Regulation of Neuronal mRNA Localization by Exclusion

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ABSTRACT

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Intra-axonal protein synthesis is important for the proper wiring of the nervous system and can have restorative or pathogenic effects in response to nerve injury and neurodegenerative stimuli. The set of axonally translated transcripts, the axonal translatome, is regulated through the control of mRNA localization, stability, and translation. Targeting the axonal translatome could result in the development of novel therapies for the treatment of neurological disorders. Yet, there are gaps in our understanding of the selective mechanism regulating the specific localization of mRNAs into axons. Currently, axonal localization of transcripts is understood to be controlled by the presence of sequence elements that direct axonal transport. In an attempt to identify novel localization motifs, I found that a well-known motif corresponding to the Pumilio Binding Element (PBE) is significantly depleted in axonally enriched mRNAs. Moreover, I found this element to be highly informative of axonal mRNA localization and translation across different neuronal types and developmental stages suggesting that it is a highly conserved regulatory motif. I found Pum2 neuronal expression and subcellular localization to be highly consistent with the way the PBE predicts mRNA regulation. I then demonstrated that interfering with Pum2 function results in increased axonal localization of PBE containing mRNAs. Finally, Pum2 downregulation was associated with gross defects in axonal outgrowth, branching, and regeneration. Altogether, this data suggests that Pum2 regulates axonal mRNA localization through an exclusion mechanism that is important during neuronal development.
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To my parents
Chapter 1. General Introduction

Randomness is an inherent property of all molecular interactions. Living organisms continuously rely on a significant number of stochastic molecular processes while achieving precise outcomes with very little error. As this comes at a tremendous energy cost, given limited resources, how are biological organisms not only able to exist but to evolve and flourish? A simple and elegant solution is to create distinct subcellular compartments which minimizes the noise generated by molecular processes and their effect on the whole system. Such cellular compartmentalization has been proposed as a passive noise filtering mechanism in biological systems (Stoeger et al., 2016). Although initially overlooked in biology, passive noise filtering by compartmentalization is very advantageous since it is highly efficient and scalable at low energy cost. One prominent biological example is passive noise filtering in transcript abundance by nuclear compartmentalization (Battich et al., 2015; Stoeger et al., 2016).

Transcription, which takes place in the nucleus, occurs in “burst” which leads to stochastic fluctuations in transcript abundance. Cytoplasmic transcript abundance has been observed to be stable and tightly regulated. Battich et al. (2015) found that regulation of nuclear retention and export is responsible for reducing the stochastic noise resulting from bursty transcription by a factor of three to four. Importantly, this comes at no additional energy cost for the cell, which would be significant if an active filtering mechanism, such as controlling mRNA degradation, was predominantly utilized. An advantageous consequence of compartmentalization is reduced cell-intrinsic phenotypic variability due to noise from stochastic transcriptional events (Battich et al., 2015). In turn, this provides benefits for multicellular organisms where extrinsic factors as well as spatial positioning drive phenotypic variability.
The evolution of complex molecular processes is thus aided by compartmentalization through increased cellular regulatory control, conferring cells an enhanced ability to specialize. Therefore, it is not surprising that compartmentalization is thought to play an essential role in the development of multicellular organisms and systems. One such striking system and the focus of this thesis work is the nervous system. Santiago Ramón y Cajal (1879-1930) was among the first to describe the complexity and diversity of nerve cells. In one of his drawings, it is easy to appreciate the extensive compartmentalization of a pyramidal neuron (Figure 1). These cells do not only contain a cytoplasm and nucleus, but processes that extend beyond the cell body called neurites. There are two distinct types of neurites: dendrites and axons. Dendrites are short and abundant, containing even smaller compartments called dendritic spines, while there tends to be only a single axon capable of extending long distances. Interestingly, both of these compartments are capable of localized protein synthesis in response to diverse stimuli and independently from somatic input. The functional role of localized protein synthesis has been underscored in the context of nervous system development, maintenance, and disease. Thus, the mRNA and protein composition of dendrites and axons must be tightly regulated, leading to the question of how selectivity and fidelity are achieved in establishing and preserving the functional and molecular differences between these compartments?

In work presented here, I will try to address this question with a focus on how axonal mRNA localization and translation are regulated in developing neurons. Since local mRNA translation is intimately connected to mRNA localization, most of my work will focus on how neurons control the later process. Unlike in nuclear compartmentalization, axons are not physically separated from the rest of the cell by a membrane. Although the axon initial segment has been shown to be capable of filtering transport of specific proteins and their cargo into the axon, its role in the regulation of mRNA entry and transport is less clear. Particularly puzzling is what determines the identity of the set of mRNAs that populate the axon. Since this has to be achieved with high
precision, a filtering mechanism should be in place. The hypothesis tested in this work is that such a filtering system exists and that it can at least be partially exemplified by the interactions of a somatically localized RNA binding protein and its target mRNAs. In the following sections, I give a comprehensive background and review of the research in the main areas that helped create and address this hypothesis: 1) local translation, 2) mRNA localization, 3) RNA binding proteins.

Figure 1.1: Drawing of Pyramidal Neuron. Representation of a pyramidal neuron by Santiago Ramon y Cajal demonstrating the extensive compartmentalization of this cell. Several dendrites with branches and dendritic spines are observed. A single axon extending downwards is remarkably different since it does not have any spines.
1.1 Local translation

Compartmentalization is at least partly responsible for neurons' ability to carry out complex signal processing and integration. Neurons are thus presented with the challenge to establish and maintain morphologically and functionally distinct compartments. To accomplish this, they must precisely regulate the local proteome with high spatiotemporal precision. Intuitively then, local translation is an elegant solution to the problems presented by local proteome homeostasis regulation (Jung et al., 2012, 2014). Local protein synthesis offers several advantages over transporting proteins synthesized elsewhere. First, the most obvious benefit is that locally synthesized proteins are produced where and when needed. This allows neurons to respond quickly and precisely to localized stimuli which can at times occur very far from the somatic compartment. Aside from avoiding a delayed response, it is also more energy efficient as it does not require coordinated active protein transport. Moreover, proteins produced on-site might be differentially post-translationally modified as well as interact with otherwise uncommon molecular partners. On the following sections, I will review the evidence for the importance and role of local translation in neuronal development, maintenance, and disease.

1.1.1 Local protein synthesis in dendrites

A substantial amount of work in neuroscience has demonstrated the role of protein synthesis in long-term memory formation, but only in the past two decades has that work focused on the dendrite as the site of synthesis (Sutton and Schuman, 2006). As early as 1965, Bodian observed for the first time that ribosomes are present in proximal dendrites (Bodian, 1965). To which he hypothesized “…that selective establishment of synaptic contacts may be determined by specific proteins synthesized at the synaptic membrane of the receptive neuron.” Seventeen years later, Steward and Levy further characterized this phenomenon by quantitatively describing the presence
of polyribosomes at the base of dendritic spines (Steward and Levy, 1982). Subsequently, a series of studies demonstrated that radiolabeled amino acids are incorporated into proteins in synaptic enriched fractions as well as in dendrites separated from the soma (Rao and Steward, 1991; Weiler and Greenough, 1991; Torre and Steward, 1992). Moreover, time course analysis revealed that the appearance of this newly synthesized proteins at synaptic sites was too fast to be accounted for by transport (Feig and Lipton, 1993; Steward et al., 1991).

The first evidence of the functional role for local translation in dendrites was provided by Kang and Schuman in 1996 when they showed that neurotrophin dependent dendritic protein synthesis was required for long-lasting enhancement of synaptic transmission (Kang and Schuman, 1996). Soon thereafter several studies provided more supporting evidence for the role of dendritic protein synthesis in the establishment of different forms of synaptic plasticity (Cracco et al., 2005; Huang and Kandel, 2005; Huber et al., 2000; Martin et al., 1997; Miller et al., 2002; Vickers et al., 2005) as well as in synapse formation (Sebeo et al., 2009). The study by Miller et al. (2002) stands out as the first demonstration of the importance of local translation in vivo. Here, Miller generated a mouse model where the dendritic localizing element of CaMKIIα was removed from the 3'UTR of the corresponding transcript. This resulted in an almost exclusive somatic localization of CaMKIIα mRNA and significantly reduced expression of its protein in dendrites. These mutant mice had significant defects in learning and memory. An et al. (2008) provided another critical example that also highlights the role of the 3'UTR in regulating the localization and translation of a mRNA in vivo. First, they reported differences in the localization of the long and short 3'UTR isoforms of BDNF, with the long 3'UTR driving the localization of BDNF mRNA into dendrites. Then, they generated a genetically modified mouse where the long 3'UTR isoform of BDNF was truncated. BDNF localization and translation in dendrites was impaired in these mice. Moreover, they showed defects
in pruning and enlargement of dendritic spines with selective impairment in LTP in dendrites but
not soma (An et al., 2008).

Lastly, an important question remained on whether local translation also played a role during
ongoing synaptic activity and not just under strong stimulated conditions. Sutton and colleagues
explored this issue by measuring the effects of inhibiting spontaneous neurotransmission in local
dendritic segments (Sutton et al., 2004, 2006). Inhibiting these minis let to an increase in local
protein synthesis with a resulting stabilizing effect on synaptic transmission.

1.1.2 Local protein synthesis in axons

Evidence for axonal protein synthesis dates back to the 1960s (Edström and Sjöstrand, 1969;
Giuditta et al., 1968; Koenig, 1967). It was met with early skepticism due to a later finding that failed
to demonstrate the presence of rRNA in axons (Lasek et al., 1973). Moreover, an electron
microscopy study of adult rats found that polysomes were restricted to the axon initial segment and
not detectable in the axon shaft of CNS neurons, thus questioning whether axonal translation could
occur in adult axons at all (Steward and Ribak, 1986). On the contrary, ribosomes had been
identified in developing axons earlier (Bunge, 1973; Tennyson, 1970; Yamada et al., 1971) and,
unlike in cultured conditions, it was found that ribosomes did not form polysomes in axons in vivo,
suggesting a predominance for monosomal translation that is spatiotemporally restricted (Jung et al.,
2012). Additionally, biochemical data was accumulating that confirmed the presence of rRNA,
mRNAs, and translating polysomes in invertebrate axons (Giuditta et al., 1980, 1986, 1991).
Subsequently, immunohistological studies identified the presence of ribosomal mRNAs and proteins
in mature PNS axons in the form of intermittently scattered plaques near the plasma membrane
(Koenig and Martin, 1996; Koenig et al., 2000; Kun et al., 2007). This finding may explain why it
was initially difficult to identify the presence of ribosomes in axons utilizing conventional
ultrastructural criteria (Jung et al., 2012). Finally, the greatest evidence for axonal protein synthesis came from experiments utilizing metabolic labeling to show that axons are capable of mRNA-dependent protein synthesis in the absence of soma (Eng et al., 1999; Koenig, 1967, 1991; Koenig and Adams, 1982; Tobias and Koenig, 1975a, 1975b). Altogether, these studies demonstrated the capacity of axons for localized protein synthesis, yet they also indicated that the protein synthesis machinery and local translation regulation might function differently from the way it was understood in the somatic and dendritic compartments.

Although axons had been shown to be capable of local protein synthesis, its functional role remained a mystery. In fact, an early study found that inhibiting local protein synthesis did not interfere with axon elongation (Eng et al., 1999). The first demonstration of the functional significance of axonal protein synthesis came from its identified role in mediating axonal growth cone chemotropic responses to Netrin-1 and Sema-3A (Campbell and Holt, 2001). In this seminal work, soma-less growth cones were shown to be able to turn towards netrin-1 gradients and away from Sema-3A gradients. Application of these guidance cues to such axons induced protein synthesis through mTOR activity. In turn, these chemotropic responses were abolished by inhibition of mTOR or protein synthesis directly. This effect was shown to be specific for these cues and mediated by local protein synthesis since the application of lysophosphatidic acid (LPA) in turn resulted in growth cone collapse independently of local mTOR activity nor protein synthesis. Since then, there has been growing evidence that axons can respond to a variety of signaling molecules such as: netrin-1 (Hengst et al., 2009; Leung et al., 2006; Tcherkezian et al., 2010; Yao et al., 2006), semaphorin-3a (SEMA3A) (Balastik et al., 2015; Campbell et al., 2001; Deglincerti et al., 2015; Nédelec et al., 2012; Wu et al., 2005), slit-2 (SLIT2) (Piper et al., 2006), engrailed 1 and 2 (EN1 and EN2) (Alvarez-Fischer et al., 2011; Brunet et al., 2005; Wizenmann et al., 2009), nerve growth factor
An interesting observation from these studies is the ability of axons to control determined growth cone behaviors such as turning or collapse, by translating only very specific mRNAs, in response to distinctive cues, even though these signals nonspecifically engage the whole translational machinery generally through mTOR pathway activation. For example, in response to a gradient of attractive cues such as NTN1 and BDNF, β-Actin is locally and asymmetrically synthesized in the side of the growth cone closest to the gradient (Leung et al., 2006; Yao et al., 2006). In this scenario, blocking β-Actin synthesis or even the asymmetric localization of its mRNA in the growth cone is enough to prevent attractive growth cone turning. On the contrary, repulsive cues such as SLIT2 and SEMA3A induce the local synthesis of actin disassembly promoting factors coflin (Piper et al., 2015) and RhoA (Wu et al., 2005) respectively, without inducing β-Actin synthesis. Moreover, interfering with axonal translation of RhoA is sufficient to block SEMA3A induced growth cone collapse, demonstrating the specificity of this pathway (Wu et al., 2005).

The prior examples demonstrated the role of local axonal translation in regulating local growth cone responses. Recently, there has been accumulating evidence to suggests that local protein synthesis can exert a broader effect on neuronal health and function through retrograde signaling of locally produced factors from the periphery to the soma (Andreassi et al., 2010; Baleriola et al., 2014; Cox et al., 2008; Ji and Jaffrey, 2012; Villarin et al., 2016). NGF application to axons alone is sufficient to promote DRG survival in a protein synthesis-dependent manner (Cox et al., 2008). This is mediated by the axonal synthesis and retrograde transport of the transcription factor cyclic AMP responsive element-binding protein (CREB) in response to NGF stimulation. In fact, somatically derived CREB does not mediate NGF-induced cell survival. This was the first evidence that axons could regulate neuronal homeostasis through the production of transcription factors. In
another example, it was found that trigeminal ganglia subtype specification was governed by the local translation and retrograde transport of SMAD1/5/8 in response to BDNF and BMP4 axonal signaling (Ji and Jaffrey, 2012). Very interesting is the finding that axonal application of β-amyloid (Aβ) leads to neuronal death mediated by axonal synthesis and retrograde transport of activating transcription factor 4 (ATF4) (Baleriola et al., 2014). It once again highlights the specificity of axonally derived responses to local stimuli by demonstrating that axons are not only capable of promoting neuronal surviving but also death. It also linked axonal dysfunction through local protein synthesis to neurodegeneration.

Although initial reports had found little evidence for translation in mature axons, further studies into the axonal response to nerve injury demonstrated increased local protein synthesis to be necessary for regeneration (Hanz et al., 2003; Verma et al., 2005; Zheng et al., 2001). Moreover, it seemed that the ability of axons to regenerate was intrinsically associated with their translational capacity as modulating mTOR activity through PTEN inhibition, both locally and globally, resulted in increased regeneration (Christie et al., 2010; Park et al., 2008). Mechanistically, axonal injury results in retrograde signaling of locally synthesized transcription factors that modulate neuronal gene expression programs to favor axon regeneration (Michaelevski et al., 2010). For example, upon axonal injury DRG axons locally synthesize and retrogradely transport STAT3 to the nucleus where it is required to promote a regenerative program (Ben-Yaakov et al., 2012). Moreover, axon injury also modulates retrograde transport itself through the local synthesis of Ran-specific GTPase-activating protein (RANBP1) (Yudin et al., 2008) and importin-β1 (Hanz et al., 2003), which in turn associate with and regulate the entry of proteins containing nuclear localization signals (NLS) into the nucleus. Finally, nerve injury also induces the local translation of vimentin which in turn promotes the retrograde transport of phosphorylated Erks (pErks) which regulate complex signaling pathways (Perlson et al., 2005).
Finally, Shigeoka and colleagues recently produced the best evidence for mature axonal translation and, most complete understanding of local translation in developing axons in a living organism (Shigeoka et al., 2016a). They developed an axon-TRAP-RiboTag mouse which allows specific isolation of ribosome-bound mRNAs in the distal compartment of retinal ganglion cells (RGC) axons in vivo. Using this approach, they characterized the axonal translatome of RGC axons at different developmental stages (E17.5, P0.5, P7.5, and adult). They found a dynamic set of mRNAs is translated at each developmental stage matching the axon specific function of that stage. For example, during the embryonic stage a significant number of mRNAs associated with “axon extension” are being synthesized, while at the adult stage there is a switch for the synthesis of mRNAs associated with “synaptic transmission”. Finally, they identified a set of motifs linking alternative splicing with isoform specific transcript translation in axons.

1.2 Neuronal mRNA localization

Although it has not always been recognized as such, increasing evidence strongly suggests that mRNA localization is a primary determinant of protein localization (Gáspár and Ephrussi, 2017a; Jung et al., 2014; Moor et al., 2017; Zappulo et al., 2017a). For example, during Drosophila embryonic development up to 71% of transcripts were found to be localized (Lécuyer et al., 2007). While in Drosophila ovarian cells a more modest 22% of mRNAs showed a subcellular localization, their localization was dynamically regulated across development, and it was cell type specific (Jambor et al., 2015). Similarly, a broad look at mRNA localization in hippocampal neurons found over 2000 mRNAs (about 30% of the total) to be localized to dendrites and or axons. This highly regulated and asymmetric localization of mRNAs is not unique to developing organisms or complex eukaryotic cells since it has been found even in bacteria (Keiler, 2011). In the following sections, I will focus on mRNA localization in neurons, with an emphasis on axonal mRNAs.
1.2.1 ‘Zipcode’ localizes mRNA

Based on several studies of mRNA localization in dendrites it was already widely known that elements contained in the 3'UTR of mRNAs were responsible for directing subcellular localization (Kuhl and Skehel, 1998). Several good examples, some of which I mentioned earlier in regards to localized translation in dendrites, include CaMKIIα (Imaizumi et al., 2000; Mayford et al., 1996), BDNF (An et al., 2008) and Arc (Kobayashi et al., 2005). This notion has been extended to axonal mRNA localization by several groups (Aronov et al., 1999; Ben-Yaakov et al., 2012; Merianda et al., 2013, 2015; Vuppalanchi et al., 2010; Wu et al., 2005; Yudin et al., 2008). Perhaps, the best characterized cis-acting element driving axonal mRNA localization is in the β-Actin 3'UTR. Actin isoforms were found to localize differentially within neurons, β-Actin localizes to axons and, γ-Actin localizes to the soma (Bassell et al., 1998). The axonal localization of β-Actin was found to be increased upon neurotrophin stimulation (NT-3) and to be dependent on its 3'UTR (Zhang et al., 1999, 2001). Specifically, the association of the Zipcode Binding Protein 1 (ZBP1), with an element termed the ‘zipcode’ in the β-Actin 3'UTR was responsible for the axonal localization of this transcript (Willis et al., 2011; Zhang et al., 2001). Moreover, transgenic expression of a mRNA with the 3'UTR of β-Actin but not γ-Actin drives its localization to axons in vivo (Willis et al., 2011). Finally, the stoichiometry of ZBP1 and β-Actin mRNA has to be tightly regulated since the introduction of an exogenous construct with the β-Actin 3'UTR, or the knockdown of ZBP1, results in a significant decrease in β-Actin mRNA localization and translation in axons with physiological and morphological effects in axon growth (Donnelly et al., 2011, 2013).
1.2.2 Axonal mRNAs are dynamic

As it became more technically feasible, researchers expanded the repertoire of axonally localized mRNAs. The axonal mRNA population was found to be highly dynamic by responding to extrinsic as well as intrinsic cues. Application of a variety of neurotrophins as well as repulsive molecules to regenerating DRG adult axons led to a differential effect in the localization of a pool of 50 mRNAs (Willis et al., 2007). The axonal mRNA content of axons in response to NGF was further characterized through SAGE analysis, revealing the presence of hundreds of mRNAs (over 11,000 tags) (Andreassi et al., 2010). Here, Impa1 was found to be the most enriched axonal mRNA and, its localization in axons was driven by an element in its 3’UTR much similarly to β-Actin. The development of a microfluidic device allowed for the isolation of matured CNS axons for the first time and, coupled with microarray analysis led to the identification of more than 300 mRNAs in axons (Taylor et al., 2005, 2009). This axonal mRNA population was altered upon axotomy (Taylor et al., 2009). Cell intrinsic changes in the axonal transcriptome were found during development from the observation that embryonic and adult axons/growth cones differ in their mRNA content (Gumy et al., 2011; Zivraj et al., 2010). Using microarray analysis, the number of mRNAs identified was 2627 in embryonic and 2924 in adult axons, with about 1400 overlapping genes which mostly encoded for ‘mitochondria’ and ‘protein synthesis’ gene enrichment categories suggesting that a core set of mRNAs might be required for general axon function (Gumy et al., 2011). Finally, comparison of growth cone vs whole axons found a distinct set of mRNAs enriched at the growth cone suggesting that even within the axonal compartment there is a mechanism driving the ‘hyper-localization’ of transcripts (Zivraj et al., 2010).

The use of high throughput RNA sequencing has led to a more comprehensive characterization of the axonal transcriptome during development (Bigler et al., 2017; Minis et al., 2014) and in disease (Baleriola et al., 2014). Sequencing of peripheral axons from rat embryonic rat...
DRGs revealed about 6118 localized mRNAs, which included about 80% of prior identified transcripts in Gumy et al. (2011), the highest number to date (Minis et al., 2014). This rich dataset provided opportunities for computational analysis to discover rules governing mRNA localization in axons. The authors did not focus their search on 3'UTRs, sites that are known to contain such regulatory elements. They found motifs that can be equally found in any region an mRNA and that are unknown to be associated with any RNA-binding protein (RBP). Therefore, their relevance is unclear. Finally, by increasing the depth of sequencing Taliaferro and colleagues discovered that distal alternative last exons drive localization of gene isoforms to neurites (Taliaferro et al., 2016).

1.3 RBPs: regulators of RNA localization and translation

As discussed in the prior sections, there is extensive evidence that axonal mRNA localization and translation occurs and, that it plays an important role in nervous system development and disease (Batista and Hengst, 2016; Jung et al., 2012, 2014). One key observation is that both axonal mRNA localization and translation are highly regulated developmentally as well as in response to extrinsic cues. This poses an interesting question, how are axonal mRNA localization and translation specified? This is believed to be mediated by RNA-binding proteins (RBPs). By interacting with select mRNAs through binding to specific regulatory motifs, RBPs form messenger ribonucleoprotein complexes (mRNPs) that are then transported to their subcellular location by motor proteins (Kiebler and Bassell, 2006). Moreover, mRNAs are translationally silent during this transport process, thus providing a dual means of regulation (Besse and Ephrussi, 2008). In the following section, I will briefly discuss the role of RBPs in regulating axonal mRNA localization and its functional consequences. Then, I will review the PUF family of RBPs in the context of mRNA localization and translation regulation, as members of this family are the focus of the work presented
in this thesis. Finally, I will discuss the computational and molecular tools that have allowed for the identification of regulatory sequence elements (motifs) and their association with RBPs.

1.3.1 Neuronal mRNPs

The life of cellular mRNAs is dictated by their interactions with RBPs. In fact, it has been hypothesized that the fate of a mRNA is sealed as soon as it is transcribed. (Haimovich et al., 2013). To understand how mRNA localization and the translation is regulated in neurons, researchers have tried to decode the mechanism by which RBPs interact with select mRNAs to form mRNPs. The complexity of this code can be astounding, as a single RBP can interact with hundreds of mRNAs (Ascano et al., 2012; Patel et al., 2012), and conversely, a single mRNA can associate with several RBPs. For example, β-Actin is known to bind to at least 10 RBPs, many of which regulate its subcellular localization and or translation (Glinka et al., 2010; Klein et al., 2013; Ma et al., 2011; Ross et al., 1997; Todd et al., 2010). Interestingly, unlike previously thought, many mRNAs are present at low or single copy numbers in RNA granules (Amrute-Nayak and Bullock, 2012; Buxbaum et al., 2014; Mikl et al., 2011). Moreover, the composition of RNA granules is more heterogeneous than initially expected (Fritzsche et al., 2013).

Subcellular transport of mRNPs is mediated by molecular motor proteins and not stochastic diffusion. This form of active transport in neurites exhibits long, processive, and oscillatory movement (Park et al., 2014; Tübing et al., 2010). Interestingly, local axonal translation can modulate transport in response to environmental cues by regulating the translation of motor protein cofactors (Villarin et al., 2016). The composition and subcellular localization of mRNPs are in turn controlled by a unique combination of cis-acting sequence elements (Jambhekar and Derisi, 2007; Shabbabian and Chartrand, 2012). RBPs have been hypothesized to serve as adapters that associate mRNPs with microtubule based motors and (Davidovic et al., 2007). There is substantial evidence suggesting that
The association of a mRNA with an RBP is required for efficient subcellular transport and, is mediated by localization sequence elements in the mRNA. For example, the number of localization elements on a mRNA has been found to correlate with the number of associated motor proteins (Sladewski et al., 2013). Increasing the number of localization elements also improves the efficiency of mRNP transport (Amrute-Nayak and Bullock, 2012). Finally, altering an RBP copy numbers or its RNA binding function can lead to defects in mRNP localization (Alami et al., 2014).

1.3.2 PUF family

The PUF (Pumilio and FBF) family of RNA-binding proteins regulates mRNA expression and localization of target mRNAs (Quenault et al., 2011). Members of this family share a highly evolutionarily conserved homology RNA-binding domain (RBD) (Wickens et al., 2002; Zamore et al., 1997; Zhang et al., 1997). This RBD is structurally and genetically modular, consisting of several repeats each of which recognizes a specific nucleotide base (Edwards, 2015; Edwards et al., 2001; Wang et al., 2001, 2002). This has allowed for the evolution of mRNA target specificity and recognition. Two Pumilio homologs (PUM1 and PUM2), sharing up to 94% of the PUM-HD, have been identified in mammals (Spassov and Jurecic, 2002). The mammalian consensus binding element often termed the Pumilio response element (PRE) consists of an octameric nucleotide sequence described initially as UGUANAUA (Galgano et al., 2008; Hafner et al., 2010; Morris et al., 2008).

In yeast, several PUFs members are responsible for regulating the localization and translation of more than 10% of the transcriptome (Gerber et al., 2004; Haramati et al., 2017). Here, specific PUFs associate with mRNAs encoding for components of distinct organelles. For example, Puf3 is associated with mRNAs encoding mitochondrial components (Gerber et al., 2004; Saint-Georges et al., 2008), while Puf4 and Puf5 are often associated with mRNAs encoding nuclear
components (Gerber et al., 2004), and Puf1 and Puf2 with mRNAs encoding membrane-associated proteins (Gerber et al., 2004; Porter et al., 2015). The function of these proteins is illustrated by the well-studied regulation of Ash1 mRNA asymmetric localization and translation in yeast. Here, Puf6 associates with Ash1 mRNA post-transcriptionally and represses its translation while promoting its localization to the distal bud tip (Deng et al., 2008; Gu et al., 2004). Interestingly, the shared RNA binding preferences among PUFs has resulted in the emergence of an RNA regulatory “super-network” whose behavior can be modulated by the stoichiometry of its members. Our understanding of the diverse roles that these RBPs perform in yeast continues to evolve as novel functions emerge (Haramati et al., 2017).

In Drosophila, together with the RBPs partners Nanos and Brain Tumor, Pumilio is responsible for regulating mRNA localization, stability, and translation with functional consequences for embryonic development, stem cell maintenance and differentiation, fertility and neurologic processes (Arvola et al., 2017). During anterior-posterior patterning, Pumilio acts together with Nanos (Nos) to repress the localization and translation of hunchback mRNA at the posterior pole to control embryonic development (Nüsslein-Volhard and Lehmann, 1987; Sonoda and Wharton, 1999; Wharton et al., 1998; Wreden et al., 1997). Pumilio is required for proper Drosophila germline development by regulating germline stem cell self-renewal and asymmetric division (Asaoka-Taguchi et al., 1999; Joly et al., 2013; Lin and Spradling, 1997). A screen for transcriptionally regulated genes during memory formation identified Pumilio as a regulator of long-term memory (Dubnau et al., 2003). Moreover, further analysis of Pumilio target mRNAs revealed an enrichment for genes that are synaptically localized and potentially locally translated (Chen et al., 2008). Finally, Pumilio regulates dendritic and synaptic morphogenesis as well as neuron excitability in Drosophila (Bhogal et al., 2016; Mee et al., 2004; Menon et al., 2004; Schweers et al., 2002; Ye et al., 2004).
Similarly, PUMs have also been implicated in the regulation of nervous system development and reproduction in mammalians. Pumilio-2 and Staufen-2 RNA granules are apically localized in progenitor cortical radial glial cells promoting self-renewal and preventing premature neuronal differentiation (Vessey et al., 2012). A more recent study demonstrated that both Pum1 and Pum2 are critical for hippocampal neurogenesis and function (Zhang et al., 2017). Additionally, Pum2 has been shown to form dendritically localized ribonucleoprotein particles and stress granules where it is involved in regulating the proper formation of dendritic spines and synaptic plasticity (Siemen et al., 2011; Vessey et al., 2006, 2010). Pum1 knockout mice develop motor dysfunction through neurodegenerative processes in the cerebellum caused by Ataxin-1 overexpression (Gennarino et al., 2015).

Mechanistically, PUMs are thought to inhibit translation and mRNA stability by recruiting specific factors to target mRNAs. Specifically, Pumilio can regulate mRNA deadenylation by the recruitment of CPEB or the CCR4-Pop2-NOT complex in a context specific and combinatorial manner that is not entirely understood (Campbell et al., 2012; Piqué et al., 2008; Van Etten et al., 2012; Weidmann et al., 2014). Pumilio proteins have been predicted to interact with miRNAs; specifically, Pum1 has been shown to repress translation of p27-mRNA through the recruitment of miR-221/222 (Kedde et al., 2010).
Chapter 2. Thesis Outline and Contributions

In the prior chapter I reviewed the evidence for localized translation and how is mainly determined by RBP regulated mRNA localization. An outstanding question that I decided to address is: how is the identity of axonally localized mRNAs globally determined in early developing neurons? Thus, I test the hypothesis that RBPs act as molecular filters in regulating the composition of asymmetrically localized mRNAs in axons of developing neurons. My findings challenge the prevailing notion that active mRNA transport is the main mode of regulation by identifying an exclusion mechanism by which mRNAs containing a specific motif are retained in the cell body. I also discover a new role for Pum2 by identifying it as the main effector in this regulatory process. In the following chapter, I describe the materials and methods used in these studies. In Chapter 4, I present the computational work to identify motifs that are informative of axonal mRNA localization and translation as well as the verification of the strongest motif in regulating this processes in-vitro. In Chapter 5, I demonstrate that this motif is strongly associated with Pum2 and test whether Pum2 regulates axonal mRNA localization and translation of endogenous mRNAs containing the motif. In Chapter 6, I test the functional consequences of disrupting this regulatory mechanism by characterizing the developmental phenotypes of developing cortical neurons in-vivo as well as regenerating axons in-vitro. Finally, in Chapter 7 I synthesize these findings in context and present ideas for further testing. The work presented in the main text of my thesis was mostly carried out by myself with one main exception. The in-vivo work was carried out in collaboration with Dan Iascone, a graduate student member of Dr. Franck Polleux’s laboratory at Columbia University. Additionally, I received some assistance from a rotation student, Helena Pernice, in the axonal injury work. Moreover, a current member of the lab, Lisa Randolph, helped test and verify some qPCR targets from the RNA sequencing results. In addition to the work presented in the main text, I collaborated
with other lab members resulting in two publications which are included in the Appendix and that I describe below (Batista et al., 2017; Villarin et al., 2016).

The work by Villarin et al. (2016) demonstrated that axons can modify the dynamics and cargo of retrograde transport in response to acute changing demands by locally synthesizing dynein cofactors. An interesting observation was that Lis1 mRNA was recruited to axons upon NGF deprivation and stimulation. Coincidentally, it had been recently demonstrated that APC functions as an RBP that transports mRNAs into axons and that Lis1 was among the targets (Preitner et al., 2014). Given my interest in the regulation of axon mRNA localization by RBPs, I decided to apply bioinformatics analysis in order to identify the binding site of APC to Lis1 mRNA. By applying CIMS (Moore et al., 2014) and PIPE-CLIP (Chen et al., 2014) analysis to the HITS-CLIP data generated by Preitner et al. (2014) I identified the binding region of APC which also contained the reported CUGU motif that was reported for APC. Additional work by Villarin et al. (2016) demonstrated the functional relevance of this binding site since blocking it with an LNA disrupted the localization and translation of Lis1 mRNA in axons. Finally, I used FISH to test whether other dynein cofactors, namely Nde1 and Ndel1, are also co-recruited to axons in response to acute demands. I found them to be enriched in axons in response to NGF withdrawal but not NGF stimulation suggesting that together with Lis1 they might be part of an RNP complex that is co-regulated. My contributions in this publication are presented in Figure 7A and Supplementary Figure 2.

Batista et al. (2017) asked whether axonal translation was required for pre-synapse formation. Using PDL-coated beads as a way to induce pre-synapses we showed that axonal synthesis of SNAP25 was indeed required to form functional pre-synapses capable of vesicle release. I was specifically interested in the recruitment and translation of SNAP25 mRNA in axons. Using FISH, I found SNAP25 mRNA to be recruited to the site of synapse formation upon simulation
with PDL-coated beads. One technically challenging question due to the low biochemical yields of axonal preparations was whether SNAP25 protein accumulated in axons over time during synapse formation. To address this, I modified and built a microfluidic device that allowed for greater quantities of protein to be extracted from axons based on a prior design (Park et al., 2009). Using this new device, I found an accumulation of SNAP25 in axons during pre-synapse formation that was blocked with local application of protein synthesis inhibitors. Finally, I also validated demonstrated that puromycilation labeling as a readout for translation correlates well with the localization of active markers of translation (p-4EBP1). My contribution to this paper are shown in Figure 3C and Figure 4A, 4B and 4C.
Chapter 3. Materials and Methods

3.1. Animal Use and Cell Culture

3.1.1. Rat Handling

Pregnant Sprague-Dawley rats (*Rattus norvegicus*) were obtained from Envigo (Indianapolis, IN) and housed for 2 days at the barrier facility at the Columbia University Institute of Comparative Medicine. All rodent procedures were approved by the Columbia University Institutional Animal Care and Use Committee.

3.1.2. Euthanasia

Rats were euthanized by gas displacement with 5% min⁻¹ CO₂ until there were no signs of breathing, approximately 6-7 min. A secondary physical means of euthanasia, bilateral thoracotomy, was performed to ensure death.

3.1.3. Microfluidic Devices

Bipartite microfluidic devices with a set of 750 µm-long microgroove barriers were designed in house and produced according to established protocol (Park et al., 2006; Taylor et al., 2005). Microfluidic chambers were produced from silicon masters using PDMS (Sylgard 184, or Qsil 216) by combining an elastomer base with curing agent in a 9:1 ratio and mixed thoroughly for 5 mins. Trapped gases were removed by placing the mixture in a vacuum desiccator for 1 h. PDMS molds were baked for at 4 h at 65°C. Individual devices were cut and reservoir holes were punched. Chambers were cleaned with vinyl tape, washed in 70% ethanol and dried before use.
3.1.4. Culture Materials

For Puro-PLA experiments glass bottom dishes (MatTek, Ashland, MA) were coated with 0.01 mg/mL PLL (Trevigen, Gaithersburg, MD) for 1 h at 37°C and then rinsed with water three times and allowed to air-dry. For the rest of the experiments glass coverslips (25 mm; Carolina Biological Supply Company, Burlington, NC) were rinsed with water twice and then coated with PLL in a 6-well Nunc cell-culture treated 6-well plate for 1 h at 37°C. Coverslips were then rinsed with water three times and allowed to air-dry.

3.1.5. DRG Neuron Culture

DRGs were harvested from E15 rat embryos and trypsinized by incubating with TrypLE Express for 30 min in a water bath at 37°C. DRGs were centrifuged to remove TrypLE solution. They were washed once with DRG growth medium (Neurobasal, 1x B27, 2 mM glutamate, 20 μM 5-FdU, 50 ng/mL NGF) and resuspended in the same media and dissociated with a fine pipette tip. About 40,000 cells were seeded per chamber and allowed to attach for 30 mins before more medium was added.

3.2. Experimental Treatments

3.2.1. Lentivirus Preparation

Lentivirus expression plasmids with shRNAs targeting Pum2 or control sequence were obtained from transOMIC (Huntsville, AL). These plasmids were slightly altered by replacing the original mCMV promoter with the hUbc for better expression in primary neurons. Also, the reporter ZsGreen1 gene and Puromycin resistance cassette were replaced by eGFP. The hairpins in these plasmids are designed using algorithms and technology that enhances specificity and potency (Auyeung et al., 2013; Knott et al., 2014). Lentiviruses are produced by transfecting HEK293T cells
with the lentiviral plasmid and packaging plasmids (pCMVΔ R8.9 and pHCMV VSVg) using Calfectin (Signagen, Rockville, MD). Medium is changed to DRG growth medium without NGF or FdU after 8h. Viruses are collected 24h later, aliquoted and stored at -80°C. The titers for every batch are calculated using a qPCR based kit from Applied Biological Materials (Richmond, Canada) according to the manufacturer’s instructions.

### 3.2.2. Neuronal Transduction

Primary DRG neurons were infected 30 mins after plating at an MOI of 40 to obtain more than 90% infectivity. Knockdown was measured 4 days after infection (Figure 3.1).

**Figure 3.1: shRNA lentiviruses are highly effective.** Pum1 or Pum2 shRNA lentiviruses were added to primary DRG neurons after plating in microfluidic chambers. Protein lysates were obtained 96h after and used for western blotting.
3.2.3. In-utero Electroporation

In utero cortical electroporation was performed according to well established protocols (Courchet et al., 2013; Hand and Polleux, 2011). shRNA plasmids at a concentration of 1 µg/µL were injected into one lateral hemisphere of E15.5 embryos and driven into progenitor cortical neurons by electroporation. Four pulses of 45 V for 50 ms with 500 ms interval were used. Animals were sacrificed 5 days or 3 weeks after birth by perfusion with 4% paraformaldehyde (PFA, Electron Microscopy Sciences).

3.3. Imaging based techniques

3.3.1. Immunofluorescence

Primary DRG neurons were fixed with 4% paraformaldehyde (PFA) in cytoskeletal preservation buffer (10 mM MES, 138 mM KCl, 3 mM MgCl2, 2 mM EGTA, 320 mM sucrose, pH 6.1) for 15 minutes at room temperature. Samples were washed with PBS (3 times for 5 minutes each) and permeabilized in PBS with 0.3% Triton X-100 for 5 minutes. Cells were rinsed briefly in PBS and blocked for 1 h at room temperature with 5% BSA in PBS-T (0.1% Tween-20 in 1X PBS). Samples were incubated overnight in 1% BSA in PBS-T at 4°C with the following primary antibodies: β-III-tubulin (1:1000, Biolegend), Pum1 (1:200, Bethyl), Pum2 (1:200, Bethyl). Samples were washed with PBS-T (3 times, 5 minutes each) and incubated for 1 hour at room temperature with fluorophore-conjugated Alexa secondary antibodies (1:1000, Thermo Fisher Scientific). Samples were washed with PBS (3 times, 5 minutes each), and mounted with ProLong Diamond antifade reagent (Thermo Fisher Scientific). Imaging was carried out using an EC Plan-Neofluar 40x/1.3 objective on an Axio-Observer.Z1 microscope equipped with an AxioCam MRm Rev. 3 camera (Zeiss, Thornwood, NY).
3.3.2. Puromycilation

Primary DRG neurons grown in microfluidic devices were incubated with 2 μM puromycin only in the axonal compartment at 37°C for 10 minutes. Cells were washed once with fresh DRG growth medium and then fixed and processed as described above for immunocytochemistry with the following modifications. Samples were incubated overnight at 4°C with α-Puromycin (1:1000, Millipore Sigma) and the next day with fluorescence conjugated β-III-tubulin (1:500, BioLegend) and Alexa-conjugated secondary for 1 h at room temperature.

3.3.3. Puro-PLA

Puro-PLA assay to detect newly synthesized proteins following a established protocol (tom Dieck et al., 2015). Briefly, the puromycilation procedure detailed above was combined with the Duolink (Sigma-Aldrich) assay according to the manufacturer’s instructions as follows. After primary antibody incubation and washes samples were incubated with Plus and Minus conjugated PLA probes for 1h at 37C in humidity chambers. Samples were then washed twice in TBS-T for 5 minutes each time. Ligation of the probes was carried out for 30 mins at 37°C and followed with 2 washes for 2 minutes with TBS-T. Amplification using the red reagent mix was performed for 100 mins at 37°C. Samples were washed with TBS and incubated with fluorescence conjugated β-III-tubulin (1:500, BioLegend) in TBS for 1 h at room temperature. Finally, samples were rinsed in TBS and mounted using Duolink Mounting Media with DAPI.

3.4. Biochemistry

3.4.1. Quantitative Real Time PCR

Total RNA was extracted from either the somatic or axonal compartment using the Single Cell RNA Purification Kit (Norgen Biotech) from 6 to 12 microfluidic chambers. Samples were
treated with DNAsel. Quantitative PCR was performed with the One-Step RT-qPCR from RNA kit (Bio-Rad) according to manufacturer’s instructions using gene specific TaqMan probes. 10ng of somatic RNA was used per reaction. Axonal RNA was too low to measure therefore loading was normalized to volume and results were normalized to a reference gene.

3.4.2. RNA-Sequencing

Total RNA was extracted from either the somatic or axonal compartment using the Single Cell RNA Purification Kit (Norgen Biotech) from 6 microfluidic chambers. Samples were submitted to GENEWIZ for RNA-Seq. mRNA was enriched by poly-A pulldown. Axonal RNA was subjected to SMART-Seq v4 Ultra Low Input RNA Kit while somatic RNA library was prepared using a standard mRNA kit. Generated libraries were pulled together and sequenced using a HiSeq 2x150bp PE configuration in a single high output lane.

3.5. Computational Analysis

3.5.1. RNA-Sequencing Analysis

Publically available datasets were accessed and downloaded from the NCBI GEO database web server. Reads were pseudoaligned to that appropriate species transcriptome (mouse or rat) using kallisto under default conditions to generate transcript specific counts (Bray et al., 2016). For differential gene expression analysis, transcript counts were imported in R using tximport (Soneson et al., 2015) and analysis was performed with DESeq2 (Love et al., 2014). Generally, a stringent threshold was set to filter lowly expressed genes from downstream analysis by excluding genes whose expression level were under the 75th quartile.
3.5.2. Motif Discovery and Testing

Motif discovery and analysis was performed using FIRE (Elemento et al., 2007) with a few modifications. Full length annotated 3'UTRs were downloaded from UCSC database using Usegalaxy webserver. Duplicates were removed and sequence homology files were prepared using scripts included in the FIRE suite. Data files containing either continuous or discrete information were prepared from the RNA-Seq analysis and provided to the program accordingly. Since the sequencing was not deep enough to accurately measure transcript isoform expression and 3'UTRs are generally under-annotated, the isoforms containing the longest 3'UTR were often used in the analysis when multiple isoforms exist. The number of bins were set to result in 200 genes per bin. For de novo motif discovery analysis minr was set at 1.

3.5.3. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc; La Jolla, CA). Unpaired t-test was performed to compare the means of two groups.
Chapter 4. The Pumilio-Binding Element is informative of neuronal mRNA localization

4.1. Rationale and Summary

To gain a more complete understanding of the full axonal transcriptome, several high-throughput studies were conducted that measured axonal mRNA from neurons grown in compartmentalized chambers. These studies revealed a core cohort of mRNAs present in every axonal transcriptome as well as other mRNAs that were neuronal type and developmental stage specific. This prompted the search for the regulatory motifs and RBPs responsible for specificity in the sorting of mRNAs into the axonal compartment. It was hypothesized that a gatekeeping mechanism that acts by selectively restricting the localization of specific mRNAs into axons might exist, yet the identity of this mechanism has remained elusive.

The idea that molecular “addresses”, termed “zipcodes”, in the form of sequence or structural motifs in the 3'UTR of mRNAs are required and sufficient for the asymmetric subcellular sorting of mRNAs is well established in biology. In the following studies, I conduct an extensive search for cis-acting elements in the 3'UTR of mRNAs, where most regulatory motifs reside, that are informative of axonal mRNA localization in developing neurons using the computational algorithm FIRE. I am able to identify the Pumilio-Binding Element (PBE) (5'-UGUAHANR-3') as a highly informative motif of axonal mRNA localization and translation. Moreover, I show that this motif is sufficient to restrict the axonal localization and translation of a transcript when present in the 3'UTR. These findings suggest that the PBE might work as a gatekeeping signal to control early mRNA composition of developing axons.
4.2. Results

4.2.1. The PBE is negatively associated with axonal mRNA localization in developing sensory neurons

To identify motifs that are informative of axonal mRNA localization, I applied the computational algorithm FIRE on discovery mode to the dataset from Minis et al. (2014). Surprisingly, all of the identified motifs are over-represented in mRNAs that are somatically localized, and under-represented in axonally enriched mRNAs (FIGURE 4.1). This indicates that there might be an important mechanism for regulating axonal mRNA localization by exclusion. The most informative motif (UGUAUAU) contains the core element (UGUA) of the mRNA motif for the PUF family of RNA binding proteins and, closely follows the binding motif recognized by the mammalian homologues Pum1 and Pum2 (UGUAHAUA), here referred to as the Pumilio binding element (PBE). I used an orthogonal approach to identify motifs regulating axonal mRNA localization by applying the computational algorithm DRIMust (Leibovich et al., 2013) to the same dataset. Interestingly, this algorithm found no motifs when using axonally enriched mRNAs as target sequences, but found two motifs, one of which is similar to the previously one identified by FIRE (UGUWU) when using somatically enriched mRNAs as target sequences (FIGURE 4.2).
Figure 4.1: Motif discovery with FIRE reveals pattern of somatic mRNA retention in neurons. FIRE was run on ‘discovery mode’ in a neuronal genome-wide subcellular localization measurement dataset (Minis et al. 2014). The top 10 motifs are shown. The following parameters were used, \( k=7 \), \( \text{ebins}=20 \), \( \text{minr}=1 \). Yellow intensity corresponds to the degree of enrichment of a motif while blue intensity refers to the degree of depletion of a motif. The left bins contain mRNAs that are relatively enriched in cell bodies, while the right bins contain mRNAs that are relatively enriched in axons. Each bin contains about 200 mRNAs.
Since both approaches identified a motif very close to the PBE and, this family of RBP is known to regulate mRNA localization, degradation, and translation, I decided to focus on this motif for further analysis. RNA immunoprecipitation and CLIP studies have demonstrated that both, Pum1 and Pum2 have an affinity for A, C, and U but not G at the fifth base the binding site. To test the specificity of the motif I ran FIRE on non-discovery mode to measure the information content and significance of changing this fifth base. I found that each instance of the motif was still highly significant when the fifth base was either A, C, or U, but non-significant when it was G as it would be for a PBE (FIGURE 4.3). Although, Pum1 and Pum2 recognize the same core motif (UGUAHAUA), several studies of the RNA binding affinities these two proteins have revealed that Pum2 is much more flexible in its nucleotide recognition at the eights base with a consensus motif of UGUAHAUW (Zhang et al., 2017). In the original de-novo motif discovery this base position was not addressed, I tested the contribution of this base to the mutual information content of the motif using FIRE and, found that the Pum2 motif is slightly more informative (FIGURE 4.4).

Altogether, this initial analysis revealed that an important mode of axonal mRNA localization control might be due to exclusion of specific classes of mRNAs. Moreover, it identified

**Figure 4.2: Motif discovery with DRIMust also identified the core of the PBE as significantly enriched in somatically retained mRNAs.** 3'UTR sequences of the top 100 somatically enriched mRNAs and the top 100 axonally enriched mRNAs were provided for comparison. Algorithm was run in the default mode allowing for motifs to be from 5 to 10 nucleotides in size and the minimum statistical threshold was set at 10^{-6}.
the PBE as a major motif contributing to this process by being highly over-represented in somatically localized mRNAs and highly under-represented in axonally enriched mRNAs. Finally, the Pum2 motif might be slightly more informative than its close relative Pum1 since it is more flexible in its RNA binding preferences.

Figure 4.3: Testing the mutual information content by varying the fifth base of the motif demonstrated high specificity for the PBE. FIRE was run on ‘non-discovery mode’ in a neuronal genome-wide subcellular localization measurement dataset (Minis et al. 2014). The following motifs were tested: UGUA[ACU]AUA, UGUA[ACU]AUA, UGUA[ACU]AUA, UGUA[ACU]AUA, UGUA[ACU]AUA. Only motifs found to be significant are shown. Yellow intensity corresponds to the degree of enrichment of a motif while blue intensity refers to the degree of depletion of a motif. The left bins contain mRNAs that are relatively enriched in cell bodies, while the right bins contain mRNAs that are relatively enriched in axons. Each bin contains about 200 mRNAs.
4.2.2 The PBE is informative of neuronal mRNA localization across different neuronal cell types

Since sensory neurons are morphologically distinct in that they are bipolar, having a central a peripheral axon but no dendrite, I decided to test whether the PBE is also informative of mRNA localization in more morphologically complex CNS neurons. Recently, Taliaferro et al. (2016) sequenced the somatic and neuritic compartments of developing cortical neurons as well as differentiated brain tumor derived N2A cells to uncover that alternative last exons confer isoform specific localization of mRNAs into neurites. I applied FIRE to their dataset to test whether the Pum1 and Pum2 binding motifs were informative of subcellular mRNA localization in these cells. I found that across all cell types both motifs were significant (FIGURE 4.5). Like in sensory neurons the motifs were over-represented in the somatically localized mRNAs and under-represented in the neurotically enriched mRNAs. Interestingly, the Pum2 motif was much more informative than the Pum1 motif across all cell types, and for primary cortical neurons the Pum1 motif was borderline

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**Figure 4.4: Pum2 specific PBE variant is slightly more informative.** FIRE was run on ‘non-discovery mode’ in a neuronal genome-wide subcellular localization measurement dataset (Minis et al. 2014). Two variants of the PBE motifs were tested: UGUA[ACU]AU[AU] (Pum2 preferred) and UGUA[ACU]AUA (Pum1 and Pum2). Yellow intensity corresponds to the degree of enrichment of a motif while blue intensity refers to the degree of depletion of a motif. The left bins contain mRNAs that are relatively enriched in cell bodies, while the right bins contain mRNAs that are relatively enriched in axons. Each bin contains about 200 mRNAs.
insignificant ($Z<10$). This data suggests that there is an exclusion mechanism by which mRNAs containing a PBE are restricted from being localized in neurites of developing neurons.

**Figure 4.5: The PBE is associated with somatically enriched mRNAs across different cell types.** FIRE was run on ‘non-discovery mode’ in primary cortical neuron genome-wide subcellular localization measurement dataset (TOP) and Neuro2A neuroblastoma derived cell line (BOTTOM) (Taliaferro et al. 2015). The two variants of the PBE were tested, UGUA[ACU]AU[AU] (Pum2 preferred) and UGUA[ACU]AUA (Pum1 and Pum2). Yellow intensity corresponds to the degree of enrichment of a motif while blue intensity refers to the degree of depletion of a motif. The left bins contain mRNAs that are relatively enriched in cell bodies, while the right bins contain mRNAs that are relatively enriched in neurites. Each bin contains about 200 mRNAs.

**4.2.3. The PBE is associated with mRNAs that are translated in adult axons**

Two interesting questions arise from these in-vitro findings: How relevant is this exclusion mechanism in-vivo and, is it developmentally regulated? To answer these questions, I tested the mutual information content of the Pumilio motifs using FIRE on the Ribo-Seq data from
developing mouse retinal axon in-vivo across different developmental stages generated by Shigeoka et al. (2016). Although this dataset is enriched in translating mRNAs there is evidence suggesting that the local translatome and transcriptome are highly correlated (Gáspár and Ephrussi, 2017b; Zappulo et al., 2017b), moreover the Pumilio family of RNA binding protein are known to regulate both mRNA localization and translation in yeast (Quenault et al., 2011). When focusing the analysis on the mRNAs translated in axons at different developmental stages (E17.5, P0.5, P7.5, and Adult) I found the Pum2 motif to be highly informative of temporal axonal mRNA translation, specifically by being highly under-represented in mRNAs that are translated early (P0.5) and over-represented in mRNAs that are translated late (P7.5 and Adult) (FIGURE 4.6.A).

Since the strongest effects were observed at P0.5 and Adult and decided to focus on this stages for more detailed analysis. I performed FIRE analysis on the measured fold changes in the translatome between these two stages which supported the prior finding that mRNAs containing the Pum2 motif are translated in adult axons but not in early development and, revealed the strength of this effect across different groups of mRNAs (FIGURE 4.6.B). Interestingly, this finding fits well with an observation described in a later chapter which shows that Pum2 protein is expression in the nervous system is high during early development and declines at later stages (FIGURE 5.2). Both of these observations together suggest that high expression of Pum2 in early developing neuron contribute to restrictive localization and translation of mRNAs containing the PBE in axons.
4.2.4. The PBE is sufficient to decrease axonal mRNA localization and translation

Based on all the computational findings I hypothesized that the PBE is restrictive of mRNA localization and translation in axons of developing neurons. To test this hypothesis I created a reporter system to measure the contribution of the PBE to the localization and translation of an mRNA in axons. I did this by adding the Actb 3'UTR, a well known axonal targeting 3'UTR, to a Gfp expression construct, and then to this I added either two PBE (UGUAUAUA) at the beginning and end of the 3'UTR, or a mutated version of the PBE (UGUAGAUA) where the fifth base is the non-preferred by Pum1/2. By measuring Gfp mRNA levels in axons using qPCR, I found that the
PBE was sufficient to reduce the localization of this transcript while the mutated PBE version did not (FIGURE 4.7). To assess the localized translation of this reporters in axons, I applied the Puro-PLA as described in Methods and by (tom Dieck et al., 2015). Similarly, I found that the presence of the PBE decreased the translation of Gfp in axons while the mutated version of the PBE did not compared to control (FIGURE 4.8).

**Figure 4.7: The PBE is sufficient to restrict axonal localization of a reporter mRNA.** Primary sensory neurons grown in microfluidic devices were infected with lentiviruses containing a Gfp reporter construct with the β-Actin 3’UTR with or without the PBE. Gfp mRNA expression levels was measured in axons using qPCR and normalized to the condition without the PBE (n=3 biological replicates). **p < 0.01.**
Figure 4.8: The PBE decreases axonal protein synthesis of reporter mRNA. Schematic of Puro-PLA technique principal to detect newly synthesized proteins (TOP). Primary sensory neurons grown in microfluidic devices were infected with lentiviruses containing a Gfp reporter construct with the β-Actin 3'UTR with or without the PBE. Axonal Gfp mRNA translation for 10 minutes was measured by using a Puro-PLA protocol (described in details in methods) (n=3 biological replicates). ** p < 0.01, *** p < 0.001.
To verify that this motif indeed binds to Pum2 I co-expressed Pum2 and each of the reporter constructs in HEK293T cells and carried and RIP with qPCR. I found that Pum2 was able to pulldown the PBE containing construct with much higher affinity (FIGURE 4.9). Altogether, these findings support the hypothesis that the PBE, possibly due to Pum2 regulation, is sufficient to decrease mRNA localization and translation in axons.

![Gfp mRNA Pulldown](image)

Figure 4.9: Pum2 protein associates with reporter mRNA containing the PBE. HEK293t cells were co-transfected with Pum2 and reporter mRNA with or without the PBE. Pum2 was immunoprecipitated and pulled-down reporter mRNA was measured using qPCR (n=3 biological replicates. ** p < 0.01.)
4.3. Interpretation

In the search for “zipcodes” that regulate axonal mRNA localization, I expected to find a positive link between the presence of these “zipcodes” and the localization of an mRNA to axons in accordance to the prevailing view that RNA localization is actively regulated in this manner by RNA binding proteins (Eliscovich and Singer, 2017; Eliscovich et al., 2013). The finding that all of the neuronal mRNA localization motifs discovered are negatively associated with axonal mRNA localization was unexpected. This suggests that a major mechanism regulating axonal mRNA sorting is through the exclusion of mRNAs containing specific motifs. Such a gating mechanism has several advantages and important implications for our understanding of mRNA localization in the developing neurons. First, it provides a molecular selectivity filter at an early time point where neuronal polarity and other physical barriers, such as the axon initial segment, are still actively developing. Secondly, it greatly increases the specificity for the cohort of mRNAs that are localized to axons by adding an extra criterion for their inclusion. Altogether, this mechanism provides a much safer system for the localization and translation of specific mRNAs in developing axons where resources, including the translational machinery, are limited, and where small errors could have a catastrophic impact on the system as a whole.

Although several motifs were initially identified, the PBE was chosen for further analysis not only because it was the most informative, but because it is associated with the well-known PUF family of RNA binding proteins. The initial computational analysis strengthened this finding by demonstrating a high specificity in the nucleotide composition of the motif for the base affinities of Pum2, which was later demonstrated to bind to a reporter containing this motif. Interestingly, the PBE was also highly informative of neurite mRNA localization across other cell types suggesting that it might be universal during nervous system development. Looking at the axonal translatome
across different developmental stages revealed that PBE containing mRNAs are translated in mature and adult axons but not early developing axons. This supports the view that axonal mRNA localization and translation is dynamic and spatiotemporally regulated, and supports a role for the PBE in restricting axonal mRNA localization and translation in early development. Finally, the introduction of the PBE in an otherwise axonal localizing 3’UTR was sufficient to significantly reduce the localization and translation of an mRNA.

There has been other attempts to identify localization motifs using genome-wide datasets mainly from the authors that generated them (Minis et al., 2014; Shigeoka et al., 2016b; Taliaferro et al., 2016; Zappulo et al., 2017b). These analyses ultimately did not result in the discovery or characterization of novel localization elements or their trans-acting partners. There are several reasons for this shortcoming. One, they did not narrow the motif search to 3’UTR regions. While regulatory elements can also be found in the 5’UTR and even the CDS, the majority are predominantly present in the 3’UTR. Including all these regions in a single analysis increases the background noise and does not take advantage of the distinct evolutionary and regulatory pressure to which they are subject. Second, they did not use algorithms that incorporate genome-wide measurements in the analysis. Rather, they relied on algorithms that search for motif enrichment in selected sequences compared to background sequences. While this approach is widely successful for the identification of regulatory elements associated with discrete processes, such as binding preferences for RBPs or transcription factors, it lacks sensitivity for the identification of regulatory elements involved in biological processes that fall in a continuous spectrum, such as mRNA stability and localization.

For these reasons, I decided to use a distinct computational approach that incorporated the information from the genome-wide mRNA localization measurements to identify motifs that are informative of axonal mRNA localization. I found such approach in FIRE which relies on the
concepts of mutual information to measure the relationship between gene expression and the presence or absence of regulatory motifs without making assumptions about the underlying relationships or structure of the data (Elemento et al., 2007). One great advantage of this approach is that it increases sensitivity and reduces the likelihood of false positives. The success of this approach is evident by the findings described above. Yet, there are some limitations that if addressed will further expand the potential for new discoveries. FIRE, utilizes a motif optimization step by which a motif seed is gradually changed until it reaches a motif with the highest possible information content. This generally results in motifs that are more similar to predicted binding elements derived from biological experiments while eliminating redundant intermediate motifs (Elemento et al., 2007). Unfortunately, there might be instances where the motif optimization process might lead to the loss of an otherwise distinct and biologically significant motif than the final product. Additionally, there might be cases where the optimized motif while being the most computationally informative it might not represent an element found in biology. A parameter in the program can be modified to alter the threshold by which a seed motif and a similar but distinct motif should be optimized into one (minr). Making this parameter very stringent leads to identification of few and highly optimized motifs, while the opposite increases the overall number of motifs. Therefore, in order to maximize sensitivity and discovery a first approach should be to apply this program with no motif optimization (minr=0). Additionally, to increase the likelihood of identifying biologically relevant motifs the program should be run in parallel on non-discovery mode to test the information content of already known RBP and miRNA motifs. Altogether, the combination of these two approaches should maximize the precision and sensitivity of the motif identification process.
Chapter 5. Pum2 negatively regulates axonal mRNA localization and translation

5.1. Rationale and Summary

The PUF (Pumilio and FBF) family of RNA-binding proteins regulates mRNA expression and localization of specific target mRNAs containing the highly conserved PBE. The two mammalian homologues, Pum1 and Pum2, have been studied in the context of translational repressors with important roles in diverse processes. Their role in mRNA localization has been less studied, but there is some evidence that at least Pum2 is involved in RNA granule formation and perhaps indirectly on mRNA localization. In progenitor cortical radial glial cells, Pum2 granules are apically localized to restrict the localization and expression of specific mRNAs which is required for self-renewal and to prevent premature neuronal differentiation. Additionally, Pum2 has been shown to form dendritically localized ribonucleoprotein particles and stress granules that are involved in regulating proper formation of dendritic spines and synaptic plasticity.

In the following studies, I decided to better characterize the subcellular as well the developmental expression of Pum1 and Pum2 in nerve cells. I then studied the role of Pum2 in axonal mRNA localization and translation. Finally, I established that Pum2 is responsible for restricting the localization and translation of PBE containing mRNAs in the somatic compartment of developing neurons.

5.2. Results

5.2.1. Pum1/2 are differentially expressed

Since Pum1 and Pum2 share a similar homology RNA binding domain and potentially recognize similar targets I decided to better characterize their subcellular localization in neurons as
well as their temporal expression in the mammalian brain. I found a striking difference between these two homologues. First, Pum2 is strictly localized to the somatic compartment of early developing embryonic primary neurons, while Pum1 is enriched in neurites and more specifically in axons (FIGURE 5.1).

Looking at different stages of mouse cortical development, I found that Pum2 protein expression peaked during the embryonic stage and progressively declined during development, while Pum1 expression briefly peaked postnatally before declining (FIGURE 5.2). This data is consistent with a recent finding by Zhang et al. (2017) that Pum1/2 are highly expressed during neurogenesis where they have a regulatory role. The subcellular expression of Pum2 and its developmental expression decline is consistent with my prior finding that PBE containing mRNAs are somatically localized and that their translation in axons occurs at the adult stage when Pum2 levels are down. Therefore, further experiments in this chapter aim at characterizing the function of Pum2 in regulating mRNA localization and translation in axons.
Figure 5.1: Pum1/2 are distinctly localized in neurons. DIV2 primary sensory (TOP) or cortical neurons (BOTTOM) were immunostained with α-Pum1 (green) or α-Pum2 (green) and α-βIII Tubulin (red).

**Sensory neurons**

![Image of sensory neuron immunostaining](image)

**Cortical neurons**

![Image of cortical neuron immunostaining](image)
5.2.2. RNA-Seq of Sensory Neurons in microfluidic devices resembles prior transcriptomes

To completely determine the regulatory role of Pum2 in neuronal mRNA localization, I measured the somatic and axonal transcriptomes of developing embryonic rat DRG neurons cultured in microfluidic chambers. By comparing the top expressed genes in my newly generated axonal transcriptome to the previously published mouse axonal transcriptome (Minis et al., 2014) and found that over 70% of my transcriptome overlapped. Gene Ontology analysis was distinct between axonal and somatically enriched transcripts with developmental programs being more prominent in the cell bodies and translation and mitochondrial processes in axons. Interestingly, somatically enriched categories coincide with enriched categories in PBE containing mRNAs (see Figure 6.1).

Figure 5.2: Pum1/2 expression peaks at different times during cortical development. Mouse brain cortical lysates from different developmental stages were immunoblotted for Pum1/2 and quantified relative to βIII Tubulin.
Figure 5.3: RNA-Seq of rat sensory neurons revealed similar class of mRNAs enriched in axons as previously identified. Venn diagram representing the overlap between the third quartile of mRNAs measured in axons from rat sensory neurons (this work) and mouse sensory neurons (Minis et al. 2014) (TOP). Gene ontology enrichment analysis results from DAVID on the set of mRNAs enriched in either cell bodies or axons as identified by the transcriptome (BOTTOM).
I wanted to test whether the PBE is informative of neuronal mRNA localization under control conditions in this newly obtained transcriptome and, whether it follows the same pattern that I previously observed in other transcriptomes. I used FIRE in non-discovery mode and found that indeed the Pum2 motif was informative of neuronal mRNA localization, more specifically it was over-represented in somatically localized mRNAs and under-represented in axonal mRNAs as previously observed in other transcriptomes (FIGURE 5.4). Thus, this finding underscores the importance of the PBE motif as a regulator of neuronal mRNA localization and validates our model system to test whether this regulation is mediated by Pum2.

**Figure 5.4: The PBE is also restrictive of mRNA localization in the newly produced subcellular transcriptome of sensory neurons.** FIRE was run on ‘non-discovery mode’ to test the information content of the PBE on the genome wide subcellular mRNA measurements obtained from sequencing rat sensory neurons growing in microfluidic devices. Yellow intensity corresponds to the degree of enrichment of a motif while blue intensity refers to the degree of depletion of a motif. The left bins contain mRNAs that are relatively enriched in cell bodies, while the right bins contain mRNAs that are relatively enriched in axons. Each bin contains about 200 mRNAs.
5.2.3. Pum2 selectively changes axonal transcriptome composition without altering overall neuronal mRNA levels.

Since it has been reported that Pum1/2 can regulate mRNA stability I first wanted to check whether knocking down Pum2 would result in major changes of the whole neuronal transcriptome. Using differential gene expression analysis on the somatic transcriptomes, I found that knocking down Pum2 did not result in any significant changes in overall mRNA expression (FIGURE 5.6). The data also shows that the hairpin shRNA was highly specific for Pum2 (FIGURE 5.6). On the contrary, when I applied differential gene expression analysis to the axonal transcriptomes, I found that Pum2 knockdown resulted in a great number of differentially expressed genes (FIGURE 5.7). Altogether, these findings suggest that Pum2 regulates axonal mRNA content without regulating mRNA stability in neuronal cell bodies.
Figure 5.5: Knock down of Pum2 did not cause major changes in the whole neuronal transcriptome. DIV7 sensory rat neurons grown in microfluidic chambers and treated with either shControl or shPumilio2 were subjected to RNA-Seq. MA Plot from DESeq2 differential expression analysis in neuronal cell bodies between the two conditions (TOP). Table showing the top changed mRNAs and whether they contain a PBE (BOTTOM).
Figure 5.6: Knock down of Pum2 resulted in major changes to the axonal transcriptome. DIV7 sensory rat neurons grown in microfluidic chambers and treated with either shControl or shPumilio2 were subjected to RNA-Seq. MA Plot from DESeq2 differential expression analysis in axons between the two conditions (TOP). Table showing the top 10 increased or decreased genes and whether they contain a PBE.
5.2.4. mRNA localization of PBE containing mRNAs in axons is increased by Pum2 knockdown.

Next, I tested the specificity of the axonal transcriptome changes upon Pum2 knockdown. Specifically, I analyzed the mutual information content of the PBE across axonal mRNA expression of shControl vs shPum2. I found that the PBE was highly under-represented in a group of mRNAs whose expression did not change across conditions, while it was over-represented in mRNAs that were enriched in axons after the knockdown (FIGURE 5.7A). I also tested this effect by directly comparing the group of mRNAs that were upregulated in axons after Pum2 knockdown to the group of mRNAs that were unchanged using FIRE in binary mode. Similarly, I observed a strong over-representation of the PBE in the set of mRNAs that were localized to axon upon knockdown (FIGURE 5.7B).

I found that the PBE is still highly informative of neuronal mRNA localization when using embryonic DRG neurons grown in microfluidic chambers, thus supporting the use of this system for current and future work on this topic, as well as supporting the main hypothesis that the PBE restricts mRNAs from localizing in axons of developing neurons. Moreover, knocking down Pum2 did not have any effects in overall neuronal mRNA stability while it resulted in a major remodeling of the axonal translatome. Specifically, Pum2 knockdown resulted in an increase of PBE containing mRNAs into axons.
5.2.5. **Pum2 increases overall translation levels in axons as well as translation of a specific target mRNA.**

Since Pum2 downregulations lead to an increase of PBE containing mRNAs in axons, I hypothesize that overall axonal translation might be affected due to their presence. I measured axonal translation capacity under naïve conditions in sensory neuron axons by measuring the...
amount of Puromycin incorporation during 10 minutes. I found that knocking down Pum2 leads to about a 30% increase in total axonal translation (Figure 5.8). To test whether this effect was at least partly due to contributions of PBE containing mRNAs that were increased in axons I carried out a Puro-PLA assay for one of the target mRNAs, Gsk3β. This mRNA was increased 3 fold in axons upon Pum2 knockdown and was chosen for downstream analysis because of its known involvement in regulating several of the neurodevelopmental processes that seem to be associated with Pum2 function. Puro-PLA analysis revealed a significant increase in the axonal translation of Gsk3β upon Pum2 knockdown.
Figure 5.8: Pum2 downregulation increases overall axonal translation. Axons of DIV7 rat sensory neurons treated with shControl or shPum2 were labeled with puromycin for 10 mins to detect newly synthesized proteins and immunostained with α-Puromycin (red) and α-βIII Tubulin (green) (n=3 biological replicates). ** p < 0.01.
5.3. Interpretation

Since Pum1 and Pum2 have an almost identical homology RNA binding domain they are often considered to have similar function and to be redundant and highly interchangeable. Yet,

**Figure 5.9: Pum2 knockdown increases translation of Gsk3\(\beta\) in axons.** Axonal Gsk3\(\beta\) translation was measured by using a Puro-PLA protocol (described in details in methods) (n=3 biological replicates). Axons were immunostained with \(\alpha\)-\(\beta\)III Tubulin (green) and Puro-PLA signal (red). * \(p < 0.05\).

5.3. Interpretation

Since Pum1 and Pum2 have an almost identical homology RNA binding domain they are often considered to have similar function and to be redundant and highly interchangeable. Yet,
Several studies have reported specific biological dysfunction when either one is knockdown alone and, embryonic lethality when both are knockdown at the same time (Zhang et al., 2017). These findings suggest that while they may complement each other and even compensate for the absence or dysfunction of the other, their functions might not completely overlap. Surprisingly, I found the subcellular localization in neurons of Pum1 and Pum2 to be strikingly different. Pum2 was highly restricted to the cell body compartment, while Pum1 was enriched in axons. This subcellular expression pattern for Pum2 had previously been reported (Vessey et al., 2006) and, Pum1 had been found to be expressed pre-synaptically and Pum2 post-synaptically (Marrero et al., 2011). Moreover, I also found these two proteins to be differentially expressed during cortical development. Specifically, Pum2 expression peaks at the embryonic stage and then gradually declines during development. This finding has been confirmed recently in the mouse nervous system (Zhang et al., 2017). Importantly, Pum2 subcellular and developmental expression patterns matches my previous findings regarding the PBE.

Several studies have now identified a similar cohort of axonally localized mRNAs. Similarly, I found that over 70% of the mRNAs localized in my RNA-Seq dataset overlap with prior described axonal transcriptome (Minis et al., 2014). This is very significant when taking into account that I utilized a different animal species, neuronal culturing method, and timing of axonal RNA extraction, suggesting a common underlying regulatory mechanism. Moreover, testing the significance of the PBE in neuronal mRNA localization on the naïve transcriptome produced the same result as previously obtained in the analysis of the Minis et al. (2014) dataset, providing more evidence for robust mRNA localization regulation mediated by the PBE.

Until now, I had hypothesized that Pum2 could act at least through two mechanisms to account for the exclusion of PBE containing mRNAs from axons. First, Pum2 could act by lowering the mRNA stability and therefore half-life of target mRNAs resulting in their degradation before
reaching the axon. Such mechanism, had actually been proposed for Drosophila Pumilio (Burow et al., 2015) where the PBE was associated with mRNA decay and enriched in neurogenesis regulating mRNAs and decreased in neurite localized mRNAs. The finding that mammalian Pum1/2 regulate neurogenesis was reproduced in the mouse nervous system (Zhang et al., 2017). But, unlike in Drosophila, Pumilio proteins did not affect mRNA stability and rather acted by regulating translation of target mRNAs. Similarly, I found that Pum2 did not regulate mRNA levels in neurons since knocking it down did not produce significant changes in the whole neuronal transcriptome. Therefore, it seems less likely that in mammals Pum2 regulates mRNA localization by regulating mRNA stability. Importantly, Pum2 knockdown led to a significant increase in PBE containing mRNAs in axons suggesting a specific function for Pum2 as a neuronal mRNA localization regulator by somatic retention of target mRNAs. Mislocalized PBE containing mRNAs may then have caused an overall increase in axonal translation output as demonstrated by an increase in total Puromycin incorporation and specifically Gsk3β translation.
Chapter 6. Pum2 dysfunction results in impaired axon growth, branching, and regeneration

6.1. Rationale and Summary

Pumilio proteins have been implicated in nervous system development. They have been shown to be important for dendritic morphogenesis, synaptic spine formation, neurogenesis, and protein toxicity induced neurodegeneration. Yet, the individual contributions and roles of each mammalian Pumilio homologue to neuron development has not been specifically addressed. Given my findings that Pum2 regulates axonal mRNA composition and translation in developing neurons, I hypothesized a role for Pum2 in axonal development. To address this experimentally, I decided to use in-utero electroporation to knockdown Pum2 in embryonic mice brains. The advantage of this technique over simply using a knockout mouse line is that allows for better temporal and spatial control of Pum2 knockdown. In collaboration with Dan Iascone, a graduate student in Franck’s Polleux laboratory at Columbia University, we observed that Pum2 is required for proper axonal extension and branching.

Axonal translation in response to nerve injury is important for axonal regeneration. Since, Pum2 knockdown alters the composition of axonal mRNAs, I expect axons to respond differently to axonal injury. I decided to use this system to study both the role of Pum2 and of axonal mRNA localization and translation in regulating axon regeneration. I found that indeed axon regeneration is impaired when Pum2 is knockdown and that this might be due to the improper localization and translation of mRNAs that negatively affect axon growth.
6.2. Results

6.2.1. Gene Ontology analysis of PBE containing mRNAs suggests a neurodevelopmental role for Pum2

To gain biological insight as to the function of PBE containing mRNAs I ran a Gene Ontology analysis on the list of these mRNAs that are both expressed in the mouse and rat DRG neuron transcriptomes. The top overall biological functions as well are listed (FIGURE 6.1). Of particular interest to the context in which we are studying the PBE motif are: mRNA processing, nervous system development, dendrite morphogenesis, neuron projection development, axogenesis, axon extension, and axon guidance. Many of these categories are shared between both transcriptomes. Moreover, they also overlap with the enriched categories of somatically enriched mRNAs previously identified suggesting that during early neuronal development.
GO Category Enrichments in PBE containing mRNAs

Mouse sensory neurons transcriptome (from Minis et al. 2015)

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Rat sensory neurons transcriptome (from this thesis)

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<td>RT</td>
<td>33</td>
<td>4.8</td>
<td>3.11-5</td>
<td>4.34E-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GOMERM_BP_DIRECT</td>
<td>multicellular organism development</td>
<td>RT</td>
<td>17</td>
<td>3.0</td>
<td>4.00-4</td>
<td>7.45E-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GOMERM_BP_DIRECT</td>
<td>forebrain development</td>
<td>RT</td>
<td>11</td>
<td>1.6</td>
<td>6.09-4</td>
<td>2.28E-1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.1: PBE containing mRNAs are enriched in nervous system development categories. PBE containing mRNAs were identified with FIRE on developing mouse and rat sensory neurons transcriptomes previously mentioned. DAVID gene ontology website was used to assess the enrichment in Gene Ontologies associated with these class of mRNAs.
6.2.2. Downregulation of Pum2 in-vivo results in impaired contralateral axon outgrowth and branching.

I decided to study the consequences of disrupting the Pum2 regulation of PBE containing mRNAs localization during neuronal development in order to better understand the biological function of this regulatory process. We used an established in-utero electroporation protocol to knockdown Pum2 in a cortical hemisphere of a developing mouse at an early stage (E15). We found a significant reduction in contralateral axonal crossing at P5 in the shPum2 condition (FIGURE 6.2). This suggests that Pum2 is required for during early axon extension. We then looked at the axonal projections to the contralateral hemisphere at P21 and found that knocking down Pum2 significantly impaired axonal targeting to the upper layers (FIGURE 6.3). Thus, Pum2 is required for proper axon targeting and or branching during neuronal development.
Figure 6.2: Pum2 knockdown results in decreased contralateral axon outgrowth. E15 mouse brains were electroporated with shControl or shPumilio2 on a single hemisphere. Contralateral axon extension was measured at P5. * p < 0.05.
Figure 6.3: Pum2 knockdown results in decreased contralateral axon branching. E15 mouse brains were electroporated with shControl or shPumilio2 on a single hemisphere. Contralateral axon extension was measured at P21. * p < 0.05, ** p < 0.01.
6.2.4. Pum2 disruption results in impaired axon regeneration after injury

Axon mRNA localization and translation are tightly regulated in response to nerve injury to establish an appropriate axon regeneration program. Since my prior findings indicate that mRNA localization and translation is regulated by Pum2 I decided to test whether disrupting this pathway would influence axon regeneration. Axons of sensory rat neurons grown in microfluidic devices were injured by aspiration with a pipette in the axonal compartment. Axon outgrowth after 24 h under shControl and shPum2 conditions was measured revealing a significant decrease in regeneration upon Pum2 knockdown.
Figure 6.4: **Pum2 knockdown results in axon regeneration in-vitro.** Axons from DIV5 rat sensory neurons grown in microfluidic devices were injured by aspiration. Axon regrowth was measured after 24 hours. * p < 0.05.
6.3. Interpretation

I conducted a gene ontology enrichment analysis for PBE containing mRNAs in sensory neurons which revealed ‘Neuron projection development’ as one of the most significant categories. A recent study that conducted CLIP-Seq of Pum1/2 in developing neurons also found this category as the second most significant in neuronal mRNA targets of Pum2 but not Pum1 (Zhang et al., 2017). Together, with my finding that Pum2 controls axonal mRNA localization and translation there was a good indication to study the role of Pum2 in regulating axonal development. Indeed, I discovered that Pum2 is required for proper axon development in mouse neurons in-vivo. By knocking down Pum2 in the developing cortex in a single brain hemisphere I found that axon growth and branching to the contralateral side was impaired. That same study by Zhang et al. (2017) had found that Pum2 was required for neurogenesis and therefore many of the developmental effects that they observed were in part due to grow morphological defects during brain development. My approach to study the role of Pum2 in axon development offers some advantages due to a high spatiotemporal control of Pum2 knockdown which only occurs after neuron and axon specification and is restricted to one brain hemisphere. Therefore, increasing the confidence that any resulting phenotype is likely due to cell-autonomous dysfunction in developing axons that were already formed.

For an axon to find the appropriate target in the contralateral hemisphere it must integrate complex signaling pathways that start by navigating through gradients of guidance cues which in turn drive responses depending on the fine spatiotemporal control of the expression of surface receptors. Local protein synthesis as well as degradation have been shown to be required during this process. One potential explanation then for the defects in contralateral outgrowth from knocking down Pum2 is due to dysregulation of axonal mRNA localization and translation. The overall effects observed were modest, it would be interesting to test whether Pum2 dysfunction equally and
gradually interferes with contralateral axon outgrowth across a broad class of cortical neurons or whether it has an ‘all or nothing’ effect on a specific subclass. Axon branching and arborization similarly requires the complex integration of signals and evidence suggests that localized translation plays a role its regulation. Specifically, mitochondrial function and cytoskeletal remodeling are modulated by local protein synthesis to respond to control branching.

One major limitation of my approach is that currently is technically unfeasible or highly difficult to test the localized protein synthesis dependence of Pum2 mediated regulation of axon outgrowth and branching in-vivo. One interesting regulator of axon branching is Gsk3\(\beta\), whose mRNA localization and translation is increased in axons upon Pum2 downregulation. Therefore, whether Pum2 exerts its function through this pathway could be tested by modulating the PI3K-AKT-GSK3 signaling axis. Additionally, another signaling pathway that I found upregulated in axons upon Pum2 downregulation, and that we are currently studying, is MAPK signaling. Small changes in the localization and translation of these enzymes can easily lead to big downstream effects given how these enzymes are capable of signal amplification.

Finally, axon regeneration after injury shares many of the signaling pathways and processes of developing axons. Specifically, Gsk3 signaling has been shown to modulate intrinsic axon growth capacity during regeneration. Moreover, axon injury induces specific recruitment of mRNAs which could be disrupted by Pum2 downregulation. Thus, it would be interesting to test how the axonal transcriptome in response to injury is affected by Pum2. The current experimental setup of carrying axon injury in microfluidic devices would also allow for the testing of local protein synthesis requirement for successful regeneration.
Chapter 7. Research Highlights: Significance and Future Directions

This dissertation provides a conceptual advance in our understanding of neuronal mRNA localization and translation regulation. It highlights a novel filtering mechanism by which mRNAs containing a specific signal are excluded from axonal localization and translation during early neuronal development. It then demonstrates the importance of this regulatory process to achieve proper contralateral axon outgrowth and branching as well as during axon regeneration in response to nerve injury. This chapter outlines the major research highlights, their significance, and future directions.

7.1. mRNA localization signals restrict axonal localization.

Significance:

A critical gap in our understanding of subcellular mRNA localization in neurons results from not knowing the extent to which localization elements globally contribute to the specificity and identity of localized mRNAs. In the case of axonal and dendritic mRNA localization, research has mostly focused on the role of localization elements in targeting a few mRNAs to their respective compartments. Such ‘positive’ localization elements have been the focus of much research although there has been difficulty in expanding and characterizing them more broadly. On the contrary, whether there exist ‘negative’ localization elements, opposing or restricting the axonal localization of an mRNA, has not been addressed. In my thesis work, I identified the PBE as a retention element that strongly restricts localization of mRNAs to the soma. This is the first time that an exclusion mechanism in the regulation of neuronal mRNA localization is reported. Although I focus on the PBE, my analysis reveals a broader pattern of regulated mRNA localization by retention since no ‘positive’ localization element was found across several transcriptomes. The extent to which the PBE repressed the translation of specific mRNAs in axons changed during development suggesting
a mechanism for stage-dependent temporal control of axonal translation. Altogether, the novel finding that the PBE is restrictive of axonal mRNA localization provides new insights into the mechanisms that regulate mRNA targeting in neurons and consequently, opens additional avenues for therapeutic targets as well as technical applications to the study and control of mRNA localization.

Future Directions:

A striking finding from my computational analysis is the absence of ‘positive’ localizing elements. While axonal mRNA localization might be strongly regulated through the exclusion of specific mRNAs, it is important to test whether current limitations in my computational analysis resulted in low sensitivity for the identification of other regulatory elements. Specifically, the search for regulatory elements in 3'UTRs is handicapped by the current status of their annotations. Recently, applying 3’end RNA-Seq to somatic and axonal mRNAs revealed that axonally localized mRNAs contain much longer 3'UTRs (Andreassi et al., 2017). Moreover, newly generated 3'UTR annotations from this study demonstrated that current annotations fall short of including whole 3'UTRs. Importantly, they found localizing elements in mRNA isoforms containing the longer 3'UTRs. Finally, this study as well as others (Taliaferro et al., 2016) have shown isoform specific 3'UTRs to be exclusively localized in axons. Incorporating improved 3'UTRs annotations with isoform specific expression utilizing more in-depth RNA-Seq will increase the likelihood of finding ‘positive’ localizing elements.

In addition to linear sequence motifs, RNA structure is known to control the binding of trans-acting regulatory factors. Much less is known about the contribution of structural RNA motifs to mRNA localization in neurons. Utilizing computational algorithms to interrogate the contributions of structural RNA motifs to the genome-wide measurements of subcellular RNA localization would not only extend the number and type of localization elements but might result in
the discovery of novel trans-acting regulators. One such algorithm, Tool for Eliciting Informative Structural Elements in RNA (TEISER), has been successfully applied to discover structural elements and their associated trans-acting factors controlling transcript stability (Goodarzi et al., 2012, 2014) as well as in genome-wide post-transcriptional regulation (Oikonomou et al., 2014). Experimentally, newly identified structural, regulatory motifs can be used as “bait” to purify neuronal and axonal RNPs (Doron-Mandel et al., 2016). Altogether, a comprehensive characterization of the cis and trans-acting elements that regulate mRNA localization in neurons would allow researchers to predict the localization of specific mRNAs in a given system.

PBE mediated somatic mRNA retention can be applied to study the role of axonally produced proteins in vivo. Currently, the best available tool to demonstrate the need and the function of localized translation is to apply RNAi locally and acutely. Unfortunately, this technique has several drawbacks and is more challenging to implement in vivo. Two alternate and complementary approaches to restrict the localization of specific mRNAs in neurons could be developed by either genetically adding PBEs to a 3'UTR of interest or by generating modular PUM binding domains that recognize specific sequence elements. Tightly controlling the induction and components of such system would be necessary, but its successful implementation could lead to insights as to the function of axonally synthesized proteins in vivo.

In the last decade, many discoveries have highlighted the importance of non-coding RNAs in regulating multiple biological processes. The majority of studies of subcellular RNA localization in neurons have only focused on mRNAs. A comprehensive understanding of RNA localization will be incomplete without the inclusion of this abundant class of RNAs. It is not just essential to answer how their localization is regulated, but also how they might control mRNA localization as well. ncRNAs contain regulatory elements that can act as "sponges" by sequestering RBPs thus lowering their effective availability to target mRNAs. For example, the ncRNA NOR-4D contains multiple
PBEs through which it sequesters Pumilio proteins to regulate genomic stability (Lee et al., 2016).

Finally, circular RNAs are an emerging subclass of ncRNAs that seem highly abundant and functional in the nervous system. There is already evidence that circRNAs are differentially localized in neuronal processes, but a genome-wide study of their subcellular localization in neurons is still needed (You et al., 2015).

7.2. Pum2 restricts axonal mRNA localization of PBE containing mRNAs.

Significance:

Studies of axonal mRNA localization have focused on the role of RBPs in positively targeting mRNAs into axons. It has been hypothesized that perhaps a gating mechanism exists that restricts the localization of mRNAs into axons (Jung et al., 2012). Here, I present the evidence for such a mechanism for the first time. As discussed earlier, mRNA retention can serve as a passive filter reducing stochastic errors in mRNA localization at a low energy cost for the cell. Moreover, I identified a novel role for Pum2 as a regulator of mRNA localization in neurons. In agreement with a recent study (Zhang et al., 2017) I found that Pum2 does not globally affect mRNA levels in neurons. This suggests that Pum2 mediated mRNA localization regulation is likely due to its exclusive expression in the somatic compartment compared to axons and not due to the control of mRNA stability as proposed in other systems (Burow et al., 2015).

Future Directions:

I chose to focus on Pum2 since its expression and subcellular localization, as well as its slightly more flexible binding preferences, matched my computational findings better than Pum1. But, both RBPs are potentially able to bind the target mRNAs, and other studies have shown a considerable overlap between the two. It would be then essential to test whether Pum1 also contributes to restricting axonal mRNA localization or whether it has the opposite effect by
competing with Pum2. Since Pum1 is highly enriched in axons, unlike Pum2, it would have to decrease transcript stability or promote their degradation to restrict their expression in axons. Additionally, there is the question of how Pum1/2 mechanistically regulate mRNA localization. To address this issue, experiments to characterize the composition of neuronal Pum1/2 mRNPs by using high throughput techniques to sequence both the protein and mRNA components should be carried out. The findings from such experiments might also help explain how Pum1 and Pum2 subcellular localization is being regulated.

7.3. Pum2 is required for proper axon development and regeneration.

Significance:

There is extensive evidence that the axonal localization and translation of specific mRNAs is required for proper axon development and maintenance. Here, I demonstrate more broadly the importance of adequately regulating axonal mRNA localization and translation globally by interfering with Pum2 function. I found a novel role for Pum2 in regulating contralateral axon extension and branching during cortical development presumably due to disruption of localized translation. Moreover, disrupting the axonal transcriptome by knocking down Pum2 impaired axonal regeneration in response to injury. Altogether, these findings suggest that the axonal transcriptome/translatome has to be tightly regulated for proper axon development and regeneration. Shigeoka et al. (2017) found targets of mTORC1 to peak in axonal translation at P0.5, the stage of axon wiring. This is not surprising given that the mTORC1 is strongly linked to the regulation of axonal translation (Campbell and Holt, 2001) and axonal branching (Spillane et al., 2013). Interestingly, at this stage, I found the highest repression of Pum2 targets in axons. Knocking down Pum2 increased the mRNA levels in axons of mRNAs that encode for known inhibitors of mTOR signaling such as Gsk3β and members of the MAPK signaling family. Thus, Pum2
morphological effects on axon development and regeneration could be explained by it having a broader function in modulating mTOR activity.

Future Directions:

Several roles have been ascribed to Pum2 during nervous system development and maintenance, yet the specific mechanisms by which Pum2 exerts its functions have not been characterized in detail. Although Pum2 has been broadly described as a translational repressor, the specific mRNA targets and signaling pathways that it regulates during neurodevelopment have not been identified. In this thesis work, I provided a first step towards the goal of increasing our understanding of the downstream mechanisms by which Pum2 acts by identifying the cohort of mRNAs that are mislocalized in axons as a result of its dysfunction. Going forward, I propose to test the way in which these changes in the axonal transcriptome disrupts the signaling mechanisms that are required for proper axon outgrowth, branching, and regeneration. As mentioned above, mTOR regulated mRNA translation plays a vital role in these processes, and some of the mRNA targets that are upregulated in axons upon Pum2 knockdown are modulators of mTOR signaling. Thus, I propose to study in more detail how components of mTOR are changed after interfering with Pum2 function and in response to nerve injury. If these elements are indeed changed, then pharmacological or molecular tools should be used to restore them in an attempt to rescue the morphological phenotypes observed. Two specific mRNA targets that were identified in the transcriptome and that should be evaluated are Gsk3β and Mapk6 both of which are involved in the regulation of axon growth and regeneration under physiological conditions.

7.4. Closing Statement

Collectively, this dissertation demonstrates a role for Pum2 in the regulation of neuronal mRNA localization by the exclusion of target mRNAs from axons during development. This novel
molecular filter is important as its disruptions leads to axon growth and branching defects as well as impaired regeneration after injury. My research findings highlight the importance of regulating axonal mRNA localization and localized protein synthesis in the nervous system. Future work should focus on further characterizing the downstream signaling pathways that are involved in this process.
References


Appendix
Local synthesis of dynein cofactors matches retrograde transport to acutely changing demands

Joseph M. Villarin¹, Ethan P. McCurdy², José C. Martínez¹ & Ulrich Hengst³,⁴

Cytoplasmic dynein mediates retrograde transport in axons, but it is unknown how its transport characteristics are regulated to meet acutely changing demands. We find that stimulus-induced retrograde transport of different cargos requires the local synthesis of different dynein cofactors. Nerve growth factor (NGF)-induced transport of large vesicles requires local synthesis of Lis1, while smaller signalling endosomes require both Lis1 and p150Glued. Lis1 synthesis is also triggered by NGF withdrawal and required for the transport of a death signal. Association of Lis1 transcripts with the microtubule plus-end tracking protein APC is required for their translation in response to NGF stimulation but not for their axonal recruitment and translation upon NGF withdrawal. These studies reveal a critical role for local synthesis of dynein cofactors for the transport of specific cargos and identify association with RNA-binding proteins as a mechanism to establish functionally distinct pools of a single transcript species in axons.

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any cellular functions rely on the ordered transport of macromolecules, proteins and organelles. Most intracellular transport is an active process mediated by motor protein complexes that move their cargos along components of the cytoskeleton: myosins transport cargos along actin filaments, while microtubule-based transport is facilitated by two families of motors, the plus-end directed kinesins and minus-end directed dynein. In contrast to the great variety of myosins and kinesins, there is only a single cytoplasmic dynein, which is complemented by an array of regulatory proteins to fulfill different functions. These multifunctional proteins bind either to the non-catalytic domains of dynein or directly to its force-generating heavy chain, thereby changing the characteristics of the dynein motor. For example, Lis1 (gene: PAFAH1B1) induces a persistent force-producing state in microtubules-attached, moving dynein by acting as a clutch linking the ATPase and microtubules-binding domains. It maintains the microtubule-bound state of dynein and is required for moving large vesicles through a constraint environment with high drag forces such as kinked axons. Together with NudE, it enhances the sustained processivity of the dynein motor and facilitates its transport capabilities to changes in demand, as, for example, in response to NGF stimulation or withdrawal, we focused on Lis1 and p150

 Activation of protein synthesis had before only been described in response to NGF stimulation or withdrawal. Inhibition of mTOR with locally applied rapamycin completely abolished these changes. To directly visualize local protein synthesis in response to changes in NGF concentrations, we performed puromycylation assays. Puromycin is a transfer RNA mimetic that gets incorporated into nascent polypeptides and can be detected with specific antibodies. NGF withdrawal and stimulation significantly increased the number of puromycylation events in axons in a protein synthesis inhibitor-sensitive manner, confirming that local protein synthesis is activated by both NGF stimulation and depletion (Fig. 1f). Puromycylation in the cell bodies was not affected by changes in NGF concentration or the addition of protein synthesis inhibitors, as expected from the phosphorylation state of 4EBP1 (Fig. 1e). The ratio of phosphorylated 4EBP1, being the largest and most important. Among several described functions, dynactin acts by increasing the processivity of the dynein motor and facilitates its binding to different cargos. Importantly, dynactin and Lis1 competitively bind the same domain of dynein, suggesting a mutually exclusive regulation of dynein by these adaptors. Thus, cofactors or adaptor proteins such as dynactin or Lis1 regulate dynein-dependent transport, but it remains unknown how their association with dynein is controlled in a spatially precise and temporally acute manner in response to extracellular signals. This question is especially relevant in axons, where essentially all microtubules are unidirectionally oriented with their plus-ends facing the cellular periphery, and dynein is anterogradely transported as cargo by kinesins. A potential solution to this question is the on-demand, local synthesis of dynein cofactors within distal axons and growth cones.

Intra-axonal protein synthesis is crucial for axon development, maintenance, synapse formation and axo-somatic communication, as well as for axonal regeneration and neurodegeneration. From these studies, a picture emerges in which local protein synthesis provides short-lived and spatially precise bursts of locally translated proteins, to react to extracellular cues, injurious insults or other changes in an axon’s environment. Therefore, it is especially interesting that messenger RNAs (mRNAs) coding for dynein regulators, including Lis1 and p150

 Here we asked whether local synthesis of dynein regulators was a mechanism to acutely match the intra-axonal retrograde transport capabilities to changes in demand, as, for example, in response to changes in extracellular trophic support. We report that axonal synthesis of Lis1 and p150

 NGF stimulation or withdrawal affect Lis1 and p150

 mRNAs coding for regulators of cytoplasmic dynein have been found in several axonal transcriptomes. To investigate which proteins might be locally synthesized in response to NGF stimulation or withdrawal, we focused on Lis1 and p150

 To directly visualize their mRNAs, Pafah1b1 and Dctn1 within axons of DRG neurons, we used fluorescence in situ hybridization (FISH). Both mRNAs were readily detectable in nascent polypeptides and can be detected with specific antibodies. NGF withdrawal and stimulation significantly increased the number of puromycylation events in axons in a protein synthesis inhibitor-sensitive manner, confirming that local protein synthesis is activated by both NGF stimulation and depletion (Fig. 1f). Puromycylation in the cell bodies was not affected by changes in NGF concentration or the addition of protein synthesis inhibitors, as expected from the phosphorylation state of 4EBP1 (Fig. 1e). The ratio of phosphorylated 4EBP1, being the largest and most important. Among several described functions, dynactin acts by increasing the processivity of the dynein motor and facilitates its binding to different cargos. Importantly, dynactin and Lis1 competitively bind the same domain of dynein, suggesting a mutually exclusive regulation of dynein by these adaptors. Thus, cofactors or adaptor proteins such as dynactin or Lis1 regulate dynein-dependent transport, but it remains unknown how their association with dynein is controlled in a spatially precise and temporally acute manner in response to extracellular signals. This question is especially relevant in axons, where essentially all microtubules are unidirectionally oriented with their plus-ends facing the cellular periphery, and dynein is anterogradely transported as cargo by kinesins. A potential solution to this question is the on-demand, local synthesis of dynein cofactors within distal axons and growth cones.

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 To directly visualize their mRNAs, Pafah1b1 and Dctn1 within axons of DRG neurons, we used fluorescence in situ hybridization (FISH). Both mRNAs were readily detectable in a punctate pattern in axons and with significantly higher intensity than the one obtained with a Gfp control probe (Fig. 2b). Using quantitative immunofluorescence, we found that the axonal abundance of Lis1 protein was significantly increased upon both NGF stimulation and withdrawal for 10 min (Fig. 2c), while in contrast p150

 Levels of each protein were not changed.
by pre-incubation with protein synthesis inhibitors under baseline conditions, but the increases in abundance upon NGF stimulation (for both Lis1 and p150\textsuperscript{Glued}) or withdrawal (Lis1 only) were abolished by the application of anisomycin or emetine to the axonal compartment. Together, these data indicate that the axonally localized transcripts of Lis1 and p150\textsuperscript{Glued} might be translated in response to changes in NGF signalling.

NGF signalling controls local Lis1 and p150\textsuperscript{Glued} synthesis. To directly test whether changes in axonal NGF signalling trigger the local synthesis of Lis1 and p150\textsuperscript{Glued}, we selectively transfected axons with siRNAs targeting their mRNAs Pafah1b1 or Dctn1, respectively, or with a non-targeting control siRNA. We had validated the siRNAs by transfecting them into rat C6 glioma cells and immunoblotting whole-cell lysates for Lis1 and p150\textsuperscript{Glued}. 

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**Figure Legends**

**Figure a:** Schematic diagram showing the experimental setup.

**Figure b:** Percentage of LysoTracker+ particles at different NGF concentrations and treatments.

**Figure c:** Percentage of LysoTracker+ particles during NGF withdrawal at different NGF concentrations and treatments.

**Figure d:** Percentage of LysoTracker+ particles during NGF stimulation at different NGF concentrations and treatments.

**Figure e:** Western blot analysis of p4EBP1 and 4EBP1 expression under different NGF concentrations and treatments.

**Figure f:** Relative puromycin labelling puncta (per 100 µm) in axons under different NGF concentrations and treatments.

**Figure g:** Relative puromycin signal in cell bodies under different NGF concentrations and treatments.
Two siRNAs against each transcript were tested individually and showed consistent phenotypes; the siRNAs were most efficacious when used together (Supplementary Fig. 1). Previously, we have demonstrated that the RNA interference (RNAi) pathway is functional in developing axons32, and that it is possible to selectively knockdown an mRNA in axons by local siRNA transfection without causing a decrease of the transcript’s abundance in cell bodies17,18,23,35. We confirmed that the effects of the local siRNA transfections were indeed restricted to axons by quantitative immunofluorescence against Lis1 and p150Glued on cell bodies whose axons had been transfected with siRNAs. No decrease of either protein was detectable in the neuronal soma (Fig. 3a,b). In the siRNA-transfected axons, the protein levels were not significantly reduced at our baseline NGF concentration, again indicating that the mRNAs are not locally translated under this condition (Fig. 3c,d). Conversely, the significant increases in Lis1 abundance in response to NGF stimulation or withdrawal were completely abolished by local siRNA application, as was the increase in p150Glued levels in NGF-stimulated axons. Together, these results demonstrate that both transcripts can be locally translated in axons, but the intra-axonal synthesis of these two dynein cofactors is differentially regulated by changes in NGF signalling.

**Lis1 synthesis is required for NGF-induced vesicle transport.** To determine whether the local synthesis of Lis1 and p150Glued in response to changes in NGF concentration impacted retrograde transport in axons, we incubated axons with LysoTracker and scored the motility of labelled vesicles as before. Axon knockdown of Pafah1b1 or Dctn1 did not significantly affect retrograde transport in the baseline condition (Fig. 4a), in line with our finding that neither protein is locally synthesized under baseline conditions. Axon-specific knockdown of Pafah1b1 abolished the significant increase in the proportion of retrogradely moving vesicles caused by NGF deprivation (Fig. 4b; Supplementary Movie 6), and it caused a reduction of retrogradely moving LysoTracker-positive particles below the baseline levels in the NGF-stimulated condition (Fig. 4c; Supplementary Movie 7). In contrast, knockdown of axonal Dctn1 mRNA did not affect the movement of LysoTracker-positive vesicles upon either NGF stimulation or withdrawal. Together, these results demonstrate that locally synthesized Lis1 is required for induced retrograde movement of these LysoTracker-labelled cargos, but p150Glued is not. This observation is reminiscent of the finding that, globally, high load retrograde transport requires Lis1 (ref. 6).

**NGF–endosome transport requires Lis1 and p150Glued synthesis.** To investigate whether the requirement for local synthesis of dynein cofactor varied between different cargos, we next visualized the retrograde transport of NGF-signalling endosomes34. Upon binding of NGF to its receptor TrkA, the receptor–ligand complex is internalized, and the resulting endosome is transported with downstream effector complexes to the soma by a dynein–dynactin complex35. Mouse 2.5S NGF was linked to red fluorescent quantum dots (QDs)36 selectively applied to axons (100 ng ml⁻¹), and movement of QD-labelled NGF-signalling endosomes was measured by live-cell microscopy (Fig. 4d). The proportion of retrogradely moving particles seen under naive and control siRNA conditions (∼27%) was consistent with previous studies37,38. Axon-specific knockdown of Pafah1b1 or Dctn1 significantly reduced the retrograde movement of QD-positive particles and increased the proportion of stationary particles. Together, these results establish that NGF stimulation triggers local synthesis of Lis1 and p150Glued, and that the stimulated transport of different cargos requires the local synthesis of different regulator proteins.

**Transport of an axonal death signal requires Lis1 synthesis.** According to the signalling endosomes hypothesis, transport of NGF bound to activated tyrosine receptor kinases in endosomes from axons to the cell body is required for the survival of neurons dependent upon target-derived neurotrophic support35,39, while another model proposes that NGF acts by suppressing a retrograde apoptotic signal, and that retrograde transport of NGF-signalling endosomes is not required for survival40. Because of the observed reduction in retrogradely moving, QD-labelled NGF-signalling endosomes upon axon-specific knockdown of Pafah1b1 or Dctn1 mRNAs, we next tested whether survival of the DRG neurons was impaired as well. NGF was withheld from both compartments or selectively applied (100 ng ml⁻¹) to the axonal compartment. To quench any residual NGF activity in the deprivation conditions, a neutralizing anti-NGF antibody was added. Contrary to what the signalling endosomes hypothesis would predict but in line with a suppressive effect of NGF on an axonal apoptotic signal in starved axons, in the NGF-replete condition axonal knockdown of either Pafah1b1 or Dctn1 did not induce apoptosis, as assessed by TUNEL-positive nuclei, nor did it reduce the number of living neurons stained by calcein acetoxymethyl (AM) (Fig. 5a,b). Moreover, in the NGF-starved condition, knockdown of Pafah1b1 completely prevented the induction of cell death by NGF deprivation. Knockdown of Dctn1 in the NGF-starved condition did not impact cell death, consistent with our finding that NGF withdrawal does not activate p150Glued synthesis.

Together, these results demonstrate that, although inhibition of local Lis1 and p150Glued synthesis greatly reduced retrograde transport of NGF-signalling endosomes, their local production is...
Figure 2 | NGF signalling differentially regulates Lis1 and p150Glued levels in axons. DRG neurons were cultured and treated as in Fig. 1. (a) Transcripts coding for dynein regulators have been found in transcriptomes derived from embryonic rat DRG axons using microarray, embryonic mouse DRG using RNAseq and embryonic rat hippocampal axons using RNAseq. Transcripts found in all three studies are highlighted in red, and Lis1 and p150Glued are outlined in blue. (b) Pafah1b1 and Dctn1 levels were measured by quantitative FISH in axons kept for 12 h at the baseline NGF level (5 ng ml⁻¹). Background fluorescence was determined using a Gfp probe and subtracted. Means ± s.e.m. of 15 optical fields per condition (n = 3 biological replicates). *P ≤ 0.05; **P ≤ 0.01. Kruskal–Wallis test with Dunn’s multiple comparison test. (c) Axons were pretreated with protein synthesis inhibitors (anisomycin and emetine) or vehicle, followed by exposure to different concentrations of NGF (0, 5 or 100 ng ml⁻¹) for 10 min. Axonal Lis1 levels were measured by quantitative immunofluorescence. Means ± s.e.m. of 15–20 optical fields per conditions (n = 3–4 biological replicates). *P ≤ 0.05. Two-way ANOVA with Dunnett’s multiple comparison test. (d) Neurons were cultured and treated as in b. Axonal p150Glued levels were measured by quantitative immunofluorescence. Means ± s.e.m. of 15 optical fields per conditions (n = 3 biological replicates). *P ≤ 0.05. Two-way ANOVA with Dunnett’s multiple comparison test. Scale bars, 5 μm. NS, not significant.
Figure 3 | NGF induces local synthesis of Lis1 and p150Glued. DRG neurons were cultured in microfluidic chambers. On DIV 3, the NGF concentration in the axonal chamber was changed to 5 ng ml\(^{-1}\), and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting *Pafah1b1* or *Dctn1*. (a,b) Twenty-four hours after transfection, axons were treated with 0, 5 or 100 ng ml\(^{-1}\) NGF for 10 min, and Lis1 (a) and p150Glued (b) levels in the cell bodies were determined by immunofluorescence. Means ± s.e.m. of 15 optical fields per conditions (n = 3 biological replicates). No significant changes. Two-way ANOVA with Dunnett’s multiple comparison test. Scale bars, 20 μm. (c,d) Neurons were cultured and treated as before, and axonal Lis1 (c) and p150Glued (d) levels were determined by immunofluorescence. Scale bars, 5 μm. Means ± s.e.m. of 20–75 optical fields per conditions (n = 4–15 biological replicates). *P < 0.05; **P < 0.01; ***P < 0.001. Two-way ANOVA with Dunnett’s multiple comparison test. See also Supplementary Fig. 1. NS, not significant.
not required for NGF-dependent survival. Rather, local Lis1 synthesis is necessary for the retrograde transport of a pro-apoptotic signal of unknown identity that is generated in NGF-deprived axons. To further characterize this retrograde death signal, we first focused on protein kinases that have been implicated in apoptotic cell death in neurons. Whole-cell death signal 40. Application of two GSK3 inhibitors, LiCl or SB216763, selectively to axons had no effect on cell death under NGF-replete conditions, but completely prevented the induction of apoptosis with NGF deprivation (Fig. 5c). Together, these results indicate that the death signal, whose transport requires local Lis1 production, involves active GSK3β.

NGF signalling regulates axonal Pafah1b1 and Dctn1 levels. In regenerating DRG axons, neurotrophins regulate the abundance of specific mRNAs through anterograde recruitment from the cell body. To investigate whether changes in neurotrophin signalling not only differentially regulate the translation of axonally localized Pafah1b1 and Dctn1 mRNAs but also their abundance, we performed quantitative FISH on axons selectively transfected with siRNAs and treated with different NGF concentrations. As before, the effect of the siRNAs was restricted to axons as neither mRNA’s abundance in the neuronal cell bodies was changed upon axonal siRNA transfection (Fig. 6a,b). Quantification of the axonal FISH signals revealed that neither mRNA was recruited in response to stimulation with NGF, but that NGF deprivation caused a significant increase in Lis1 transcript levels (Fig. 6c,d). Similar results were obtained for FISH against the transcripts of NudE and its paralogue NudEL, two proteins can form a trimeric complex with dynein and Lis1 (Supplementary Fig. 2), indicating that NGF might co-regulate mRNAs of proteins that frequently function in a complex. The FISH signal was specific for the targeted mRNAs as transfection of axons with siRNAs targeting either transcript reduced the FISH signal to background levels. The results of the FISH experiments were confirmed by quantitative real-time PCR with reverse transcription (RT–PCR) performed on RNA collected from axonal compartments (Fig. 6e). The siRNAs failed to reduce the levels of their target transcripts under NGF conditions that do not trigger the translation of these mRNAs (baseline for both mRNAs; NGF withdrawal). This effect is likely due to the tight packaging in RNA granules of silenced mRNAs in axons rendering them inaccessible for the RNAi machinery, an effect we had observed previously. Together, these results demonstrate that the intra-axonal expression of the dynein

Figure 4 | NGF-induced changes in axonal trafficking require local synthesis of Lis1 or p150Glued. DRG neurons were cultured in microfluidic chambers. On DIV 3, the NGF concentration in the axonal chamber was changed to 5 ng ml⁻¹, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting Pafah1b1 or Dctn1. (a–c) After 24 h, fresh medium was added to the axonal chamber containing 5ng ml⁻¹ NGF, no NGF or 100 ng ml⁻¹ NGF together with LysoTracker Green for 15 min. Live-imaging time-lapse series of axonal fields were acquired, with images being taken every 13 s for 4 min. Kymographs of representative 100-μm-long axonal segments are shown. Scale bar, 10 μm. LysoTracker-positive particles with diameters ≥ 1 μm were scored as anterograde, retrograde, bidirectional or stationary. Means ± s.e.m. of 12–18 optical fields per conditions (n = 3–6 biological replicates). **P ≤ 0.01; ***P ≤ 0.001. One-way ANOVA with Bonferroni’s multiple comparisons test. (d) On DIV 4, axons were treated with 100 ng ml⁻¹ QD-NGF for 15 min and live imaged as above. QD-labelled particles < 1-μm diameter were scored as anterograde, retrograde, bidirectional or stationary. Means ± s.e.m. of nine optical fields per conditions (n = 3 biological replicates). **P ≤ 0.01; ***P ≤ 0.001. Kruskal–Wallis test with Dunn’s multiple comparison test. NS, not significant.
cofactors Lis1 and p150Glued is differentially regulated both translationally and through recruitment of their mRNAs.

APC-binding sorts Pafah1b1 into functionally distinct pools. The finding that the one mRNA species, Pafah1b1, is locally translated in response to both NGF stimulation and withdrawal, and, further, is recruited into axons only upon NGF deprivation but not stimulation, strongly suggested that distinct regulatory mechanisms exist that control Pafah1b1 localization and translation under different signalling conditions. Recently, Pafah1b1 has been found to be part of the adenomatous polyposis coli (APC) interactome. APC is a microtubules plus-end tracking protein, also referred to as +TIP49, and by binding a specific subset of mRNAs, APC might provide a platform for the local synthesis of dynein regulators, including Lis1, at the distal end of axonal microtubules. Thus, we wondered whether association with APC was required for Pafah1b1 regulation in axons. To address this question, we used a locked nucleic acid (LNA) oligomer designed to interfere with Pafah1b1–APC

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**Figure 5** | Pro-apoptotic signalling from NGF-deprived axons requires axonally synthesized Lis1 and active GSK3β. (a) DRG neurons were cultured and transfected with siRNAs as in Fig. 4. On DIV 4, the medium in the somatic compartment was changed to NGF-free medium containing NGF-neutralizing antibody, and axonal compartments were changed to 100 ng ml⁻¹ NGF or NGF-free medium with NGF-neutralizing antibody plus vehicle for 24 h. Cell death was assessed by TUNEL assay. Means ± s.e.m. of 15–25 optical fields per conditions (n = 3–5 biological replicates). ***P ≤ 0.001. Two-way ANOVA with Dunnett’s multiple comparison test. (b) Neurons were cultured and treated as in a. Survival was assessed by calcein AM staining. Means ± s.e.m. of 15 optical fields per conditions (n = 3 biological replicates). ***P ≤ 0.001. Two-way ANOVA with Dunnett’s multiple comparison test. (c) DRG neurons were cultured as in Fig. 4. On DIV 4, the medium in the somatic compartment was changed to NGF-free medium containing NGF-neutralizing antibody, and the medium in the axonal chamber was changed to 100 ng ml⁻¹ NGF or NGF-free medium with NGF-neutralizing antibody plus the mixed lineage kinase inhibitor, CEP-1347, the p38 MAP kinase inhibitor, SB239063, or the GSK3β inhibitors, LiCl or SB216763, or vehicle for 24 h. Cell death was assessed by TUNEL assay. Means ± s.e.m. of 15–25 optical fields per conditions (n = 3–5 biological replicates). ***P ≤ 0.001. Two-way ANOVA with Dunnett’s multiple comparison test. Scale bars, 20 μm. NS, not significant.
Figure 6 | NGF signalling regulates axonal transcript levels of dynein regulators. (a–d) DRG neurons were cultured in microfluidic chambers for 3 DIV, at which time the NGF concentration in the axonal chamber was changed to 5 ng ml$^{-1}$, and axons were selectively transfected with a non-targeting control siRNA or siRNAs targeting Pafah1b1 (a,c) or Dctn1 (b,d). Twenty-four hours after the transfection, the NGF concentration in the axonal chamber was adjusted to 0, 5 or 100 ng ml$^{-1}$ NGF for 12 h, and cell body Pafah1b1 (a) or Dctn1 (b) or axonal Pafah1b1 (c) or Dctn1 (d) mRNA levels were determined by FISH. Means ± s.e.m. of 15–25 optical fields per condition (n = 3–5 biological replicates). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Two-way ANOVA. Scale bars, 20 μm (a,b); 5 μm (c,d). (e) Neurons were cultured and axons treated with NGF in microfluidic chambers as before. Axonal RNAs were collected after the 12 h NGF treatment, and Pafah1b1 and Dctn1 levels were determined by quantitative real-time RT–PCR. Relative quantification with Gapdh as reference was done using the 2$^{-\Delta\Delta C_{T}}$ method. The means of the 5 ng ml$^{-1}$ NGF conditions for Pafah1b1 and Dctn1 were defined as 1.0. Means ± s.e.m. of 3–5 biological replicates. *P ≤ 0.05. Kruskal–Wallis test with Dunn’s multiple comparison test. NS, not significant.
association by binding the putative APC-binding site, a CUGU motif in the 3'-untranslated region (UTR) of Pafah1b1 (ref. 48). To determine which of the several CUGU motifs in the 3'-UTR of Pafah1b1 to target, reads from the APC-CLIP study48 were collapse and quality-filtered50 and mapped to the mouse genome (mm10). Mapped reads were analysed for cluster enrichment using PIPE-CLIP51. Three clusters in the 3'-UTR of Pafah1b1 were found to be significantly enriched in APC-binding, but only two of these clusters had a significant fold change compared with a control mRNA-seq data set. We chose the cluster with the lowest P value (1.41 × 10^{-11}), which also contained a CUGU motif. A second LNA, binding Pafah1b1 13 bases upstream of the CUGU LNA, was used as a control (Fig. 7a). To confirm whether the CUGU LNA was able to interfere with APC–Pafah1b1 interaction, we transfected the LNAs in dissociated DRG and performed anti-APC RNA immunoprecipitation. Pafah1b1 was
detectable in the precipitate and its abundance reduced in RIPs from CUGU LNA-transfected DRGs (Fig. 7b). Next, the LNAs were transfected in the cell body compartment, and mRNA levels in axons were determined by quantitative FISH 12 h after different NGF treatments as before (Fig. 7c). The control LNA had no discernible effect compared with naive axons (two-way analysis of variance (ANOVA) P = 0.7585), while transfection with the CUGU LNA caused reduced Pafah1b1 levels in all three conditions (P = 0.008). The differences in Pafah1b1 abundance at 5 and 0 ng ml \(^{-1}\) or 0 and 100 ng ml \(^{-1}\) NGF were significant in both naive and CUGU LNA-transfected axons and extremely similar (5 and 0 ng ml \(^{-1}\): 0.56 versus 0.50; 0 and 100 ng ml \(^{-1}\): 0.58 versus 0.68). These results indicate that APC association is required for the axonal localization of a fixed amount of Pafah1b1, but that the recruitment of additional Pafah1b1 into axons in response to NGF withdrawal is APC-independent.

To determine whether Lis1 protein levels in axons were similarly affected by interference with Pafah1b1–APC binding, we transfected DRGs with LNAs as before and performed quantitative immunofluorescence against Lis1 (Fig. 7d). No significant change in Lis1 abundance was detected at 5 ng ml \(^{-1}\) NGF, again confirming that it is not locally synthesized under this condition. The increase in axonal Lis1 abundance in CUGU LNA-transfected DRGs at 0 ng ml \(^{-1}\) was indistinguishable from naive and control LNA neurons, while in NGF-stimulated axons transfection with the CUGU LNA prevented the increase in Lis1 levels.

Finally, we investigated the requirement of Pafah1b1–APC association for stimulation-induced retrograde transport of LysoTracker-positive cargos. Transfection of the CUGU LNA had no effect on transport at the baseline NGF condition or upon NGF withdrawal, but completely prevented the increase in retrograde transport triggered by NGF stimulation (Fig. 7e–g).

Together, the results from the LNA experiments reveal the existence of two distinct modes of Pafah1b1 localization in axons: one that is constitutively active and APC-dependent, and other that is APC-independent and particularly responsive to NGF deprivation (Supplementary Fig. 3). The transcripts that are localized in the APC-dependent mode are translocated in response to stimulation with NGF, while the APC-independent pool is locally translated with NGF withdrawal and is boosted by recruitment into NGF-starved axons. Thus, the two diametrically opposed triggers of axonal Lis1 synthesis, NGF withdrawal and stimulation, act on two separate pools of Pafah1b1 mRNA that each are solely responsible for the increase in local Lis1 levels under either condition.

**Discussion**

Association with various accessory proteins allows cytoplasmic dynein to fulfil a multitude of functions in cells and to transport a wide variety of different cargos. Here we provide evidence that in distal axons, stimulus-induced changes in dynein-dependent transport are regulated through local synthesis of Lis1 and p150\(^{\text{Glued}}\). The unidirectional nature of microtubules in axons poses special challenges for the acute regulation and initiation of dynein-dependent transport, and local translation of its cofactors can solve this problem. As the unidirectional orientation of microtubules is not unique to axons but occurs also in distal dendrites or during neuronal cell migration, it is possible that this mechanism is utilized in these circumstances as well. Meanwhile, local synthesis of motor complex proteins might not be restricted to Lis1 and p150\(^{\text{Glued}}\). p150\(^{\text{Glued}}\) is only one of several subunits of dyactin. The transcript for another subunit, p50, is also consistently found in transcription, while the localization of transcripts coding for other subunits is less clear. It remains unknown whether the entire dyactin complex can be locally synthesized or whether it locally assembles upon on-demand synthesis of p150\(^{\text{Glued}}\) and potentially p50.

Changes in intra-axonal transport have long been recognized as hallmarks of many neurological and neurodegenerative disorders\(^{32,33}\). In addition, alterations in neurotrophin signalling have been implicated in neurodegenerative and psychiatric disorders\(^{34}\). Therefore, it will be important to investigate whether the processes uncovered here are disrupted in disease and whether they might present novel targets for therapies. For example, our previous finding that soluble oligomeric \(\alpha\)-\(\beta\)-\(\delta\) upregulates protein synthesis in axons of mature hippocampal neurons\(^{23}\) indicates the possibility that local translation has an impact on dynein-dependent transport in Alzheimer's disease brain.

It is worth noting that axonal production is not merely supplementary to global synthesis of these dynein cofactors. Neuron-wide knockdown of Lis1 expression reduces retrograde stimulation-independent transport of LysoTracker-positive vesicles in axons\(^{35}\), while we found that axon-specific knockdown of Lis1 or p150\(^{\text{Glued}}\) prevented only induced changes of transport. Why is induced but not constitutively active transport dependent on local translation? A possible explanation might be that in response to stimulation, previously inactive dynein motor complexes get activated and coupled to their cargos. We found that Lis1 synthesis in response to NGF stimulation requires the association of Pafah1b1 with APC. As a + TIP, APC is well situated to mediate the activation of dynein through local production of regulatory proteins. In fact, the
recruitment of dynactin by + TIPs has been found to be required for the initiation of retrograde axonal transport of various cargos. In *Aspergillus nidulans*, the Lis1 homolog has also been described as an initiation factor for dynein-mediated transport that is absent from and is unnecessary for dynеin–cargo complexes once they are in motion. Local synthesis of Lis1 or p150

[90]head at very precise loci in axons or growth cones could, therefore, be a tuning or initiation mechanism for dynein-based transport.

In essentially all instances, intra-axonal protein synthesis has been seen to be stimulus-dependent. Our finding that NGF deprivation triggers axonal Lis1 synthesis within 10 min leads to the question as to how withdrawal of a ligand can be a stimulus for translation. The extremely short time needed to induce translation rules out that it might be a consequence of neuronal degeneration caused by the lack of trophic support. Rather, translation appears to be triggered by a signalling pathway that is active in the absence of NGF and suppressed by NGF-TrkA binding. For example, TrkA has been proposed to act as a dependence receptor that triggers cell death in the absence of its ligand.

Our study provides additional support for the dependence receptor hypothesis and, further, an experimental paradigm in which to dissect the underlying cell intrinsic death pathway downstream of TrkA.

Our finding that association with APC establishes distinct pools of axonally localized *Pafah1b1* mRNA that differ as to whether they are translated in response to NGF stimulation or withdrawal provides mechanistic insight into the differential regulation of axonally localized mRNAs. APC localizes mRNAs to microtubule plus-ends, and spatially orchestrates protein synthesis in axons and growth cones.

It remains unknown how many of these translational hubs exist in axons. The finding that the netrin receptor DCC binds components of the protein synthesis machinery and regulates local translation suggests that APC is not unique. In the surface Ustilago maydis, polycytes are actively translationally active there. If the same occurs in neurons, it would therefore be possible that cargos might be able to hitch a ride on dynein by locally synthesizing adapter proteins on their surface.

In conclusion, through these studies, we provide a mechanistic explanation for how a unidirectional motor can be tuned to fulfil changing transport needs far away from the cell soma, and we further reveal that transcripts of the same gene exist in axons in functionally distinct pools based on their association with translational hubs.

**Methods**

**Compartmentalized DRG culture.** All reagents were from Thermofisher Scientific (Waltham, MA) unless otherwise noted. To apply NGF, inhibitors or siRNAs specifically to distal axons and growth cones without affecting the cell bodies, DRG neurons were prepared from Sprague-Dawley embryonic day 15 rat embryos of both sexes. All work involving animals was performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals, and was approved by the Institutional Animal Care and Use Committee of Columbia University. Embryonic rat DRGs were grown in tripartite microfluidic chambers composed of three compartments (width of middle compartment: 500 μm; side compartments: 1,500 μm, width: 10 μm; side compartments: 1,500 μm) connected by two microgroove barriers (microgroove length: 500 μm, width: 10 μm, height: 3 μm).

Microfluidic chambers were produced according to published protocols. The microfluidic chambers were coated with 100 μg/ml N-cyanoacrylate (Trevigen, Gaithersburg, MD), the plating medium (Neurobasal, 1 x B27, 2 μM glutamate, 20 μM 5-fluorodeoxyuridine, 100 ng/ml -1 NGF) was completely exchanged for 5 ng/ml -1 NGF in both axonal compartments after 48h. siRNA transfection in the axonal compartments or LNA transfection in the somatic compartment was performed on DIV 3, and all experiments were performed on DIV 4. Whenever stated, the axonal compartments only were treated with anisomycin (1 μM, Sigma-Aldrich), emetine (2 μM, EMD Millipore), rapamycin (10 nM, Sigma-Aldrich), CEP-1347 (0.5 μM, Sigma-Aldrich), SB239063 (1 μM, Sigma-Aldrich), lithium chloride (15 mM) or SB216763 (10 μM, Sigma-Aldrich).
Supplementary Fig. 4. Images have been cropped for presentation. Full-size images are presented in horseradish peroxidase-conjugated secondary antibodies were used at 1:2,000. ImageJ (NIH) software. The primary antibodies used for loading controls were: secondary antibodies (Pierce) and visualized with SuperSignal West Pico primary antibodies followed by incubation with horseradish peroxidase-conjugated lysed in RIPA buffer and proteins were resolved by 4–12% SDS–polyacrylamide gel.

Quantitative immunofluorescence imaging. Axons of neurons grown in microfluidic chambers were exposed to 4% paraformaldehyde in PBS for 20 min at room temperature. Following three washes with PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS and washed twice more with PBS. The coverslips were incubated with a total of 100 ng digoxigenin-labelled riboprobes (20 ng each of five distinct riboprobes) in 30 µl hybridization buffer (50% formamide, 2 × SSC, 0.2% bovine serum albumin (BSA), 1 mg/ml 1 E. coli cell transfert RNA and 1 mg/ml salmon sperm DNA) overnight at 37 °C. The coverslips were washed with constant agitation at 37 °C, first with 50% formamide in 2 × SSC for 30 min followed by 50% formamide in 1 × SSC for another 30 min. An additional three washes were done at room temperature with 1 × SSC for 15 min each. The coverslips were washed three times with PBS containing 0.1% Tween-20 for 5 min each, blocked with 3% BSA in PBS for 30 min, and incubated with anti-digoxigen (Sigma-Aldrich, DI-22; 1:500) and anti-β-III tubulin (Abcam, ab41489; 1:1,000) antibodies in blocking solution overnight at 4 °C. The coverslips were washed three times with PBS, blocked and incubated with fluorescein-conjugated Alexa secondary antibodies (1:2,000 for 1 h at room temperature, and washed and mounted with ProLong Gold antifade reagent. β-III tubulin staining was used to generate a mask within which the intensity of the FISH signal was quantified. Average fluorescence intensity of axonal fields that were incubated with a Gfp probe was subtracted from the fluorescence intensities resulting from hybridization with Pafah1b1 or Dctn1 riboprobes.

siRNA and LNA transfection. Axon-specific silencing of Pafah1b1 and Dctn1 mRNAs was achieved by transfecting siRNAs into axons concomitant with the axonal medium change on DIV 3 using NeuroPORTER (Genlantis, San Diego, CA) and concentrated using the RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA). A total amount of 2 ng was generally isolated from axonal lysates, which was concentrated to 10 µl for reverse transcription. Reverse transcription was performed using SuperScript III First-Strand Synthesis SuperMix for qRT–PCR. Real-time RT–PCR was performed with TaqMan Gene Expression master mix in a StepOnePlus Real-Time PCR instrument using the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 1 min. Pafah1b1 and Dctn1 levels were normalized to Gapdh.

RNA immunoprecipitation. CUGU and control LNAs were transfected into dissociated DRGs and 24 h later the DRGs were lysed in RIPA buffer (150 mM KCl, 25 mM Tris-HCl, 5 mM EDTA, 0.5 mM dithiothreitol, 0.5% NP40, protease inhibitors). The cleared lysate was incubated with an APC antibody (Santa Cruz Biotechnology, sc-896; 1:500) overnight at 4 °C. Antibody–protein–RNA complexes were precipitated by incubation under agitation with Dynabeads for 1 h at 4 °C. The beads were washed five times in ice-cold RIPA buffer. RNA elution was done at the beads, RNA was purified using the Direct-zol RNA MicroPrep kit (Zymo Research) with DNasel treatment. Complementary was synthesized using the iScript Reverse Transcription Supermix for RT–qPCR. RT–PCR was run according to the guidelines for TaqMan Fast Advance Master Mix.

Statistical analyses. All experiments were performed at least three biological replicates to gain sufficient power for meaningful statistically analyses. Two means were compared by t-tests, whereas multiple means were compared using one-way ANOVAs with multiple comparisons testing. When comparing multiple groups in experiments with more than one variable, two-way ANOVA was performed. For all comparisons, normal distribution and variance were determined and appropriate statistical tests chosen.

Data availability. Data supporting the findings of this study are available within the article (and its Supplementary Information files) and from the corresponding author on reasonable request.

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Author contributions

J.M.V. with E.P.M. and J.C.M. performed and analysed all the experiments. U.H. conceived the project, and U.H. and J.M.V. designed the experiments and wrote the manuscript.

Additional information

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Intra-axonal Synthesis of SNAP25 Is Required for the Formation of Presynaptic Terminals

Highlights

- Protein synthesis is rapidly induced at contact sites with presynaptic organizers
- The presynaptic protein SNAP25 is locally synthesized at induced presynaptic sites
- Local SNAP25 synthesis is required for formation of presynapses
- Inhibition of local SNAP25 synthesis interferes with vesicle release

In Brief

Batista et al. find that, during the assembly of presynaptic terminals, mRNA translation is upregulated at the nascent presynapses and required for the clustering of presynaptic proteins. Inhibition of local SNAP25 synthesis prevents proper formation of presynaptic terminal and interferes with synaptic vesicle release.

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Intra-axonal Synthesis of SNAP25 Is Required for the Formation of Presynaptic Terminals

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SUMMARY

Localized protein synthesis is a mechanism for developing axons to react acutely and in a spatially restricted manner to extracellular signals. As such, it is important for many aspects of axonal development, but its role in the formation of presynapses remains poorly understood. We found that the induced assembly of presynaptic terminals required local protein synthesis. Newly synthesized proteins were detectable at nascent presynapses within 15 min of inducing synapse formation in isolated axons. The transcript for the t-SNARE protein SNAP25, which is required for the fusion of synaptic vesicles with the plasma membrane, was recruited to presynaptic sites and locally translated. Inhibition of intra-axonal SNAP25 synthesis affected the clustering of SNAP25 and other presynaptic proteins and interfered with the release of synaptic vesicles from presynaptic sites. This study reveals a critical role for the axonal synthesis of SNAP25 in the assembly of presynaptic terminals.

INTRODUCTION

During the development of the nervous system, axons project over long distances to their cognate targets, until upon contact with target-derived adhesive or soluble factors the assembly of a presynaptic terminal is initiated (Chia et al., 2013; Jin and Garner, 2008). Application of these presynaptic organizing molecules to isolated axons is sufficient to induce presynapse formation from components that have been transported from the neuronal soma. An alternative source for at least some of the presynaptic proteins might be the axon itself through the process of local translation. Protein synthesis in axons is required for proper axon development (Campbell and Holt, 2001; Gracias et al., 2014; Hengst et al., 2009; Wu et al., 2005) by providing a spatially and temporally tightly restricted source of protein to response to extracellular signals (Batista and Hengst, 2016). Transcripts coding for several presynaptic proteins have been found in developing cortical axons (Taylor et al., 2009), and, in Aplysia, protein synthesis is required for the formation of presynapses (Schacher and Wu, 2002). Recently, the importance of presynaptic protein synthesis in the control of neurotransmitter release was reported for the mature mammalian brain (Younts et al., 2016), but the role of local translation in the formation of presynapses remains poorly understood. Specifically, it is unknown whether axonal protein synthesis is required for the assembly of presynaptic terminals. So far, only one locally synthesized protein has been described that accumulates at nascent presynapses, β-catenin, where it regulates the release of synaptic vesicles (Taylor et al., 2013). Here, we report the induced intra-axonal synthesis of the t-SNARE protein synaptosomes-associated protein 25 (SNAP25) as a necessary, early step for the clustering of presynaptic proteins and the formation and function of presynapses.

RESULTS

Presynaptic Proteins Cluster within 1 hr at Contact Sites with PDL-Coated Beads

To investigate whether the formation of presynapses requires local protein synthesis, we cultured embryonic hippocampal neurons in tripartite microfluidic chambers that allow the fluidic isolation of axons from cell bodies and dendrites (Figure 1A) (Galeriola et al., 2014; Taylor et al., 2005). Poly-D-lysine (PDL)-coated latex beads were applied selectively to the axonal compartments to induce the clustering of presynaptic proteins (Lucido et al., 2009; Taylor et al., 2013). Immunostaining revealed significantly increased levels of several presynaptic proteins (β-catenin, synaptophysin, GAP-43, SNAP25) and tau at contact sites between axons and PDL-coated beads after 24 hr of incubation, while β-III tubulin levels were unchanged (Figure 1B). As local protein synthesis is frequently an acute reaction to an external stimulus, we next investigated how early after addition of the PDL-coated beads we could detect clustering of presynaptic proteins. SNAP25 levels were significantly increased after 1 hr of contact with the PDL-coated beads (Figure 1C).

Inhibition of Axonal Protein Synthesis Prevents Clustering of β-Catenin and SNAP25 at 1 hr

Previously, it has been reported that the clustering of β-catenin at 3 hr after addition of PDL-coated beads requires local protein synthesis (Taylor et al., 2013). To determine whether the clustering of other presynaptic proteins is likewise dependent on
Figure 1. Clustering of Presynaptic Markers at PDL-Coated Beads in Axons

(A) Scheme of a tripartite microfluidic chamber used to selectively treat axons. Embryonic hippocampal neurons were seeded in the upper compartment of the chamber (teal) and axons cross through two microgroove barriers (500-μm-long) into the middle and lower axonal compartments (yellow). After DIV10, PDL-coated or uncoated beads were added to the axonal compartments for 15 min to 1 hr, and clustering of presynaptic proteins (i.e., β-catenin, SNAP25) adjacent to the bead-axon contact sites was determined by immunofluorescence (IF).

(B) Axons were incubated with PDL-coated beads for 24 hr and immunostained for presynaptic and axonal proteins. Levels of β-catenin, synaptophysin, SNAP25, GAP43, and tau are significantly increased at axon-bead contact sites. IF and IF merged with differential interference contrast (DIC) images are shown; yellow dashed circles outline beads. Quantifications are relative to off-bead fluorescence values. Mean ± SEM of 30–120 axonal fields (n = 3 biological replicates per condition). Unpaired t tests. n.s., not significant; **p < 0.01; ****p < 0.0001.

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axonal translation, we focused on SNAP25 and added the protein synthesis inhibitor emetine selectively to the axonal compartment during the treatment with uncoated or PDL-coated beads (Figure 2A). We quantified the fluorescent intensity within axons along a 30-μm-long line starting at the center of the beads (Figure 2B). Within 1 hr of treatment with PDL-coated beads, β-catenin and SNAP25 were significantly increased in the first 5 μm from the beads’ centers, i.e., at contact sites (Figures 2C–2E). The clustering of β-catenin and SNAP25 did not show any bias for either the proximal (i.e., toward the cell body) or distal side of the beads (Figure S1); thus, for our analyses we did not distinguish between the proximal and distal sides. Uncoated beads did not induce clustering, and addition of emetine completely prevented the clustering of β-catenin and SNAP25. Neither tau nor β-III tubulin clustered at this early time point (Figures 2F and 2G). To determine whether the observed protein synthesis dependency of β-catenin and SNAP25 clustering at 1 hr was limited to this early time point, we analyzed their clustering at 3, 6, and 12 hr after addition of beads and emetine to the axons. While SNAP25 clustering required protein synthesis at all time points tested, β-catenin clustering was significantly affected by emetine only at 3 hr (as previously reported by Taylor et al., 2013, but not at the later time points [Figure 2H]).

PDL-Coated Beads Induce Protein Synthesis at Axonal Contact Sites within 15 min
The requirement for local translation for clustering after 1 hr of incubation with PDL-coated beads indicated that this treatment might trigger protein synthesis directly and acutely at contact sites with axons. We used puromycylation, also known as SUnSET (Figure 3A) (Schmidt et al., 2009), to detect protein synthesis events in axons. Puromycin is a tRNA analog that gets incorporated into the nascent protein chain (Yarmolinsky and Haba, 1959), allowing the detection of protein synthesis in situ with an anti-puromycin antibody. We detected a significant increase in the number of puromycin-positive puncta in axons at contact sites within 15 min of addition of PDL-coated beads (Figure 3B). The increase of the puromycin signal was accompanied by an increased presence of phospho-4EBP1, a marker for active translation, at contact sites with PDL-coated but not uncoated beads (Figure 3C). To investigate whether the immediate induction of protein synthesis was sustained over a longer time period, we added the beads for 1 hr and shifted the puromycylation time window to the last 10 min of the assay. As before, the addition of PDL-coated beads was associated with a significantly higher number of puromycylation-positive puncta in their immediate vicinity compared to uncoated beads, and addition of the protein synthesis inhibitor anisomycin completely abolished this effect (Figure 3D).

Detection of SNAP25 mRNA in Axons by Fluorescent In Situ Hybridization
mRNA encoding SNAP25 has previously been found in axons of cortical neurons (Taylor et al., 2009), but it was below the detection threshold in other axonal transcriptome datasets (Baleriola et al., 2014; Zivraj et al., 2010). Here, we used single-molecule inexpensive fluorogenic in situ hybridization (smiFISH; Tsanov et al., 2016) to directly visualize SNAP25 transcripts in axons of dissociated embryonic hippocampal neurons and to determine whether their intra-axonal localization changes in response to treatment with PDL-coated beads (Figure 4A). SNAP25 mRNA FISH-positive puncta were readily detectable at contact sites with PDL-coated beads, indicating that contact with PDL-coated beads recruits SNAP25 transcripts. The axonal smiFISH signal for SNAP25 mRNA was specific as transfection of axons with a SNAP25 small interfering RNA (siRNA) greatly reduced the number of positive puncta (Figure 4B).

SNAP25 Is Synthesized Locally at Contact Sites of Axons with PDL-Coated Beads
The protein synthesis-dependent clustering of SNAP25 and the presence of its mRNA in axons at contact sites with PDL-coated beads indicated that SNAP25 itself might be locally produced. To test this hypothesis, we adopted a different design of microfluidic chambers (Figure 4C) (Park et al., 2009). In these circular chambers, axons grow into the central open compartment, allowing the collection of axonal material in quantities required for biochemistry. SNAP25 protein was detectable by immunoblot in lysates of axons treated with uncoated beads. Application of PDL-coated beads for 24 hr greatly increased the presence of SNAP25 in axons and treatment of axons with emetine reduced SNAP25 below detection limit without affecting SNAP25 expression in the cell body compartment (Figure 4C). To directly visualize SNAP25 synthesis at contact sites with PDL-coated beads, we combined puromycylation with a proximity ligation assay (puro-PLA) (Figure 4D) (Tom Dieck et al., 2015). Axons that were not treated with PDL-coated beads showed very few SNAP25 puro-PLA puncta, while treatment with PDL-coated beads induced the local synthesis of SNAP25 (Figure 4E). As before, we tested how persistent the induction of SNAP25 synthesis was by incubating the axons for 1 hr with PDL-coated beads and adding puromycin for the last 10 min. SNAP25 puro-PLA puncta were readily detectable, and their presence was abolished by the presence of the protein synthesis inhibitor anisomycin (Figure 4F). Together, these results establish that SNAP25 is locally synthesized upon contact of axons with PDL-coated beads.

SNAP25 Is Locally Synthesized at Synapses
So far, our approach has been to induce formation of presynap- tic proteins in the absence of a postsynaptic cell, but it is necessarily artificial. To test whether SNAP25 is locally synthesized at synapses rather than at induced presynaptic specializations, we cultured embryonic hippocampal neurons in regular dissociated cultures and performed SNAP25 puro-PLA assays on DIV 5, 10,
Figure 2. Clustering of β-Catenin and SNAP25 at PDL-Coated Beads Requires Local Protein Synthesis

(A) Experimental design: on DIV11 axons were treated with PDL-coated or uncoated beads in the presence of a protein synthesis inhibitor (emetine, 100 nM) or vehicle. Cultures were fixed at different time points after treatment.

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Knockdown of SNAP25 restricted to axons (Figure 5B). The knockdown of SNAP25 we transfected axons with a SNAP25 siRNA, the effect was in cias et al., 2014; Hengst et al., 2009; Villarin et al., 2016). When expression in dendrites or cell bodies (Baleriola et al., 2014; Gra- down using locally applied siRNAs without affecting protein expression in dendrites or cell bodies (Baleriola et al., 2014; Gra- cias et al., 2014; Hengst et al., 2009; Villarin et al., 2016). When we transfected axons with a SNAP25 siRNA, the effect was restricted to axons (Figure 5B). The knockdown of SNAP25 mRNA in axons was only partial, likely because of the tight pack- aging of axonal mRNAs in granules (Buxbaum et al., 2014), which makes them inaccessible for the RNAi machinery while silenced but susceptible to siRNA under conditions that activate their trans- lation, as we have observed previously (Baleriola et al., 2014; Vil- larin et al., 2016). Knockdown of axonal SNAP25 transcripts signifi- cantly reduced the appearance of SNAP25 puro-PLA puncta at contact sites with PDL-coated beads and increased that percent- age of beads that had no puncta in their vicinity (Figure 5C). Together, these results establish that axonal transfection of SNAP25 siRNA is an efficient method to prevent the local translation of SNAP25 at contact sites with PDL-coated beads.

Clustering of SNAP25 and β-Catenin Requires the Presence of Their Transcripts in Axons

Next, we used this approach to investigate whether the local translation of SNAP25 or β-catenin was required for the clustering of these proteins. As before, we selectively knock down SNAP25 or β-catenin transcripts in axons by locally applied siRNA and measured protein clustering 1 hr after the application of PDL-coated beads (Figure 5D). Knockdown of axonal SNAP25 mRNA significantly prevented the clustering of SNAP25 at contact sites with PDL-coated beads (Figure 5E). Knockdown of β-catenin mRNA did not prevent clustering of β-catenin directly at the contact sites (0–5 μm) but led to broad- ening of the peak with increased β-catenin levels at 5–10 μm (Figure 5F). This result indicates that the vast majority of β-cate- nin protein clustering at PDL-coated beads is not derived from acutely triggered local synthesis but rather is of somatic origin. However, a small amount of β-catenin whose local synthesis is prevented by the siRNA appears to be required to induce the clustering of β-catenin directly at contact sites. The β-catenin siRNA used here efficiently knocks down its target mRNA when applied to dissociated hippocampal neurons (Figure 5G).

Together, these results suggest that most of the SNAP25 required during the early stages of presynapse formation is derived from local protein synthesis.

Axon-Specific Knockdown of SNAP25 mRNA Reduces SNAP25 Synthesis at Contact Sites with PDL-Coated Beads

To investigate the requirement of localized SNAP25 or β-catenin mRNAs, we selectively transfected the axons of hippocampal neurons grown in microfluidic chambers with siRNA (Figure 5A). Previously, we have found that RNA is functional in axons (Hengst et al., 2006) and that localized mRNAs can be selectively knocked down using locally applied siRNAs without affecting protein expression in dendrites or cell bodies (Baleriola et al., 2014; Gra- cias et al., 2014; Hengst et al., 2009; Villarin et al., 2016). When we transfected axons with a SNAP25 siRNA, the effect was restricted to axons (Figure 5B). The knockdown of SNAP25 mRNA in axons was only partial, likely because of the tight pack- aging of axonal mRNAs in granules (Buxbaum et al., 2014), which makes them inaccessible for the RNAi machinery while silenced but susceptible to siRNA under conditions that activate their trans- lation, as we have observed previously (Baleriola et al., 2014; Vil- larin et al., 2016). Knockdown of axonal SNAP25 transcripts signifi- cantly reduced the appearance of SNAP25 puro-PLA puncta at contact sites with PDL-coated beads and increased that percent- age of beads that had no puncta in their vicinity (Figure 5C). Together, these results establish that axonal transfection of SNAP25 siRNA is an efficient method to prevent the local translation of SNAP25 at contact sites with PDL-coated beads.

Clustering of SNAP25 and β-Catenin Requires Each Other’s Local Synthesis

Next, we used the same experimental approach to test the reciprocal requirement of SNAP25 and β-catenin synthesis for the clustering of these proteins. Clustering of β-catenin protein was significantly reduced in axons depleted of SNAP25 mRNA (Figure 6A). In axons with β-catenin mRNA knockdown, clustering of SNAP25 protein was reduced as well, and the peak for SNAP25 was broadened as we had seen before for β-catenin protein itself (Figure 6B).

Knockdown of Axonal SNAP25 mRNA Interferes with Vesicle Release from Induced Presynaptic Sites

Last, we performed functional assays on induced presynaptic sites in axons transfected with siRNA similar to a previously described approach (Taylor et al., 2013). To study the release of synaptic vesicles at induced presynaptic sites, we used FM4-64. FM dyes are lipophilic dyes that can be endocytosed and incorporated into synaptic vesicles. We stimulated cells with a high potassium solution in the presence of FM4-64 and let dye endocytosis occur. Cells were then imaged and stimu- lated a second time with high potassium. As vesicles are exocy- tosed, dye molecules are released and rapidly diffuse, resulting in nerve terminal destaining. In axons transfected with the siRNA targeting SNAP25, release of synaptic vesicles as measured by the disappearance of fluorescence was significantly slower than in scrambled siRNA-transfected axons, while the loading was not different (Figures 7A and 7B). Again, this effect was limited to the siRNA-treated axons, as the unloading dynamic was unchanged at synapses in the cell body compartment (Figure 7C). As an additional control, we transfected axons with an siRNA targeting another presynaptic protein, piccolo (Fenster et al., 2000), whose transcript is absent from axonal transcriptome or transla- tome datasets (Shigeoka et al., 2016; Taylor et al., 2009).

(B) A three-pixel-wide line was drawn along the axon, starting at the center of the bead. Fluorescence along this line was quantified for 30 μm and normalized against the average fluorescence in last 15 μm. Beads have a diameter of 5 μm.

(C–G) Axon were immunostained for β-catenin (C and D), SNAP25 (C and E), tau (F), and β-III tubulin (G) after 1 hr of treatment. β-catenin and SNAP25 increased in the direct vicinity of the beads in a protein synthesis inhibitor sensitive manner, while tau and β-III tubulin levels remained unchanged. IF and IF merged with DIC images are shown; yellow dashed circles outline beads. Fluorescence intensities obtained in the lines scans were averaged and normalized. The area under the curve (AUC) was then calculated for the first 5 μm and 5–15 μm from the bead center. Mean ± SEM of 30–120 beads (n = 3 biological replicates per condition).

(H) Axons were immunostained for β-catenin or SNAP25 after 3, 6, or 24 hr of treatment. SNAP25 levels were significantly increased at bead contact sites at all times tested. The effect for β-catenin was significant only at 3 hr, and emetine did not affect β-catenin levels at 24 hr.

One-way ANOVA with Tukey’s multiple comparison tests and unpaired t tests. n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001. Scale bars, 5 μm. See also Figure S1.
Figure 3. PDL-Coated Beads Induce Axonal Protein Synthesis

(A) Puromycylation assay: ribosomes incorporate puromycin into nascent polypeptide chains, leading to elongation termination and premature chain release. An antibody against puromycin is used to label nascent proteins in situ.

(B) Axons were treated with PDL-coated beads and puromycin or puromycin alone for 15 min and immunostained for puromycin and β-III tubulin. The level of puromycylation, indicating de novo protein synthesis, is significantly increased at contact sites with PDL-coated beads. Mean ± SEM of 30 axonal fields per condition (n = 3 biological replicates). Unpaired t test. *p < 0.05.

(C) Axons were treated with uncoated or PDL-coated beads and puromycin for 15 min and immunostained for phospho-4EBP1, β-III tubulin, and puromycin. The signal for p-4EBP1, indicating activation of translation, co-localizes with the puromycin signal at contact sites with PDL-coated beads.

(D) Uncoated beads, PDL-coated beads, or PDL-coated beads and the protein synthesis inhibitor anisomycin (10 μM) were added to the axonal compartments. Puromycin was added to axons during the last 10 min of the assay. The number of puromycin-positive puncta in a circle of 5 μm around each bead center and the percentage of all beads imaged in each condition with no puncta or at least one puncta in the 5-μm circle were plotted. Means ± SEM of 40–142 beads in 30 axonal fields (n = 3 different biological replicates). One-way ANOVA with Tukey’s multiple comparison test. *p < 0.05; ***p < 0.001.

Yellow dashed line represents bead location. Scale bars, 5 μm.
**A** DIV 11 24 h

Axonal treatment: PDL-coated beads

DIV 11

Axonal treatment: PDL-coated beads

β-III tubulin smiFISH merge

PDL-coated beads

PDL-coated beads + anisomycin

PDL-coated beads + siRNA

DIV 5

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 10

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 15

β-III tubulin

DAPI

Puro-PLA

Snap25

**B** β-III tubulin Snap25 smiFISH

No beads

PDL-coated beads

PDL-coated beads + siRNA

DIV 11

Axonal treatment: PDL-coated beads ± anisomycin

50'

fix

β-III tubulin puro-PLA for SNAP25 merge

**C** DIV 11 24 h

Axonal treatment: uncoated beads or PDL-coated beads ± emetine

uncoated beads

PDL-coated beads

emetine

DIV 11

Axonal treatment: harvest of axons and cell bodies

No beads

PDL-coated beads

PDL-coated beads + siRNA

DIV 5

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 10

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 15

β-III tubulin

DAPI

Puro-PLA

Snap25

**D** DIV 11

Axonal treatment: puromycin ± PDL-coated beads

puromycin

no beads

puromycin + PDL-coated beads

**E** DIV 11

Axonal treatment: puromycin ± PDL-coated beads

puromycin

no beads

puromycin + PDL-coated beads

**F** DIV 11

Axonal treatment: puromycin ± PDL-coated beads

puromycin

no beads

puromycin + PDL-coated beads

**G** DIV 5

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 10

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 15

β-III tubulin

DAPI

Puro-PLA

Snap25

**H** β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 10

β-III tubulin

DAPI

Puro-PLA

Snap25

DIV 15

β-III tubulin

DAPI

Puro-PLA

Snap25

(legend on next page)
Neuron-wide knockdown of piccolo causes enhanced synaptic vesicle exocytosis rates (Leal-Ortiz et al., 2008), but axon-specific delivery of piccolo siRNA had no effect on synaptic vesicle release, again demonstrating that the siRNAs act only locally in axons and do not interfere with protein expression and transport from the cell body (Figure 7D).

**DISCUSSION**

Our results establish that SNAP25 synthesis is locally activated by a presynaptic organizing signal and required for presynaptic terminal assembly. Previously, it has been reported that β-catenin is locally synthesized at nascent presynaptic sites and that the locally produced protein regulates the release dynamics of synaptic vesicles (Taylor et al., 2013). The question of whether local protein synthesis was required for the assembly of presynaptic terminal was not directly addressed. Here, we demonstrate that localized protein synthesis is a required step in the formation of presynaptic sites.

In accordance with Taylor et al. (2013), we also detect that β-catenin is locally synthesized. We find, however, differences in the requirement for local SNAP25 and β-catenin synthesis. While inhibition of protein synthesis prevents the accumulation of SNAP25 protein at contact sites with PDL-coated beads at all time points tested (1–12 hr), the accumulation of β-catenin is only affected until the 3-hr time point, indicating that at this time anterograde transport sufficiently meets the demand for β-catenin at presynapses. This difference is not easily explained by differences in the stability of SNAP25 and β-catenin: the half-life of SNAP25 during synaptogenesis in cerebellar granule neurons was reported to be 16 hr (Sanders et al., 1998), slightly longer than the half-life of β-catenin in PC12 cells of 12 hr (Bareiss et al., 2010). Instead, the role of the locally synthesized proteins appears to differ. While axonal-specific siRNA treatment prevents the clustering of SNAP25 directly at contact sites with PDL-coated beads by around 50%, the analogous treatment leads to a broadening of the profile for β-catenin: the amount of protein found directly at beads is not significantly reduced but instead more β-catenin accumulates in the vicinity of the beads (5- to 15-μm distance from the bead center). These findings indicate that 1 hr after contact with the beads nearly half of presynaptic SNAP25 protein is derived from local synthesis. In contrast, nearly all β-catenin accumulates at or near the beads independently of local protein synthesis, i.e., by anterograde transport from the cell body. The small amount of locally produced β-catenin is required to cluster the anterogradely transported protein directly at presynaptic sites. Thus, while local production of β-catenin is required only during the first steps of presynapse assembly and does not generate the bulk of presynaptic β-catenin protein, SNAP25 synthesis persists at least until 12 hr after initiation of presynapse formation, it generates a substantial amount of synaptic SNAP25 proteins, and it continues to be required even in established synapses, as demonstrated by the effect of axonal SNAP25 mRNA knockdown on synaptic vesicle release.

A recent translome analysis of retinal ganglion cells axons identified SNAP25 as highly expressed not only in developing, but also in mature axons (Shigeoka et al., 2016), and inhibition of protein synthesis at established presynaptic terminals deregulates GABA release (Younts et al., 2016). The stability of SNAP25 at presynapses is controlled by activity induced ubiquitination and proteasome-dependent degradation (Sheehan et al., 2016). SNAP25 synthesis at established synapses might therefore be an important mechanism for the control of synaptic SNAP25 levels and synapse function.

mRNA localization to axons is generally understood to be controlled by RNA-binding proteins that associated with mRNAs...
Figure 5. Effect of Local siRNA on β-Catenin and SNAP25 Clustering
(A) Experimental design for (C): hippocampal neurons were cultured in microfluidic chambers for 10 days. siRNA was applied only to the axonal compartments for 24 hr before PDL-coated beads were added for 1 hr. Puromycin was added to the axons for the last 10 min before fixation and processing for SNAP25 puro-PLA.
(B) SNAP25 mRNA levels were quantified by RT-PCR in lysates obtained from the axonal and the cell body compartment 48 hr after transfection of the axons with siRNA. SNAP25 levels were reduced significantly reduced in axons but not in the cell bodies, demonstrating that the axonally applied siRNAs act exclusively locally. Mean ± SEM (n = 3 biological replicates).

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through sequence elements nearly always found in the 3′ UTRs of the transcripts. Interestingly, several SNPs located in the 3′ UTR of SNAP25 are linked to adult attention deficit disorder (ADHD) (Barr et al., 2000; Brophy et al., 2002; Kustanovich et al., 2003; Mill et al., 2004). In the context of our findings, it is tempting to speculate that these mutations might interfere with the local synthesis of SNAP25 at presynaptic sites. Future investigation of the potentially changes in subcellular localization and synaptic translation of SNAP25 mRNA might provide a mechanistic understanding of how these mutations are linked to hyperactive disorders.

In conclusion, we describe an alternative source for presynaptic proteins, intra-axonal synthesis, that is required for the formation presynaptic terminals.

EXPERIMENTAL PROCEDURES

Compartmentalized Culture of Embryonic Hippocampal Neurons

All work involving animals was performed in accordance with NIH guidelines for the care and use of laboratory animals and was approved by the Institutional Animal Use and Care Committee (IACUC) of Columbia University. All reagents were purchased from Thermo Fisher Scientific unless otherwise noted. Hippocampal neurons were harvested from Sprague-Dawley embryonic day 17/18 rat embryos (Kaech and Banker, 2006). Embryonic rat neurons were grown in triplicate microfluudic chambers with 500-μm-long microgrooves connecting the three fluidically isolated compartments. Microfluidic chambers were produced according to published protocols (Gracias et al., 2014; Park et al., 2008). Primary hippocampal neurons (50,000–60,000 cells per chamber) were cultured in one of the side compartments, and axons were allowed to grow into the other two compartments. Chambers were coated with 0.1 mg mL⁻¹ poly-D-lysine (Sigma-Aldrich) and 2 μg mL⁻¹ laminin (Trevigen). After 24 hr, plating medium (neurobasal, 10% fetal bovine serum, 100 μM glutamine, 1 mM sodium pyruvate, 100 IU mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin) was completely exchanged for growth medium (neurobasal, 1 x B27, 100 mM glutamine). Half of this growth medium was replaced with fresh growth medium on DIV4 and 8. All experiments were performed at DIV9–11. Whenever stated, axonal compartments were treated with emetine (100 nM, EMD Millipore) or anisomycin (10 μM, Sigma-Aldrich).

Presynaptic Clustering with PDL-Coated Beads

Bead preparation and treatments were performed as described (Lucido et al., 2009; Taylor et al., 2013). Sulfactant-free aliphatic amine latex microspheres 4–5 μm in diameter (Invitrogen) were coated in 50 μg mL⁻¹ PDL (Sigma-Aldrich) at 37°C for at least 2 hr, rinsed twice with water, and diluted in growth medium. Uncoated beads were incubated in water. Around 150,000 PDL-coated beads were added to each axonal compartment through the side access ports. As adhesion of uncoated beads to axons is much lower for uncoated than for PDL-coated beads they were added at five to ten times excess.

Immunofluorescence and Line Scans

Neurons grown in microfluidic chambers were treated on DIV9–11 and fixed for 20 min at room temperature in 4% paraformaldehyde (PFA) in cytoskeleton buffer (10 mM 2-(N-morpholino)ethanesulfonic acid [MES], 3 mM MgCl₂, 138 mM KCl, 2 mM EGTA, 0.32 M sucrose [pH 6.1]). Neurons were washed with PBS, permeabilized, and blocked for 30 min with 3% gelatin, 0.1% BSA, 100 mM glycine, and 0.25% Triton X-100. Coverslips were incubated overnight at 4°C with primary antibodies: rabbit anti-β-catenin (1:500, Invitrogen, 71-2700), mouse anti-synaptophysin (1:500, BioLegend, SY38), mouse anti-GAP-43 (1:500, Invitrogen, 7B10), mouse anti-SNAP25 (1:1,000, BioLegend, SMI 81), rabbit anti-tau (1:500, GenScript, phospho-Ser235), rabbit anti-ßIII-tubulin (1:1,000, BioLegend, Polyclonal, P08020), and mouse anti-ßIII-tubulin (1:500, Abcam, TU-20). Neurons were washed with PBS and incubated with fluorophore-conjugated Alexa secondary antibodies (1:200) for 1 hr at room temperature. Samples were mounted with ProLong Diamond Antifade (Invitrogen), and images were acquired using a 63x/1.3 oil objective on an AxioObserver.Z1 microscope equipped with an AxioCam MRm Rev. 3 camera (Zeiss). Acquisition settings were kept the same for all samples in any given experiment. Five random axonal fields containing beads were imaged per coverslip. To quantify average fluorescence intensity at axon-beads contact sites, a 5-μm-diameter circle around the bead center was drawn in AxioVision, and average pixel intensity was determined inside that circle. For off-bead values, average pixel intensity was determined in a 5-μm circle that encompassed axons not in proximity with beads. For each image, background fluorescence intensity was determined in an area with no axons and subtracted from all bead and off-bead values. To quantify fluorescence along the axons, starting at the center of the bead, a three-pixel-wide line was drawn along the axon using ImageJ. Average pixel intensity in that line was determined for 30 μm. The intensity along the last 15 μm of each segment was averaged, and the resulting off-bead mean axonal fluorescence intensity was subtracted from all values.

Immunoblot Analysis

Hippocampal neurons were culture in circular microfluudic chambers modified after Park et al. (2009), in which the axon grows across a 500-μm-long microgroove barrier into the inner open compartment (6 mm diameter). For protein isolation, medium was carefully removed from axonal compartment, and axons were collected in 50 μL of RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1% SDS, 1 mM EDTA, supplemented with protease and phosphatase inhibitors [Pierce], the chamber was then removed, and somatic material was collected in 100 μL of RIPA buffer. The material from six different chambers was collected this way, using the same buffer to increase the amount of protein. 20 μL of lysate from axons and 2 μL of lysate from cell bodies were used for western blotting. Nitrocellulose membrane was incubated with anti-SNAP-25 (1:1,000, BioLegend, 836304) or anti-ßIII tubulin (1:10,000, BioLegend, 802001) at 4°C overnight in TBS-T with 4% milk. For detection, blots were incubated with respective secondary antibodies (1:10,000, anti-Ms-horseradish peroxidase [HRP] or anti-Rb-HRP, Invitrogen) and developed with 1-Shot Digital-ECL (KinnexBio, R1003), and images were taken with the KwikQuant Imager (KinnexBio, D1001).

Puromycylation and Puromycylation-Proximity Ligation Assays

To detect newly synthesized proteins, puromycin (1.8 μM, Sigma-Aldrich) or growth medium was added to axons in compartmentalized cultures or to dissociated neurons in regular cultures for 10–15 min, depending on the experiment, in the absence or presence of the protein synthesis inhibitor anisomycin (10 μM for axons, 40 μM for dissociated cultures). After incubation, cells were
Fluorescence values were quantified for 30 μm bars, 5 μm starting at the bead center and mean AUC for the 0- to 5-μm and 5- to 15-μm regions are plotted. Mean ± SEM of 80–100 beads (n = of 3 different biological replicates). Unpaired t test. *p < 0.05 ***p < 0.0001. Yellow dashed lines represent bead outline. Scale bars, 5 μm.

Single-Molecule Inexpensive Fluorescence In Situ Hybridization

Oligonucleotide probes were designed using Oligostan software (Tsanov et al., 2016). For Snap25, we obtained 30 probes, while for Egrp we designed 15 probes due to the smaller coding sequence (Table S1). The probes were hybridized to a digoxigenin-labeled FLAP oligonucleotide to create FLAP-structured duplex probes (Tsanov et al., 2016), smiFISH was performed as described (Tsanov et al., 2016), with minor changes. On DIV10, beads were added to the axonal compartments of hippocampal neurons grown in microfluidic devices. Cells were fixed after 24 hr for 20 min in 4% PFA in cytoskeleton buffer. Coverslips were washed in PBS, permeabilized with 0.3% Triton X-100, and blocked with 10% dextran, 350 ng mL⁻¹ yeast tRNA, 0.2 mg mL⁻¹ BSA, 2 mM vanadyl ribonucleoside complex overnight at 37°C. Coverslips were washed twice with PBS, blocked with PBS, and permeabilized in 3 mg mL⁻¹ BSA, 0.25% Triton X-100 and 0.25% Triton X-100 in PBS for 5 min, and washed again with PBS. Coverslips were equilibrated at 37°C in 15% formamide, 1 x saline sodium citrate (SSC). Samples were incubated overnight at 4°C with mouse anti-purmerin antibody (1:250, Millipore, MABE343) and rabbit anti-SNAP25 antibody (1:250, Sigma-Aldrich, S9684) for the puro-PLA assay. Detection of newly synthesized SNAP25 was performed according to the manufacturer’s recommendations, using rabbit PLA⁺ and mouse PLA⁻ probes and red Duolink detection reagents (Sigma-Aldrich). Images were acquired in a Zeiss LSM 800 confocal microscope using a 40x oil objective and Zen Blue 2.1 software.

Figure 6. Reciprocal Effects of Local β-Catenin and SNAP25 Knockdown on Clustering

Hippocampal neurons were cultured and treated as in Figure 5D. (A) Axons were transfected with scrambled or SNAP25 siRNA. 24 hr later, PDL-coated beads were added for 1 hr, and β-catenin clustering was determined by IF. Fluorescence values were quantified for 30 μm starting at the bead center and mean AUC for the 0- to 5-μm and 5- to 15-μm regions are plotted. (B) Axons were transfected with scrambled or β-catenin siRNA. 24 hr later, PDL-coated beads were added for 1 hr, and SNAP25 clustering was determined by IF.
Figure 7. Knockdown of Axonal SNAP25 mRNA Affects Vesicle Release of Newly Formed Synaptic Terminals

(A) Experimental design: hippocampal neurons were cultured in microfluidic chambers for 10 days. Axons were transfected with siRNAs 24 hr before adding PDL-coated beads. 24 hr after bead incubation, cells were loaded with FM dyes and imaged.

(B) Sequential imaging and stimulation of FM4-64 puncta in scrambled or SNAP25 siRNA-treated axons at contact sites with PDL-coated beads. SNAP25 siRNA slows vesicle release after stimulation with potassium but does not impede FM4-64 loading. Pictures were taken every 15 s, high potassium was added after 30 s.

(legend continued on next page)
(0.1% Tween 20) for 5 min, blocked with 3% BSA in PBS-T for 30 min, and incubated with goat anti-digoxigenin (1:500, Vector Laboratories) overnight at 4°C. Samples were washed three times with PBS-T for 5 min and incubated with Alexa 488 anti-goat secondary (1:1,000), and anti-fluorescein-tubulin was conjugated to Alexa 594 (Abcam, ab201740) for 1 hr at room temperature. They were then washed with PBS and mounted with ProLong Diamond Antifade Mountant (Invitrogen). Eif4f fluorescence was used as a control for nonspecific hybridization and subtracted from all Snap25 values. The specificity of the Eif4f probes was verified by performing smiFISH on cells transfected by a Gfp plasmid or control (data not shown). The specificity of the Snap25 probe was verified by RNA in axons (Figure 4B).

**siRNA Transfections**

Axon-specific silencing of Snap25, ctnnb1, and pclo mRNAs was achieved using the following siRNAs: Snap25 (NM_001270575.1) 5'-CGUGUGGCAA GAAGGGAUGAACCAUA-3' and 5'-UAUUGUUCAGUCCUCUCUUGCAGCAG-3'; ctnnb1 (NM_053357.2) 5'-UCUGAUCGGCUCACUAGUGUC UC-3' and 5'-GAGGUGUCAAGGAUGAAGCACCAUA-3' and pclo (NM_0200981.1) 5'-CACCUUCGGUCGUCUCACACAAUUAU-3' and 5'-AAUAUUGAUGAGACCA GCAAGGUGU-3'; Negative control siRNA was purchased from Thermo Fisher Scientific (Stealth RNAi siRNA Negative Control Med GC Duplex #3). siRNAs were transfected into axons of DIV10 neurons grown in microfluidic chambers using NeuroPORTER transfection reagent (Genlantis). Final siRNA concentration was 50 nM. Beads were added 24 hr after transfection.

**Real-Time RT-PCR**

Total RNA from cell bodies (four chambers or 150,000 cells) and axonal compartments (from a minimum of six chambers) was extracted with TRIzol, and RNA was purified using the Direct-zol RNA MiniPrep kit (Zymo Research). Axonal RNA was eluted in 10 μl of nuclease-free water and reverse transcribed to cDNA using the Superscript VILO cDNA synthesis kit. For cell body RNA, a total of 100 ng was reverse transcribed to cDNA. Axonal cDNA was preamplified using the TaqMan PreAmp kit, following manufacturer’s instructions for 14 cycles preamplification. Real-time RT-PCR was performed with TaqMan Fast Advanced master mix in a StepOnePlus Real-Time PCR instrument, using pre-designed TaqMan probes. Amplification conditions were as follows: initial denaturing step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and extension at 60°C for 1 min. Snap25, ctnnb1, and pclo levels were normalized to gapdh.

**FM4-64 Release Assay**

After DIV11, the chamber medium was exchanged with Tyrode’s solution (125 mM NaCl, 2 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 30 mM glucose, 25 mM HEPES [pH 7.4]), and cells were allowed to equilibrate for at least 30 min. Then, FM4-64 (15 μM, N-[3-Tritylammoniumpropyl]-4-[4-(diethylamino) Phenyl] Hexatrienyl] Pyridinium Dibromide; Invitrogen) was loaded for 90 s in a high K+ solution (37 mM NaCl, 90 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 30 mM glucose, 25 mM HEPES [pH 7.4]) with 0.5 mM advasep-7 (Sigma-Aldrich), CNQX, and DL-AP5 and three times in Tyrode’s solution with CNQX and DL-AP5. Unloading was then evoked with high K+ solution. During imaging, neurons were kept in a CO2 and humidity controlled incubation chamber at 37°C. Images were acquired every 15 s, for a total of 4 min. High K+ was added after 30 s. The intensity of FM4-64 puncta around beads was quantified over time. Relative fluorescence values were plotted and entered in SPSS (IBM). A monoeXponential fit was calculated for each individual bead data with the equation, \( F(t) = F_{\text{max}} \cdot e^{-\tau \cdot (t - t_{\text{max}})} + F_{\text{final}} \). The means of multiple groups with two independent variables were compared using two-way ANOVA with Bonferroni’s multiple comparisons tests.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes one figure and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.08.097.

**AUTHOR CONTRIBUTIONS**

A.F.R.B. and U.H. designed the experiments. A.F.R.B. and J.C.M. performed the experiments and data analysis. A.F.R.B. and U.H. wrote the paper.

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