal cultures. Rab6B+/−, n = 207 axons / 2 independent cultures; Rab6B−/−, 167/2.
(B) Electron micrograph analysis of axons from ELKS1α/β control or cKO hippocampal cultures that were infected with Cre recombinase at DIV 5 and were harvested either by high pressure freezing or chemical glutaraldehyde fixation protocols. Higher pressure freezing, ELKS1α/β control, n = 209 axons / 2 independent cultures; ELKS1α/β cKO, 221/2; chemical fixation, n = 1 culture for both genotypes with approximately 50-100 axons per genotype.
(C) Electron micrograph analysis of axons from ELKS1α/β control or cKO hippocampal cultures that were infected with Cre recombinase at DIV 1 and were harvested either by high pressure freezing or chemical glutaraldehyde fixation protocols. In either preparation, both genotypes are n = 1 culture with approximately 50-100 axons per genotype.

All data are means ± SEM and p-values are set as * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Student’s t-test.
Supplemental Figure 3

Synaptic enrichment of mitochondrial (relating to Ch.3 Fig.7.J):

Synaptic enrichment of mitochondrial was determined using three different thresholding for association between Bassoon (synaptic marker) and GFP (reporter for mito-Rab6). Quantifications show the fraction of Bassoon objects that contained mito-Rab6 (m6BQL or m6BTN) signal using the following three co-localization area thresholds: > 0 μm², > 0.1 μm², > 0.25 μm². ELKS1αB + m6BQL, n = 19 fields of view / 3 independent cultures (fields of view = 50 μm x 10 μm, each field of view typically containing about 30-60 Bassoon objects). ELKS1αBΔ955-971 + m6BQL, n = 21/3; ELKS1αB + m6BTN, n = 18/3.

Experiments were conducted blind. All data are mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001. analyzed by one-way ANOVA, followed by Holm-Sidak post hoc comparison to mito-ELKS1αB condition to ‘ELKS1αB + mito-Rab6BQL’ condition.
(A) Control fluorescent images from live imaging experiment testing for bleed-through. Given that transfections can lead to extreme overexpressions of fluorescent signals swelling boutons and saturating signal, I tested for any potential bleed-throughs. Neurons were transfected with a singular DNA construct to express either Cerulean-Rab6\textsubscript{WT}, Cerulean-ELKS1\textsubscript{aB}, or tdTomato-SV2A alone, then were imaged using both channel 550 (for tdTomato) and channel 435 (for Cerulean) using LED light sources.

(B) Representative axonal traces and their corresponding kymographs comparing mobilities of overexpressed Cerulean-tagged Rab6\textsubscript{aB}, Rab6B\textsubscript{WT}, Rab3\textsubscript{aW}, and ELKS1\textsubscript{aB}. 
Supplemental Figure 5:

A

![Pie chart]

B

<table>
<thead>
<tr>
<th>bait</th>
<th>peptide fragments</th>
<th></th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP-1a</td>
<td>E2 (C-term)</td>
<td>103</td>
<td>162</td>
</tr>
<tr>
<td>MAP-1b</td>
<td>E2 (C-term)</td>
<td>40</td>
<td>42</td>
</tr>
<tr>
<td>MAP-2</td>
<td>E2 (C-term)</td>
<td>114</td>
<td>234</td>
</tr>
<tr>
<td>MAP-9</td>
<td>E2 (C-term)</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>MARK1</td>
<td>E1 (N-term)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>MARK2</td>
<td>E1 (N-term)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>MARK3</td>
<td>E1 (N-term)</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>JKP1, Jakmip1</td>
<td>E1, E2 (C-term)</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>JKP2, Jakmip2</td>
<td>E2 (C-term)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>JKP3, Jakmip3</td>
<td>E2 (C-term)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>CLAP-2</td>
<td>E2 (C-term)</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>DYN1/DNM1</td>
<td>E1, E2 (C, N-term)</td>
<td>45</td>
<td>54</td>
</tr>
<tr>
<td>DCTN1</td>
<td>E1 (N-term)</td>
<td>96</td>
<td>147</td>
</tr>
<tr>
<td>MAST3</td>
<td>E1 (N-term)</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>KIF5-A</td>
<td>E1 (N-term)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>KIF5-B</td>
<td>E1 (N-term)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>KIF5-C</td>
<td>E1 (N-term)</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>KIF2-A</td>
<td>E1, E2 (C, N-term)</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>AP2-A1</td>
<td>E1, E2 (C, N-term)</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>AP2-A2</td>
<td>E1, E2 (C, N-term)</td>
<td>36</td>
<td>44</td>
</tr>
<tr>
<td>AP2-B1</td>
<td>E1, E2 (C, N-term)</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>AP1-B1</td>
<td>E1, E2 (C, N-term)</td>
<td>18</td>
<td>24</td>
</tr>
</tbody>
</table>
Supplemental Figure 5:

An interaction screen suggests ELKS to be in complex with microtubule binding proteins (A and B) Mass spectrometry analysis of products from a brain pulldown assay with ELKS1 and ELKS2 baits. GST-tagged ELKS1 and ELKS2 fragments were used from their N- and the C-terminal regions in a pulldown assay where purified adult mouse brains were used as input. The interaction assay was then run in an SDS-PAGE gel electrophoresis, and bands from the gel at different sizes were submitted for mass spectrometric analysis. (A) The pie-chart shows the category of proteins identified in the mass spectrometry analysis. (B) The table shows a list of example proteins that are microtubule associating or trafficking proteins. The left column shows the gene names. The bait-column indicates which bait protein pulled out the protein: ELKS1αB 2-208 (E1 N-term), ELKS1αB 697-992 (E1 C-term), ELKS2αB 657-921 (E2 C-term). The peptide fragment column indicates the number of peptides (unique fragments and the total number of peptide fragments) that were identified in total.
REFERENCES


