From nucleus to axon: Spatial regulation of bclw mRNA promotes neurotrophin-dependent axon survival

From nucleus to axon: Spatial regulation of *bclw* mRNA promotes neurotrophin-dependent axon survival

A dissertation presented
by
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to
The Division of Medical Sciences

in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Neuroscience

Harvard University
Cambridge, Massachusetts

February 2015

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From nucleus to axon:

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Abstract

During development, dorsal root ganglion (DRG) neurons depend on target-derived neurotrophins to establish a functional circuit capable of conveying sensory information to the central nervous system. Neurotrophins signal from the periphery via dynein-dependent retrograde transport of signaling endosomes to activate a nuclear transcriptional response critical for neuronal survival. Importantly, neurotrophins support the health of the entire DRG neuron, but it is not well understood how neurotrophins specifically function to promote survival of axons. Previous research demonstrated that target-derived neurotrophins induce expression of the anti-apoptotic Bcl2 family member, Bclw, and *in vivo* studies demonstrate that Bclw is a critical survival factor for sensory axons. Here we describe multi-step regulation of *bclw* mRNA from the nucleus to the axon to support the health and long-term maintenance of axons. We find that neurotrophins induce transcription of *bclw* mRNA, which is rapidly transported to axons. There it is locally translated to prevent caspase-dependent apoptosis and axon degeneration. We identify the RNA-binding protein splicing factor proline-glutamine rich (SFPQ) as a critical regulator that specifically interacts with *bclw* mRNA. SFPQ is required for nuclear export of *bclw* mRNA and for generating an axonal pool of *bclw* mRNA. Interestingly, we find that SFPQ binds and regulates additional mRNAs also

required for axonal survival. Therefore we propose that SFPQ orchestrates an RNA regulon for coordinated regulation of functionally-related mRNAs including, *bclw* and *laminb2*, and thereby enables neurotrophin-dependent axonal health. Lastly, to investigate neurotrophin regulation of *bclw* translation in axons we describe development of a novel tool called spaceSTAMP. SpaceSTAMP allows for drug-dependent labeling of newly synthesized protein within distinct cellular compartments for both live cell imaging and biochemical analysis. We believe spaceSTAMP will be a rigorous method for studying local protein synthesis that will also provide critical information about the functional significance for localized translation in axons. Together, these studies demonstrate that spatial regulation of *bclw* mRNA mediates neurotrophin-dependent axonal viability and contribute towards our understanding of how neural circuits are established and maintained throughout life.

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Acknowledgements

This thesis is dedicated to the memory of two beloved scientists: my godfather, physicist Ashley Carter, and my grandfather, physicist Joseph Hershkowitz. They are forever in my heart as I follow my passion for science.

I am filled with gratitude for the opportunity to study and do research in Roz's lab for my graduate training. When Roz recruited me to the neuroscience program, I had no idea that I would end up joining her lab, but it was one of the best decisions I have ever made. Roz has a contagious passion for science and endless curiosity that is evident by her involvement and dedication to people and projects in the lab. I am extremely thankful for Roz's mentorship, which has provided me with the skills and confidence to move forward in my career. She has provided constant support and enthusiasm throughout my work and given me invaluable experience becoming a part of the scientific community, both locally and around the world. She has also created a fun and collaborative environment to do science, in which I've had many wonderful teachers and colleagues. I would particularly like to thank the women of "Team Neurotrophin" (Kat, Maria, and Sarah) for their spirit, collaboration, support, and friendship. So happy and thankful that our paths crossed here.

Thank you to my Dissertation Advisory Committee: Bernardo Sabatini, Tom Schwarz, and Susan Dymecki, for the support, ideas, and contributions throughout my work. Thank you to my Defense Exam Committee: Tom Schwarz, Melissa Moore, David Ginty, and Matt Pecot, for the thoughtful discussion and appreciated contribution to my thesis. Thank you to Mike Greenberg for wonderful advice over the years. A special thank you to Rachel Wilson for the invaluable guidance and generous mentorship.

I am extremely grateful for the important mentors who influenced my decision to pursue graduate school and inspired me about a life in science and education: Ron Hart, Jim Millonig, Matthew Heintzelman, Kelly Knox, Chris Boyatzis, and Owen Floody.

Thank you to Christiana, Sung Hee, Kristy, Kathryn and Allison. Your support through phone calls, coffee dates, yoga classes, adventures, and our laughter together make life always wonderful and get me through any challenging moment.

Thank you to the amazing yoga teachers and friends of SASY and BBY, for giving me a life-changing practice that supports me each day. In particular, I thank Caitlyn, Jess E, Erica, Kim, and Jess R for always challenging me, both physically and mentally. You give me strength, balance, perspective, patience, gratitude, and breath.

Lastly, I thank my family. Thank you for your unwavering love and support. Thank you for being passionate about what you do and inspiring me to chase after my dreams. To my fiancé, Bryan, thank you for literally being there each day to listen, laugh and celebrate with. You inspire me to work harder and learn more. I can't imagine a better partner to pursue my dreams alongside and I'm beyond excited to see where our paths lead together. Finally, I thank my parents. Thank you for the tremendous amount of support and encouragement you have given me over the last 6 years, and throughout all of the years before. Thank you to my Dad for sharing your passion for science and teaching, from the early days taking pictures of the sun through all of the thoughtful and interesting conversations we continue to share. Thank you to my Mom for inspiring me to follow my heart, to do what I love, and for always reminding me that I can do it. Together, your values, beliefs, and practices have shaped me in more ways than you will ever know, and it is with these that I have completed my Ph.D. Thank you for providing all of the opportunities that have helped me to reach this point. I am forever grateful for having the two most wonderful parents a woman could ask for. Full of admiration and love for the both of you. Thank you.

Chapter 1:

Introduction

To survive and thrive in a competitive and changing environment, living organisms require the ability to take in information from the environment and generate an appropriate response. This could be a hawk observing and capturing its prey or a person checking an intersection before walking out into the street. The nervous system has evolved across many species to process different types of sensory stimuli and to produce precise behavioral responses.

To perform a great diversity of functions, the nervous system is made up of multiple interconnected components. The vertebrate central nervous system is composed of the brain and spinal cord, and the human brain contains approximately 100 billion neurons. Together, groups of neurons make precisely wired circuits to process different types of information. The peripheral nervous system includes sensory nerves, which relay information from the extremities to the central nervous system.

During embryonic and postnatal development, the cells that make up the nervous system must be specified to generate precise neuron types and to integrate into the correct circuits. This requires cell migration to a specific location, polarization of a neuron to generate an appropriate morphology, and selection of precise target cells with whom to establish connections. Both cell intrinsic and extrinsic properties function during development to set up a properly connected and functioning nervous system. For example, numerous extracellular cues guide cell migration and axon outgrowth. This thesis seeks to understand the molecular mechanisms by which trophic factors promote

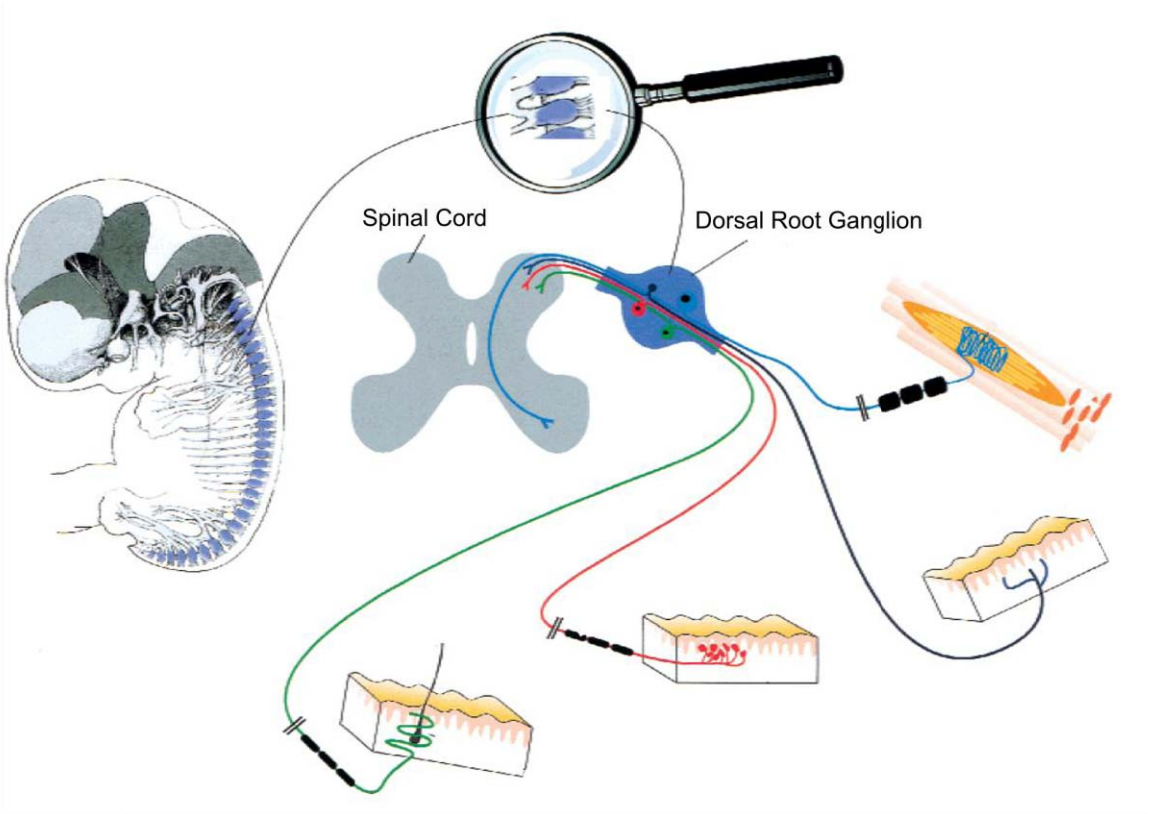
survival and maintenance of peripheral sensory neurons within a properly wired circuit for transmission of sensory information.

Neurotrophins and Dorsal Root Ganglion (DRG) Neuron Survival

Neurotrophins are a family of secreted proteins that play critical roles in neuronal survival and function. The well-characterized neurotrophins in vertebrates are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) and these trophic factors have a high affinity for specific tropomyocin-receptor kinases (Trks): NGF mediates its effects by interacting with TrkA receptors, BDNF and NT-4 with TrkB, and NT-3 with TrkC and to a lesser extent TrkA and TrkB (Segal, 2003). Neurotrophin binding to Trks leads to receptor dimerization, phosphorylation, and activation of downstream signaling cascades Ras/MAPK, PI3K/Akt, or IP3-dependent $Ca2^{+}$ release. These neurotrophins can also interact with the p75 neurotrophin receptor which can modulate the response to neurotrophins.

Dorsal root ganglion (DRG) neurons are primary sensory neurons that depend on neurotrophins for survival (Fig. 1.1). DRG neurons reside within the dorsal root alongside the spinal cord and are pseudounipolar. They extend a single process which bifurcates, sending one axon to innervate skin or muscle targets in the periphery and one axon into the spinal cord. This allows DRG neurons to convey sensory information from the periphery to the central nervous system. DRG neurons are a heterogeneous population of cells with unique properties for carrying distinct types of sensory information,

Figure 1.1



Adapted from Bibel & Barde, 2000

Figure 1.1. Neurotrophins promote survival of sensory neurons.

Dorsal root ganglion (DRG) neurons innervate both spinal cord and peripheral targets, such as skin and muscle. Neurotrophins secreted by peripheral targets regulate survival of DRG sensory neurons. Specific subpopulations of sensory neurons (denoted by green, red, black, and blue) require different neurotrophins for survival.

including touch, pain, and temperature. The subpopulations express different Trk receptors and thus rely upon different target-derived neurotrophins for their survival.

While neurotrophins are now appreciated to have numerous roles in development and neuronal functioning within both the central and peripheral nervous systems, they were originally identified as target-derived survival factors. Early embryological and transplantation studies by Viktor Hamburger and Rita Levi-Montalcini established a relationship between peripheral tissues and developing sensory and sympathetic neurons, suggesting the existence of a diffusible factor that could be accessed by peripheral axons (Hamburger and Levi-Montalcini, 1949; Levi-Montalcini, 1952, 1987). This led to the discovery and isolation of the first neurotrophin, NGF, in work done together with Stanley Cohen in the 1950s. The neurotrophic factor hypothesis, which is now widely accepted model of how neuronal circuits are formed, explains that as developing neurons send out axons, they compete for a limited amount of neurotrophin from the target cell or tissue to survive. Neurons are produced in excess during development and cell death is a naturally occurring part of development (Oppenheim, 1989). By default, neurons undergo a process of programmed cell death and thus require target-derived neurotrophins to block this pathway and promote survival. Those neurons that make accurate connections with the target tissue and receive sufficient neurotrophin survive, while aberrant targeting neurons that do not receive an adequate quantity of neurotrophin do not survive. This ensures wiring of a nervous system with the proper number of neurons to match the size requirements of a particular organism. This hypothesis is supported by studies showing that loss of NGF or its receptor leads to a significant reduction in the number of DRG

neurons. Similar studies demonstrate that specific populations of neurons require corresponding target-derived neurotrophins for survival during development (Snider, 1994).

Since the peripheral branch of DRG neurons can extend up to a meter in length, the nerve terminal may be positioned a great distance from the cell body, creating a unique challenge for neurotrophin signaling. To study the molecular mechanisms of neurotrophin signaling, researchers have made use of compartmented *in vitro* systems (Fig. 1.2), first established by Robert Campenot (Campenot, 1977). These and newer compartmented cultures (Taylor et al., 2003) create fluidically isolated chambers for cell bodies and axons allowing selective neurotrophin treatment of distal axons to mimic the environment *in vivo* (See Appendix I). Using compartmented cultures, a substantial amount of work has led to our current understanding for how target-derived neurotrophins can act over long distances to induce a response in the cell body and promote survival.

The signaling endosome hypothesis described by Mobley and colleagues proposes that activated Trk receptors at the nerve terminal are endocytosed and retrogradely transported the cell body to promote a functional response (Beattie et al., 1995). Signaling endosomes containing activated receptors have been identified in axons and are necessary to mediate the survival response to target-derived neurotrophins (Segal, 2003). Studies show that activated Trk receptors from the terminal are transported by dynein

Figure 1.2

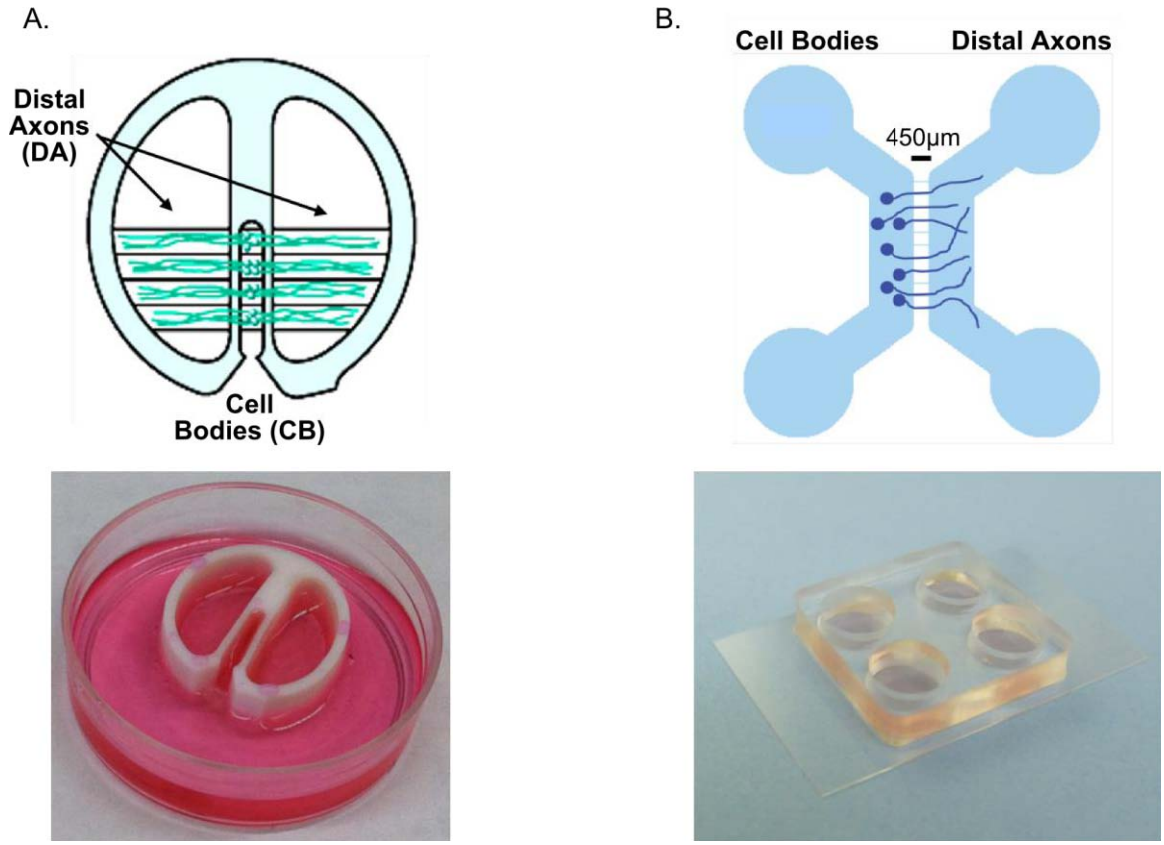


Figure 1.2 Compartmented cultures for studying DRG neurons.

A. Schematic of Campenot culture (Campenot, 1977) wherein embryonic rat DRG neurons are plated in center (cell body) compartment and axons grow beneath a Teflon divider into side (distal axons) compartments. Image of prepared culture with media.

B. Schematic of microfluidic culture (Taylor et.al. 2003) with DRG neuron cell bodies in left compartment and axons extending into right compartment. Image of assembled culture on glass coverslip and filled with media. See Appendix I for additional information about set up and use of both cultures systems.

motors to the cell body in response to neurotrophin stimulation of axon terminals (Tsui-Pierchala and Ginty, 1999; Watson et al., 1999; Heerssen et al., 2004).

In addition to immediate early gene induction (Ginty et al., 1994; Watson et al., 1999), a unique transcriptional program is activated upon this retrograde signaling endosome reaching the cell body of DRG neurons (Pazyra-Murphy et al., 2009). Specifically, *mef2d* and *bclw* have been identified as retrograde response-genes, genes that are selectively induced by neurotrophin stimulation of distal axons. Their induction is dependent on the transcription factor myocyte-specific enhancer factor 2D (MEF2D) and distinct extracellular signal-regulated kinase (ERK5)/MAPK pathway (Watson et al., 2001). Importantly, induction of Mef2d and Bclw is required to promote survival when neurons rely upon target-derived trophic factors, as absence of either gene results in increased apoptosis (Pazyra-Murphy et al., 2009).

Axon survival and degeneration

Maintaining healthy axons during development and throughout life requires a diverse set of mechanisms. Axon viability depends greatly on dynamic and healthy mitochondria for energy production and an intact cytoskeleton for transport of protein, mRNA, and organelles. External cues such as neurotrophins promote the survival of axons during development and support their health into adulthood. Axon degeneration is also a critical component of development used to refine and prune neural connections, while pathological axon degeneration can be caused by injury, disease and other stressors.

Understanding the balance of factors that promote axon survival or degeneration will lead to better therapeutics for trauma and neurodegenerative disease.

Recent evidence suggests that axons use specialized pathways for mediating survival and degeneration (Yan et al., 2010; Neukomm and Freeman, 2014; Pease and Segal, 2014). Local neurotrophin withdrawal from axons activates a caspase cascade and axon degeneration, that is blocked by loss of the proapoptotic regulator Bax (Nikolaev et al., 2009; Simon et al., 2012). Activation of caspase-9 results in cleavage and activation of caspase-3, which directly activates caspase-6 in axons to cause local degeneration (Fig. 1.3)(Simon et al., 2012). Interestingly, while binding of cytochrome C to apoptotic protease activating factor 1 (Apaf1) activates caspase-9 in cell bodies, Apaf1 may not be required for axonal degeneration (Cusack et al., 2013). Thus, overlapping but distinct mechanisms for regulating degeneration exist within cell bodies and axons.

Target derived-neurotrophins promote Bclw expression, and genetic studies *in vivo* demonstrate that loss of Bclw leads to a progressive degeneration of sensory axons (Pazyra-Murphy et al., 2009; Courchesne et al., 2011). Bclw is expressed during late embryonic and early postnatal development, as sensory axons are innervating peripheral targets, and remains at high levels throughout adulthood. Interestingly, Bclw is the prosurvival Bcl2 family member that is both expressed highly within axons and regulated by neurotrophins (Courchesne et al., 2011), suggesting that Bclw is a prosurvival factor specialized for axonal maintenance. The Bcl2 family of proteins is made up of both anti-apoptotic and pro-apoptotic factors that are tightly controlled to regulate caspase-

Figure 1.3

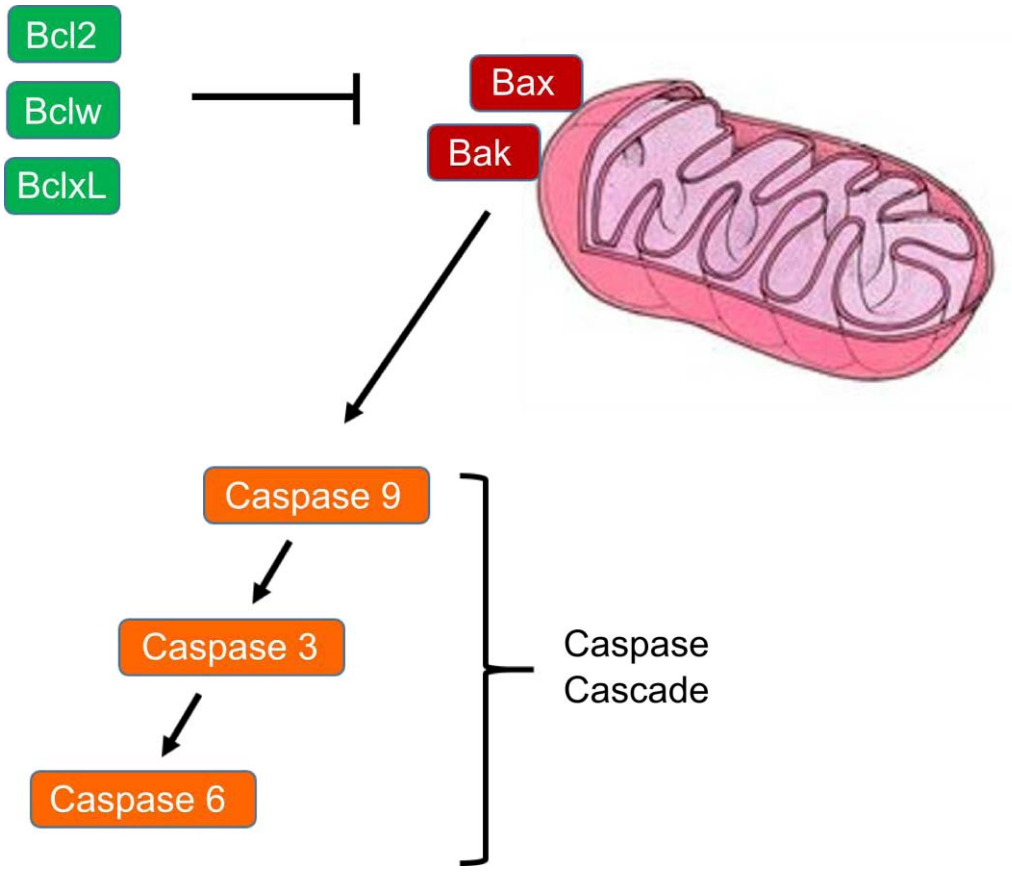


Figure 1.3. Bcl2 family proteins and model for regulation of axon degeneration. Bcl2 pro-survival factors (green) function by binding and inhibiting Bcl2 pro-apoptotic factors (red) at mitochondria to promote survival. Activation of pro-apoptotic factors within axons leaves to activation of an axonal caspase cascade that results in axon degeneration (Simon et al. 2012, Cusack et.al. 2013).

dependent apoptosis (Fig. 1.3) (Gross et al., 1999; Brunelle and Letai, 2009). The anti-apoptotic family members Bcl2, BclxL, and Bclw localize to mitochondria (Kaufmann et al., 2004) where they sequester pro-apoptotic factors that promote cytochrome C release and caspase activation. The studies presented in this thesis aim to understand how neurotrophins regulate the axonal survival factor Bclw and how Bclw supports the health of sensory axons.

In response to injury, axons undergo a very distinctive and stereotyped form of degeneration called Wallerian degeneration with massive fragmentation and clearing of the axonal debris. The Wld^S (Wallerian degeneration slow) mutation produces a chimeric protein consisting of the N-terminal of Ube4b (E4 ubiquitin ligase) and the NAD^+ biosynthesis enzyme Nmnat1 (nicotinamide mononucleotide adenylyltransferase 1) that protects severed axons, likely by acting within axons themselves (Babetto et al., 2010). Studies of Wld function highlight that healthy metabolism and energy production are critically important for supporting axon viability.

Axons have high metabolic demands and are therefore filled with dynamic mitochondria, and disruptions in mitochondrial transport and quality control make axons vulnerable to degeneration (Schwarz, 2013). Unsurprisingly, mutations in various mitochondrial proteins result in neurodegenerative diseases of axons, including Parkinson's disease and Charcot-Marie-Tooth disease (Court and Coleman, 2012). Interestingly, loss of Bclw results in longer axonal mitochondria and reduced ATP levels, indicating that the neurons are energetically compromised (Courchesne et al., 2011). A second component studied

here, the nuclear matrix protein LaminB2, also localizes to axonal mitochondria and following depletion, mitochondria are longer and exhibit reduced membrane potential (Yoon et al., 2012). Thus, Bclw and LaminB2 are both required for axonal maintenance and appear to be critical for mitochondrial health and size.

mRNA localization

The classical notion that all protein is synthesized and then delivered to required sites within a cell has been challenged in recent years as subcellular localization of mRNA has been found to be prevalent across a vast array of cell types and species (Holt and Bullock, 2009). In the *Drosophila* embryo, 70% of expressed mRNAs are found to be asymmetrically distributed using high-resolution in situ hybridization (Lecuyer et al., 2007) and localization of several of these mRNAs is important for establishing a patterning within the developing embryo (Johnstone and Lasko, 2001). Numerous localized mRNAs have also been visualized and studied within yeast, *Xenopus* oocyte, fibroblasts, and neurons.

Subcellular mRNA localization provides an efficient mechanism for extremely precise spatial and temporal control of gene expression (Martin and Ephrussi, 2009). Firstly, it is efficient because single localized mRNAs can give rise to many protein copies for local function. Secondly, mRNA localization allows for regulated translation in response to local cues. Finally, localized mRNA enables generation of spatially restricted proteins that could be functionally unique compared to those proteins synthesized with a different subcellular domain.

It is obvious that mRNA localization would be particularly advantageous in highly polarized neurons where the cell body can be quite distant from synapses and nerve terminals. High-throughput assays in neurons have revealed that thousands of mRNAs are localized to axons and dendrites (Zivraj et al., 2010; Cajigas et al., 2012). It is now widely accepted that mRNA localization in the developing and adult nervous system is required for numerous neuronal processes. In the adult brain mRNA localization is critical for synaptic plasticity, and neuronal activity promotes dendritic mRNA localization (Martin and Zukin, 2006; Wang et al., 2009; Farris et al., 2014). During development, mRNA localization in axons is required for axon growth and guidance (Wu et al., 2005; Leung et al., 2006), as well as health and maintenance (Yoon et al., 2012). Selective axonal localization of mRNAs is regulated by extracellular stimuli such as growth factors and guidance cues (Willis et al., 2007; Andreassi et al., 2010). Localized mRNAs are also required for injury response and regeneration (Zheng et al., 2001; Verma et al., 2005; Vogelaar et al., 2009; Yoo et al., 2009; Perry et al., 2012). Large-scale analysis of purified axons has identified mRNAs that encode protein within a variety of functional classes including cytoskeletal components, translational machinery, transport & signaling molecules, and nuclear-encoded mitochondrial components (Taylor et al., 2009; Vogelaar et al., 2009; Andreassi et al., 2010; Zivraj et al., 2010; Gumy et al., 2011; Cajigas et al., 2012; Minis et al., 2014). The composition of axonal mRNAs is highly dynamic and changes across development and adulthood.

How is mRNA localized?

Subcellular localization of mRNA depends upon *cis*-acting sequences called localization elements that are commonly found within the untranslated regions (UTRs) of mature mRNAs but can also found within the coding region itself. Both the primary sequence and secondary structure (Li et al., 2010; Laver et al., 2013; Jambor et al., 2014) of a localization element can be important for targeting an mRNA to a particular subcellular domain. Localization elements are recognized and bound by specific *trans*-acting factors, RNA-binding proteins (RNA-BPs) (Fig. 1.4). Following nuclear export, these messenger ribonucleoprotein complexes (mRNPs), containing both mRNA and interacting RNA-BPs, are assembled into cytoplasmic granules and localized by microtubule- or actin-dependent transport to sites for translation (Martin and Ephrussi, 2009; Xing and Bassell, 2013).

Localized mRNAs in axons or dendrites are usually targeted by sequences within their 3'UTRs (Zhang et al., 2001; Andreassi and Riccio, 2009; Andreassi et al., 2010; Aschrafi et al., 2010; Vuppalanchi et al., 2010; Perry et al., 2012; Baraban et al., 2013; Merianda and Twiss, 2013). The localization element responsible for *β-actin* mRNA targeting was identified by Singer and colleagues as the 54-nucleotide "zipcode" sequence within its 3'UTR (Kislauskis et al., 1994) and neurotrophins regulate localization of *β-actin* in axons (Zhang et al., 1999; Willis et al., 2007), whereas the mRNA for isoform γ-actin is restricted to the soma (Bassell et al., 1998). There is some evidence that longer 3'UTRs target more distally within neurons (An et al., 2008; Andreassi et al., 2010) and alternative polyadenylation sites can give rise to differentially

Figure 1.4

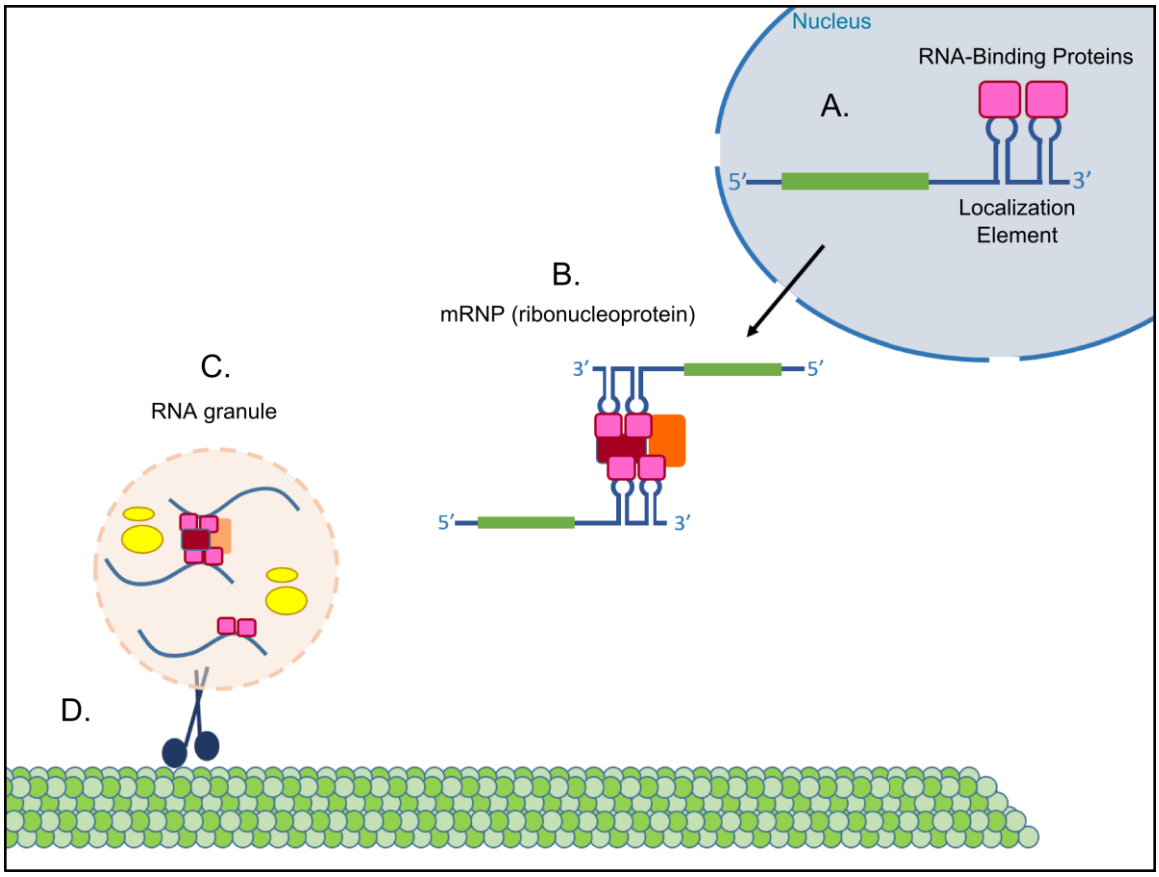


Figure 1.4. mRNA localization is a multistep process.

A. mRNA containing *cis*-acting localization elements is transcribed and spliced (ORF in green, UTRs in blue) in the nucleus. RNA-BPs bind the mRNA to form a messenger ribonucleoprotein (mRNP) complex. **B**. Following nuclear export, mRNPs are remodeled to include additional proteins and can form oligomers with other mRNPs in the cytoplasm. **C**. mRNPs are assembled into non-membrane bound RNA granules, which may contain other mRNPs and translation machinery, such as ribosomal subunits (yellow). **D**. RNA granules associate with molecular motors for transport along cytoskeleton to their final destination. (Martin and Ephrussi, 2009)

localized mRNA isoforms (Epstein et al., 2014). Unfortunately, little sequence homology among identified axonal localization elements has been found, leading to the idea that many distinct regulatory mechanisms exists for localizing particular mRNAs to axons.

To understand how specific mRNAs are localized, many studies have sought to identify the RNA-BPs which bind localization elements required for post-transcriptional mRNA regulation. The zipcode binding protein (ZBP1) binds the *β-actin* mRNA zipcode (Ross et al., 1997) and mediates neurotrophin-dependent localization of *β-actin* mRNA in axons (Zhang et al., 2001). While regulation of *β-actin* mRNA has been relatively well-characterized, our knowledge of other RNA-BPs that mediate mRNA localization in neurons remains quite limited, partly due to challenges in identifying their mRNA binding sites. Recently, new tools have been developed to identify binding sites within mRNAs precipitated by specific RNA-BPs (Licatalosi et al., 2008; Hafner et al., 2010). Use of these tools generate preferred binding motifs and lists of hundreds or even thousands of putative mRNA targets (Darnell et al., 2011; Ascano et al., 2012) that require additional study to validate and understand the functional role for the RNA-BP in the cell.

RNA-BPs have diverse functions throughout the lifetime of an mRNA, as nuclear mRNA processing splicing events are tightly coupled to cytoplasmic localization and translational regulation (Moore and Proudfoot, 2009). Splicing factors have been implicated in nuclear export and cytoplasmic localization of mRNA (Le Hir et al., 2001; Hachet and Ephrussi, 2004) and RNA-BPs required for mRNA transport often also

regulate translation of localized mRNA (Huttelmaier et al., 2005). RNA BPs can also coordinate post-transcriptional regulation of multiple functionally related mRNAs for a desired cellular response (Keene, 2007). RNA-BPs and other factors with roles in mRNA processing, localization and translation are implicated in a number of neuronal diseases (Lyons et al., 2009; Hirokawa et al., 2010; Tolino et al., 2012; Dix et al., 2013). Thus, post-transcriptional mRNA regulation appears more complex than previously appreciated and continued study is critical for identifying these mechanisms and for understanding disease.

Local protein synthesis

Localized protein synthesis is now recognized as a common method for spatially regulating gene expression to establish cell polarity or sequestered protein activity. For protein synthesis to occur in neurites they must contain the required translational machinery. Steward and Levy first reported the presence of ribosomes beneath dendritic spines and suggested the idea that mRNAs could be localized and translated in response to cues at the synapse (Steward and Levy, 1982). In axons, ribosomes and active polyribosomes are present in within the squid giant axon (Giuditta et al., 1991) (Sotelo et al., 1999; Bleher and Martin, 2001) and electron microscopy shows ribosomes within myelinated peripheral axons (Tennyson, 1970). Using immunostaining for ribosomal protein and RNA, clusters of axonal ribosomes have been identified along the periphery of the axoplasm termed “peri-axoplasmic ribosomal plaques” (Koenig and Martin, 1996; Koenig et al., 2000) and have been observed to contain mRNA and kinesin motors

(Sotelo-Silveira et al., 2004; Sotelo et al., 2014). It is this localization close to the membrane that is partially thought to explain why ribosomes had been observed so infrequently within axons until more recently (Holt and Schuman, 2013). It has even been suggested that ribosomes are transferred from neighboring glia into axons (Court et al., 2008; Twiss and Fainzilber, 2009; Court et al., 2011). Interestingly, localization and translation of mRNAs for transmembrane and secreted proteins have been detected within axons (Spencer et al., 2000; Brittis et al., 2002; Willis et al., 2007) and studies suggest that there is also a form of ER and Goli present in axons that may be capable of protein targeting and secretion (Merianda et al., 2009). Finally, axons contain translation initiation factors that can be activated by cues that promote local translation (Campbell and Holt, 2001), as well as the mRNA for initiations factors that are locally translated in axons (Kar et al., 2013). Thus, axons contain the required machinery to support translation of a variety of mRNAs within axons.

It is now appreciated that local translation within axons serves a diversity of functions including of axon growth, guidance, and maintenance, synapse formation and regeneration (Deglincerti and Jaffrey, 2012). The first functional evidence for axonal translation showed that growth cone responses to guidance cues is prevented by application of translation inhibitors (Campbell and Holt, 2001). Since then is has been demonstrated that translation of *β-actin* and *rhoA* mRNA within growth cones mediates responses to growth promoting and inhibitory cues (Zhang et al., 1999; Wu et al., 2005; Leung et al., 2006). During mRNP transport, RNA-BPs translationally repress mRNA until they reach desired sites where local cues can stimulate mRNA release and

translation (Krichevsky and Kosik, 2001; Besse and Ephrussi, 2008). In addition, there is substantial evidence that axons contain miRNAs for regulation of axonal mRNAs (Aschrafi et al., 2008; Natera-Naranjo et al., 2010; Aschrafi et al., 2012; Hancock et al., 2013; Kaplan et al., 2013), thus numerous mechanisms exist by which the local proteome can be altered.

Metabolic labeling has been widely used to demonstrate local synthesis of protein and to identify the locally synthesized proteome of axons. A challenge to studying local protein synthesis is that the large excess of protein is synthesized within neuronal cell bodies can confound the analysis. To avoid this, many studies use metabolic labeling methods within axons that have been severed from their cell bodies, although this causes significant injury. More recently, the use of cultures where cell bodies and axons are contained within fluidically isolated compartments (Eng et al., 1999; Taylor et al., 2010) has been extremely useful and convincing for metabolic analysis. Photo-convertible fluorescent reporters flanked by UTRs that contain important regulatory elements are also used to investigate local synthesis by observing the appearance of new signal following conversion of total signal within axons or growth cones. Another creative way to identify locally translated mRNAs is to precipitate axonal ribosomes and collect the associated mRNAs, which has been used successfully in vivo (Heiman et al., 2008; Sanz et al., 2009; Yoon et al., 2012).

Local synthesis of several proteins within axons is shown to function in axonal health and maintenance. Neurotrophins promote localization and translation of *Impa1* in sympathetic

axons, and selective silencing of localized *Impa1* mRNA causes axon degeneration (Andreassi et al., 2010). LaminB2 is locally synthesized within retinal ganglion cell axons, and loss of axonal *laminb2* mRNA in vivo results in degeneration of axons innervating the optic tectum (Yoon et al., 2012). As previously mentioned, loss of LaminB2 interferes with mitochondria function, suggesting that protein synthesis within axons maybe particularly important for supporting local mitochondria. In fact, numerous nuclear encoded mRNAs mitochondrial components are localized to axons, including CoxIV and ATP synthase, and local protein synthesis is required for mitochondrial function and axon viability (Hillefors et al., 2007; Aschrafi et al., 2010; Natera-Naranjo et al., 2012; Kar et al., 2014). Taken together, this suggests that intra-axonal translation is critical for local energy metabolism, which is required to maintain healthy axons.

Somewhat surprisingly, proteins with known nuclear roles are also locally synthesized within axons. Local synthesis of the transcription factor CREB within sensory axons is required for neurotrophin-dependent neuronal survival (Cox et al., 2008). These studies report that CREB is transported retrogradely from the synapse to the cell body to promote nuclear accumulation of pCREB and activation of transcription. It is intriguing to imagine that a locally synthesized TF could be made to appear unique to the cell body and activate of a distinct cellular response, although future work is required to address these questions. In addition to CREB, there is compelling evidence that retrograde transport of locally synthesized STAT3 and Importin is required for response to axon injury (Ben-Yaakov et al., 2012; Perry et al., 2012).

The studies presented within this thesis identify and describe mechanisms by which target-derived neurotrophins promote health of peripheral axons. We find that neurotrophins promote spatial regulation of mRNA from the nucleus to the axon to mediate protection of axons. In response to target-derived neurotrophins, *bclw* mRNA is transcribed and localized to sensory axons, where it is locally translated and functions to prevent axon degeneration. We identify an RNA-BP, SFPQ, which is required for trafficking of *bclw* mRNA, as well as another localized mRNA for axon survival. To study local translation of *bclw* mRNA and investigate its functional significance, we describe development of a novel tool for labeling locally synthesized protein in living cells. Together, this work suggests that multistep regulation of *bclw* mRNA and other axonal survival factors for translation locally within axons is a critical mechanism for neurotrophin-dependent axon survival and maintenance.

Chapter 2:

Target-derived Neurotrophins Coordinate Transcription and Transport of Bclw to Prevent Axonal Degeneration

Publication:
Cosker KE, Pazyra-Murphy MF, Fenstermacher SJ, Segal RA. Target-derived neurotrophins coordinate transcription and transport of Bclw to prevent axonal degeneration. *J. Neuroscience*. 2013; 33(12):5195-5207.

Contributions:
Katharina E. Cosker wrote and prepared manuscript and generated data for all Figures.
Maria Pazyra-Murphy generated data for Figures 2.6-2.9.
Sara J. Fenstermacher established microfluidic culture protocol, generated cultures for Figure 2.1, and generated data for Figure 2.6.
Rosalind A. Segal wrote and edited manuscript with K.E.C.

Acknowledgements:
This work was supported by National Institute of Health (NIH) Grant R01 NS050674 (R.A.S.) and Grant F31NS077620 (S.J.F). K.E.C. is a David Mahoney Fellow at Harvard Medical School and a recipient of a Neurodegenerative Disease Pilot Study Grant from MGH Neurology Clinical Trials Unit and Harvard NeuroDiscovery Center. S.J.F. is a Victoria Quan Fellow at Harvard Medical School. Confocal microscopy imaging was performed at the Harvard NeuroDiscovery Center Optical Imaging Program. Thanks to Stephanie Courchesne for help in generating *in vivo* samples, Jose Alfaro and Emily Chadwick for assistance with *bclw*$^{-/-}$ mice and Eugene Vaios for assistance with designing probes for ISH.

Abstract

Establishment of neuronal circuitry depends on both formation and refinement of neural connections. During this process, target-derived neurotrophins regulate both transcription and translation to enable selective axon survival or elimination. However, it is not known whether retrograde signaling pathways that control transcription are coordinated with neurotrophin-regulated actions that transpire in the axon. Here we report that target-derived neurotrophins coordinate transcription of the anti-apoptotic gene *bclw* with transport of *bclw* mRNA to the axon, and thereby prevent axonal degeneration in rat and mouse sensory neurons. We show that neurotrophin stimulation of nerve terminals elicits new *bclw* transcripts that are immediately transported to the axons and translated into protein. Bclw interacts with Bax and suppresses the caspase6 apoptotic cascade that fosters axonal degeneration. The scope of *bclw* regulation at the levels of transcription, transport and translation provides a mechanism whereby sustained neurotrophin stimulation can be integrated over time, so that axonal survival is restricted to neurons connected within a stable circuit.

Introduction

Formation of a working sensory nervous system relies on axonal guidance for innervation of appropriate targets and on the selective survival of neurons that are integrated into functional circuits. These critical events are regulated by neurotrophins both during development and into adulthood (Segal, 2003; Skaper, 2011).Target-derived neurotrophins initiate retrograde signaling cascades that induce transcriptional changes enabling neuronal survival (Cosker et al., 2008; Pazyra-Murphy et al., 2009; Chowdary et al., 2011), while lack of neurotrophin support prevents outgrowth, triggers local axonal degeneration and eventually causes neuronal apoptosis (Luo and O'Leary, 2005). It is currently not known whether neurons are able to coordinate transcriptional events in the cell body with localized mechanisms that function within the axons to promote axon outgrowth and survival pathways.

During axonal outgrowth, neurotrophins regulate subcellular localization and translation of specific mRNAs (Yoon et al., 2008). The most extensively characterized of these is *β-actin* mRNA, which is both targeted to axons and locally translated there in response to neurotrophins to facilitate growth cone motility and neurotrophin-dependent axon guidance (Zhang et al., 1999; Zhang et al., 2001; Willis et al., 2005; Yao et al., 2006; Willis et al., 2007). It has been proposed that local protein synthesis of *β-actin* allows axonal turning responses to occur rapidly and independently of the remote cell body.

More recently it has been reported that mRNA localization and local translation may also play a role in neurotrophin-dependent axonal survival. One such mRNA is *Impa1*, which

is targeted to sympathetic neuron axons and locally translated in response to nerve growth factor (NGF) stimulation of distal axons (Andreassi et al., 2010). The *Impa1* gene product is critical for synthesizing membrane lipids and thereby promotes axonal maintenance. Similarly, local synthesis of Lamin B2 is critical for axon maintenance in *Xenopus* retinal ganglion cell axons (Yoon et al., 2012). It is currently unknown if local protein synthesis enables axonal survival to be regulated independently of the cell body, or whether neurotrophins coordinate survival pathways in the cell body and axons.

Bcl2 family members are critical determinants of neurotrophin survival responses. In sensory neurons, Bclw [Bcl2l2] is the only anti-apoptotic Bcl2 family member selectively regulated by target-derived neurotrophins (Pazyra-Murphy et al., 2009). Furthermore, Bclw is the Bcl2 family member that exhibits substantial protein expression in axons and prevents axonal degeneration *in vivo* (Courchesne et al., 2011). Here we show that mRNA encoding *bclw* is present in peripheral axons as well as in cell bodies of sensory neurons, and neurotrophin stimulation of distal axons increases levels of *bclw* mRNAs in both locations. Our studies indicate that a retrograde neurotrophin signaling cascade leads to transcription of *bclw* mRNA, which is immediately transported back to the stimulated axons. There, *bclw* is translated in response to continuous neurotrophin exposure and thereby suppresses axon degeneration mediated by Bax and caspase6. Thus target-derived neurotrophins coordinate transcription, mRNA transport and translation in space and time to promote neuronal survival of cell bodies and axons.

Materials and Methods

Microfluidic chambers. Cover glass (Fisherband Microscope Cover Glass; 24x40-1) was coated with 0.25 mg/ml Poly-d-lysine in HBSS overnight at room temperature. After washing with ddH_20, the microfluidic chamber (Xona Microfluidics) was attached to the cover glass. Laminin (10 μg/ml) was added to each channel at 37 °C for 3 h. DRGs from embryonic day 15 (E15) rats of either sex were dissected, trypsinized and plated directly into the channel at 100,000 cells/microfluidic chamber in 5 μl of media (DMEM with 5% horse serum, 1% penicillin-streptomycin and 0.3 μM cytosine arabinoside (AraC) and 50 ng/ml NGF+BDNF (Peprotech). After allowing cells to attach for 10 min, 100 μl of media with 50 ng/ml NGF+BDNF was added to cell body wells, and 100 μl of media with 100 ng/ml NGF+BDNF to axonal wells. Media was changed daily, removing AraC, and cells were fixed after 4 d in culture with ice cold MeOH for 2 min and 4% PFA for 20 min.

Fluorescent in situ hybridization. DRGs from E15 rats of either sex were cultured in microfluidic chambers as described above. Cells were permeabilized in 0.5% Triton X-100 for 10 min and refixed in 4% PFA for 5 min. Cells were acetylated in 0.25% acetic anhydride in 0.1M TEA for 10 min and washed in PBS. Neurons were pre-hybridized for 1 h, before being incubated in 1 μg/ml probe at a final concentration of 4% Dextran Sulfate overnight at 65 °C. Cultures were washed in 0.2X SSC at 65 °C, cooled down and equilibrated in TNT buffer (100mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween) for 5 min. Cells were incubated in blocking buffer (100mM Tris pH 7.5, 150 mM NaCl, 0.5% blocking reagent) for 1 h and anti-DIG-POD (1:500) and mouse anti-Tuj1 (1:400; Covance) in blocking buffer overnight at 4 °C. Cultures were washed with TNT buffer

and incubated in biotin-tyramide (1:50; PerkinElmer) in amplification plus solution for 8 min. After washing in TNT, cells were incubated with 546-Strepdavidin and goat anti-mouse 488 in blocking buffer for 1 h at room temperature. Cells were washed with TNT, and mounted. Images were obtained using NIS Imaging software and a 60X oil objective.

In situ hybridization. Probes for *bclw, β-actin* and *γ-actin* were amplified with gene-specific sets of PCR primers and cDNA templates prepared from P0 rat brain. Digoxigenin (DIG)-labeled riboprobes were generated with DIG-RNA labeling mixture (Roche) and T7 RNA polymerase. Whole DRG with central and peripheral nerves were dissected from P0 rats of either sex and fixed overnight in 4% PFA in DEPC PBS. After fixation, embryos were dehydrated stepwise into 100% MeOH. Before hybridization, DRGs were rehydrated, refixed and prehybridized in hybridization buffer for 1 h at 65 °C. Prehybridization buffer was replaced with hybridization buffer containing 1.5 ug/ml probe and hybridized overnight at 65 °C. After washes, DRGs were blocked in TBST+10% lamb serum for 2 h at room temperature, then incubated in TBST+10% lamb serum containing 1/2000 dilution of alkaline phosphatase-conjuaged sheep anti-digoxigenin Fab fragments overnight at 4 °C. After washing in TBST, and alkaline phosphatase buffer, DRGs were incubated in BM Purple (BMB/Roche) until signal was detected. The reaction was stopped by washing in PBST/5mM EDTA, DRGs were postfixed in 4% PFA for 24 h, washed and mounted.

Cell cultures. Compartmented chamber cultures (Campenot cultures) were prepared as previously described (Heerssen et al., 2004) (Pazyra-Murphy and Segal, 2008). Briefly, DRGs from embryonic day 15 (E15) rats or E13 *bclw*$^{+/+}$ and *bclw*$^{-/-}$ mice of either sex were dissected, trypsinized and plated in the center compartment of a Teflon divider

(Camp10; Tyler Research) (Campenot, 1982). Cultures were maintained in media consisting of DMEM with 5% horse serum, 1% penicillin-streptomycin and 0.3 μM cytosine arabinoside (AraC) at 37°C, 7.5% CO_2; BDNF+NGF (Peprotech) were added to the cell body compartment at a concentration of 10 ng/ml and to the axon compartment at a concentration of 100 ng/ml for 2 d. On day 3, media was replaced and the AraC was omitted. On day 5, neurotrophins were removed from the cell body compartment and reduced to 1 ng/ml in axon compartments for 3-7 d. For each experiment, neurons were starved for 2 h in serum and neurotrophin-free media, and then vehicle control (100 ng/ml BSA in PBS) or neurotrophins (100 ng/ml BDNF+NGF) were applied to distal axons or cell body compartments for the indicated time. Mass cultures consisting of 3 x 10^5 DRG neurons were grown on laminin-coated p35 cultures dishes (Nunc) in neurotrophin-enriched (100 ng/ml NGF+BDNF) media with 0.3 μg/ml AraC.

Quantitative reverse transcription-PCR. RNA was extracted from DRG neurons in compartmented chamber cultures using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using the cDNA archive kit (Applied Biosystems) according to the manufacturer's specifications. Quantitative real-time RT-PCR was performed using Taqman Gene expression assays (Applied Biosystems) to assess the expression of *c-fos* (Rn02105452_s1), *bclw* (Rn00821025_g1), *β-actin* (Rn00667869_m1), and *γ-actin* (Rn01470738_g1). For each sample, data was normalized to the expression level of *gapdh* (Applied Biosystems).

Western blotting. For analysis of Bclw protein in DRG cell bodies and distal axons, E15 rat DRGs were grown in compartmented chamber cultures as described above. Cells and axons were lysed in non-ionic detergent and protein lysates were separated by 4-12%

SDS-PAGE and blotted with the following antibodies: anti-pTrk (1:1000; pY-490) (Segal et al., 1996), anti-phospho Erk1/2 (1:1000; Cell Signaling), anti-pan-actin (1:1000; Cell Signaling), anti-Bclw (1:200; Stressgen), anti-His (1:1000; Novagen), anti-Bax (1:1000; Cell Signaling) and anti-GAPDH (1:1000; Cell Signaling). Bands were visualized with secondary antibodies conjugated to HRP (1:10,000; Bio-Rad) and SuperSignal chemiluminescent substrate kit. Using NIH ImageJ software, protein levels were quantified and levels of protein were normalized to GAPDH.

mRNA stability. E15 rat DRG neurons were grown in mass cultures as described. After 2d in culture, 4-thiouridine nucleoside (20 μM; Sigma) was added to cultures for 24h for mRNA labeling. Total RNA was extracted at 0 h, 4 h and 8 h after 4-thiouridine removal. Total RNA (10 μl) was biotinylated with Biotin-HPDP for 3 h at room temperature in the dark. Labeled RNA was precipitated and washed with 75% EtOH. Biotinylated mRNA was purified using Dynabeads MyOne Streptavidin C1 (Invitrogen), blocked with 10 mg/ml yeast tRNA for 1 h at room temperature. Biotinylated RNA was added to the beads (10 μl RNA to 100 μl beads) and incubated for 15 min at room temperature. Beads were collected, washed 3 times with MPG buffer (1M NaCl, 10 mM EDTA, and 100 mM Tris-HCl, pH 7.4, in RNase-free H_20) and bound RNA was eluted by addition of 5% BME. The eluted thiolated RNA was precipitated with 2-propanol, spun, washed with 75% EtOH, spun, and resuspended in 20 μl RNase-free H_2O. Reverse transcription was performed using the cDNA archive kit (Applied Biosystems), according to manufacturer's specifications for analysis by quantitative RT-PCR.

Drug treatment. Distal axons and cell bodies of DRG neurons grown in compartmented chamber cultures were treated either with 200 nM K252A (Calbiochem), 10 μM U0126

(Calbiochem), 20 μM actinomycin D, 10 μg/ml cycloheximide (Calbiochem), 10 μM anisomycin (Sigma), 500nM ABT-263 (Selleck Chemicals) or DMSO vehicle control. Drugs were applied 10 min before neurotrophin stimulation or throughout the duration of neurotrophin deprivation.

4-thiouridine labeling. E15 rat DRGs were grown in compartmented chamber cultures as described. For pulse-labeling experiments, 4-thiouridine nucleoside (20 μM; Sigma) was added to cell body compartments during 2 h of distal axon NT stimulation, and then removed for an additional 2 h of NT stimulation. For control experiments, 4-thiouridine nucleoside was added to cell body compartments 24 h before NT stimulation, during which time it was removed. After NT stimulation, total RNA was extracted from cell bodies and distal axons using Trizol (Invitrogen). Total RNA (10 μl) was biotinylated and precipitated as described. A 3 μl RNA sample was removed and reverse transcription was performed using the cDNA archive kit (Applied Biosystems) to check for neurotrophin induction of total-biotinylated mRNA by quantitative RT-PCR. Total biotinylated mRNA was captured on Dynabeads MyOne Streptavidin C1 (Invitrogen) and purified 4-thiouridine biotinylated RNA was eluted as described. The eluted thiolated RNA was precipitated with 2-propanol, spun, washed with 75% EtOH, spun, and resuspended in 20 μl RNase-free H_2O. Reverse transcription was performed using the cDNA archive kit (Applied Biosystems), according to manufacturer's specifications for analysis by quantitative RT-PCR.

His pulldown of recombinant Bclw protein. E15 rat DRGs were grown in mass cultures as described. After 2 d in culture, Recombinant His-tagged Bclw protein was introduced using Chariot reagent. Cells were lysed in non-ionic detergent and transferred onto

Dynabeads for His-Tag Isolation & Pulldown (Life Technologies) according to manufacturer's instructions. Input and pulldown were analysed by western blotting.

Axonal degeneration assay. E15 rat DRGs were grown in compartmented chamber cultures. After 6 d in culture, the cell body compartment was maintained in 10 ng/ml NGF+BDNF, and the axonal compartment was starved in DMEM alone for 10 h before fixation and staining. Compartmented chamber cultures were fixed in ice cold MeOH for 2 min and 4% PFA for 20 min, blocked in 2% BSA and 0.5% Triton X-100 for 1 h at room temperature, incubated with mouse anti-Tuj1 (1:400, Covance) overnight at 4 °C and then with goat anti-mouse Alexa Fluor 488 (1:1000; Invitrogen) for 1 h at room temperature, and counterstained with DAPI. Images of distal axons were obtained using a 40X objective and NIS Elements software. To quantify axonal degeneration, we used the method described by Sasaki et al. (2009). Images were binarized so that axonal areas were converted to black and background areas were converted to white. To detect fragmented (degenerating) axons, the particle analyzer function of NIH ImageJ software was used to determine the area of the fragments (size, 5-1000 pixels). The degeneration index was then calculated as the ratio of the area of fragmented axons over the total axon area.

Caspase6 Immunostaining. For *in vitro* analysis, compartmented chamber cultures were fixed in ice cold MeOH for 2 min and then in 4% PFA for 20 min. Cultures were blocked in 5% normal goat serum and 0.1% TritonX for 1 h. Cultures were incubated overnight at 4 °C in primary antibody (1:100 anti-Active/Cleaved Caspase6; Imogene, and 1:400 anti-Tuj1; Covance) followed by incubation in secondary antibody (1:1000 Alexa Fluor 546 and Alexa Fluor 488; Invitrogen) for 1 h at room temperature, and counterstained with

DAPI. Active caspase6 was quantified using Image J software, taking the average of the mean fluorescence within each axon as defined by Tuj1 staining.

Protein introduction. Recombinant His-tagged Bclw (R&D Systems) was introduced into E15 DRG sensory neurons using the Chariot protein transfection system (Active Motif). β-galactosidase protein was used as a control. Bclw or β-galactosidase (1 μg/μl) was introduced into mass cultures using 6 μl of Chariot or into the cell body or into distal axon compartments of compartmented chamber cultures by using 2 μl of Chariot reagent.

Footpad innervation. *bclw*$^{+/+}$ and *bclw*$^{-/-}$ animals of either sex were killed with isoflurane, and then footpad tissue from hindpaws was removed, fixed overnight in Zamboni's fixative at 4°C, and cyropreserved in 30% sucrose overnight at 4°C. Footpads were frozen, and 30 μm floating sections were prepared. Tissue sections were blocked in 10% normal goat serum and 0.1% Triton X-100 in PBS for 1 h at room temperature and then incubated in anti-tuj1 (1:400; Chemicon) or anti-substance P (1:100; R&D Systems) and anti-Active/Cleaved Caspase6 (1:100; Imgenex) overnight at 4°C. Sections were then incubated in goat anti-mouse Alexa Fluor 488 (1:200; Invitrogen) and goat anti-rabbit Alexa Fluor 568 (1:200; Invitrogen) and DAPI (1:1000) for 2 h at room temperature and mounted on gelatin-coated slides. Confocal images were obtained using a Carl Zeiss LSM 510 META upright confocal microscope, with a 40x oil objective. Caspase6 staining was quantified using Image J software, taking the average of the mean fluorescence per pixel within each z-stack of an axon outlined by Tuj1 or Substance P staining.

Statistics. Data are expressed as mean ± s.e.m. To assess statistical significance, data was analysed by unpaired two-tailed Student's *t*-test or by z test for a difference from 1. For

multiple comparisons, data was analysed by one-way ANOVA with post-hoc Bonferroni or Dunnett correction. Significance was placed at $p<0.05$ unless otherwise indicated.

Animal use. Timed pregnant rats were purchased from Charles River. *bclw*$^{-/-}$ mice were a generous gift from Grant MacGregor (University of California, Irvine, CA) (Ross et al., 1998). Genotyping for the wild-type *bclw* gene and/or *lacZ* gene were performed by Transnetyx using the Bclw targeting sequence GCTCTGAACCTCCCCATGACTTAAATCCGTTGCTCTTTCTTGGCCCTGCCCAG TGCCTCTGAGCATTTCACCTATCTCAGGAGC and the lacZ sequence CGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAATGAGTCAGGC CACG-G. All experimental procedures were done in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

Results

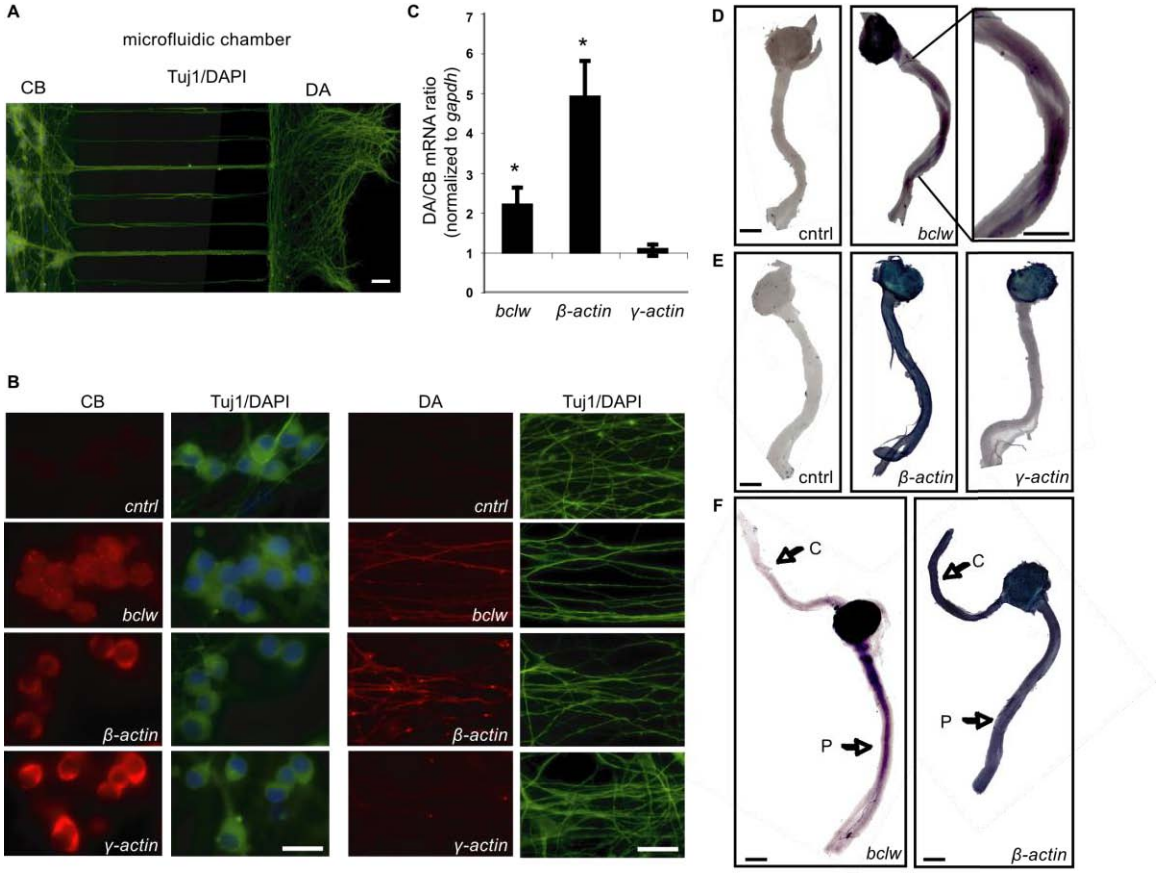
Target-derived neurotrophins regulate axonal levels of bclw mRNA

Bclw promotes neurotrophin-dependent maintenance of sensory nerve fibers (Courchesne et al., 2011). As Bclw protein is expressed in axons, we asked whether *bclw* mRNA is also localized to axons of sensory neurons. Fluorescent *in situ* hybridization (FISH) (Hafezparast et al., 2003) of cultured dorsal root ganglion (DRG) neurons in microfluidic chambers (Fig. 2.1*A*) demonstrates the presence of *bclw* transcripts in axons as well as cell bodies (Fig. 2.1*B*). This pattern is similar to *β-actin* mRNA, which is known to be localized to axons, and in contrast to *γ-actin* mRNA, which is restricted to cell bodies (Willis et al., 2007) (Fig. 2.1*B*). By quantitative RT-PCR from DRG neurons grown in

Figure 2.1. *Bclw* mRNA is localized in peripheral axons of sensory neurons.

A. Tuj1 immunostaining (green) and DAPI staining of E15 DRG neurons grown in microfluidic chambers. Scale bar 60 μm. **B**. FISH assay of *bclw*, *β-actin* and *γ-actin* mRNA (red) in cell body (CB) and distal axon (DA) compartments. Counterstained images show Tuj1 antibody (green) and DAPI. Scale bars 20 μm. **C**. mRNA from CB and DA of E15 DRG neurons grown in compartmented chamber cultures for *bclw, β-actin* and *γ-actin* analyzed by qRT-PCR. Data is presented as DA mRNA/CB mRNA ratio, normalized to *gapdh* mRNA. Statistical analysis by z-test, $*p<0.05$ for a difference from 1; n=7. **D**. ISH in P0 DRGs and peripheral nerves with anti-sense *bclw* and non-sense control probes. Scale bars 500 μm. **E**. ISH in P0 DRGs and peripheral nerve with anti-sense *β-actin* and *γ-actin* and non-sense control probes. Scale bar 500 μm. **F**. ISH in P0 DRGS and associated central (C) and peripheral (P) nerve with anti-sense *bclw* and *β-actin* probes. Scale bar 500 μm.

Figure 2.1 (continued)



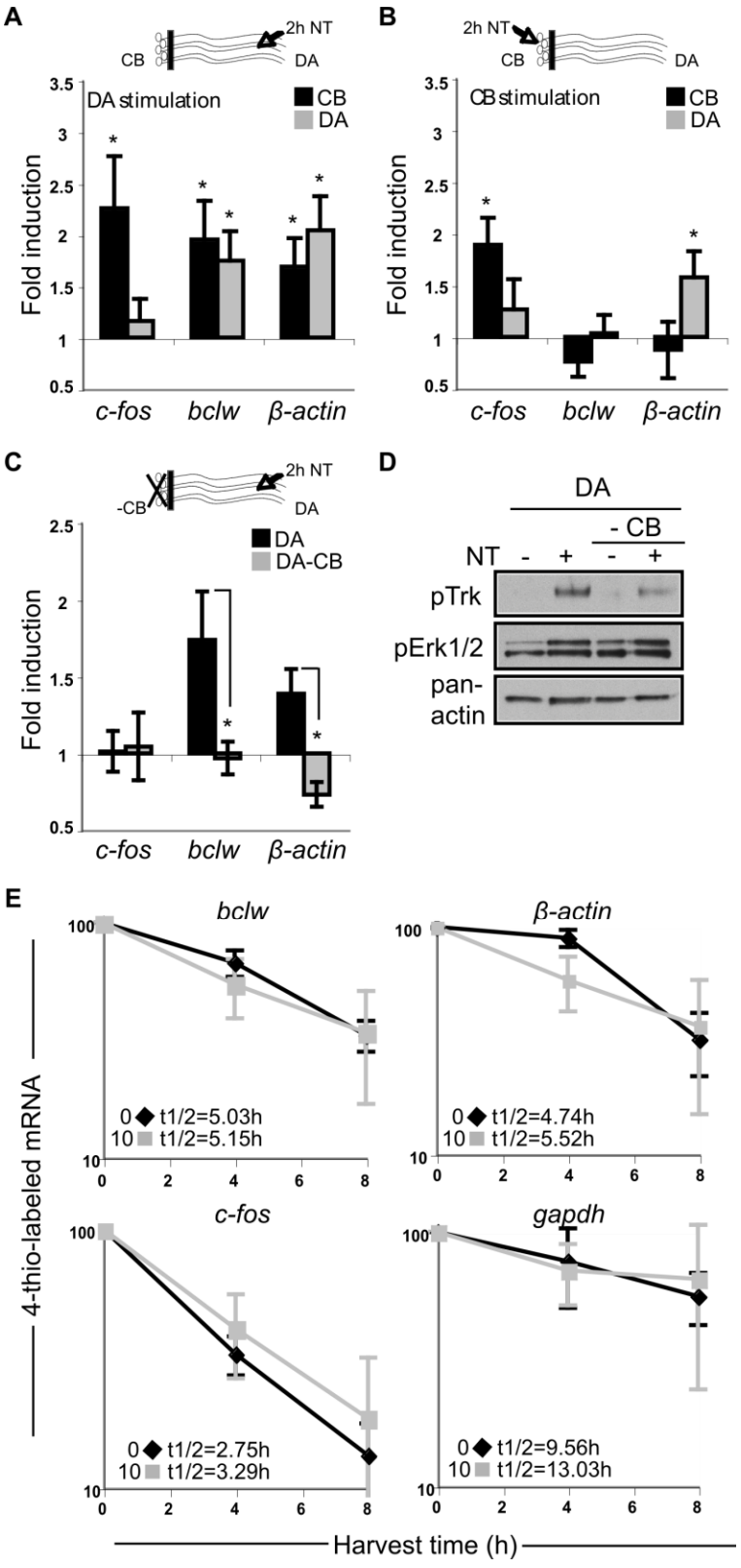
compartmented chamber cultures, *bclw* mRNA shows a relative enrichment in axonal compartments as compared to cell body compartments (Fig. 2.1*C*). Similarly, *in situ* hybridization in whole DRG and associated peripheral nerves demonstrates the presence of *bclw* and *β-actin* transcripts in cell bodies and in peripheral nerves, consistent with expression of *bclw* mRNA in axons (Fig. 2.1*D,E*). Together these data indicate that *bclw* mRNA, like *β-actin* mRNA, is localized to axons of developing sensory neurons. *In vivo*, the central and peripheral axons of DRG neurons can be clearly distinguished from one another. We find that *bclw* mRNA is clearly evident in cell bodies and peripheral nerves, but is barely detectable in the central projections (Fig. 2.1*F*). In contrast, *β-actin* mRNA shows a high level of expression in both central and peripheral nerves (Fig. 2.1*F*). This suggests that *in vivo, bclw* mRNA is primarily localized to peripherally extending axons that encounter target-derived neurotrophins.

As a retrograde response gene, *bclw* transcription is selectively induced in response to distal axon neurotrophin stimulation (Courchesne et al., 2011). Using compartmented chamber cultures, we find that selective distal axon neurotrophin stimulation for 2 h not only increases *bclw* levels in the cell bodies but also increases *bclw* mRNA levels in axon compartments (Fig. 2.2*A*). In contrast, *bclw* mRNA levels do not change in either cell bodies or distal axons in response to neurotrophin stimulation of cell bodies (Fig. 2.2*B*). In these experiments, the transcription factor *c-fos* serves as a critical control. Expression of *c-fos* is induced in cell bodies in response to neurotrophin stimulation of either cell bodies or distal axons, and so provides a measure of successful stimulation, while *c-fos* mRNA levels in axons are barely detectable and do not change with neurotrophin

Figure 2.2. Neurotrophins regulate *bclw* mRNA in cell bodies and distal axons.

A. Compartmented chamber cultures were stimulated with neurotrophins (NT; 100 ng/ml NGF+BDNF) at distal axons (DA) for 2 h. *Bclw* and *β-actin* mRNA is upregulated in cell bodies (CB) and distal axons (DA). Expression is compared with neurons treated with vehicle (100 ng/ml BSA). Fold induction of *c-fos* mRNA is a control. Statistical analysis by z-test, $*p<0.05$ for a difference from 1; n=8. **B.** Expression of *c-fos, bclw* and *β-actin* mRNA in CB and DA in response to 2 h NT stimulation of CB. Statistical analysis by z-test, $*p<0.05$ for a difference from 1; n=8. **C.** Severing of cell bodies during NT stimulation of DA prevents increases in *bclw* and *β-actin* mRNA in DA. **D.** Cell bodies were severed from axons and DA lysates were blotted with pTrk and pErk1/2. After 6 h, axons still respond to NT stimulation. Pan-actin was used as a loading control. **E.** Decay kinetics of *bclw* and *β-actin* mRNA and control mRNAs *c-fos* and *gapdh.* Data is presented as amount of 4-thio-labeled mRNA, normalized to no thiol control; n=5.

Figure 2.2 (continued)



stimulation (Fig. 2.2*A*,*B*). In these experiments *β-actin* serves as a positive control, as neurotrophins induce transport of *β-actin* mRNA from cell bodies to axons (Willis et al., 2007), and so levels of *β-actin* mRNA in axons increase in response to neurotrophin stimulation of distal axons (Fig. 2.2*A*). Interestingly, cell body stimulation also increases *β-actin* mRNA levels in distal axons (Fig. 2.2*B*).

Neurotrophin-dependent increases in axonal *bclw* mRNA levels could be due to transport of mRNA from the cell body, or could reflect an increase in mRNA stability within the axon. To distinguish these possibilities, we removed the cell bodies of sensory neurons grown in compartmented chamber cultures, then stimulated distal axons with neurotrophins for 2 h. In these experiments, axons are still responsive to neurotrophins in the absence of cell bodies for at least 6 h after cell body removal, as shown by activation of Trk and Erk (Fig. 2.2*D*). After removal of cell bodies, increases in axonal *bclw* and *β-actin* mRNA are abrogated, indicating that increased axonal mRNA is not simply due to local events that cause neurotrophin-dependent stabilization of *bclw* mRNA in axons (Fig. 2.2*C*). To investigate further whether neurotrophins regulate *bclw* mRNA stability, we studied mRNA decay kinetics. We labeled endogenous mRNA with 4-thiouridine for 24 h (Zeiner et al., 2008), then removed 4-thiouridine and measured changes in levels of labeled mRNA over time by quantitative RT-PCR. Half-life was quantified by obtaining the decay rate constant from the line of best fit of a semilogarithmic plot of mRNA concentration (Chen et al., 2008). The half-life of *bclw* is 5 h, consistent with prior studies in non-neuronal cells (Danckwardt et al., 2011) and the relative decay kinetics of *bclw* mRNA over 8 h are unaffected by neurotrophins (Fig. 2.2*E*). We compared *bclw* to

β-actin mRNA, which shows similar decay rates to previous studies (Leclerc et al., 2002) and found no significant difference in decay kinetics between *β-actin* and *bclw* mRNA. Half-life measurements for *c-fos* and *gapdh* mRNA, which are respectively rapidly and very slowly degraded, served as controls (Guhaniyogi and Brewer, 2001). Together, these data indicate a mechanism whereby neurotrophins induce transport of *bclw* mRNA from cell bodies to axons, with no effect of mRNA stability.

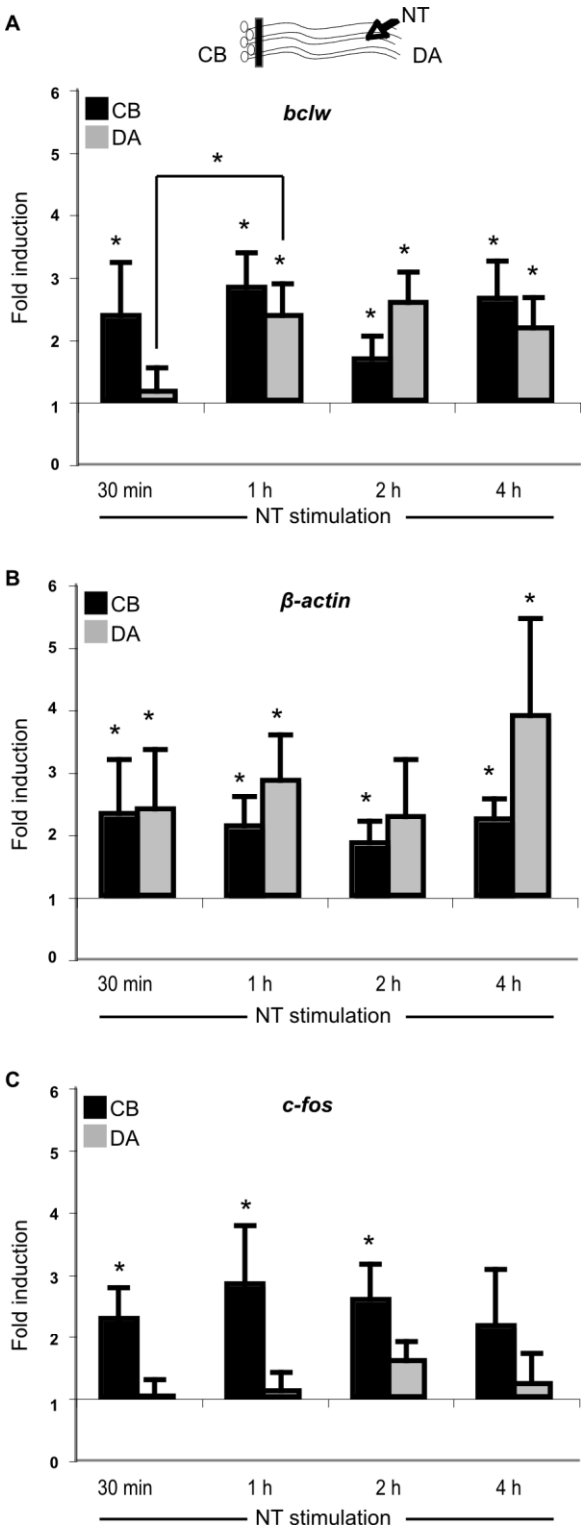
We analyzed the temporal relationship between increases in cell body mRNA levels and those seen in the axon following axonal neurotrophin stimulation of axons. Increased levels of *bclw* mRNA are seen in the cell body after 30 min of stimulation (Fig. 2.3*A*). These data are in line with the time course seen for other early transcriptional changes induced by neurotrophin stimulation of these cultures (Riccio et al., 1997; Watson et al., 1999). Intriguingly, there is no increase in *bclw* mRNA levels in distal axons until 1 h after stimulation (Fig. 2.3*A*). This temporal pattern suggests that a retrograde signal must first reach the cell body and increase transcription of *bclw* mRNA, before *bclw* mRNA in the distal axon can be affected. This is strikingly different from *β-actin* mRNA, where there is a concomitant increase in mRNA levels in both cell bodies and distal axons by 30 min (Fig. 2.3*B*). At each time point, *c-fos* induction serves as a control for successful stimulation (Fig. 2.3*C*).

Induction of *bclw* mRNA in cell bodies by neurotrophin stimulation of distal axons depends on a retrograde Trk-Erk5 pathway culminating in Mef2D-dependent transcription of *bclw* (Pazyra-Murphy et al., 2009). Since *bclw* mRNA levels are

Figure 2.3. *Bclw* mRNA increases in cell bodies before distal axons.

Compartmented chamber cultures were stimulated with neurotrophins (NT) at distal axons (DA) for 30 min, 1 h, 2 h and 4 h. **A.** After 30 min NTs induce *bclw* mRNA expression increases only in cell bodies (CB). Statistical analysis by Student's *t*-test, **p*<0.05 of 30 min versus 1 h; n=10. At 1 h, 2 h and 4 h, *bclw* mRNA increases in both CB and DA. Statistical analysis by z-test, **p*<0.05 for a difference from 1; n=10. **B.** Expression of *β-actin* mRNA increases at 30 min, 1 h, 2 h, and 4 h both in CB and DA. Statistical analysis by z-test, **p*<0.05 for a difference from 1; n=10. **C.** Expression of *c-fos* mRNA is induced only in CB. Statistical analysis by z-test, **p*<0.05 for a difference from 1; n=10.

Figure 2.3 (continued)



increased in axons after cell bodies, we asked whether axonal levels of bclw mRNA are also regulated through retrograde Trk-Erk signaling, or through a distinct regulatory mechanism. Previous studies indicate that Trk kinase and Erk kinase activity are needed in both compartments for induction of *bclw* transcription (Watson et al., 1999; Watson et al., 2001). This reflects the role of transported signaling endosomes containing activated Trks in these long-range responses (Ginty and Segal, 2002). Addition of the Trk inhibitor K252a or the Erk inhibitor UO126 at either location completely abrogates gene induction of *bclw* in cell bodies (Fig. 2.4*A,D*). Similarly, *c-fos* induction is blocked in cell bodies by this intervention (Fig. 2.4*B,E*). Interestingly, the increase in *bclw* mRNA in distal axons is also abrogated when K252a or UO126 is applied to cell bodies (Fig. 2.4*A,D*), despite robust activation of Trk and Erk in distal axons (Fig. 2.4*C,F*). This suggests that a Trk-Erk signal from the distal axon must reach the cell body in order to increase axonal *bclw* mRNA.

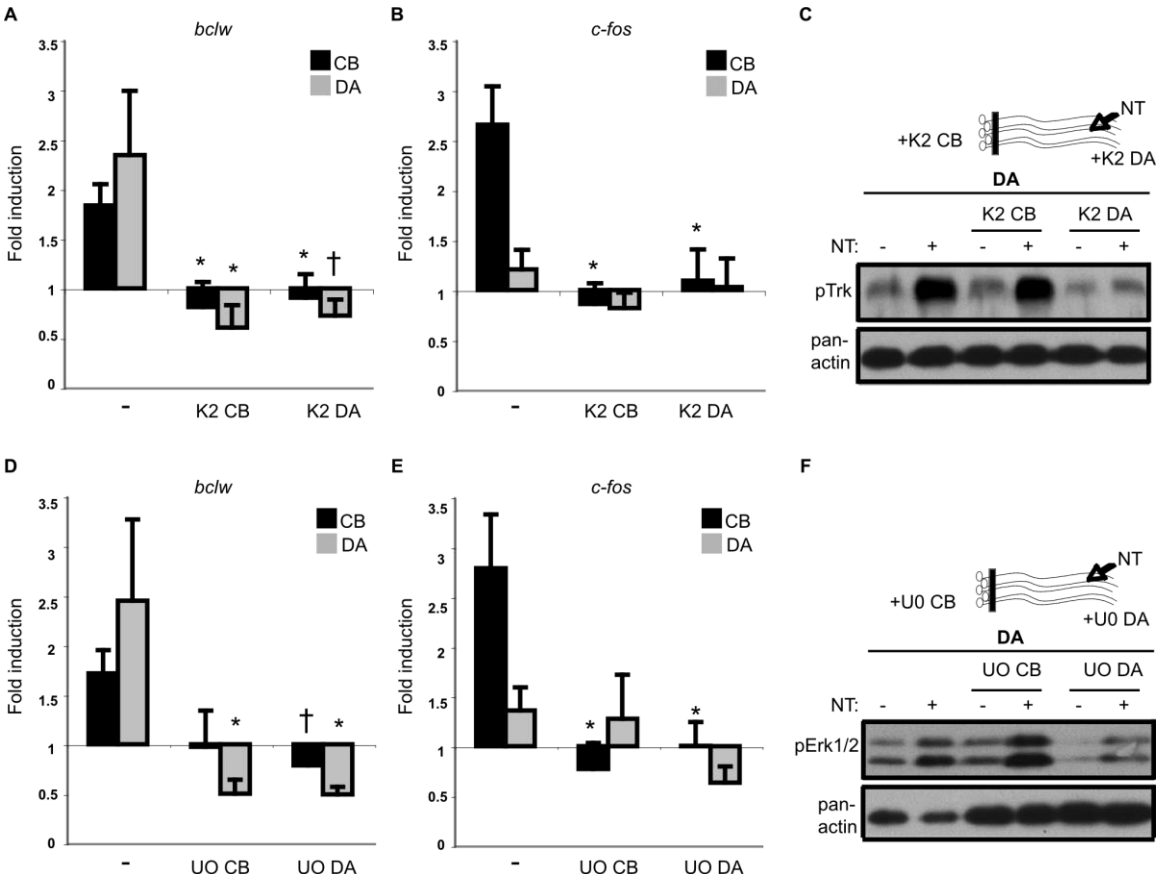
Newly transcribed bclw mRNA is targeted to axons following distal axon neurotrophin stimulation

To determine whether transport of *bclw* mRNA from cell bodies to distal axons requires transcription of *bclw*, distal axons were stimulated with neurotrophins while the transcription inhibitor actinomycin D (actD) was added to the cell body compartment. Addition of actinomycin D to cell bodies prevents neurotrophin-dependent increases of *bclw* mRNA in axons. In contrast, neurotrophin-dependent increases in axonal *β-actin* mRNA are unaffected by actinomycin D (Fig. 2.5*B*). Control experiments showed that actinomycin D blocks the increased expression of *c-fos, bclw* and *β-actin* in cell bodies,

Figure 2.4. Inhibition of a Trk-Erk retrograde signal blocks induction of axonal *bclw* mRNA.

A. Addition of Trk inhibitor K252a (K2; 200nM) to cell bodies (CB) or distal axons (DA) blocks neurotrophin (NT) induction of *bclw* mRNA in CB and DA. **B.** *c-fos* induction is blocked by addition of K2 to CB or DA. **C.** Western blot of NT-induced pTrk activation in DA. **D.** Addition of Erk inhibitor U0126 (U0; 10 μM) to CB or DA blocks NT induction of *bclw* mRNA in CB and DA **E.** *c-fos* induction is blocked by addition of U0 to CB or DA. **F.** Western blot of NT-induced pErk1/2 activation in DA. Statistical analysis by one-way ANOVA with Bonferroni correction; *$p<0.05$; †$p<0.1$; n=7.

Figure 2.4 (continued)

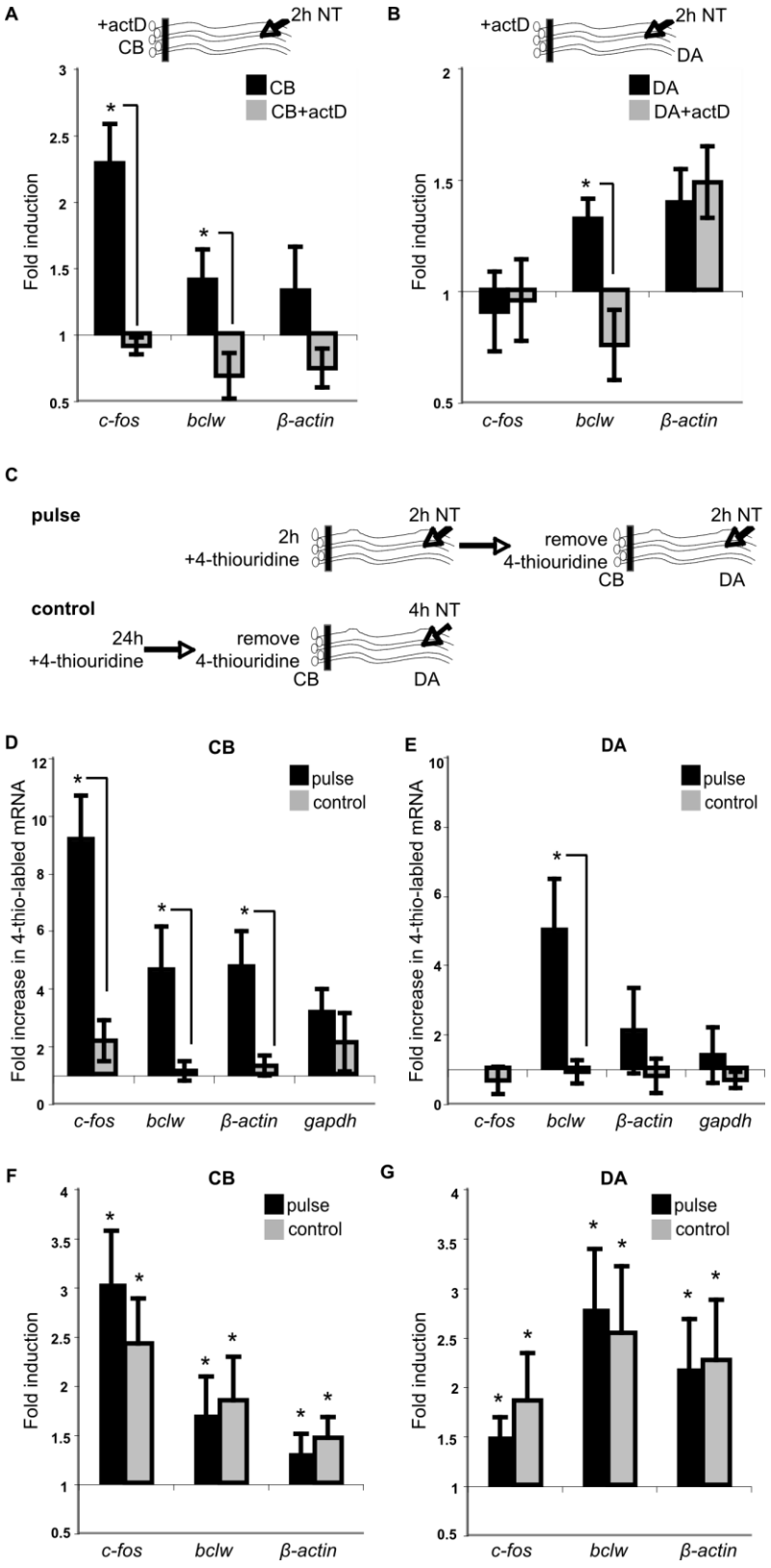


indicating successful inhibition of transcription (Fig. 2.5*A*). Taken together with the time course studies, these data indicate that *bclw* mRNA is regulated differently from *β-actin* mRNA and that changes in axonal *bclw* mRNA levels depend on neurotrophin-induced transcription, while *β-actin* mRNA does not.

Previous studies show *β-actin* mRNA can be transported to axons from pre-existing pools of mRNA in cell bodies without new transcription (Willis et al., 2007). It is possible that transcription of *bclw* mRNA replenishes cytoplasmic mRNA pools available for axonal transport. Alternatively, neurotrophins could regulate transcription of *bclw* mRNA such that the new *bclw* transcripts are immediately targeted to axons. In order to follow newly made mRNA from the cell bodies to the distal axons, we pulse-labeled mRNA with 4-thiouridine (Zeiner et al., 2008) during 2 h of distal axon neurotrophin stimulation and followed the labeled mRNA for an additional 2 h of distal axon neurotrophin stimulation (Fig. 2.5*C*). The newly transcribed thiouridine-labeled mRNA was isolated from each compartment by biotin-Streptavidin pull-down, and the neurotrophin-induced changes in newly transcribed mRNA were assessed by quantitative RT-PCR. As expected, neurotrophin stimulation induces increases in labeled *c-fos, bclw,* and *β-actin* mRNA in cell bodies (Fig. 2.5*D*). We then analyzed newly transcribed mRNA in distal axons. Neurotrophin stimulation dramatically increases labeled *bclw* mRNA in axons, indicating that newly made *bclw* mRNA is rapidly transported to axons (Fig. 2.5*E*). In contrast, neurotrophin stimulation does not increase labeled, newly transcribed *β-actin* mRNA in axons, supporting previous studies showing transport of *β-actin* mRNA from pre-existing cytoplasmic pools (Fig. 2.5*E*). No labeled *c-fos* mRNA is detected in axons. In control

Figure 2.5. Newly transcribed *bclw* mRNA is immediately targeted to distal axons. **A.** Addition of actinomycin D (actD) to cell bodies (CB) inhibits transcription of *c-fos, bclw* and *β-actin* mRNA in CB in response to distal axon (DA) neurotrophin (NT) stimulation. Statistical analysis by Student's *t*-test, **p*<0.05; n=6. **B.** Addition of actD to CB blocks *bclw* mRNA increase in distal axons (DA). Statistical analysis by Student's *t*-test, **p*<0.05; n=6. **C.** To label new mRNA, 4-thiouridine was added to CB during 2 h of DA NT stimulation and removed for a further 2 h DA NT stimulation. In control experiments, 4-thiouridine was added for 24 h and removed prior to 4 h DA stimulation. **D.** Fold increase in newly transcribed *c-fos, bclw,* and *β-actin* mRNA in cell bodies. Levels of newly transcribed *gapdh* mRNA levels do not change. Statistical analysis by student's *t*-test, **p*<0.05; n=5. **E.** Fold increase in newly transcribed *bclw* mRNA in distal axons. Statistical analysis by student t-test, **p*<0.05; n=5. **F.** Total mRNA levels of *c-fos, bclw,* and *β-actin* in CB are upregulated in pulse and control experiments. **G.** Total mRNA levels of *c-fos, bclw,* and *β-actin* in DA are upregulated in pulse and control experiments. Statistical analysis by z-test, **p*<0.05 for a difference from 1; n=5.

Figure 2.5 (continued)



experiments, distal axons were stimulated for 4 h with no 4-thiouridine during the period of stimulation, following 24 h incubation with 4-thiouridine to label transcripts generated prior to neurotrophin stimulation (Fig. 2.5*C*). In these control experiments, where neurotrophin-induced mRNA is not labeled with 4-thiouridine, there is no increase in labeled mRNA for *c-fos, bclw* or *β-actin* in cell bodies, and there is no increase in labeled mRNA for *bclw* in the axons, verifying the specificity of our assay (Fig. 2.5*D*). Thus *bclw* mRNA represents a new "class" of axonal mRNAs that are transcribed and immediately targeted to axons in response to target-derived neurotrophin stimulation.

Axonal Bclw suppresses the caspase6 apoptotic pathway to prevent axon degeneration

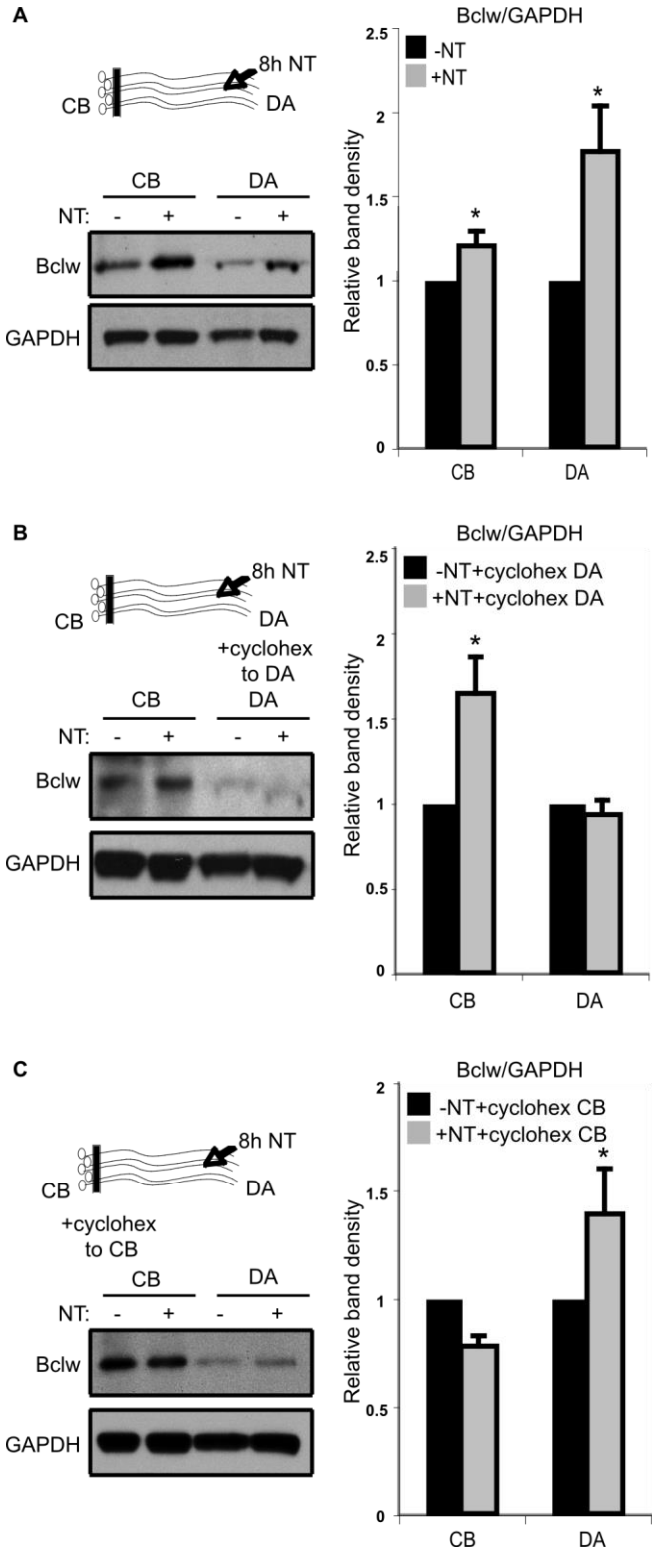
To address the functional significance of neurotrophin regulation of *bclw* mRNA in axons, we asked whether neurotrophins also regulate axonal Bclw protein. In response to 8 h of neurotrophin stimulation, Bclw protein levels are increased in both cell body and in distal axon compartments (Fig. 2.6*A*). To test whether this is due to local translation of Bclw in the axon, we selectively inhibited protein synthesis in axons only, or in cell bodies only, and then analyzed Bclw levels. When the translation inhibitor cycloheximide is added to distal axons, neurotrophins do not increase Bclw in axons (Fig. 2.6*B*). In contrast, when cycloheximide is added to cell bodies, neurotrophins still increase axonal Bclw (Fig. 2.6*C*). Taken together, these data suggest that neurotrophins regulate Bclw at multiple steps: transcription, transport and perhaps local translation.

We previously demonstrated that Bclw is critical for neurotrophin-dependent axonal survival; in cultures from *bclw*$^{-/-}$ mice, withdrawal of neurotrophins results in increased

Figure 2.6. Neurotrophins regulate local translation of *bclw* in distal axons.

A. Western blot analysis and quantification of Bclw in cell body (CB) and distal axon (DA) lysate after 8 h of DA neurotrophin (NT) stimulation. Results represent the mean ± s.e.m. normalized to GAPDH; n=5. **B.** Western blot analysis and quantification of Bclw in CB and DA lysate after 8 h of DA NT stimulation with cycloheximide (10 mg/ml) added to DA. Results represent the mean ± sem normalized to GAPDH; n=3. **C.** Western blot analysis and quantification of Bclw in CB and DA lysate after 8 h of DA NT stimulation with cycloheximide added to CB. Results represent the mean ± sem normalized to GAPDH; n=3. Statistical analysis by Student's *t*-test, $*p<0.05$.

Figure 2.6 (continued)



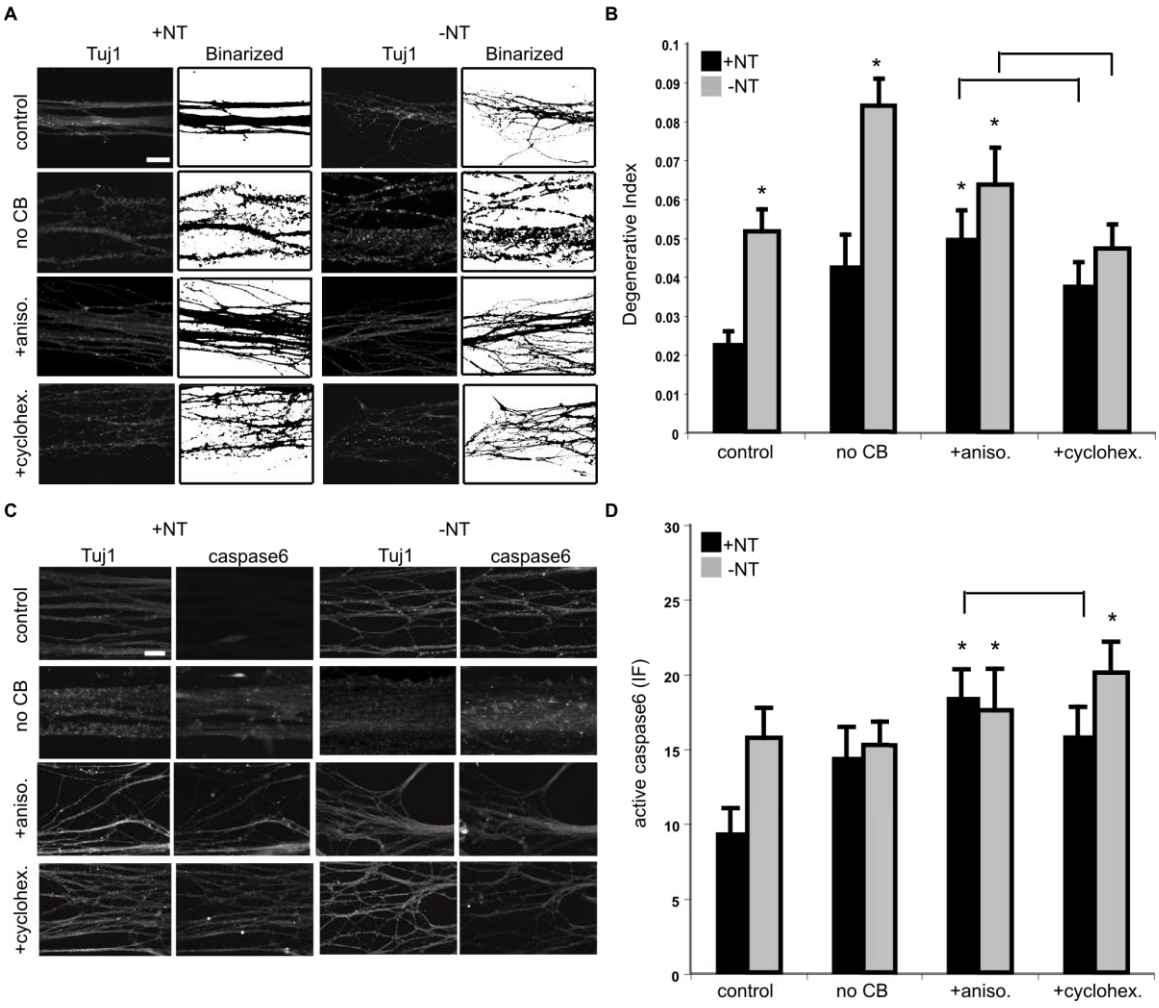
axonal degeneration (Courchesne et al., 2011). Therefore we asked whether manipulations that alter Bclw regulation affect neurotrophin-dependent axonal survival. Withdrawal of neurotrophins from distal axons for 10 h induces selective axonal degeneration (Fig. 2.7*A,B*). When severed from cell bodies, axonal degeneration is increased. This would be expected, since many survival factors are transported from cell bodies, including the Wallerian Degeneration slow component, Nmnat (Gilley and Coleman, 2009). Withdrawal of neurotrophins further increases axonal degeneration in isolated axons severed from cell bodies, suggesting that neurotrophins act locally to protect axons from degeneration, perhaps due to local translation (Fig. 2.7*A,B*). Indeed, addition of translation inhibitors anisomycin or cycloheximide to axons that remain attached to their cell bodies, interrupts the ability of neurotrophins to prevent axon degeneration. (Fig. 2.7*A,B*). Neurotrophin withdrawal can activate an APP/DR6/caspase6 pathway to foster axonal degeneration (Nikolaev et al., 2009; Vohra et al., 2010), and we observed activated caspase6 in degenerating axons following neurotrophin withdrawal (Fig. 2.7*C,D*), and inhibition of translation in axons by anisomycin or cycloheximide leads to increased levels of activated caspase6. Taken together, these data indicate that proteins translated locally in axons prevent activation of caspase6 and promote axonal viability.

To determine whether Bclw itself works within the axon to promote neurotrophin-dependent axon survival, we utilized a protein transfection system to selectively introduce Bclw into axons. We efficiently and selectively transfected recombinant Bclw-His protein in axonal compartments (Fig. 2.8*A*). Introduction of Bclw in axons protects

Figure 2.7. Local translation prevents axon degeneration and caspase6 activation

A. Tuj1-labeled axons in the presence (+NT; left) and absence of (-NT; right) neurotrophins. Axons were severed from cell bodies (no CB) or treated with anisomycin or cycloheximide as indicated and deprived of NT for 10 h. Binarized images show fragmented axons defined by the Analyze Particle function in NIH ImageJ software. Scale bar 40 μm. **B.** Quantification of axonal degeneration (ratio of area of fragmented axons to total axon area). Statistical analysis by one-way ANOVA ($p<0.0001$) with Dunnett correction; *$p<0.05$ versus control+NT; n=27-36 axonal fields from 5 experiments. Quantification for cycloheximide is not significantly different from anisomycin. **C.** Axons in the presence (+NT; right) and absence of (-NT; left) neurotrophins stained for actived caspase6. Caspase6 activation is detected in axons severed from cell bodies (no CB), or treated with anisomycin or cycloheximide. Scale bar 40 μm. **D.** Quantification of activated caspase6 in DA shown in C. Statistical analysis by one-way ANOVA ($p=0.03$) with Dunnett correction; *$p<0.05$ versus control+NT; n=17-24 axonal fields from 3 experiments. Quantification for cycloheximide is not significantly different from anisomycin.

Figure 2.7 (continued)



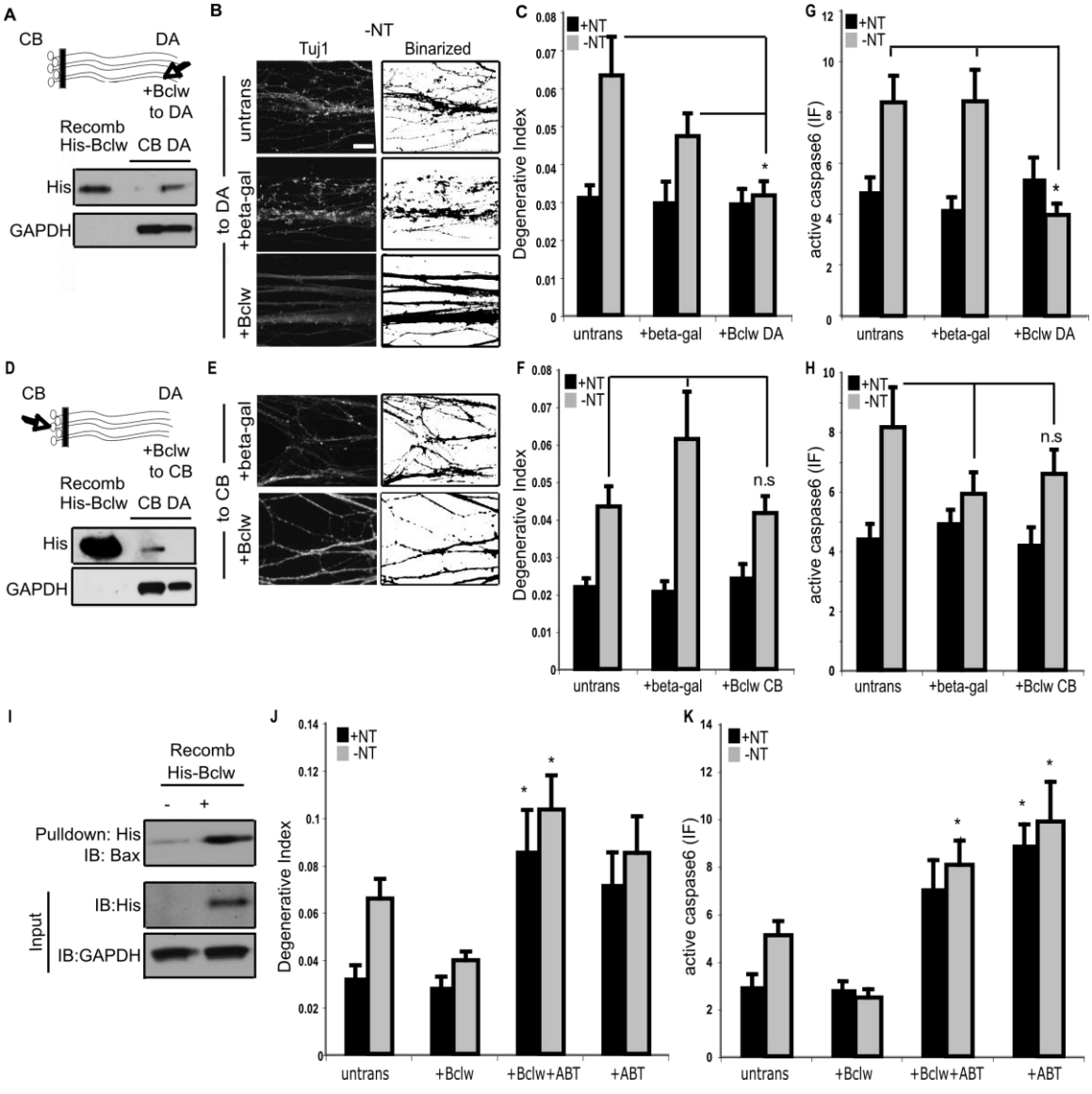
against axonal degeneration caused by removal of neurotrophins (Fig. 2.8*B,C)*, which is readily observed in control untransfected or beta-galactosidase transfected conditions (Fig. 2.8*B,C*). Importantly, Bclw introduced to cell body compartments rather than to axons (Fig. 2.8*D)*, cannot prevent axonal degeneration in response to neurotrophin deprivation (Fig. 2.8*E,F)*. To determine whether Bclw protection of axon viability is associated with reduced caspase6 activation, we measured activated caspase6 in axons after Bclw protein was introduced. Transfection of Bclw into axons dramatically reduces levels of activated caspase6 (Fig. 2.8*G*), while transfection of Bclw into cell bodies does not (Fig. 2.8*H*). These results indicate that neurotrophin regulation of local, axonal synthesis of Bclw is critical for supporting axonal survival and that Bclw functions within the axon to suppress the caspase6 apoptotic pathway.

It has been shown that Bclw binds to the BH3-binding protein Bax in order to prevent caspase activation (Yan et al., 2000; Kim et al., 2012). We find that recombinant Bclw protein introduced into DRG sensory neurons binds to Bax (Fig 2.8*I)*. To determine whether Bclw protects axons from degeneration through binding and sequestration of Bax, we applied the Bcl2/Bcl-xL/Bclw inhibitor ABT-263 to axons transfected with recombinant Bclw protein. ABT-263 inhibits binding of Bcl2 family members to BH-3 domain proteins including Bax (Tse et al., 2008; Mérino et al., 2012)and we see a reduction in recombinant Bclw protein binding to Bax following treatment with 500 nM ABT-263 (data not shown). Addition of ABT-263 to axons abbrogates the ability of recombinant Bclw to prevent axon degeneration and caspase activation after neurotrophin

Figure 2.8. Axonal Bclw binds to Bax and prevents axon degeneration and caspase6 activation

A. Western blot for His after selective introduction of His-tagged Bclw protein into distal axons (DA) of DRG neurons grown in compartmented chamber cultures. **B.** Tuj1-labeled axons following introduction of β-galactosidase (beta-gal) protein or Bclw protein to DA following 24 h neurotrophins deprivation (-NT). Scale bar 40 μm. **C.** Quantification of axonal degeneration after introduction of beta-gal or Bclw protein to DA after removal of NT. Statistical analysis by one-way ANOVA ($p=0.0018$) with Bonferroni correction; *$p<0.05$; n=19-36 axonal fields from 4 experiments. **D.** Western blot for His after selective introduction of His-tagged Bclw protein into cell bodies (CB). **E.** Tuj1-labeled axons following introduction of β-galactosidase (beta-gal) protein or Bclw protein to CB following 24 h neurotrophins deprivation (-NT). Scale bar 40 μm. **F.** Quantification of axonal degeneration after introduction of beta-gal or Bclw protein to CB after removal of NT. Statistical analysis by one-way ANOVA. **G,H.** Quantification of activated caspase6 in DA after introduction of beta-gal protein or Bclw protein to DA **(G)** or CB **(H)**, in the presence (+NT) and absence (-NT) of neurotrophins. Statistical analysis by one-way ANOVA with Bonferroni correction, *$p<0.05$; n=29-51 axonal fields from 4 experiments. **I.** Binding of His-tagged recombinant Bclw to Bax after His pulldown. **J.** Quantification of axonal degeneration after addition of ABT-263 to axons with Bclw protein introduced to axons. Statistical analysis by one-way ANOVA with Bonferroni correction, *$p<0.05$; n=10-24 axonal fields from 3 experiments. **K.** Quantification of activated caspase6 in DA after addition of ABT-263 to axons with Bclw protein introduced to axons. Statistical analysis by one-way ANOVA with Bonferroni correction, *$p<0.05$; n=8-17 axonal fields from 3 experiments.

Figure 2.8 (continued)



withdrawal (Fig. 2.8*J,K*). Together, these data indicate that Bclw in axons binds to Bax and thereby suppresses the caspase6 apoptotic pathway.

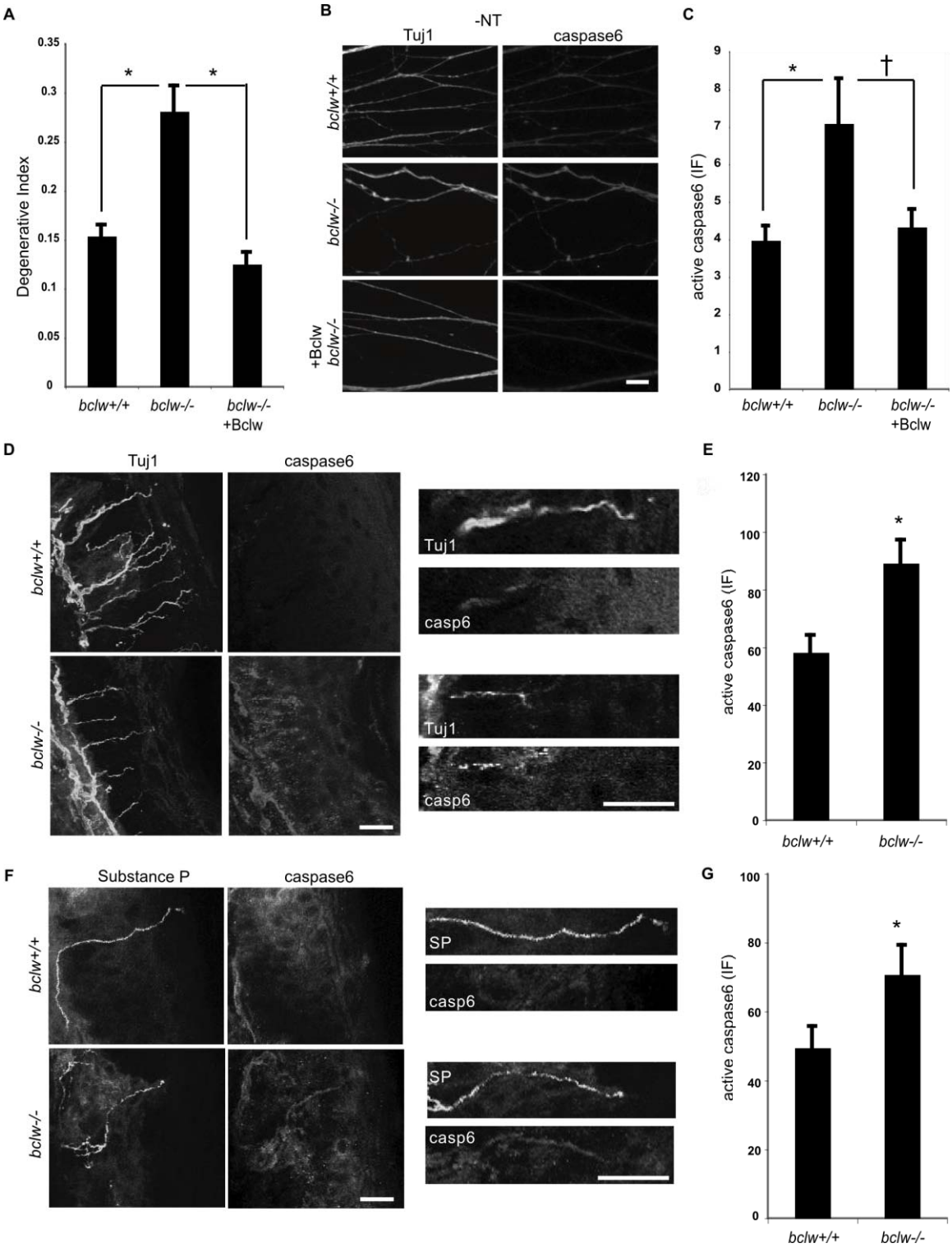
Previous studies have shown that axons cultured from *bclw*$^{-/-}$ mice exhibit increased axon degeneration after neurotrophin withdrawal (Courchesne et al., 2011)(Fig. 2.9*A*). We examined whether recombinant Bclw protein introduced into axons deficient for *bclw* can rescue the axon degeneration phenotype. Introduction of Bclw protein into axons of sensory neurons from *bclw*$^{-/-}$ mice grown in compartmented chamber cultures protects axons from degeneration after neurotrophin withdrawal (Fig. 2.9*A*). We then examined caspase6 activation in sensory axons from *bclw*$^{-/-}$ mice and find increased levels of caspase6 activation following neurotrophin withdrawal as compared to *bclw*$^{+/+}$ axons (Fig. 2.9*B,C*). The increase in activated caspase6 is rescued with addition of recombinant Bclw protein to *bclw*$^{-/-}$ axons (Fig. 2.9*B,C*).

In vivo, Bclw is critical for sustained survival of sensory axons (Courchesne et al., 2011). At 6 months of age, *bclw*$^{-/-}$ mice show a progressive nociceptive neuropathy associated with a loss of epidermal innervation and axonal degeneration (Courchesne et al., 2011). We examined activated caspase6 staining in axons of nociceptors that innervate the skin in *bclw*$^{+/+}$ and *bclw*$^{-/-}$ mice at 6 months of age. We find significantly elevated levels of activated caspase6 staining in nerve endings stained with Tuj1 in *bclw*$^{-/-}$ mice (Fig. 2.9*D,E*). Nociceptor axons in the epidermis include both peptidergic, TrkA-expressing and non-peptidergic, TrkA-independent fibers (Silos-Santiago et al., 1995; Molliver and Snider, 1997; Marmigère and Ernfors, 2007). To determine whether degeneration and

Figure 2.9. Loss of Bclw leads to increased axon degeneration and caspase6 activation in NGF-responsive axons *in vitro* and *in vivo*

A. Quantification of axonal degeneration after introduction of Bclw protein to DA of *bclw*$^{-/-}$ after removal of NT. Statistical analysis by one-way ANOVA ($p<0.0001$), *$p<0.05$. **B.** Tuj1-labeled axons and activated caspase6 staining from *bclw*$^{+/+}$, *bclw*$^{-/-}$ and *bclw*$^{-/-}$ axons transfected with recombinant Bclw protein (+Bclw) in DRG neurons grown in compartmented chamber cultures in absence of NT. Scale bar 40 μm. **C.** Quantification of activated caspase6 in DA shown in B. Statistical analysis by ANOVA ($p=0.009$) with Bonferroni correction, *$p<0.05$; †$p<0.1$; n=38 axonal fields from 3 *bclw*$^{+/+}$ mice, n=39 axonal fields from 3 *bclw*$^{-/-}$ mice and n=28 axonal fields from 3 *bclw*$^{-/-}$ mice transfected with recombinant Bclw protein. **D.** 6 month old *bclw*$^{-/-}$ mice show increased activated caspase6 staining in axons innervating the skin, co-stained with Tuj1. Scale bars 50 μm. **E.** Quantification of activated caspase6 in axons of 6 month old *bclw*$^{-/-}$ and *bclw*$^{+/+}$ mice. Statistical analysis by Student's *t*-test, *$p<0.05$; n=3 animals per genotype, 3-5 sections per animal. **F.** 6 month old *bclw*$^{-/-}$ mice show increased caspase6 staining in substance P positive fibers innervating the skin. Scale bars 50 μm. **G.** Quantification of activated caspase6 in substance P positive axons of 6 month old *bclw*$^{-/-}$ and *bclw*$^{+/+}$ mice. Statistical analysis by Student's *t*-test, *$p<0.05$; n=3 animals per genotype, 3-5 sections per animal.

Figure 2.9 (continued)



caspase6 activation occur in axons of peptidergic neurons in *bclw*$^{-/-}$ mice, we examined caspase6 staining in substance P-positive fibers. Activated caspase6 staining is increased in subtance P positive-nerve endings in *bclw*$^{-/-}$ mice (Fig. 2.9*F,G*), demonstrating that *in vivo* Bclw is critical for maintenance of NGF-responsive, peptidergic axons. Together, these studies suggest that neurotrophins induce increased axonal Bclw through regulated transcription, mRNA transport and translation, resulting in suppression of the Bax-caspase6 apoptotic cascade and thus preventing axonal degeneration.

Discussion

The retrograde response gene Bclw is transcriptionally upregulated in response to distal axon neurotrophin stimulation and is critical for viability. Our studies have elucidated a mechanism by which neurotrophin stimulation of distal axons induces transcription of *bclw* mRNA and subsequent transport of those newly made transcripts from the cell body to the axon. Furthermore Bclw protein functions within the axon to inhibit caspase6-mediated axonal apoptosis and promote neurotrophin-dependent axonal survival. We postulate that the scope of Bclw regulation, at the level of transcription, transport and translation, reflects a mechanism by which signaling cascades are integrated over time, thus promoting axonal maintenance only under conditions of sustained neurotrophin stimulation.

Retrograde neurotrophin signaling has traditionally been thought of as a mechanism by which target-derived neurotrophins communicate to a remote cell body to elicit transcriptional and translational changes required for neuronal survival (Cosker et al.,

2008; Chowdary et al., 2011). In this way, only neurons that correctly reach their targets survive and are incorporated into a functional circuit. Since a functional neural connection depends on the maintenance of the axon as well as the cell body, it makes sense that an axon-derived neurotrophin signal would selectively induce expression of genes required for axonal survival. Our data reveal a bidirectional mechanism wherein retrograde neurotrophin signaling from the axon activates a specific transcriptional program of retrograde response genes that in turn increases expression of axon-targeted mRNA and protein critical for axonal maintenance. It seems likely that additional retrograde response genes may also encode mRNAs that localize to the axon and generate protein products whose roles are primarily in the axon.

Intriguingly, it appears that *in vivo*, *bclw* mRNA is predominantly evident in axons that extend to peripheral, neurotrophin-expressing targets, rather than in centrally projecting branches. Indeed, in *bclw*$^{-/-}$ mice at 6 months of age, there is considerable loss of peripheral nerves in the epidermis, while nerves that extend centrally into the spinal cord show no deficit (Courchesne et al., 2011). Together, these findings suggest that the mechanisms regulating survival and maintenance of centrally and peripherally projecting axons differ from one another. There is evidence in sensory neurons that there are indeed differences between the central and peripheral axons; for example tyrosine-hydroxylase appears to be differentially expressed in peripheral and central axons (Brumovsky et al., 2006). Further studies will be needed to demonstrate whether there is an intrinsic localization element within *bclw* that targets the mRNA preferentially to peripheral axons, or whether selective targeting of *bclw* depends on the local NGF stimulus present in the

periphery. Loss of Bclw results in increased activated caspase6, and degeneration of the peptidergic, NGF-responsive sensory fibers in the epidermis, indicating that Bclw functions to support axon NGF-dependent axon maintenance *in vivo*. Other trophic factors in target tissues may similarly regulate Bclw for maintenance of long axons in neuronal subtypes that do not express TrkA.

Regulated transport of mRNA to distal axons in response to neurotrophins requires localization elements often found within the 3'UTR (Andreassi and Riccio, 2009; Donnelly et al., 2010). The best characterized is the 3' zipcode element in the 3'UTR of *β-actin* mRNA, which contains a 54-nucleotide sequence, "zipcode", responsible for axonal localization (Ross et al., 1997). This is achieved through binding to the mRNA binding protein (RBP) ZBP1 (zipcode binding protein). ZBP1 associates with *β-actin* mRNA during transcription (Oleynikov and Singer, 2003; Pan et al., 2007), and also during transport to the axons. However, *β-actin* transcription and mRNA transport appear to be separately regulated. Thus *bclw* and *β-actin* may represent two distinct classes of neurotrophin-regulated mRNA; those whose transcription and transport into axons are co-regulated, and those whose transport into axons is independent of transcriptional regulation. As *bclw* does not contain the same "zipcode" within its 3'UTR as *β-actin*, other *cis*-localization elements and *trans*-binding RBPs are likely to be responsible for targeting *bclw* mRNA to axons. The long 2.7 kB 3'UTR of *bclw* transcripts may contain several as yet unidentified localization motifs (Andreassi and Riccio, 2009; Cosker and Segal, 2010). Identification of such localization elements and mRNA-binding proteins that interact with *bclw* mRNA will give further understanding into how neurotrophins

regulate axonal *bclw* mRNA levels. It will be interesting to investigate whether mRNAs whose transcription and transport are closely linked might share mRNA-binding proteins and whether this group of mRNAs share transport mechanisms.

Neurotrophin stimulation increases axonal mRNA for both *bclw* and *β-actin*, and abundant evidence indicates that this is due, at least in part, to mRNA transport (Willis et al., 2007). However, it is not known whether neurotrophin-dependent changes in mRNA stability might also contribute to regulation of axonal mRNAs. Here we measured the half-life of *bclw* mRNA and showed that it is not regulated by neurotrophins in DRG neurons. We also measured *β-actin* mRNA stability and found that neurotrophins lead to slight changes in stability. These results cannot exclude the possibility that the stability of mRNAs within axons is differentially regulated than elsewhere in the cell. Nonetheless, these data strongly suggest that the neurotrophin-dependent increase in axonal *bclw* and *β-actin* mRNA are predominantly due to regulated mRNA transport.

Like other Bcl-2 family members, Bclw is associated with mitochondria (O'Reilly et al., 2001). A significant proportion of axonally localized mRNAs encode mitochondrial-associated components (Zivraj et al., 2010; Gumy et al., 2011), and local protein synthesis in axons is critical for mitochondrial health (Hillefors et al., 2007). Failure to maintain healthy mitochondria in axons is associated with neurodegenerative diseases and results in axonal degeneration (Court and Coleman, 2012). Mitochondria in *Bclw*$^{-/-}$ axons show increased length and impaired membrane potential (Courchesne et al., 2011). Abnormal mitochondrial morphology and impaired energy production in *bclw*$^{-/-}$ mice

may contribute to the selective axonal degeneration observed. Intriguingly, *laminb2,* another mRNA that is locally translated in axons, also associates with the mitochondria (Yoon et al., 2012), and loss of LaminB2 also leads to elongated mitochondria and decreased membrane potential. While the role for LaminB2, a nuclear-associated intermediate filament protein, at axonal mitochondria is currently unknown, these observations lend further support to the idea that axonal mitochondria require a local supply of new proteins. Local translation of mRNAs such as *bclw* or *laminb2,* may be a mechanism by which healthy mitochondria are maintained in long axons to prevent axonal degeneration. Further work will be needed to definitively demonstrate local translation of Bclw and to show that newly synthesized protein functions to support mitochondrial health in axons.

As a Bcl2 family member, Bclw functions to promote cell survival through inhibition of the apoptotic cascade and so is likely to play a direct role in regulating axonal survival. Recent studies suggest that axonal degeneration after neurotrophin withdrawal is mediated through a distinct apoptotic pathway that requires activation of Bax and caspase3 and the downstream effector caspase6 (Nikolaev et al., 2009; Vohra et al., 2010). Bclw is known to bind to Bax and inhibit its mitochondrial pore-forming ability (Yan et al., 2000). Here we have shown that Bclw binds to Bax and thereby suppresses activation of caspase6 and protects axons from degeneration. Thus Bclw appears to be the axonal anti-apoptotic Bcl2 member critical for neurotrophin-dependent axonal survival. Bax is also required for axon degeneration after axonal injury, which occurs independently of caspase activation (Schoenmann et al., 2010). Thus, Bclw can prevent

axon degeneration induced by neurotrophin deprivation, and could also play a protective role against axon degeneration after injury.

This work provides new understanding of the mechanisms whereby neurotrophins coordinate signals between distal axons and cell bodies to maintain and preserve connected neurons within a functioning circuit. Our findings suggest that target-derived neurotrophins induce transcription and transport of Bclw to regulate axonal levels of *bclw* mRNA and that the function of Bclw protein in axons is critical for suppression of the caspase6 apopotic cascade and long-term axonal survival. Understanding how Bclw is regulated in developing neurons provides insight into axonal viability during normal development of neuronal circuitry and into axonal degeneration in progressive neurodegenerative disorders.

Chapter 3:

RNA-binding protein SFPQ orchestrates a neuronal RNA regulon for axonal survival

Contributions:
Sara J. Fenstermacher wrote chapter, prepared figures, and generated data for Figures 3.1-3.4.
Katharina E. Cosker generated data for Figures 3.1-3.3, 3.5.
Maria Pazyra-Murphy generated data for Figures 3.1 & 3.5.
Rosalind A. Segal edited chapter.

Acknowledgements:
Thank you to Hunter Elliot of the Harvard Image and Data Analysis Core (IDAC) for help with quantification of FISH data. Thank you to the Segal lab and Goodrich lab for helpful comments. This work was supported by National Institute of Health Grant RO1 NS050674 to R.A.S. and F31 NS077620 (NINDS) to S.J.F.

Introduction

Living cells achieve an accurate spatiotemporal pattern of gene expression through precise localization of mRNA and subsequent protein synthesis. This mechanism is particularly important in highly polarized neurons, where localized mRNAs are translated in axons and dendrites, often at far reaching distances from the cell body and nucleus (Holt and Schuman, 2013; Jung et al., 2014). mRNAs are packaged co- or post-transcriptionally with specific interacting RNA-binding proteins (BPs) to form messenger ribonucleoprotein complexes (mRNPs). After nuclear export, mRNPs with mRNAs destined for subcellular localization are assembled into granules for transport into axons or dendrites by motor proteins (Martin and Ephrussi, 2009; Mitchell and Parker, 2014). Thus, the interconnected steps of mRNA regulation require RNA-BPs that are multi-functional, regulating RNA during early steps of splicing and polyadenylation, through nuclear export and transport through the cytoplasm, to final steps of mRNA stabilization and translation (Moore, 2005; Mitchell and Parker, 2014).

In neurons, mRNA localization and translation within axons is critical for axonal outgrowth and steering, response to injury, as well as for axonal survival and maintenance (Deglincerti and Jaffrey, 2012). Previous studies have provided evidence that regulated local translation of *bclw* and *laminb2* mRNAs in axons support axonal survival (Yoon et al., 2012; Cosker et al., 2013). Interestingly, both *bclw* and *laminb2* mRNAs contain an upstream sequence element (USE) within their 3'UTRs. USE motifs bind to a small set of RNA-BPs that includes SFPQ (spicing factor proline-glutamine rich) (Brackenridge and Proudfoot, 2000; Danckwardt et al., 2007).

SPFQ, also known as PSF, the polypyrimidine tract-binding protein-associated splicing factor, is an RNA-BP and member of the DBHS (*Drosophia* Behavior Human Splicing) family of proteins (Patton et al., 1993). SFPQ is a multi-functional protein with known nuclear roles in transcription, splicing, and 3' end processing (Shav-Tal and Zipori, 2002; Danckwardt et al., 2007; Dong et al., 2007; Hall-Pogar et al., 2007; Hirose et al., 2013; Cho et al., 2014; Imamura et al., 2014). SFPQ is highly expressed in the developing and mature nervous system and is required for neuronal survival and normal brain development (Chanas-Sacré et al., 1999; Lowery et al., 2007). Aberrant subcellular localization of SFPQ has been observed in brains of Alzheimer's patients (Ke et al., 2012). In addition to its described nuclear functions, SFPQ has been isolated from a kinesin-bound dendritic RNA granule in the brain (Kanai et al., 2004), observed with MAPK component JNK in the cytoplasm of cortical neurons (Sury et al., 2014), and found to associate with and regulate IRES activity (Cobbold et al., 2008; Sharathchandra et al., 2012). Together, these data suggest that SFPQ also functions in mRNA localization and translation within the nervous system.

Many studies document either a single mRNA that is bound and regulated by one RNA-BP or a full transcriptome analysis of RNAs and their binding-protein partners. However, can distinct mRNAs be regulated post-transcriptionally in concert? The RNA regulon model proposes that mRNAs encoding functionally related proteins are coordinately regulated as mRNPs (Keene, 2007) and while RNA regulons have been described in yeast, fruitfly, and mammalian cells, little is known about whether RBPs bind and regulate multiple functionally related mRNAs in neurons. Neurotrophin signaling in

dorsal root ganglia (DRG) neurons directs RNA localization and translation in axons to promote axonal health. Here, we sought to determine whether the multifunctional RNA-BP SFPQ functions to promote axonal survival of sensory neurons by regulating axonal mRNAs. We find that SFPQ binds and regulates *bclw* and *laminb2* mRNA which localize to axons and promote axonal survival. Together these studies identify an RNA regulon that coordinates the localization of mRNAs to promote the health and maintenance of long sensory axons.

Materials and Methods

Animal Use. Timed-pregnant Sprague-Dawley rats were purchased from Charles River. All experimental procedures were done in accordance with the National Institutes of Health guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

Neuronal cultures. Dorsal root ganglia (DRG) neurons were dissected from rats embryonic day 15 (E15) and dissociated in trypsin. Neurons were grown in compartmented cultures as previously described (Pazyra-Murphy and Segal 2008, Appendix I). Compartmented cultures were maintained in DMEM, 5% heat-inactivated horse serum, 1% penicillin streptomycin, 0.3 μM Cytosine ß-D-Arabino Furanoside (AraC) at 37°C and 7.5% CO_2, with 10 ng/mL NGF + BDNF in the center compartment and 100 ng/mL NGF + BDNF in the side compartments. At 8DIV compartmented cultures were starved in serum and neurotrophin-free DMEM for 2 hours, followed by stimulation of side compartments with 100 ng/mL NGF + BDNF or 100 ng/mL BSA vehicle control. For transcription inhibition, Actinomycin D (20 μM) or DMSO vehicle

control applied 10 min before neurotrophin stimulation or throughout the duration of neurotrophin deprivation. Microfluidics cultures (Xona Microfluidics) were maintained in Neurobasal, 2% B-27, 1% glutamax, 1% penicillin streptomycin, 0.08%, 0.3 μM AraC, with 10 ng/mL NGF + BDNF in the cell body side and 100 ng/mL NGF + BDNF in the distal axon side (see methods chapter for detailed protocol). Mass cultures on matrigel-coated (1:45 in DMEM) tissue culture plates were maintained in Neurobasal, 2% B-27, 1% glutamax, 1% penicillin streptomycin, 0.08%, 0.3 μM AraC, with 100 ng/mL NGF + BDNF.

Immunocytochemistry. Microfluidic cultures were fixed on 5DIV with 4% paraformaldehyde for 20 minutes. Cells were permeabilized in 0.5% Triton 100-X for 10 minutes and blocked in 3% BSA and 0.1% Triton 100-X for 1 hour. Mouse anti-SFPQ [B92] (1:100, Abcam 11825) and rabbit anti-β-Tubulin (Tuj1, 1:300, Covance MRB-435P) were added in block overnight at 4°C, followed by goat anti-mouse AlexaFluor488 (1:1000) and goat anti-rabbit AlexaFluor546 (1:1000) for 1 hour at room temperature, and DAPI (1:1000) for 1 minute. Images were acquired using Nikon C2 S*i* laser-scanning confocal microscope with 40x oil objective.

Whole-mount immunostaining. Whole DRG with central and peripheral nerves were dissected from P0 rats of either sex and fixed overnight in 4% PFA in PBS. After fixation, DRGs were washed in PBS, permeabilized in 0.5% Triton X-100 for 1 hour and blocked in 5% BSA and 0.5% Triton X-100 for 4 hours. DRGs were incubated for 48 hours at 4C in primary antibody (1:100) and washed overnight in PBS. DRGs were then incubated in secondary antibody (1:1000) at R.T for 2 hours and conterstained with DAPI.

Lentiviral shRNA knockdown. Lentiviral shRNA constructs were obtained from the Broad Institute RNAi Consortium (TRC). Virus conditioned media was collected from 293T packaging cells transfected using Fugene6 reagent (Promega) in complete DMEM supplemented with 10% fetal bovine serum. E15 rat DRGs were grown in compartmented chamber cultures for 4 DIV and then infected with concentrated shRNA lentivirus in the cell body compartment for 24 hours. Infected cells were selected with 0.5 μg/ml puromycin and cultured for a further 7 DIV before RNA or protein collection.

Axon degeneration assay. E15 rat DRGs were grown in compartmented chamber cultures. After lentiviral shRNA knockdown for 6 days in culture, the cell body compartment was maintained in 10 ng/ml NGF+BDNF, and the axonal compartment was starved in DMEM alone for 12 hours before fixation and staining. Compartmented chamber cultures were fixed in ice-cold MeOH for 2 min and 4% PFA for 10 min, blocked in 2% BSA and 0.5% Triton X-100 for 1 hour at RT. Cultures were incubated with mouse anti-Tuj1 (1:400, Covance) overnight at 4C and then with goat anti-mouse Alexa-Fluor 488 (1:1000) for 1 hour at RT, and counterstained with DAPI. Images of distal axons were obtained using a 40X objective and axonal degeneration was quantified as previously described by (Cosker et al., 2013).

RNA motif analysis. The rat *bclw* mRNA (NM_021850.2) and rat *laminb2* mRNA (XM_216850.6) were searched for each of the highly-ranked SFPQ preferred RNA-binding sites (Ray et al., 2013) and the polyA signal sequence (AAUAAA). The USE identified within *bclw* is UUAUUUUUGUGU (Danckwardt et al., 2007) and in *laminb2* is UUUCUUUUAUAAGAUGCAUGCCAAACGUGUUCCACUUUUUCUUUUCUG

CCUAUGAUUUGUAA UAUACAUUUUACUACUGGGAACUUUUGU

(Brackenridge (Brackenridge and Proudfoot, 2000).

qRT-PCR. RNA from DRG neurons grown in compartmented cultures was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed using the cDNA archive kit (Applied Biosystems) according to the manufacturer's specifications. Quantitative real-time RT-PCR was performed using TaqMan gene expression assays (Applied Biosystems) to assess the RNA levels of rat *bclw* (Rn00821025_g1), *β-actin* (Rn00667869_m1), *laminB2* (Rn01408653_g1), and custom gene expression assays for *egfp* and the *bclw*, *bcl2*, β-*act*, and *gact* 3'UTRs. For each sample, average c_t values were normalized to the expression level of rat *gapdh* (Applied Biosystems). To present data as fold induction, the values for neurotrophin stimulation were normalized to vehicle control condition.

Western Blotting. Protein from DRG neurons grown in compartmented cultures was collected in lysis buffer (20mM Tris-HCl pH 7.4, 140mM NaCl, 10% glycerol, 1% Triton X-100) with Complete Mini protease inhibitor cocktail tablet (Roche) for analysis by western blot. Lysates were made up with Invitrogen NuPAGE LDS sample buffer (4X) and reducing agent (10X) and separated by 4-12% Bis-Tris SDS-PAGE. Protein was transferred to methanol-activated PVDF membrane and blocked with 5% milk in 1X TBST for 1hr at room temperate. Membranes were probed with the following antibodies overnight at 4°C: mouse anti-SFPQ [B92] (1:100, Abcam 11825), rabbit anti-GAPDH (1:1000, Cell Signaling #2118), rabbit anti-Histone H3 (1:1000, Cell Signaling #9715), rabbit anti-Bcl-w [31H4] (1:1000, Cell Signaling #2724), rabbit anti-FMR1 [H-120] (1:500, Santa Cruz 28739), rabbit anti-Musashi (1:1000, Cell Signaling D46A8), rabbit

anti-IGF2BP1 (IMP1) (1:1000, MBL International RN007P), rabbit anti-Kinesin 5A+B+C (1:1000, Abcam 62104), rabbit anti-Kinesin 3B (1:1000, Cell Signaling #13817), and rabbit anti-LaminB2 (1:1000, Abcam 138516). Membranes were washed 3 times with 1X TBST and probed with goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibodies (1:10,000; Biorad) in 5% milk for 1hr at room temperature. Blots were washed 3 times with 1X TBST and developed with ECL detection reagent (VWR).

RNA pulldowns. Rat *bclw* mRNA sequences were amplified by PCR using the following primers to add a T7 promoter to the 5' end of the PCR product:

5'UTR frwd CTTCCTTTCCTCCCTCTC, 5'UTR rev TTGCTCACCATGGTGGGCG (upstream of 5'UTR within vector used as PCR template),

ORF frwd ATGGCGACCCCAGCCTCAA, ORF revTCACTTGCTAGCAAAAAAGG,

3'UTR-A frwd GAAAGTCTAGGGTCAGGTG,

3'UTR-A rev AAAGCTCCTATCCACCCAGGCTCCA,

3'UTR-B frwd TTGGTACCTAGAGTGTTGCCT,

3'UTR-B rev TAGGACACTTCGGGGCTCTGCCA,

3'UTR-C frwd TGTAGCTGTCTTATGTATCGGGGT,

3'UTR-C rev ATTGACAACTGCAGGACTGACTTGCCC.

LaminB2 was amplified using the primers ORF-A frwd TAGCTCAGCGGATCCTC,

ORF-A rev GCTGCCTTGTCATTCTGGTCCGA,

ORF-B frwd CGTGGAGTCCCTCAGCTAC, ORF-B rev CTTGAGGTAGTCCTTG,

3'UTR-A frwd GGATGATGTGAAACCTGC,

3'UTR-A rev GATCCTGAATACACAACGCCCAC,

3’UTR-B frwd AGGCTAGCTGTGCAACTG,

3’UTR-B rev GTGGAACACGTTTGGCATGCATC.

β-actin 3’UTR was amplified using the primers frwdGCGGACTGTTACTGA and revCGGTCTCACGTCAGTGTACAGGCC, and control GFP was amplified using frwdGTAAACGGCCACAAGTT and revTCGTCCATGCCGAGAGTGATCC primers. DNA bands were purified using the QIAquick Gel Extraction Kit (Qiagen) and confirmed by sequencing. In vitro transcription with T7 RNA polymerase (Promega) was used to generate RNA from PCR products according to manufacturer’s protocol and was purified using the RNeasy MinElute Cleanup Kit (Qiagen). RNA was biotinylated using the RNA 3’ End Biotinylation Kit (Pierce) and extracted with trizol. DRG neuron lysate was collected from 6DIV mass cultures in polysome extraction buffer (20mM TrisHCl pH7.5, 100mM KCl, 5mM $MgCl_2$ and 0.5% NP-40) with protease inhibitor, RNasin (1:100), 0.2mM PMSF, 2mM NaOVa, and 10mM NaF. Protein lysate was pre-cleared with MyOne streptavidin C1 beads (Invitrogen), yeast tRNA, and *in vitro* transcribed GFP RNA. Bclw, βact, and GFP RNAs were conjugated to streptavidin beads in 10mM Tris-HCl, 1mM EDTA, and 2M NaCl. RNA-beads were incubated with protein lysate, washed, eluted by boiling in SDS buffer, and resolved by western blot.

Formaldehyde crosslinking. Trk PC12 cells were differentiated in low serum media before starvation in DMEM alone for 2 hours followed by NGF (100 ng/ml) stimulation or vehicle control (BSA 100 ng/ml). Cells were collected in HBSS, before crosslinking with 1% wt/vol formaldehyde (AR grade, 37% wt/wt) in PBS for 10 min at R.T. The crosslinking reaction was stopped with 1M glycine to a final volume of 0.25M glycine for 5 minutes at R.T and washed 2x with ice-cold PBS. The cells were resuspended in

RIPA buffer (50mM Tris-HCl pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.05% SDS, 1mM EDTA, 150mM NaCl +protease inhibitors +RNasin inhibitors), and sonicated for 5x 3 sec bursts. Lysate was centrifuged at 13,000rpm to remove insoluble material and incubated overnight in antibody (αSFPQ 1:100, abcam) or no-antibody control at 4C overnight. Beads (A/G plus-agarose, Pierce) were added for 4 hours at 4C and collected by centrifugation at 2500rpm and washed 4x with RIPA buffer). Beads were resuspended in 50mM Tris-HCl pH7, 5mM EDTA, 10mM DTT, 1% SDS and incubated at 70C for 45 min to reverse crosslinks. Immunoprecipitated RNAs were isolated with Trizol and analyzed by qRT-PCR after reverse transcription.

Single-molecule FISH. DRG neurons grown in microfluidic cultures were starved in neurotrophin-free Neurobasal and for 2 hours, followed by stimulation of axon compartment with 100 ng/mL NGF + BDNF on 6 DIV. Cultures were fixed in 3.7% formaldehyde for 20 minutes followed by 3 washes with 1X PBS (without calcium or magnesium), dehydration to 100% EtOH and stored at -20°C. On day of FISH, cultures were rehydrated and incubated in PBS for 10 minutes at RT, followed by 1XPBST for 10 minutes at RT. RNAscope (Advanced Cell Diagnostics) kit was used for FISH with custom probes designed for bclw and laminb2 mRNAs. Cell were incubated with Pretreatment 3 (protease) diluted in 1XPBS at 1:15 for 10 minutes at RT, followed by 2 washes with 1XPBS. Probe incubation was performed for 2hr at 40°C followed by washing and amplification steps according to manufacturer's protocol. Microfluidic coverslips were washed by dunking into beakers with wash buffer ~10 times between steps. Tuj1 staining was performed overnight at 4°C followed by DAPI staining and coverslip mounting with ProLong Gold antifade reagent. Images were acquired using

Nikon C2 S*i* laser-scanning confocal microscope and PlanApo 60x (NA 1.4) oil objective with 2.29 zoom to obtain a pixel size of 0.09µm.

FISH colocalization analysis. Manual per-cell region-of interest selection was combined with thresholding and morphological post-processing to segment cell areas. Robust point source detection and gaussian PSF-model fitting were then performed within the cell area to identify puncta with a localization precision ranging from approximately 15-70nm (Aguet et al., 2013). A nearest-neighbor co-localization measure was then calculated as described previously (Lachmanovich et al., 2003; Mendoza et al., 2011). Briefly, closest distances between points in the two image channels were calculated, and a frequency vs. search radius curve generated. This curve is then normalized by ratioing to the mean frequency of nearest-neighbor distances observed in 1000 rounds of randomly generated point positions. These 1000 rounds of randomization are repeated twice: Once holding image channel one (bclw) fixed while randomizing channel two (laminb2), and a second time randomizing channel one while holding channel two fixed. Alternately randomizing the two channels controls for the fact that spatial patterns in the points in one channel can potentially induce spurious indications of colocalization in the other channel. These normalized curves are calculated for each cell, and then averaged across cells. A 99% confidence interval was then calculated at each distance by 5000 bootstrap repetitions, by sampling from the individual cell curves with replacement. Significance was determined by comparing these confidence intervals to 1, the normalized frequency at any given distance which would be expected purely by chance. The colocalization code is available upon request from Image and Data Analysis Core at Harvard Medical School (http://idac.hms.harvard.edu/).

3'UTR localization. Constructs were generated by inserting myrEGFP with a 5' Kozak sequence from J. Twiss with BamH1 and Not1 sites into the pcDNA3.1 (Invitrogen) mammalian expression vector, positioned 5' to a synthesized rat *bclw* 3'UTR sequence (Genscript). Egfp-bclw3'UTR or egfp was transfected using Lipofectamine LTX and PLUS Reagent (Life Techonlogies) into the cell bodies of DRG neurons grown in compartmented cultures on 5 DIV. Transfection reagents and DNA were made up in Opti-MEM (Life Technologies) and DMEM, and added to cells for 3 hours in 10nm/mL NGF/BDNF. Three days later, cultures were starved and distal axons were stimulated with 100 ng/mL NGF + BDNF or vehicle control (100 ng/mL BSA). To access levels of *egfp* within each compartment RNA was extracted and analyzed by qRT-PCR as described previously.

Protein transfection. Recombinant His-tagged Bclw (R&D Systems) was introduced into E15 sensory neurons using the Chariot protein transfection system (Active Motif). β-Galactosidase protein was used as a control. Bclw or β-Galactosidase (1μg/μl) was introduced into the distal axon compartments of compartmented chamber cultures by using 2 μl of Chariot reagent.

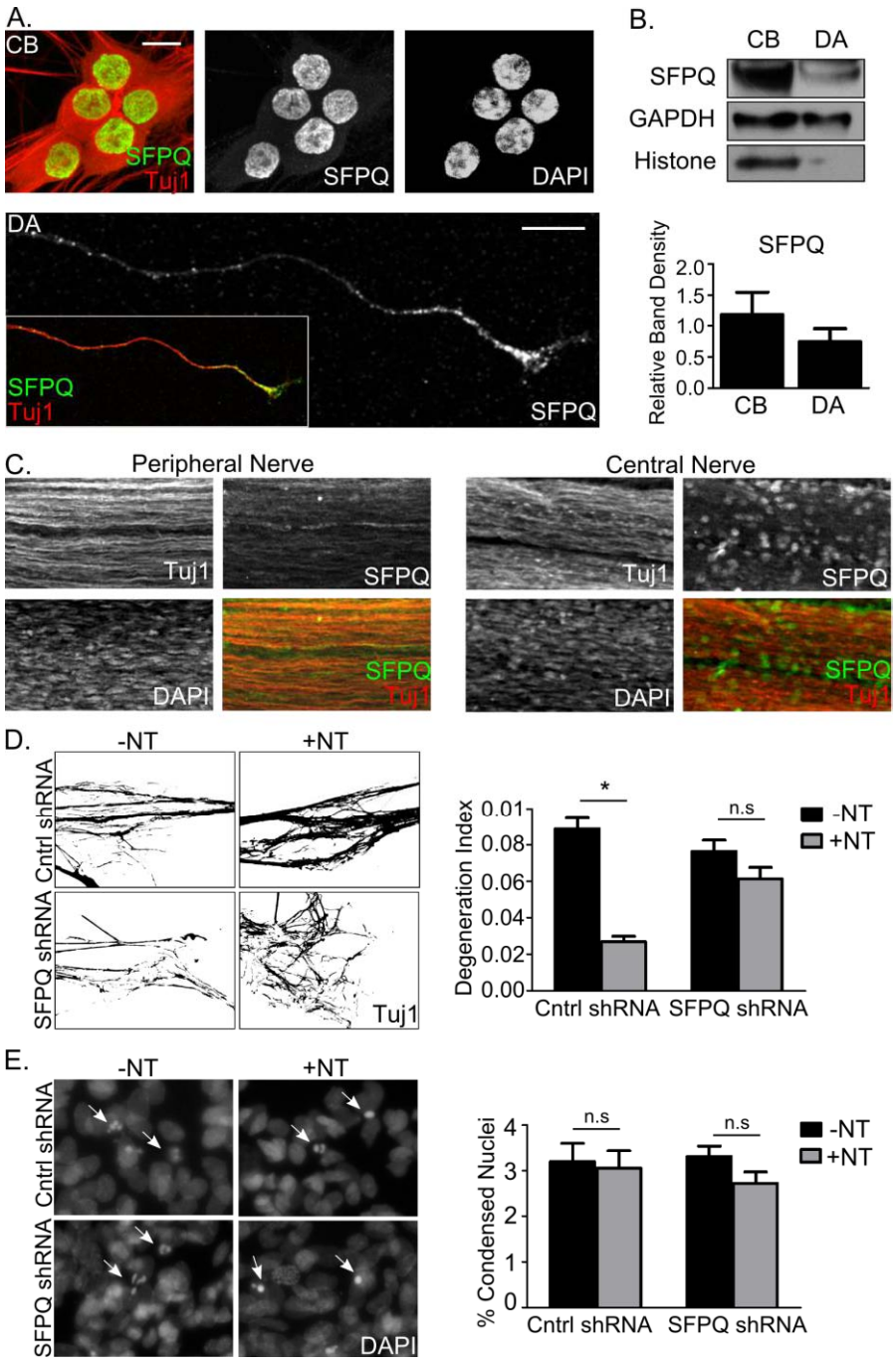
Results

RNA-binding protein SFPQ is required for neurotrophin-dependent axon survival

To determine whether SFPQ is capable of regulating RNA both within the nucleus and in the long axonal process of DRGs neurons, we first examined subcellular localization of SFPQ. Immunostaining and western blot analysis of DRG neurons grown in compartmented cultures demonstrate that SFPQ is highly expressed within the nuclei of

Figure 3.1. SFPQ is expressed in DRG neurons and is required for axonal survival. **A**. SFPQ immunostaining of neuronal cell bodies (CB) and distal axons (DA) in microfluidics cultures. Scale bars CB and DA 10μm. **B**. Western blot of CB and DA lysates from compartmented cultures. Histone restricted to CBs demonstrates compartmentalized cultures. Relative band density is SFPQ normalized to loading control GAPDH; mean values ± SEM, n=3. **C**. SFPQ immunostaining of wholemount peripheral and central nerves of the DRG age P0. D. Compartmented cultures binarized Tuj1 (anti-neuron-specific beta-tubulin) images and quantification of degeneration following infection with control or SFPQ shRNA. Distal axon compartments starved or maintained in NT for 24hr after 7 days in culture. Mean values ± SEM, n=3; one-way ANOVA with Bonferroni posttests, *p<.05. E. DAPI stained CB compartment cultures in **D**. Quantification of percent condensed nuclei; mean values ± SEM, n=3; one-way ANOVA with Bonferroni posttests, n.s.= not significant.

Figure 3.1 (continued)



cell bodies and is also localized to the distal axons and growth cones (Fig. 3.1A,B). DRGs with the accompanying peripheral and central nerves from P0 rats were analyzed by whole mount immunostaining to determine SFPQ localization *in vivo*. SFPQ is present within the nuclei of DRG neurons *in vivo* (Supp. Fig. S1A) and preferentially within the axons of the peripheral nerve, as compared to axons of the centrally projecting nerve (Fig. 3.1C). SFPQ is highly localized to the Tuj1-positive axons within the peripheral nerve, which innervates peripheral targets and receives high concentrations of neurotrophins, but SFPQ is instead restricted to glial cell nuclei in the central process.

The extracellular milieu of the peripheral axons differ from that of the central axons, in particular neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), stimulate peripheral axons. We find that SFPQ is required for neurotrophin-dependent axonal survival (Fig. 3.1D). The addition of neurotrophins to the distal axons of DRG neurons grown in compartmented cultures leads to a reduction in the axon degenerative index, but this protective effect is blocked with SFPQ knockdown (Fig 3.1D, Supp. Fig. S1B,C). In contrast, quantification of condensed apoptotic nuclei to access cell body health, shows that loss of SFPQ does not affect cell body survival (Fig. 3.1E). Therefore, SFPQ is specifically required for neurotrophin-mediated axonal survival.

SFPQ binds the mRNA of axonal survival factors Bclw and LaminB2

Bclw and *laminb2* are two axonal mRNAs that have been shown to promote survival of axons (Yoon et al., 2012; Cosker et al., 2013). We have previously described

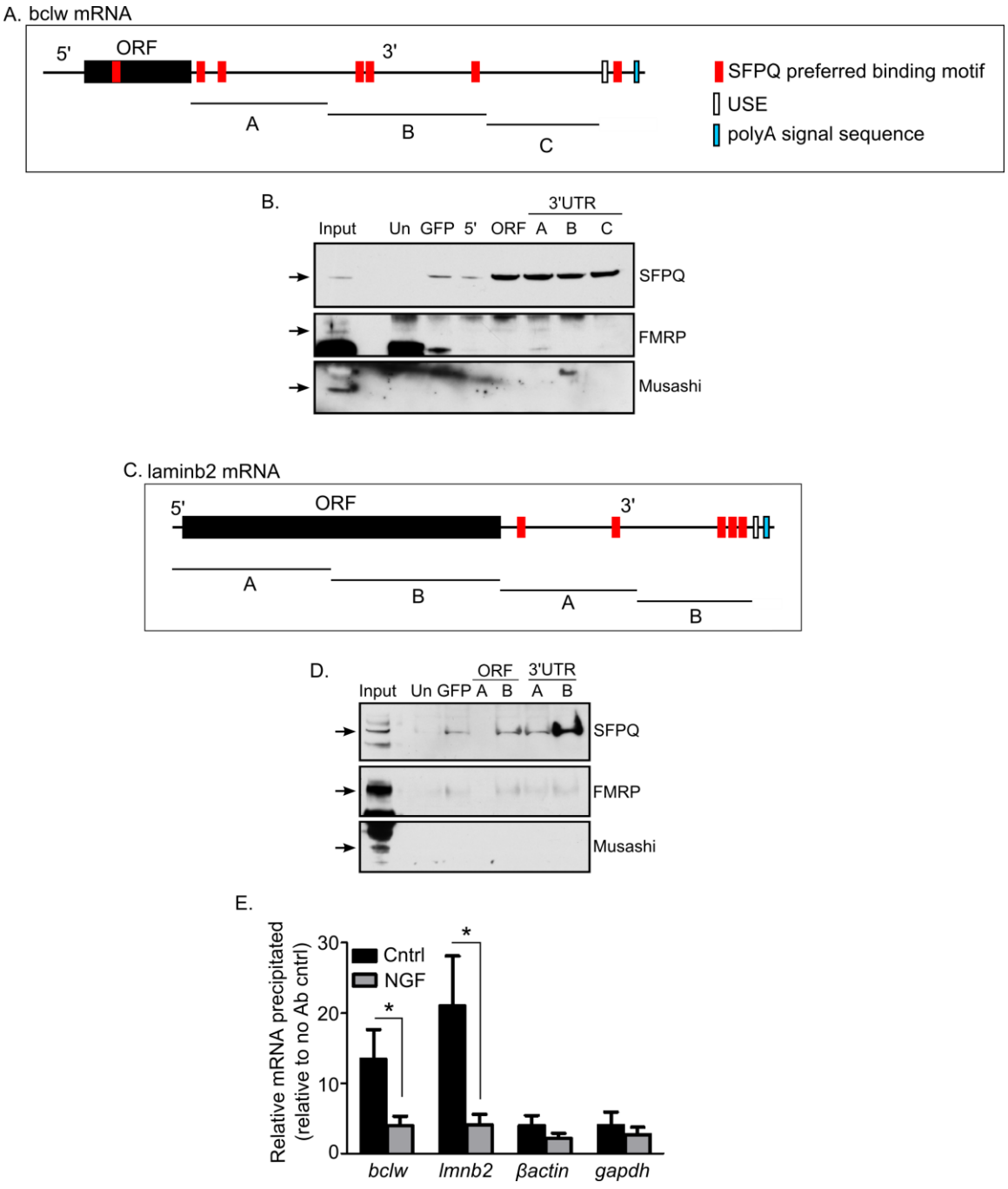
neurotrophin regulation of Bclw, a Bcl2 prosurvival family member that is specialized for axonal health *in vivo* (Courchesne et al., 2011) and showed that neurotrophins induce new transcription and axonal localization of *bclw* mRNA (Cosker et al., 2013). Holt and colleagues showed that the mRNA for LaminB2 localizes to axons and local translation of *laminb2* mRNA in retinal ganglion cell axons is required for axon maintenance (Yoon et al., 2012). Both *bclw* and *laminb2* mRNAs contain an upstream sequence element (USE) within their 3'UTRs that has been implicated in proper 3'end formation by SFPQ or other factors (Brackenridge and Proudfoot, 2000; Danckwardt et al., 2007), Fig. 3.2A,C, Supp. Fig. S2A), suggesting that SFPQ may regulate *bclw* and *laminb2* mRNA. In addition, a recent data set of predicted SFPQ binding motifs (Ray et al., 2013) identified several highly-ranked binding motifs within the *bclw* open reading frame (ORF) and long 2.7kb 3'UTR, as well as within the 3'UTR of *laminb2* mRNA (Fig. 3.2A,C).

We performed RNA pulldown experiments using *in vitro* synthesized biotinylated RNAs and DRG protein lysates to determine whether SFPQ is capable of binding *bclw* and *laminb2* mRNA. The *bclw* ORF and 3'UTR sequences, where predicted motifs are located, precipitated SFPQ, but not other neuronal RNA BPs, FMRP and Musashi (Fig. 3.2B, Supp. Fig. S2A). Similarly, the 3' end of the *laminb2* 3'UTR precipitates SFPQ, but not FMRP or Musashi (Fig. 3.2D). These data demonstrate that SFPQ can bind *bclw* and *laminb2* mRNA. Strikingly, formaldehyde crosslinking and immunoprecipitation of SFPQ pulls down both *bclw* and *laminb2* mRNA, but not *β-actin* or *gapdh* mRNA, and NGF stimulation reduces the amount of bound *bclw* and *laminb2* mRNA (Fig. 3.2E).

Figure 3.2. Neurotrophin-regulated binding of SFPQ to *bclw* and *laminb2* mRNA.

A. Schematic of rat *bclw* mRNA 5'UTR, ORF, and 3'UTR regions with predicted SFPQ preferred binding motifs (Ray 2013), upstream sequence element (USE) (Danckwardt 2007), and polyA signal sequence. **B**. Biotinylated RNA pulldown of *bclw* RNAs, *GFP* RNA, and unbiotinylated (Un) control RNA from DRG protein lysates. Western blot to detect protein pulled-down with RNAs (representative of 3 experiments). Input is 10% of lysate used for IP. **C**. Schematic of rat *laminb2* mRNA 5'UTR, ORF, and 3'UTR with domains described in A. **D**. Biotinylated RNA pulldown of *laminb2* RNAs, *GFP* RNA, and unbiotinylated (Un) control RNA from DRG protein lysates. Western blot to detect protein pulled-down with RNAs (representative of 3 experiments). Input is 10% of lysate used for IP. **E**. Crosslinking of differentiated Trk PC12 cells following control (BSA) or NGF stimulation and SFPQ immunoprecipitation. qRT-PCR analysis of precipitated mRNA with SFPQ. Normalized values ± SEM, Unpaired t-test for each mRNA, n=7, *$p<.05$.

Figure 3.2 (continued)



Interestingly, the *bclw* 3'UTR, the region where SFPQ binds, is able to promote neurotrophin-dependent localization of RNA to axons (Supp. Fig. S2C). Together, these experiments demonstrate specific interactions between SFPQ and the *bclw* and *laminb2* mRNAs, suggesting that SFPQ could regulate both mRNAs.

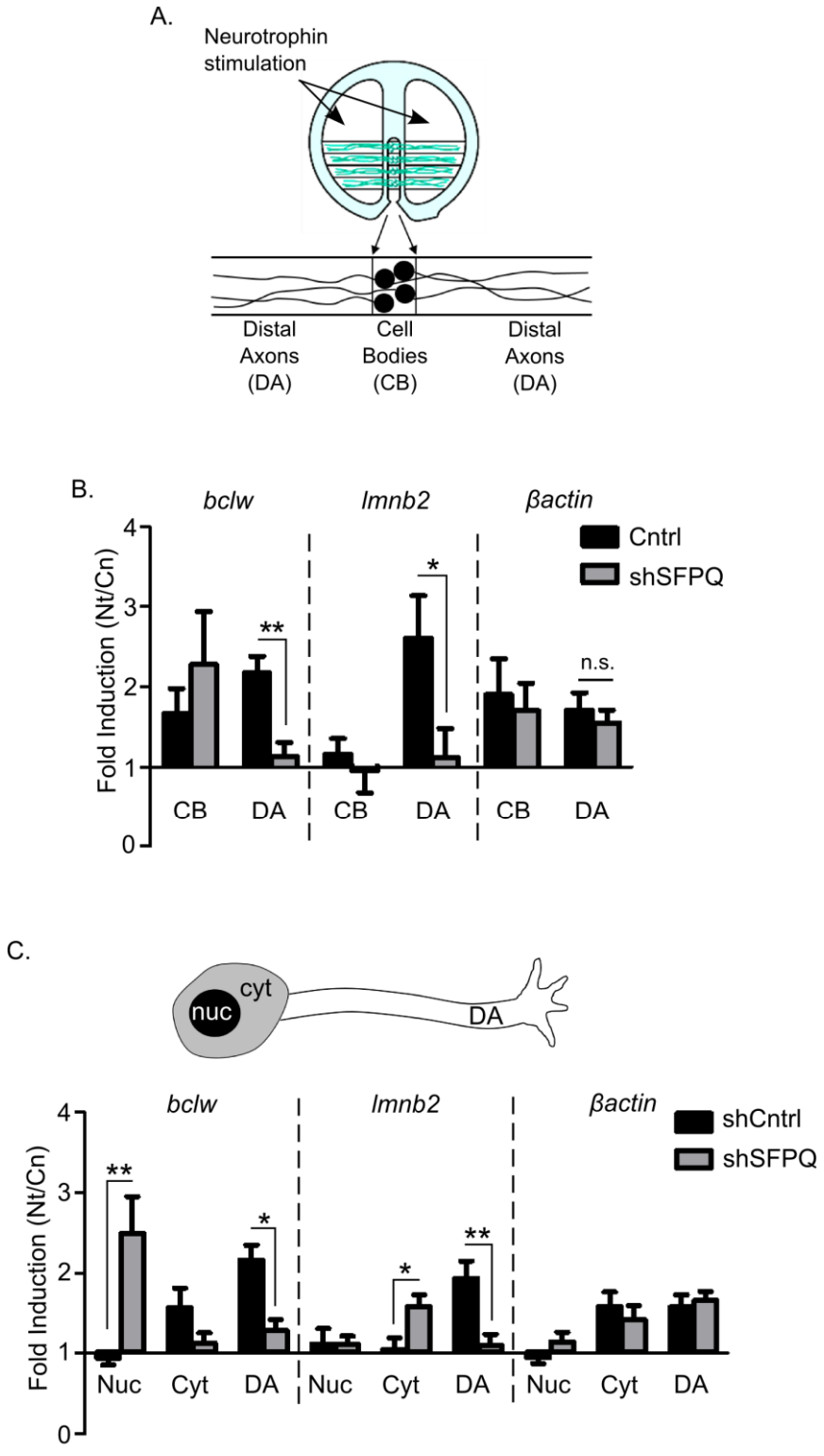
Regulation of axonal bclw and laminb2 mRNA depends upon SFPQ

To determine whether SFPQ regulates *bclw* and *laminb2* mRNA in neurons, we used shRNA to reduce endogenous SFPQ levels in DRG neurons grown in compartmented cultures (Fig. 3.3A, Supp. Fig. S3A). Distal axons of neurons were stimulated with neurotrophins, followed by collection of cell bodies (CB) and distal axons (DA) for analysis of RNA by qRT-PCR. Neurotrophin stimulation leads to transcription-dependent induction of *bclw* mRNA in CB and DA (Fig. 3.3B, Supp. Fig. S3D), but knockdown of SFPQ blocks induction of *bclw* mRNA in DA. Interestingly, *bclw* mRNA is still induced by neurotrophins within CB in the absence of SFPQ, suggesting that SFPQ is not required for neurotrophin-induced transcription of *bclw*. Similarly, *laminb2* mRNA is induced in DA by neurotrophins and blocked by knockdown of SFPQ (Fig. 3.3B). In contrast to *bclw*, neurotrophins regulate *laminb2* in a transcription-independent fashion, as the transcription inhibitor actinomycin does not block neurotrophin induction of *laminb2* mRNA in CB or DA (Supp. Fig. S3D). Loss of SFPQ has no effect on neurotrophin induction of *β-actin* mRNA in CB or DA, suggesting that SFPQ specifically regulates a group of axonal mRNAs that include *bclw* and *laminb2*. Together these data indicate that SFPQ is required for localization of both *bclw* and *laminb2* mRNA to axons.

Figure 3.3. SFPQ is required for trafficking of *bclw* and *laminb2* mRNA.

A. Schematic of DRG neurons grown in fluidically isolated compartmented culture where cell bodies (CB) are plated in the center compartment and axons extend outward into both side, distal axon (DA) compartments. Neurotrophin stimulation of the DA recapitulates target-derived neurotrophins received by axon terminals in the periphery *in vivo*. **B**. qRT-PCR analysis of *bclw*, *β-actin*, and *laminb2* mRNA levels in CB and DA of neurons grown in compartmented cultures with SFPQ knockdown following a 2hr neurotrophin stimulation of DA. Fold induction (Nt/Cn) values ± SEM, Unpaired t-test for each mRNA, n=5, *p<.05, **p<.01. **C**. Nucleus and cytoplasm fractionation of CB following 2hr neurotrophin stimulation of DA. qRT-PCR analysis of *bclw*, *β-actin*, and *laminb2* mRNA levels from nucleus, cytoplasm, and DA. Fold induction (Nt/Cn) values ± SEM, t-test for each mRNA, n=8, *p<.05, **p<.01

Figure 3.3 (continued)



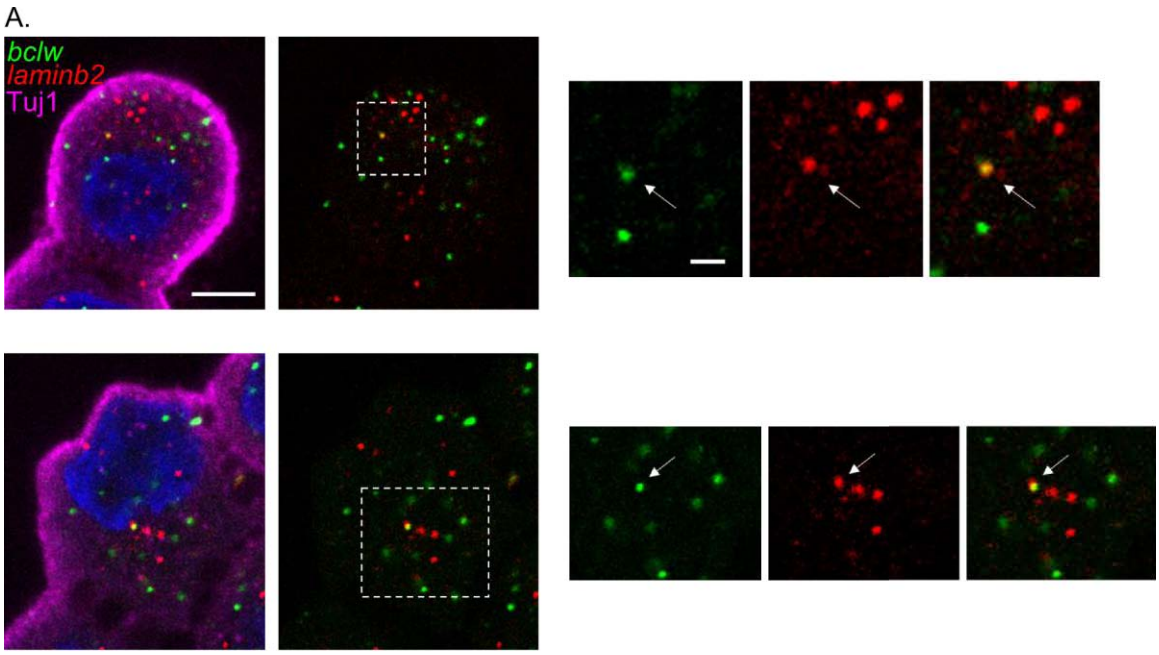
SFPQ has numerous roles in nuclear mRNA regulation and also promotes mRNA cytoplasmic transport (Kanai et al., 2004). To determine where within the post-transcriptional pathway SFPQ is functioning, we performed cellular fractionation of DRG neurons (Fig. 3.3C, Supp. Fig. S3B,C). In response to neurotrophin stimulation *bclw* mRNA is increased in the cytoplasmic fraction and DA. In contrast, following knockdown of SFPQ, neurotrophins increase *bclw* mRNA in the nuclear fraction, but not within the cytoplasmic fraction or DA, suggesting that in the absence of SFPQ, newly transcribed *bclw* mRNA cannot undergo nuclear export (Fig. 3.3C). While knockdown of SFPQ also prevents axonal localization of *laminb2* mRNA, the *laminb2* mRNA accumulates in the cytoplasm, not in the nucleus. By comparison, knockdown of SFPQ does not alter localization of *β-actin* mRNA. Thus, SFPQ acts to promote nuclear export and cytoplasmic transport of the target mRNAs *bclw* and *laminb2*.

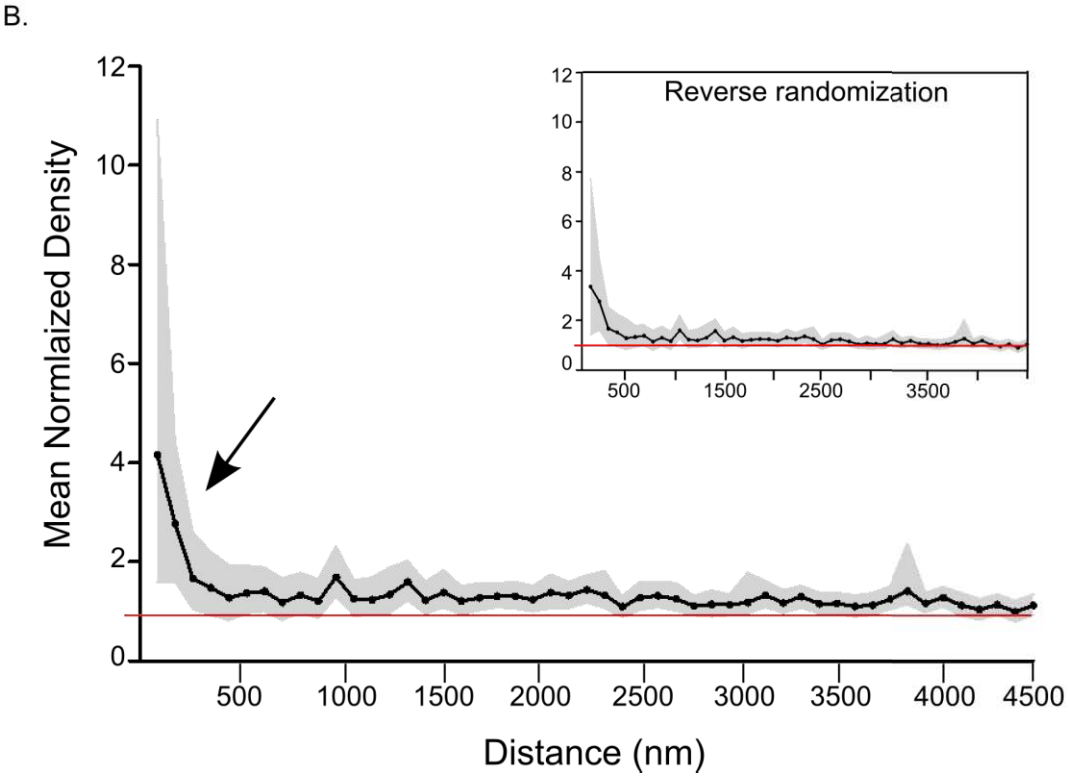
It is unknown how distinct mRNAs are packaged within cytoplasmic transport granules. To determine whether *bclw* and *laminb2* mRNA are transported together in the cytoplasm we performed single-molecule fluorescent *in situ* hybridization (FISH) in DRG neurons grown in microfluidic cultures (Fig. 3.4A, Supp. Fig. S4). Electron microscopy studies estimate granules to be roughly 100-250nm in diameter (Krichevsky and Kosik, 2001; Batish et al., 2012), thus we performed analysis of the distances between neighboring *bclw* and *laminb2* mRNA to determine if they are frequently present within this proximity. Analysis of mRNAs was performed using point source detection software that compared the distances between neighboring *bclw* and *laminb2* mRNAs in cell bodies to

Figure 3.4. Colocalization of *bclw* and *laminb2* mRNA in DRG neurons.

A. Single-molecule FISH for *bclw* and *laminb2* mRNA with DRG neurons grown in microfluidic cultures following neurotrophin stimulation of DA. Neurons labeled with Tuj1 immunostaining. Scale bar is 5µm. Zoom-in of white dotted area on right with 1µm scale bar. **B**. Quantification of distances between neighboring *bclw* and *laminb2* mRNAs within neuronal cell bodies (n=51 cells). Normalized density is frequency of *bclw* and *laminb2* neighbors at each distance on x-axis normalized to the frequency of *bclw* and randomized *laminb2* points at each distance. Inset graph shows data where *bclw* and *laminb2* data are normalized to frequency of *laminb2* and randomized *bclw* points. The two graphs appear very similar as they both show the frequency of neighboring *bclw* and *laminb2* mRNAs, each with normalization to a different set of randomized points (averaged over 1000 randomizations). Grey shaded region shows 99% confidence intervals and red line is at y=1. Arrow points to distances less than ~250µm where normalized frequency is greater than 1, indicating *bclw* and *laminb2* mRNA localize within this distance more than expected by random chance.

Figure 3.4 (continued)





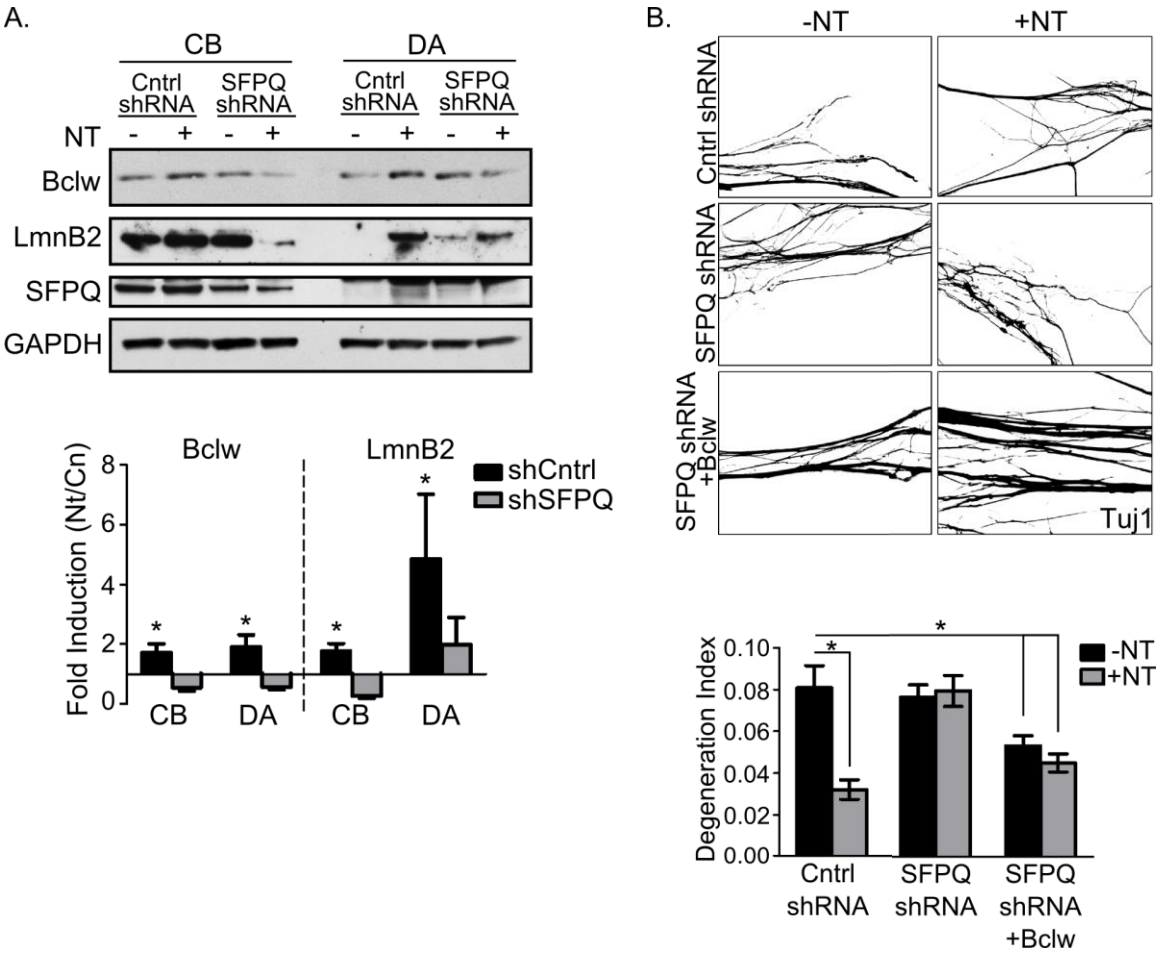
the distances between one mRNA (bclw or laminb2) and a set of randomized points. The normalized density is the frequency of neighboring *bclw* and *laminb2* mRNAs detected within a particular distance, normalized to the frequency of one mRNA with randomized points measured at that distance. Thus, a normalized value greater than 1 indicates that the occurrence of neighboring *bclw* and *laminb2* mRNA at a particular distance is more than would be expected by random chance, while a value of 1 suggests the occurrence of mRNA spots at a certain distance is no different than chance. Importantly, the analysis was performed twice, first to compare with randomization of *laminb2* points (Fig. 3.4B) and second to compare with randomization of *bclw* points (Fig. 3.4B inset graph of reverse randomization) and yield similar results. Our analysis finds that *bclw* and *laminb2* mRNAs within proximities of ~250nm or closer occur more frequently than by chance (Fig. 3.4B), suggesting that they can be packaged together within a single mRNP complex and possibly a single granule for cytoplasmic transport.

It has been proposed that localized protein synthesis of Bclw and LaminB2 occurs in axons to promote axonal survival. Knockdown of SFPQ blocks neurotrophin-induced increases in Bclw and LaminB2 protein in both CB and DA (Fig. 3.5A). Introduction of recombinant Bclw protein into axons rescues axon degeneration due to SFPQ knockdown (Fig. 3.5B). Thus, SFPQ mediates neurotrophin-dependent survival by post-transcriptional regulation of Bclw and LaminB2 that affect levels of functional protein within axons.

Figure 3.5. SFPQ regulates Bclw and LaminB2 in axons to prevent axon degeneration

A. Western blot analysis of DRG neurons grown in compartmented cultures with SFPQ or control shRNA and following 8hr NT stimulation of DA. Quantification of Bclw levels normalized to GAPDH ± SEM, Z-test comparison with 1, n=3, *p<.05. **B**. Degeneration assay showing binarized Tuj1 images for DA of neurons grown in compartmented cultures and starved or maintained in NT for 24h. DRGs infected with SFPQ or control shRNA. Bclw protein transfection to DA. Ratio degenerated particles to total Tuj1-labeled area) ± SEM, one-way ANOVA with Dunnetts Multiple Comparison Test, n=3, *p<.05.

Figure 3.5 (continued)



Discussion

Our results provide evidence for a novel neuronal RNA regulon that functions to promote axonal survival in response to target-derived neurotrophins. The RNA-BP SFPQ binds the mRNA of known axonal survival factors, Bclw and LaminB2, and regulates their mRNA and protein levels in axons of sensory neurons. SFPQ regulation coordinates nuclear export and axonal transport of target mRNAs to promote neurotrophin-dependent axonal survival. Thus, SFPQ orchestrates the spatial localization of gene expression for key survival factors to support axonal health and maintenance. While SFPQ is often implicated in nuclear functions, including pre-mRNA splicing, SFPQ has recently been shown to have an extra-nuclear role in cytoplasmic trafficking.

mRNA binding proteins and motifs

RNA-BPs are required throughout the life of an mRNA, regulating events from transcription through translation and decay (Moore, 2005; Mitchell and Parker, 2014). RNA BPs mediate binding through multiple RNA-binding domains that recognize both primary mRNA sequence and secondary structure. SFPQ contains two C-terminal RNA-recognition motifs (RRMs) (Patton et al., 1993) which mediate regulation of mRNAs, including Neat1 mRNA in nuclear paraspeckles for transcriptional repression (Fox and Lamond, 2010; Hirose et al., 2013). The precise mRNA binding sequence or structure that SFPQ recognizes has not yet been identified and studied *in vivo*, but large-scale analysis of RNA-BP motifs using short nucleotide sequences identified a number of preferred 7-mers for SFPQ (Ray et al., 2013). We identified several of these motifs within the sequences of *bclw* and *laminb2* mRNA (Fig. 3.2A,C & Supp. Fig. S2A) and

the binding we observed for *bclw* and *laminb2* RNA is consistent with the position of these predicted 7-mer binding sequences. SFPQ has also been shown to interact with AU-rich elements (AREs) (Buxade et al., 2008), which are present within the 3'UTRs of both *bclw* and *laminb2* mRNA. Additional mutation studies will be necessary to precisely identify the functionally important SFPQ binding motifs *in vivo*.

It is possible that SFPQ regulates mRNAs via the 3'UTR USE, with which SFPQ has been shown to interact (Danckwardt et al., 2007). Both *bclw* and *laminb2* mRNAs contain a USE (Danckwardt et al., 2007), Brackenridge 2000) that is usually required for 3' end cleavage and polyadenylation. Without proper 3'end formation an mRNA will not be exported from the nucleus (Carmody & Wente, 2009; Hocine et al., 2010). Interestingly, we find that loss of SFPQ causes retention of *bclw* mRNA in the nucleus. In contrast, SFPQ is not required for nuclear export of *laminb2* mRNA and the USE sequence within *laminb2* mRNA differs from that of bclw mRNA. This may explain the difference in nuclear export regulation by SFPQ. There may be different *tran*-acting factors which regulate nuclear export of *laminb2* mRNA, but SFPQ is required for localization of the mRNA to axons. This suggests that SFPQ is capable of regulating different mRNAs through distinct mechanisms, possibly requiring unique binding sites and additional proteins. It will be useful to determine the full set of SFPQ-bound mRNAs to characterize the binding motifs in detail and explain functional differences between different binding motifs.

Nuclear proteins and mRNA localization

There are numerous examples of nuclear proteins involved in cytoplasmic mRNA localization (Moore and Proudfoot, 2009). To localize *Vg1* mRNA in *Xenopus* oocyte, a core mRNP is assembled in the nucleus with hnRNP I and Vg1RBP/vera. Upon entry into the cytoplasm the complex is reorganized to include XStau which interacts with a molecular motor (Kress et al., 2004; Yoon and Mowry, 2004). The exon junction complex promotes efficient nuclear export of spliced mRNAs by promoting recruitment of the export factor TAP/p15 (Le Hir et al., 2001). Furthermore, this complex undergoes significant remodeling following nuclear export which may be critical for regulation of nonsense mediated decay, subcellular localization, or translation of many mRNAs. Splicing factors are also required for cytoplasmic localization of *oskar* mRNA in the Drosophila oocyte, thus nuclear events and components can impart critical information to an mRNA for subcellular localization following mRNA processing and nuclear export (Hachet and Ephrussi, 2004). Finally, a large scale analysis of RBPs find many splicing factors with conserved binding motifs within the 3'UTR, suggesting that splicing factors may be more multifunctional that previously appreciated (Ray et al., 2013). In fact, Muscleblind-like RNA binding proteins have extensive role in splicing and have recently been suggested to regulate mRNA transport (Wang et al., 2012).

In neurons, *β-actin* mRNA is localized to axons by a 54-nucleotide "zipcode" within the 3'UTR (Kislauskis et al., 1994, 1997). ZBP1 (Zipcode binding protein 1) regulates localization of *β-actin* mRNA (Ross et al.,1997; Zhang et al., 2001) and phosphorylation of ZBP1 by protein kinase Scr promotes local translation of *β-actin* mRNA (Huttelmaier

et al., 2005). ZBP1 is a predominantly cytoplasmic protein, but the second zipcode binding protein ZBP2 is predominantly a nuclear protein, a homologue of the human KD domain-containing splicing regulatory protein (KSRP) (Pan et al., 2007). Interestingly, Singer and colleagues find that ZBP2 binds nascent *β-actin* mRNA co-transcriptionally, facilitating recruitment of ZBP1 and formation of an mRNP for localization (Pan et al., 2007) and that ZBP2 likely shuttles between the nucleus and cytoplasm (Gu et al., 2002). It is unclear whether ZBP2 is component of the cytoplasmic *β-actin* mRNP or whether ZBP2 and ZBP1 are involved in a "handover" mechanism to promote *β-actin* mRNA localization. Either scenario implies that the mRNA processing events and mRNP formation that begin within the nucleus are the intimately linked to cytoplasmic localization of mRNAs (Hachet and Ephrussi, 2004; Kress et al., 2004; Miki et al., 2004; Giorgi and Moore, 2007), supporting a model for SFPQ shuttling between the nucleus and cytoplasm to regulate localization of axonal survival factors. As recent studies find that SFPQ functions within the cytoplasm and neurites (Kanai et al., 2004; Sury et al., 2014), this also supports the ideas that nuclear splicing factors can and do have additional extra-nuclear roles.

mRNA trafficking and transport granules

The final stages of RNA transport granule assembly likely occur within the cytoplasm to include additional translation machinery and attachment to motor proteins, but relatively large mRNPs destined for localization in the cytoplasm can form within the nucleus. While nuclear export has traditionally been shown to occur through pores in the nuclear membrane, recent studies have suggested an alternative pathway for delivering mRNPs

into the cytoplasm. Budnik and colleagues show evidence for a "budding" mechanism through the inner and outer layers of the nuclear membrane, similar to the exit pathway that viruses use (Speese et al., 2012). Tyrosine-phosphorylated SFPQ has been identified with the nuclear membrane (Otto et al., 2001) and the authors concluded that SFPQ-dependent splicing activity likely occurs close to the membrane. Our studies suggest that SFPQ is actually accompanying mRNAs into the cytoplasm and it will be interesting to determine whether SFPQ-bound mRNAs are exported via the canonical nuclear pore mechanism or within larger mRNPs via nuclear budding.

Our findings show that SFPQ is needed to obtain neurotrophin-dependent increases in *bclw* and *laminb2* mRNA in axons, but cellular fractionation experiments suggest that SFPQ initiates a regulatory role for these two mRNAs at different points. Without SFPQ, *bclw* mRNA is not exported from the nucleus, suggesting a role in upstream mRNA processing or in nuclear export. We do not detect splicing defects in *bclw* following knockdown of SFPQ (data not shown). SFPQ interacts with USE elements (Danckwardt et al., 2007), and may thereby promote 3'end formation and polyadenylation, functions required for nuclear export. In contrast to *bclw* mRNA, our data suggest that SFPQ primarily promotes axonal transport of *laminb2* mRNA, although it is unknown when and where SFPQ first interacts with the mRNA. In addition, it is unknown whether SFPQ accompanies *bclw* mRNA in the axon for transport. It will be interesting to determine whether SFPQ is a required component of RNA transport granules to regulate localization and translation of *bclw* and *laminb2* mRNAs in axons.

While studies agree that RNA transport granules contain mRNPs and translation machinery (Krichevsky and Kosik, 2001; Kanai et al., 2004; Fritzsche et al., 2013), little is known about how many mRNAs are contained within distinct granules and whether distinct mRNAs are co-assembled into single granules. Using single-molecule FISH and colocalization studies of multiple dendritic mRNAs, it has been suggested that mRNAs are transported separately into dendrites (Batish et al., 2012). Our studies suggest that *bclw* and *laminb2* mRNA can be transported within a single granule. SFPQ-bound *bclw* mRNA may exit the nucleus and then reorganize to form a granule with SFPQ-bound *laminb2* mRNA and/or other axonally-targeted mRNAs. Additional studies are needed to determine whether SFPQ granules containing *bclw* and *laminb2* are present within axons.

The RNA regulon model proposes that functionally related mRNAs are regulated together by RNA-BPs, so that signaling pathways efficiently orchestrate gene expression for a targeted functional response (Keene, 2007). Years of work demonstrate that target-derived neurotrophins activate a transcriptional response to promote neuronal survival, but it is now becoming clear that post-transcriptional regulation is extremely complex and is highly regulated to promote a variety of cellular functions. Here we describe coordinated post-transcriptional regulation of known axonal survival factors, Bclw and LaminB2, by SFPQ and loss of SFPQ causes selective degeneration of axons. Together, this suggests that neurotrophins activate a distinct pathway to promote axonal health, whereby SFPQ coordinates the spatial expression of mRNAs that are locally translated to prevent axon degeneration.

Bclw is a prosurvival Bcl2 family member that is required *in vivo* for the health and long-term maintenance of sensory axons (Courchesne et al., 2011). We have previously shown neurotrophin-induction of Bclw is dependent on local protein synthesis and that Bclw protein binds Bax to block caspase6 activation and axon degeneration (Cosker et al., 2013). The Bcl2 family contains both prosurvival and proapoptotic factors that act at mitochondria to tightly regulate mitochondrial membrane potential and release of cytochrome C in response to a variety of stimuli to promote the appropriate cell survival or death response (Brunelle and Letai, 2009). The mechanism by which LaminB2, a nuclear intermediate filament, functions in axonal health is less understood, but it also localizes to mitochondria and loss of both Bclw and LaminB2 protein results in defects in axonal mitochondria length and function, as well as axon degeneration (Courchesne et al., 2011; Yoon et al., 2012).

Interestingly, this study is the first to demonstrate neurotrophin regulation of LaminB2, which was previously shown to be induced by the guidance factor Engrailed-1 in retinal ganglion cells (Yoon et al., 2012). Neurotrophin regulation of LaminB2 may be a more widely used mechanism to promote mitochondrial health for neurotrophin-dependent axonal survival. The health and functional activity of mitochondria in axons for viability, requires local protein synthesis in axons (Hillefors et al., 2007; Kar et al., 2014; Aschrafi et al., 2010; Natera-Naranjo et al., 2012). Numerous nuclear-encoded mRNAs for mitochondria-associated proteins localize to developing and mature axons and mechanisms exist for specific localization of mRNAs to mitochondria (Gerber et al., 2004; Garcia-Rodriguez et al., 2007; Aschrafi et al., 2010). Thus, future experiments will

be necessary to determine whether the mRNAs encoding proteins for axonal mitochondria are localized and translated in close proximity to the intended axonal mitochondria. In addition to *bclw* and *laminb2*, it will be exciting to show whether SFPQ coordinates subcellular expression of additional mRNAs as part of an even larger RNA regulon for axonal survival.

Chapter 4:

Tagging and tracking of locally synthesized protein by spaceSTAMP

Contributions:
Sara J. Fenstermacher wrote chapter, prepared figures, and generated data for all Figures. Rosalind A. Segal edited chapter.

Acknowledgements:
Thank you to Michael Lin for a wonderful collaboration, and for providing constructs and helpful advice throughout this work. Thank you to Jennifer Waters of the Harvard Nikon Imaging Center for help with TIRF imaging. Thank you to Maria Pazyra-Murphy for help with Campenot culture experiments and to Katharina Cosker and Sarah Pease for helpful discussion. This work was supported by National Institute of Health Grant RO1 NS050674 to R.A.S. and F31 NS077620 (NINDS) to S.J.F.

Introduction

Localization of mRNA and localized protein synthesis are used widely across species and cell types to generate spatially restricted gene expression. It is intuitive that such a mechanism would be particularly advantageous for a polarized neuron with highly separated and sometimes distant subcellular compartments. Sensory neurons that transmit information from the periphery to the central nervous system can be a meter long in humans. While protein translocation provides for a significant portion of protein localization, the ability to synthesize protein locally in response to extracellular cues is efficient, allows for site-specific changes in protein composition, and creates additional places for regulation. Diverse cues and signaling pathways can regulate mRNA splice forms for precise subcellular localization, as well as targeted mRNA stabilization and translation to induce distinct cellular responses.

While thousands of mRNAs have been shown to localize to neuronal axons and dendrites, it has been challenging to rigorously demonstrate local translation and even harder to prove functional significance for the localized mRNA or locally generated protein. Several methods have been extensively used to study local translation over the last two decades. Myristolated GFP (myrGFP) was first used to investigate translation of CamKII mRNA in dendrites and has since been used to study numerous axonal mRNAs (Aakalu et al., 2001). MyrGFP is commonly fused to the 3' and 5'UTRs of mRNAs that exhibit distinctive intracellular localization. Upon translation, myristolation promotes interaction with the membrane and thus limits GFP diffusion to reveal where the mRNA was translated. The photoactivatable protein Kaede, which is irreversibly converted from

green to red fluorescence with ultraviolet light, has also been used as a translation readout in living cells (Leung et al., 2006). This is done by converting all Kaede signal to red and observing where newly synthesized green fluorescence appears within the cell. Methods known as BONCAT (bioorthogonal non-canonical amino acid tagging) and FUNCAT (fluorescent non-canonical amino acid tagging) use incorporation of amino acid analogs to label sets of newly made protein in subcellular compartments in vitro or in vivo (Dieterich et al., 2006; Dieterich et al., 2010; Hodas et al., 2012). While these methods have provided significant evidence for localized translation, none is capable of labeling a specific newly synthesized protein for both biochemical analysis and fluorescent live imaging and of gaining biological insight for the locally synthesized proteins. Thus, we sought to develop a novel method for studying local translation that would label protein synthesized only within a particular subcellular compartment and allow us to follow a particular protein of interest to understand the biological significance for its localized synthesis.

Lin and colleagues developed a method for drug-dependent labeling of protein called TimeSTAMP (time-specific tagging for the age measurement of proteins) (Lin et al., 2008; Butko et al., 2012). This allows fluorescent and epitope tagging of a specific protein to study distribution of newly synthesized protein in living cells within a particular time period. The tags are removed by a sequence-specific protease, but retained in the presence of a non-toxic, selective protease inhibitor. TimeSTAMP has demonstrated that newly synthesized PSD-95 protein localizes to stimulated dendritic synapses (Butko et al., 2012), but has not been used to investigate local translation. To

achieve this goal, we took advantage of compartmented cultures for the *in vitro* growth of neurons wherein cell bodies and distal axons are separated within fluidically isolated compartments. Microfluidics cultures (Taylor et al., 2005) can be used for live imaging of neurons and compartmented "Campenot" chambers (Campenot, 1977) can be used for biochemical analysis of cell bodies and distal axons of neurons (see Appendix I). With fluidically isolated compartments, protease inhibitor can be added solely to the distal axon compartment to selective label protein synthesized within axons. Thus, we call this method spaceSTAMP because it will enable precise spatial tagging of newly made proteins to investigate localized translation in neurons.

Extracellular stimuli regulate localization and translation of mRNAs in axons. For example, local translation of *β-actin* in growth cones is required for turning in response to a Netrin-1 gradient (Leung et al., 2006). To understand the purpose for locally synthesizing *β-actin*, it would be extremely useful to visualize the newly made protein molecules and follow them in real time to observe where new proteins are incorporated into polymerizing actin filaments within the growth cone to facilitate the turning response. We have previously shown that neurotrophins regulate axonal localization of mRNA for Bclw, the prosurvival Bcl2 family member, to promote survival of long sensory axons (Cosker et al., 2013). While we demonstrated that local protein synthesis is required for neurotrophin induction of Bclw protein in axons, we would like to show directly that *bclw* mRNA is translated in axons and observe where locally synthesized Bclw protein localizes within axons. Bclw protein is a critical survival factor for dorsal

root ganglion (DRG) neuron axons *in vivo* (Courchesne et al., 2011) that functions at mitochondria to prevent apoptosis.

Here, we develop a method called spaceSTAMP to study local translation of *bclw* in DRG neurons. spaceSTAMP tagging of Bclw is dependent on protease inhibitor and Bclw protein localizes to axonal mitochondria. Although we cannot yet reliably detect local Bclw synthesis in axons, we hope to use spaceSTAMP to visualize where locally synthesized Bclw is made within axons and how it is distributed amongst axonal mitochondria. Our studies suggest that spaceSTAMP may be used in the future to study local synthesis of β-actin and many other localized mRNAs to increase our understanding for the functional significance of localized translation.

Materials and Methods

293T cells. 293T cells were grown in DMEM media with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. FuGENE 6 Transfection Reagent (Promega) was used to transfect cells in a 24-well plate with 300ng BclwSS/BclwSS^{mut} and 60ng mCherry plasmids for 4hr. Following transfection, media was replaced without FBS and with 1uM BMS-650032. Cells were collected 24 hours following drug addition in lysis buffer (20mM Tris-HCl pH 7.4, 140mM NaCl, 10% glycerol, 1% Triton X-100) with Complete Mini protease inhibitor cocktail tablet (Roche) for analysis by western blot. Transfected cells for live imaging were changed into media with .5% FBS and 1uM BMS-650032 following transfection and imaged 24hr following. Living imaging was performed using

an inverted Nikon microscope with temperature and CO_2 controlled incubator to assay for YFP and mCherry expression.

*Neuronal cultures***.** Dorsal root ganglion (DRG) neurons were dissected from E15 rat embryos in Hank's Balanced Salt Solution (HBSS) and dissociated in 0.3mg/ml trypsin in HBSS for 50min at 37ºC. Cells were transfected with 5ug BclwSS/SS^{mut} and 0.6ug mCherry using the Nucleofector (Lonza; Program G-30). Cells are plated in Neurobasal with 2% B-27 Supplement, 1% Glutamax, 0.4% Glucose, and 100ng/ml NGF and BDNF. Tissue cultures plates are pre-coated with Matrigel Basement Membrane Matrix (BD Biosciences) diluted 1:45 in DMEM. One day after cell plating, media was changed to include 1% penicillin-streptomycin and 0.3uM cytosine arabinoside (AraC). Cycloheximide (EMD Biosceinces) was used at 10ug/ml to inhibit translation in BclwSS mass cultures. BMS-650032 (obtained from M. Lin) was added to mass cultures or microfluidics add desired concentration.

*Microfluidic cultures***.** Microscope cover glass 24X40 #1.5 (Fisher) was washed in EtOH, autoclaved, and coated with 0.2mg/mL poly-D-lysine in HBSS overnight at room temperature. Cover glass was washed 3 times with sterile water and coated with laminin 10µg/ml in HBSS for 3 hours at 37ºC prior to plating neurons. Following DRG neuron transfection, cells were plated into the left compartment of each microfluidic device in Neurobasal with 2% B-27 Supplement, 1% Glutamax, 0.4% Glucose, and 50ng/ml NGF and BDNF. The right, compartment was filled with 100ng/ml NGF and BDNF media. One day after plating, the left compartment was changed to media with 1% penicillin-streptomycin, 0.3uM AraC, and 10ng/ml NGF and BDNF and the right compartment to media with 100ng/ml NGF and BDNF. On the second and third days after plating, the

right cell body compartment was changed into media with 1ng/ml NGF and BDNF and the right compartment into media with 10ng/ml NGF and BDNF.

*Western Blotting***.** 293T cell and DRG neuron lysates were made up with Invitrogen NuPAGE LDS sample buffer (4X) and reducing agent (10X) and separated by 4-12% Bis-Tris SDS-PAGE. Protein was transferred to methanol-activated PVDF membrane and blocked with 5% milk in 1X TBST for 1hr at room temperate. Membranes were probed with the following antibodies overnight at 4 ºC: anti-HA Tag (1:1000, Millipore), anti-pan-actin (1:1000, Cell Signaling Technology), rabbit anti-Bcl-w [31H4] (1:1000, Cell Signaling #2724). Membranes were washed 3 times with 1X TBST and probed with goat anti-rabbit or goat anti-mouse HRP-conjugated secondary antibodies (1:10,000; Biorad) for 1hr at room temperature. Blots were washed 3 times with 1X TBST and developed with ECL detection reagent (VWR).

*Plasmids***.** BclwSpaceSTAMP (BclwSS) was synthesized by GenScript using the timeSTAMP YFP sequence (spaceSTAMP cassette) from M. Lin (Butko et al., 2012) and rat ORF and UTR sequences obtained from NCBI (NM_021850.2). The spaceSTAMP sequence was positioned upstream of the Bclw ORF and downstream of the 5'UTR. The GenScript sequence was flanked by Not1 at the 5' end and Xho1 on the 3'end. These sites were used to clone BclwSS into pcDNA3.1 (Invitrogen) for expression in mammalian cells. The QuikChange Site-Directed Mutagenesis Kit from Stratagene was used to make the mutated spaceSTAMP construct by mutating a single amino acid generating a serine to alanine transition at aa 362 in the spacestamp cassette. PAGE purified primers were used in the mutagenesis reaction to convert TCG to GCT (5'-3' frwd cttgaaaggctcc**gct**gggggtccgctg and rev agcggacccccc**agc**ggagcctttcaag). Pncs-

mcherry was provided by M. Lin. Mcherry flanked by BamH1 and EcoR1 was cloned into pcdna3.1 (Invitrogen) for our experiments.

*Mitotraker***.** Neuronal cultures were incubated in media with MitoTracker Red CMXRos (1:5000) for 15min at 37ºC, followed by 3 washes with HBSS and 1min light fix with iced 100% MeOH. Cultures were changed into PBS and imaged immediately.

*Live imaging and fluorescence quantification***.** Images were acquired using an inverted Nikon Eclipse T*i* fluorescent microscope and Photometrics Cool SNAP HQ^2 Camera within a humidified chamber maintained at at 37° for live imaging. A 20X PlanApo (NA 0.75) or 60X PlanApo (NA 1.4) oil objective was used for imaging live and fixed samples. ImageJ was used for quantification of YFP and mCherry signal. mCherry positive cells were identified and threasholded to contain the whole neuron cell body. The mean YFP intensity was then calculated within each mCherry theasholded region. A mean background intensity value was subtracted from the mean YFP value for each cell to produce a relative intensity value for each cell.

*Metabolic labeling***.** DRG mass cultures were starved of methionine on 7 DIV in DMEM without methionine for 1hr. L-Azidohomoalanine (AHA) or methionine was added at 200uM to cells in DMEM. One hour later cells were stimulated with 100ng/ml NGF and BDNF and incubated for 24hr at 37ºC. Cells were collected in lysis buffer (20mM TrisHCl , 140mM NaCl, 10% glycerol, 1% SDS, and protease inhibitor) lysed at 4ºC on nutator for 30min, sonicated briefly in a water bath, and spun down for 5min at 4ºC. Lysates were pre-cleared with MyOne Streptavidin T1 Dynabeads. Click Chemistry was performed according to manufacturer's protocol using Click-iT Metabolic Labeling Reagents for Proteins (Invitrogen) and 200µM alkyne biotin (Invitrogen B10185) with

100ug protein of each sample. To remove excess biotin, lysates were run through Bio-Spin P6 columns according to manufacturer's protocol. Lysates were incubated with pre-blocked (1% BSA) Dynabeads for 20min on nutator at room temperature. Beads were removed by magnet, washed 3 times with 0.1% BSA, and boiled in sample buffer with reducing (Invitrogen). Total precipitated protein was analyzed by western blot and 40ug for input lane.

Viral expression of BclwSS. A replication-deficient herpes simplex virus (HSV) for bicistronic expression of BclwSS (same as described above for transfection) and mCherry was generated by Rachael Neve at the Viral Core Facility of the Picower Institute at MIT. The highly concentrated virus was estimated to be $3x10^8$ transducing units per ml. Virus was used at 1:4000 in 10ng/ml NGF and BDNF media and added to cell bodies in microfluidic cultures, while axons were maintained in 100ng/ml NGF and BDNF. BMS-650032 was added to mass cultures or microfluidics at desired concentration.

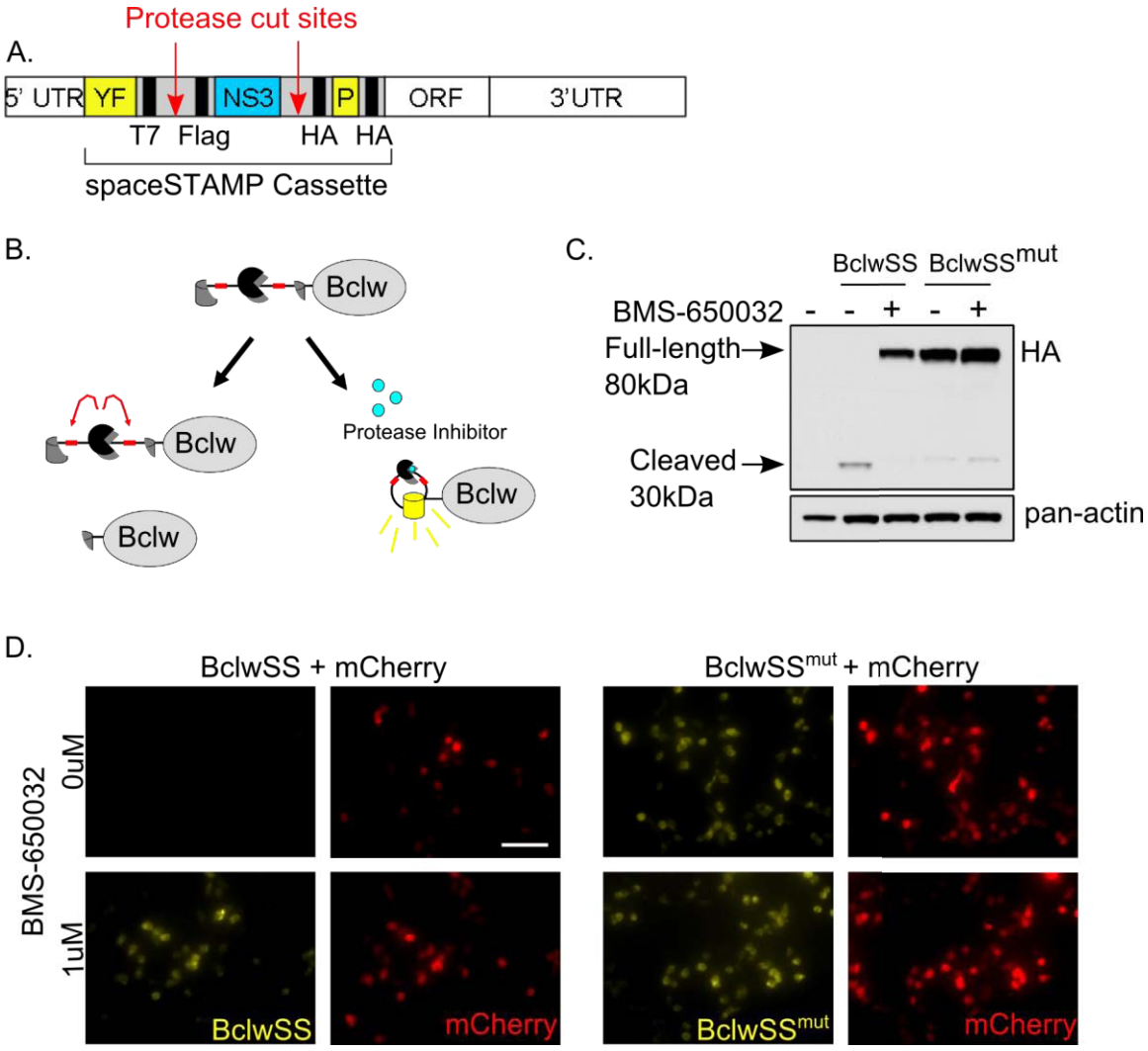
Results

To investigate translational regulation of Bclw in DRG neurons, we generated spaceSTAMP-tagged Bclw (BclwSS) to allow drug-dependent labeling of Bclw protein (Fig. 4.1A). Since the C-terminal of Bclw is critical for its activity (Hinds et al., 2003) and localization at mitochondria, the Bclw is tagged on the N-terminus. In addition, this placement allows for contiguity between the *bclw* mRNA ORF and 3'UTR, which is important for localization of *bclw* mRNA to axons of DRG neurons (Supp. Fig. S2C-E). The SS cassette contains a split YFP molecule, the NS3 protease derived from the hepatitis C virus, sequence-specific NS3 protease cleavage sites, and several other small

Figure 4.1. SpaceSTAMP schematic and function in 293T cells.

A. The spaceTAMP cassette contains a split YFP molecule, NS3 protease (Hepatitis C Virus), T7, Flag, HA tags, and protease cleavage sites. This cassette is placed between the coding and UTR sequences for the gene of interest, Bclw. **B**. Detection of YFP-tagged Bclw is dependent upon the addition of a specific NS3 protease inhibitor (BMS-650032). Protein synthesized in the absence of the inhibitor (left arrow) generates a non-fluorescently-tagged protein, as the active NS3 protease expressed within the cassette cleaves at the two recognition sites. Protein synthesized in the presence of the protease inhibitor (right arrow) generates YFP-tagged protein, as cleavage is blocked and a mature fluorescent YFP molecule forms. **C**.Western blot for HA to detect BclwSpaceSTAMP (BclwSS) in 293T cells with and without BMS-650032 treatment. BclwSS^{mut} contains a mutation in the protease rendering it inactive. No HA is detected in the untransfected neurons (left lane).Western blot for pan-actin is a loading control. **D**. Live imaging of YFP fluorescence generated by BclwSS and BclwSS^{mut} with BMS-650032 treatment, in mCherry co-transfected cells. Scale bar is 100μm.

Figure 4.1 (continued)



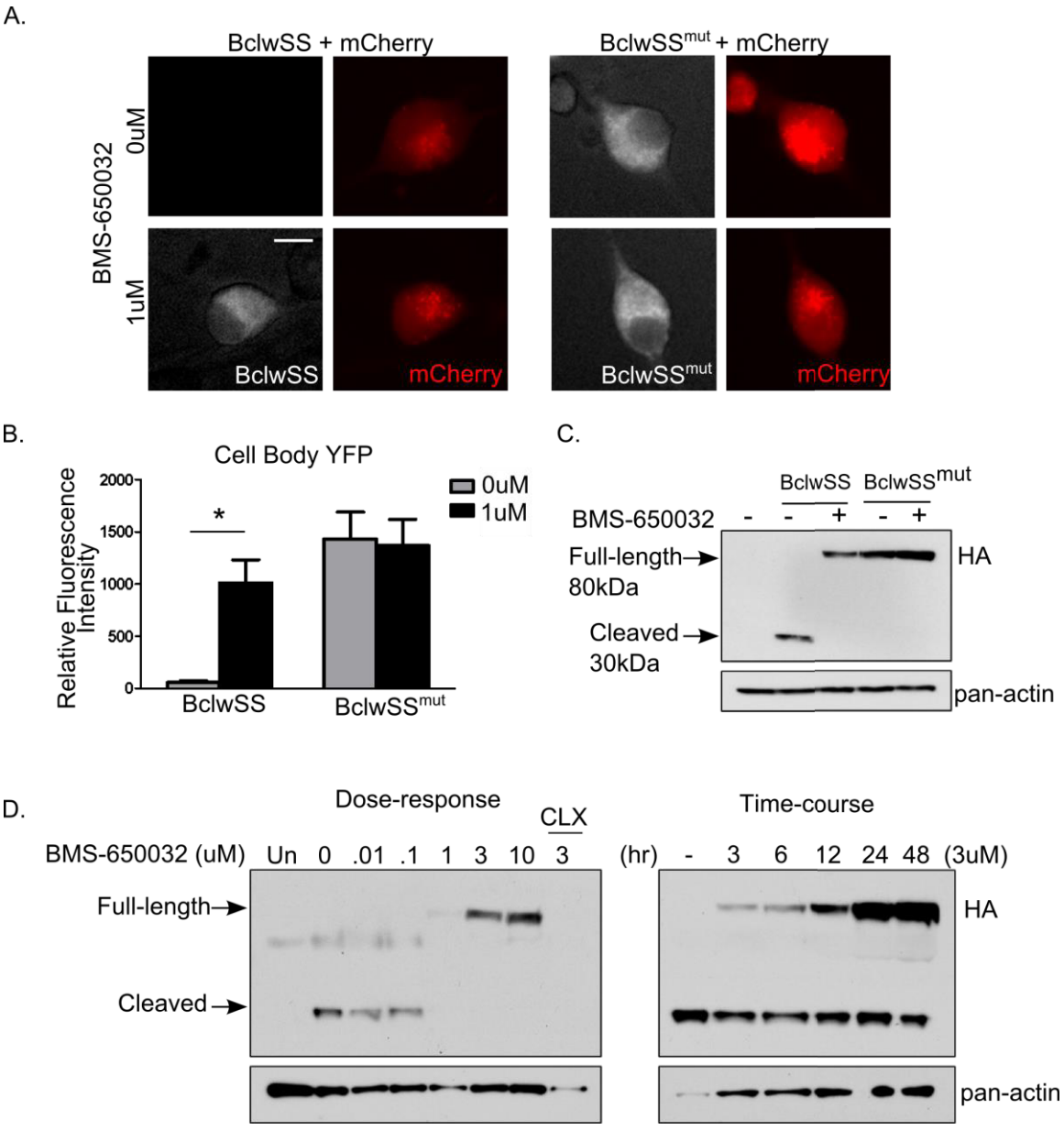
epitope tags. By default, as the protein is synthesized, the protease cuts at the two recognition sites leaving a non-fluorescent Bclw that is HA-tagged (Fig. 4.1B). Upon addition of the specific NS3 protease inhibitor, BMS-650032, the protease activity is blocked and the split YFP generates a mature YFP molecule by bimolecular fluorescence complementation. This produces YFP-tagged Bclw protein with HA and additional epitope tags. BMS-650032 (Asunaprevir) is currently in clinical trial use and displays no toxicity in cultured neurons at ≤10uM. In HEK cells, the uncleaved BclwSS protein that generates YFP is only present following addition of protease inhibitor (Fig. 4.1C,D). In contrast, BclwSS^{mut} contains a point mutation within the protease rendering it inactive and thus full-length, YFP-tagged Bclw is generated in the absence or presence of inhibitor (Fig. 4.1C,D). This demonstrates that YFP labeling is dependent upon protease activity.

In DRG neurons co-transfected with BclwSS and mCherry, YFP is only detected following inhibitor treatment, while BclwSS^{mut}-generated YFP is independent of treatment (Fig. 4.2A,B). By western blot, a 30kDa, cleaved protein product is detected without protease inhibitor, but with inhibitor treatment the 80kDa, uncleaved BclwSS protein is detected (Fig. 4.2C). A BMS-650032 dose-response curve shows production of full-length BclwSS with inhibitor doses above 1uM that can be blocked by the protein synthesis inhibitor cycloheximide (Fig. 4.2D). The protease activity is blocked by BMS-650032 as newly synthesized full-length BclwSS is detected following a 3hr treatment (Fig. 4.2D). Thus, BclwSS is a novel tool that can fluorescently label newly synthesized Bclw protein in neurons within the period of BMS-650032 treatment.

Figure 4.2. SpaceSTAMP labels Bclw in DRG neurons.

A. Live imaging of YFP generated by BclwSS (left) and BclwSS^{mut} (right) in DRG neurons with 24hr BMS-650032 treatment (1μM). Neurons co-transfected with mCherry. Scale bar is 10μm. **B**. Quantification of YFP signal in (A.). Mean YFP intensity minus background fluorescence intensity in neuronal cell bodies ±SEM; unpaired t-test, n=17 cells, *p<0.001. **C**. Western blot for HA tag to detect BclwSS and BclwSS^{mut} with BMS-650032 treatment (1μM, 48hr). Pan-actin is a loading control. **D**. Western blot for HA to detect BclwSS with BMS-650032 (24hr) dose-response (left) and time-course (right). Cycloheximide (10ug/mL, CLX) treatment used to inhibit protein synthesis during BMS-650032 treatment. Pan-actin is a loading control.

Figure 4.2 (continued)



To determine whether BclwSS can be used to label Bclw protein in axons, we performed live imaging of DRG neurons grown in microfluidic cultures, where axons grow away from the DRG cell bodies into a separate distal axon compartment. BclwSS^{mut} generates YFP throughout axons with a non-uniform expression pattern (Fig. 4.3A). Since Bclw is known to localize and function at mitochondria, MitoTracker was used to visualize axonal mitochondria in BclwSS^{mut} neurons. BclwSS^{mut} YFP colocalizes with mitochondria in axons (Fig. 4.3B). Importantly, BclwSS also shows inhibitor-dependent YFP expression that colocalizes with mitochondria in axons (Fig. 4.3C). Thus, YFP-tagged Bclw localizes properly to axons and can be labeled in a drug-dependent manner.

To investigate localized translation of *bclw*, DRG neurons were grown in fluidically isolated microfluidic cultures to allow restricted application of protease inhibitor to spatially separated neurons (Fig. 4.4B). BclwSS functions such that only the protein synthesized in the presence of BMS-650032 will be tagged with YFP. We have previously shown that *bclw* mRNA localizes to DRG axons (Cosker et al., 2013). Thus, *BclwSS* mRNA that is present in DRG cell bodies or axons will only give rise to YFP-labeled Bclw protein if BMS-650032 is present in the media at the time of translation. Fluidically restricted microfluidic cultures can maintain a local high concentration of protease inhibitor within the media on one side of the culture due to the hydrostatic pressure elicited by putting an unequal volume of media on each side. We find that BclwSS generates YFP in DRG cell bodies following 24hr inhibitor (BMS) treatment to the cell body compartment (Fig. 4.4A top rows, 4.4C). Importantly, YFP is not detected within cell bodies following 24hr inhibitor treatment to the distal axons. Thus, protease

Figure 4.3

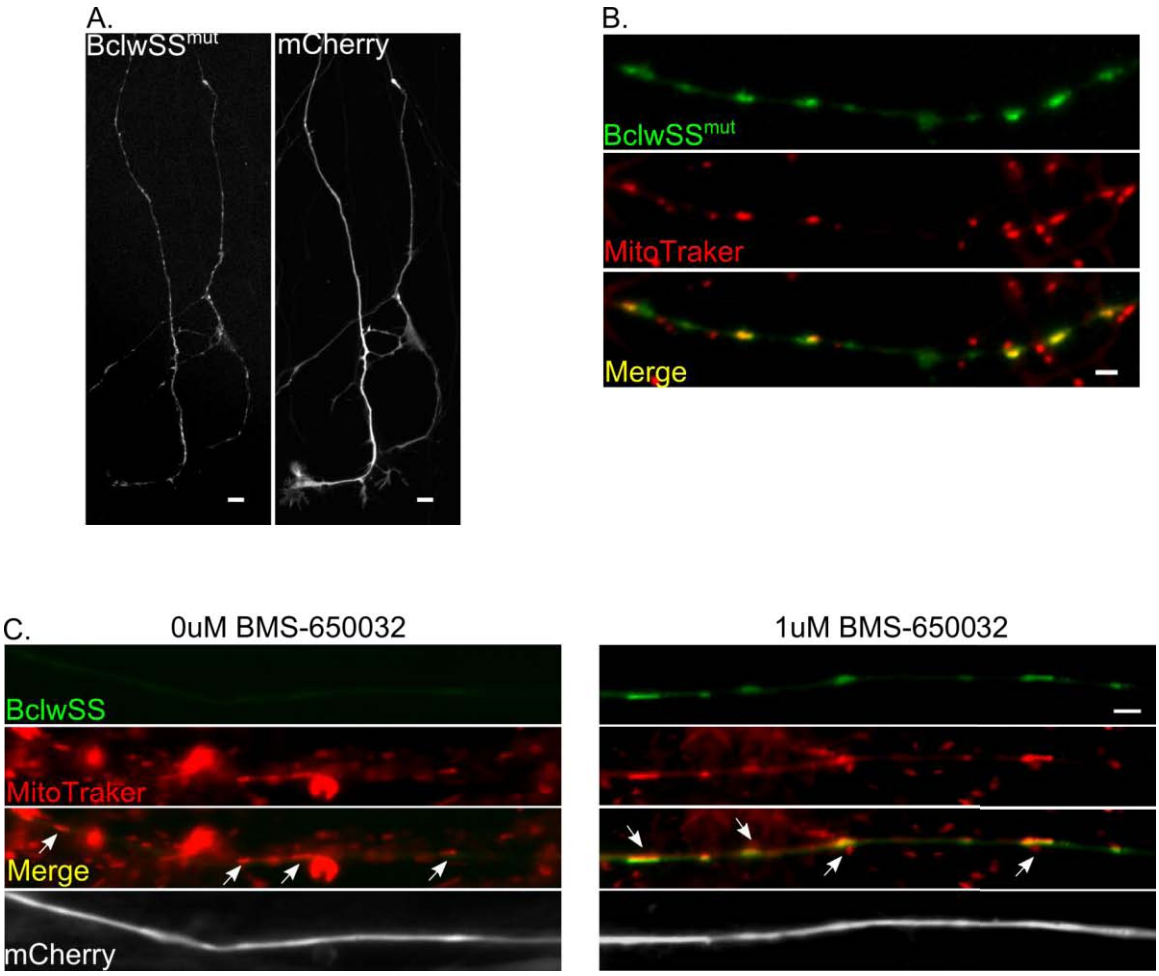


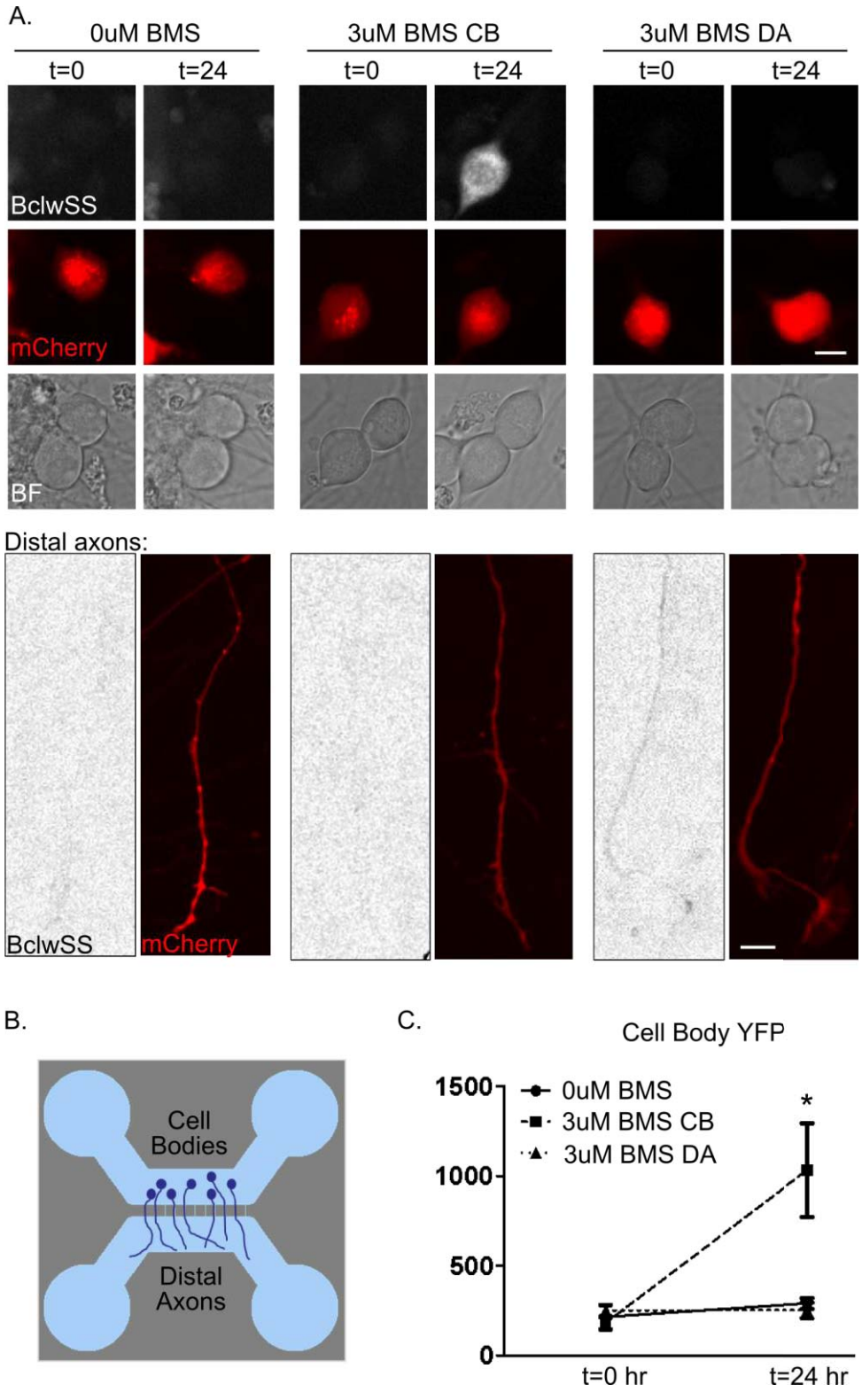
Figure 4.3. Localized BclwSS expression at mitochondria within DRG axons.

A. Live imaging of YFP expression in BclwSS^{mut} DRG neurons grown in microfluidic cultures. Scale bar is 10µm. **B**. BclwSS^{mut} neurons labeled with MitoTracker to visualize axonal mitochondria with BclwSS YFP. Scale bar is 2µm. **C**. BclwSS neurons with 24hr BMS-650032 treatment and MitoTracker to visualize BclwSS YFP with mitochondria in axons. Arrows mark mitochondria within axon. Scale bar is 2µm.

Figure 4.4. BclwSS for detection of local protein synthesis by live imaging.

A. BclwSS DRG neurons grown in microfluidic cultures with no treatment (left column), 3uM BMS-650032 treatment (24hr) to cell bodies (CB) (middle column), or 3uM BMS-650032 treatment (24hr) to distal axons (DA) (right column). Live imaging of YFP generated by BclwSS in mCherry expressing neurons. Cell bodies shown in upper half and distal axons for each condition shown below. BclwSS signal in distal axons is inverted. BF- brightfield. CB and DA scale bars are 10μm. **B**. Schematic of microfluidic culture to grow DRG neurons with fluidic isolation of cell bodies (CB) and distal axons (DA). **C**. Quantification of BclwSS YFP in cell bodies between 0 and 24hr protease inhibitor treatment shown in A.

Figure 4.4 (continued)



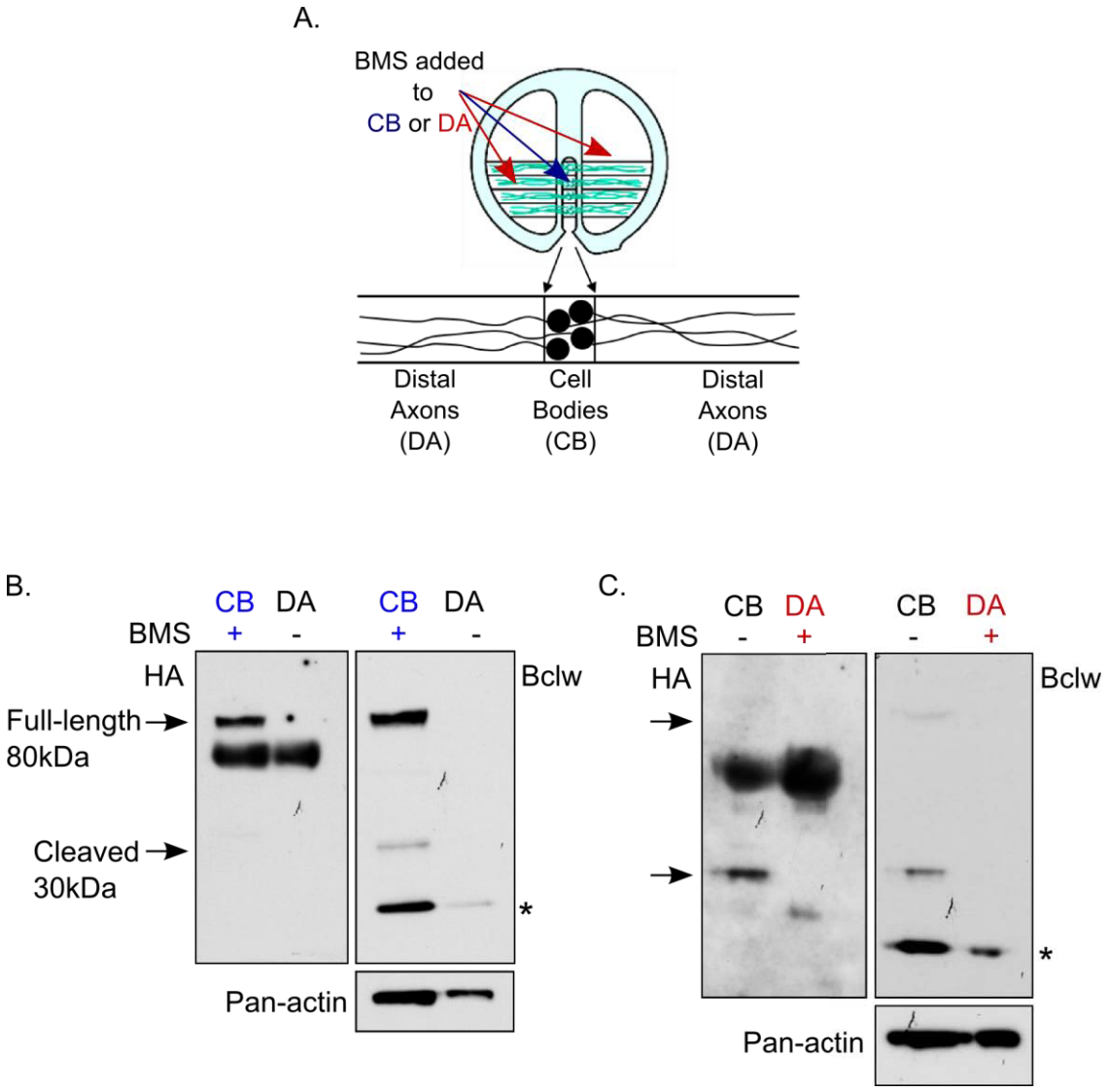
inhibitor does not leak across compartments, eliminating the possibility that cell body-synthesized YFP could contribute to axonal signal within this time frame. To determine whether BclwSS can label Bclw protein synthesized in locally in axons, we imaged distal axons following inhibitor treatment to that compartment. Thus far, we have been unable to detect local increases in axonal YFP under these conditions, as any axonal YFP that is detected can be followed throughout the entire axon and back into the cell body. Figure 4.4A shows the axon (DA right panel) of a rare cell overexpressing high levels of BclwSS protein in the cell body (not shown), and thus one cannot conclude that the YFP detected in the axon was synthesized locally. In addition, we have been unable to detect a dose-dependent effect on axonal YFP levels and local cycloheximide treatment does not block YFP generation. Thus, this approach is not yet working as originally desired.

While the BclwSS YFP mechanism is ideal for live imaging, it is also extremely well-suited for biochemical assays. Thus, we performed additional experiments to investigate local protein synthesis using a different compartmented culture system (Fig. 4.5A). Here, cell bodies are plated within a center compartment and axons extend beneath a Teflon divider into two side, distal axon, compartments (Pazyra-Murphy and Segal, 2008). These cultures are also fluidically isolated to allow local inhibitor treatment and permit separate collection of cell body and distal axon material for analysis. We find that protease inhibitor treatment to cell bodies generates uncleaved BclwSS protein in cell bodies, but this is not seen with treatment to the distal axons (Fig. 4.5B). Unfortunately, with inhibitor treatment to distal axons, thus far we do not detect full-length BclwSS with distal axons (Fig. 4.5C).

Figure 4.5. BclwSS for biochemical detection of local protein synthesis.

A. Schematic of compartmented "Campenot" culture with Teflon divider used to fluidically isolate cell bodies (CB) and distal axons (DA) for separate BMS-650032 treatment. **B**. BclwSS DRG neurons grown in compartmented cultures with 3uM protease treatment (24hr) to cell bodies (CB) and analyzed by western blotting for HA, Bclw and pan-acting (loading control). Small (~20kDa) band is endogenous Bclw (marked by *). **C**. BclwSS neurons treated with 2uM protease inhibitor (24hr) to the distal axons (DA) and analyzed by western blot.

Figure 4.5 (continued)



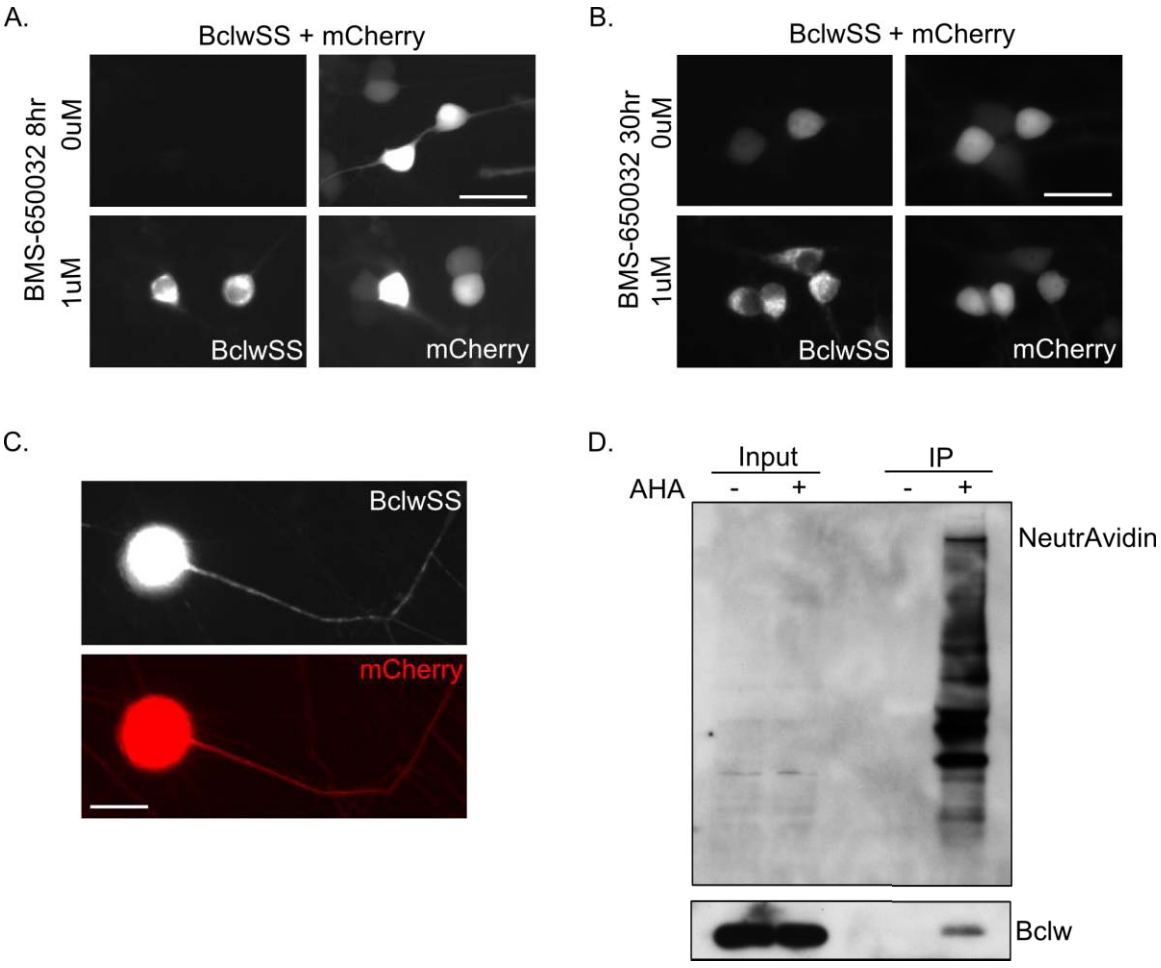
Since we have previously shown that local cycloheximide treatment blocks neurotrophin-induced Bclw protein in axons, we were surprised to not detect BclwSS synthesis in axons. Concern surrounding expression variability and health of neurons transfected by electroporation, led us to generate a bicistronic Herpes simplex virus vector for BclwSS and mCherry overexpression. Virally expressed BclwSS functions very similarly to overexpression by electroporation. Generation of YFP in DRG cell bodies is BMS-650032-dependent (Fig. 4.6A), yet by 30hr infection and inhibitor treatment low levels of YFP can be detected in the absence of inhibitor (Fig. 4.6B). Importantly, YFP protein localizes to proximal axons following bath inhibitor treatment (Fig. 4.6C). This method of overexpressing BclwSS is currently being used with both compartmented culture systems to determine whether locally synthesized Bclw protein can be detected using spaceSTAMP.

To rigorously investigate axonal synthesis of Bclw, we sought to use a conventional method for labeling protein alongside development of spaceSTAMP. Labeling newly synthesized protein by incorporation of non-canonical amino acids has been widely used in studies of local translation. Here, DRG neurons mass cultures were incubated with L-Azidohomoalanine (AHA), the methionine analog, followed by ligation with biotin and pulldown of analog-incorporated proteins. Western blotting was used to resolve biotinylated proteins. We find that biotinylated proteins are generated from cells incubated with (AHA), but not in the control methionine condition (Fig. 4.6D). In addition, we find that Bclw protein is present within the input for both AHA and control conditions, but only pulled down in the AHA condition. Without AHA incubation,

Figure 4.6 HSV BclwSS and metabolic labeling of Bclw.

A. Live imaging of YFP expression in DRG neurons infected with HSV BclwSS and BMS-650032 treatment for 8hr. Scale bar is 50µm. **B**. Live imaging of YFP expression in DRG neurons infected with HSV BclwSS and BMS-650032 treatment for 30hr. Scale bar is 50µm. **C**. Living imaging of non-uniform YFP expression within DRG axons with 30hr BMS-650032 (3uM) treatment. Scale bar is 20µm. **D**. Labeling of newly synthesized protein with Azidohomoalanine (AHA). DRG neurons incubated with (AHA) or (-) control methionine, biotin ligation by click chemistry, and pulled-down with magnetic beads. Western blotting with NeutrAvidin-HRP to bind and resolve biotinylated proteins and with anti-Bclw antibody to detect Bclw protein in input and pulldown.

Figure 4.6 (continued)



protein will not be ligated to biotin and therefore not pulled down. Thus, Bclw protein synthesis can be studied using AHA labeling and will be used next within compartmented cultures to label and resolve protein synthesized within cell bodies or axons.

Discussion

Our study seeks to develop a novel method for detecting local translation within axons and for following locally synthesized proteins to inform function. We still hope that Bclw spaceSTAMP can be modified to selectively label protein synthesized. Thus far, we find that BclwSS generates YFP within neurons in a protease inhibitor-dependent manner and BclwSS localizes to axonal mitochondria. Importantly, we also show that protease activity can be restricted within compartmented cultures to allow labeling of protein within cell bodies or distal axons. Additional experiments will be performed to modify our approach and to determine whether axonally synthesized Bclw can be detected biochemically or by live imaging.

How does spaceSTAMP compare with other methods?

Among the currently available methods for visualizing local translation with fluorescent proteins, there is not a method that conditionally labels protein within a subcellular region of the neuron. The photoconvertible Kaede is closest in that newly synthesized protein appears different following photoconversion of all protein from green to red, yet it relies on exposing neurons to additional UV light. This can be particularly worrisome if continual UV stimulation is used to maintain conversion within the cell body to avoid axonal contamination of cell body synthesized proteins. Alternatively, others have

severed the cell bodies from axons to remove chance of contamination when performing studies of axonal protein synthesis. While severed axons appear healthy and maintain active signaling for at least 12 hours in culture, it would be extremely useful to have a method to selectively label proteins synthesized in axons of un-severed neurons and without time restriction. The mRNAs for several transcription factors (TF), including CREB and SMAD1/5/8, have been detected within axons and it has been suggested that retrograde trafficking of locally synthesized TFs provide a unique cue to promote neuronal survival and differentiation (Cox et al., 2008; Ji and Jaffrey, 2012). While the theoretical biological functions of locally synthesized TF are interesting to discuss, spaceSTAMP would allow investigators to observe whether locally synthesized TFs are transported back to the nucleus or remain in the axon with a potentially novel axonal function. Importantly, since spaceSTAMP only labels protein where the inhibitor is present it would allow you to follow labeled protein from the axon trafficked back to the cell body without presence of any cell body signal.

This introduces another important difference between conventional fluorescent labeling methods and spaceSTAMP. In studies of local translation, the fluorescent reporter is most often used with only the 3' and/or 5'UTRs for the mRNA of interest, thus the protein that is locally synthesized is only a reporter protein and not the actual protein of interest. Thus, it is impossible to study localization or function of the locally synthesized protein. SpaceSTAMP is designed as a protein tagging system to add or remove a YFP tag to the protein of interest. We find that BclwSS localizes appropriately to axonal mitochondria (Fig. 3), thus we are able to follow a tagged, synthesized protein throughout the neuron.

To investigate the function of local protein synthesis, it is necessary to have the protein present to mediate the protein-protein interactions that promote localization and function.

Metabolic labeling techniques currently provide a method for labeling sets of newly synthesized protein but cannot be used in living cells to investigate a single protein of interest. Proteins labeled by BONCAT (Dieterich et al., 2006) in severed or compartmented axons are resolved and identified by immunoblotting or mass spectrometry to identify specific axonally synthesized proteins (Yoon et al., 2012; Kar et al., 2013). In contrast, FUNCAT allows for fluorescent visualization of newly made proteins in fixed cells and can be used with methionine surrogates to investigate pools of protein synthesized within different time intervals (Dieterich et al., 2010). While these methods have been critical for establishing strong evidence of local protein synthesis, novel methods are needed to investigate regulation and importance of each locally translated mRNA.

Local synthesis of Bclw in axons

Previous biochemical studies using compartmented cultures and local cycloheximide treatment suggest that a large proportion of neurotrophin-induced Bclw protein in axons is locally synthesized (Fig. 2.6). There are several possible explanations for our inability to detect local spaceSTAMP labeling of Bclw in axons. Firstly, the amount of axonally synthesized YFP could be below the level of detection for wide-field microscopy. YFP is not bright enough for single-molecule imaging and if only small amounts of Bclw are synthesized across the entire axon on this time scale, it may not be detected. To increase

the ability of detecting low levels of axonal YFP, additional experiments were performed (data not shown) with TIRF imaging set to illuminate a significant portion of axons and eliminate background, but these also did not detect inhibitor-dependent YFP.

Secondly, while we know that endogenous *bclw* mRNA is localized to DRG axons, the overexpressed BclwSS mRNA may not be localized to axons at endogenous levels. The increased length (~1.6kb) of BclwSS mRNA over the endogenous may reduce the localization efficiency or interfere with the mRNP formation and transport granule assembly. Using a probe designed to the SS sequence, we performed RT-qPCR to determine whether BclwSS mRNA is localizing to axons (data not shown) and results were highly variable across cultures. Therefore, we assume that the expression levels and localization vary highly across transfections, which is evident also in the highly variable fluorescent intensities observed using nucleofection. We find that YFP expression is much more consistent using viral expression and hope this may increase the chance of detecting axonal BclwSS.

Finally, if locally synthesized BclwSS is below the limit of detection within single axons or extremely variable across cells, western blot analysis of collective distal axon lysate may increase detection ability. We plan to use virally-expressed BclwSS in compartmented cultures with local inhibitor treatment (as in Fig. 4.5) to further investigate local translation. Interestingly, we find that the mutant BclwSS, which generates YFP within distal axons (Fig. 4.3A), expresses more efficiently than the inhibitor-dependent form (Fig. 4.2C). This suggests that BclwSS may in fact be

expressing just below the limit of detection. Future experiments will test a new lot and higher dose of protease inhibitor to maximally label locally synthesized protein for live imaging and biochemical detection. If we detect localized synthesis of Bclw in axons, local treatment with protein synthesis inhibitors will be used to show that axonal YFP is indeed dependent on local protein synthesis. And lastly, we will use BclwSS to determine whether neurotrophins regulated local synthesis of Bclw protein in axons.

Alternative uses for BclwSS

Previous studies show that neurotrophins induce transcription of *bclw* mRNA that is subsequently localized to sensory axons (Cosker et al., 2013), but it has been challenging to determine whether neurotrophins directly regulate steps downstream of transcription. For example, we find that neurotrophin stimulation leads in increase Bclw mRNA and protein in DRG cell bodies and axons, but this is dependent upon new transcription. Thus, it is possible that neurotrophins only directly regulate transcription of *bclw* that is then localized and translated. Alternatively, neurotrophins could signal locally within axons to directly regulate translation of *bclw* mRNA. Flanagan and colleague report that the Netrin receptor forms a complex with translation machinery in the axon to regulate protein synthesis (Tcherkezian et al., 2010).

Since BclwSS overexpression is independent of transcriptional regulation, it can be used to study neurotrophin regulation of translation. Experiments in compartmented cultures to investigate local translation of *bclw* mRNA will be performed with and without neurotrophin stimulation to determine whether neurotrophins directly regulate translation

in axons. Additionally, BclwSS can be used to investigate neurotrophin regulation of cell body protein synthesis in a similar manner. Used in this way, spaceSTAMP could be a powerful tool for studying cue-dependent protein synthesis and localization of newly made protein.

Chapter 5:

Conclusion

Summary of Findings

Neurotrophins promote the survival of DRG cell bodies, as well as growth and viability of long axonal processes. Recent studies have suggested that axon viability may be regulated by distinct, yet partially overlapping, mechanisms compared to cell body survival. Here, I describe a distinct mechanism by which target-derived neurotrophins act to promote axonal survival. The studies presented here indicate that neurotrophins regulate *bclw* mRNA along the pathway from the nucleus to the axon where it is locally translated to support axon health.

Chapter 2 demonstrated that target-derived neurotrophins regulate transcription of *bclw* mRNA and show that newly transcribed *bclw* mRNA is targeted back into axons. Importantly, neurotrophin stimulation increases levels of Bclw protein in axons and this requires local protein synthesis. These findings suggest that blcw mRNA is locally translated in axons following neurotrophin stimulation. Furthermore, by introducing Bclw protein directly into axons, I show that Bclw protein functions within axons to prevent axon degeneration that is caused by neurotrophin deprivation. Finally, axonal Bclw binds proapoptotic Bax to inhibit activation of the caspase-6 apoptotic pathway, thereby promoting axon survival.

Chapter 3 identified the RNA-BP, SFPQ, that binds and regulates trafficking of *bclw* mRNA within sensory neurons. These studies demonstrate that SFPQ is required for nuclear export of *bclw* mRNA, and is also required for axonal localization of another

axonal survival factor, *laminb2*. I show that *bclw* and *laminb2* mRNAs can be packaged together within cytoplasmic mRNPs, suggesting they could be transported within a single axonal RNA granule. SFPQ is required for neurotrophin-dependent axon survival and introduction of Bclw into axons prevents degeneration due to loss of SFPQ, suggesting that Bclw acts downstream of SFPQ to promote axon health. Together, these findings suggest that SFPQ coordinately regulates mRNAs that are locally translated to promote axon viability in response to neurotrophins: an RNA regulon for axonal survival.

In Chapter 4, I describe development of a novel method for investigating local protein synthesis in axons. Thus far, I show that spaceSTAMP labels Bclw with YFP in a drug-dependent manner and that the tagged protein localizes appropriately to axonal mitochondria. I am currently performing experiments to study local translation of *bclw* and determine whether neurotrophins directly regulate local synthesis of Bclw in axons. I believe spaceSTAMP will provide a very useful tool to label and follow locally synthesized proteins throughout the neuron.

Together, these data suggest a model whereby target-derived neurotrophins activate Trk receptors at the axon terminal and initiate a retrograde signal to induce transcription of *bclw* mRNA (Fig. 5.1). SFPQ-bound *bclw* exits the nucleus and is transported along with SFPQ-bound *laminb2* into sensory axons where these mRNAs are translated and function to promote axonal survival. The studies presented in this thesis demonstrate that spatial regulation of *bclw* and *laminb2* mRNA within sensory neurons is an important mechanism for neurotrophin-dependent axon survival and long-term maintenance.

Figure 5.1

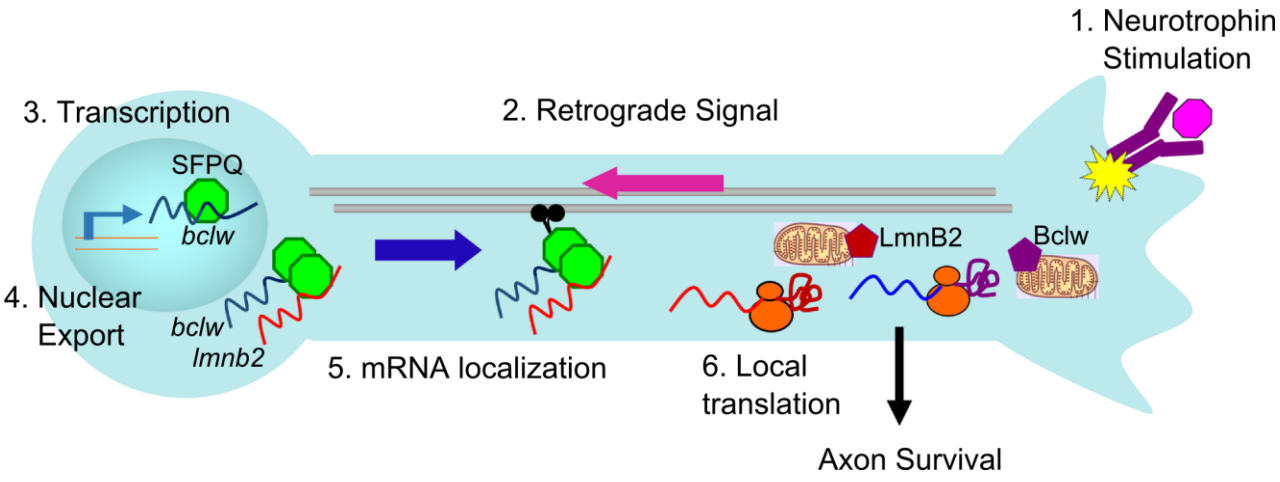


Figure 5.1. Model for spatial regulation of mRNA to promote axon survival

Neurotrophin stimulation of peripheral axon terminals (1) initiates a dynein-dependent retrograde signal (2) transporting activated Trk receptors to the cell body and inducing transcription of *bclw* mRNA (3). SFPQ-bound *bclw* mRNA is exported from the nucleus (4) and is transported into axons with SFPQ-bound *laminb2* mRNA (5). *Bclw* and *laminb2* mRNA are locally translated in axons (6), where their proteins function at mitochondria to promote neurotrophin-dependent survival of axons.

Discussion

Neurotrophins and axon survival

A significant body of work has described the dynein-dependent mechanisms by which target-derived neurotrophins signal back over long distances to the nucleus to induce changes in gene expression for neuronal survival. Neurotrophins induce activation of transcription factors, such as CREB and MEF2D, to induce immediate early genes and anti-apoptotic genes to promote neuronal survival (Ginty et al., 1994; Riccio et al., 1999; Watson et al., 1999; Watson et al., 2001; Pazyra-Murphy et al., 2009). It remained unknown how target-derived neurotrophins coordinate a transcriptional response to specifically support the health of long peripheral axons. Previous studies show that neurotrophins induce Bclw in DRG neurons (Pazyra-Murphy et al., 2009) and that Bclw is a critical survival factor for DRG axons *in vivo* (Courchesne et al., 2011). Here, I provide evidence that post-transcriptional regulation of *bclw* mRNA from the cell body to the axon is needed to support axonal health. In addition, I identify an RNA-BP that coordinately regulates multiple mRNAs and thereby enables expression of axonal survival components.

I find that neurotrophins induce transcription of *bclw* mRNA that is immediately transported into axons and locally translated. However, it is challenging to determine whether neurotrophins directly regulate the multiple steps downstream of *bclw* transcription. Since induction of *bclw* mRNA relies upon a dynein-dependent retrograde signal to the nucleus, we cannot perturb the microtubule cytoskeleton to investigate whether neurotrophins directly promote transport of endogenous *bclw* mRNA into axons.

Overexpression studies using the *bclw* 3'UTR tethered to GFP suggest that neurotrophins do promote localization of this mRNA (Supp. Fig. S2C). In the absence of cell bodies in vitro, neurotrophins still provide some protection from axon degeneration, suggesting that neurotrophins do act locally within axons to promote survival. Furthermore, inhibition of local translation in axons results in axon degeneration. Together, these data suggest that local neurotrophin regulation of translation in axons provides a likely method for protecting axons.

During development, neurotrophins are secreted from peripheral tissues to promote survival and maintenance of DRG axons that correctly innervate their target. Our findings that SFPQ is selectively localized *in vivo* to the peripheral nerve branch of the DRG is quite intriguing, especially in light of our previous finding that *bclw* mRNA is restricted to the peripheral and not central branch of the DRG (Fig. 2.1F). This raises the important question of how a neuron recognizes two different axons and choses to localize molecules to one side or another. While tyrosine hydroxylase (TH) is differentially expressed in DRG branches (Brumovsky et al., 2006), there are few examples of selective localization in DRG axons and almost nothing known about the mechanisms which execute this selectivity. An obvious possibility is that neurotrophins are received by the peripheral branch and play a role in this process. However, future work will be necessary to define the mechanisms by which central vs. peripheral axons are distinguished and maintained during the lifetime of an organism.

While neuronal connections must be established properly during development, they also must be maintained throughout life. Neurotrophins clearly play a pivotal role establishing the peripheral sensory circuitry, but neurotrophins continue to be expressed by peripheral targets in adulthood and likely continue to function in supporting axonal health (Lewin and Barde, 1996). Interestingly, loss of Bclw *in vivo* results in a progressive loss of sensory axons and thermosensation in adulthood, suggesting that neurotrophin regulation of Bclw throughout development and adulthood is required for sensory circuit maintenance. Bclw is also shown to be protective against beta-amyloid-induced cell death, suggesting that regulation of Bclw in the mature brain may be important to prevent neurodegeneration, such as in Alzheimer's disease (Zhu et al., 2004; Yao et al., 2007). Since mature axons are capable of synthesizing protein, it is likely that regulation of *bclw* mRNA within axons is important for mediating a protective effect on axons throughout life.

Axonal mRNA and studying RNA localization

Localization of mRNA is an efficient method for generating regulated local pools of protein. While extracellular cues, such as guidance factors and neurotrophins, have been shown to regulate local protein synthesis, it is also clear that there is regulated localization of mRNA to subcellular domains. Twiss and colleagues demonstrate that select mRNAs are localized in response to growth-promoting and growth-inhibitory stimuli in injured adult sensory neurons (Willis et al., 2005; Willis et al., 2007). Interestingly, their experiments are performed in the presence of transcription inhibitors. Under these conditions transcription is uncoupled from transport and regulated

localization is clearly evident. Martin and colleagues describe that without any cues, mRNA can be localized to multiple neurites, but that the formation of a synapse can redistribute mRNA for local regulated translation at a particular synapse (Lyles et al., 2006). Martin refers to this uncoupling between transcription and translation as a way for the cell to be in a "state of readiness" for local cues to stimulate translation. In contrast to this model, our findings indicate that neurotrophin induction of *bclw* mRNA in axons is dependent upon transcription, pointing towards a model whereby sustained neurotrophin stimulation activates this transcription and post-transcriptional regulation of *bclw* to continuously provide the axons with the factors it requires to survive.

Study of endogenous mRNA localization is most commonly performed using PCR to amplify mRNA within isolated axons, as well as fluorescent *in situ* hybridization (FISH) in fixed neuronal tissue or neuronal cultures. FISH provides additional spatial information about the subcellular localization in comparison to other localized proteins and organelles. Recent work by Buxbaum et. al. (2014) shows protease treatments are critical for visualizing axonal mRNA, as they are surrounded by many proteins within tightly-packed granules. Buxbaum describes this step as RNA "unmasking" and suggests that neuronal activity promotes biological unmasking of mRNA and ribosomes as a mechanism to regulate local translation (Buxbaum et al., 2014). Interestingly, our single-molecule FISH studies label *bclw* and *laminb2* mRNA within DRG cell bodies, but were unable to detect signal within axons. A possible explanation is that the mRNP granules containing *bclw* and *laminb2* mRNA within axons maybe remodeled in such a way that prevents probe access to the RNA targets. Classical FISH studies and qPCR from pure

axon factions clearly demonstrate the presence of the mRNAs within axons, thus future studies with various protease treatment or "unmasking" protocols will be useful to study the axonal mRNA granules with higher spatial resolution.

The MS2 bacteriophage system has been very prominent in studies of mRNA dynamics within living cells (Buxbaum et al., 2015). This method uses the bacteriophage coat protein (MCP) which binds to a distinct RNA hairpin MS2-binding site. Overexpression of an mRNA containing multiple MS2-binding sites can be visualized by co-expressed, interacting MCPs tethered to fluorescent protein. Singer and colleagues have generated an MS2 knock-in mouse for β-actin that labels the mRNA with multiple fluorescent proteins to enable visualization of endogenous *β-actin* mRNA *in vivo* with very high signal to noise ratio (Lionnet et al., 2011). Studies using this tool show that *β-actin* mRNA is transported at an average rate of 3μm/s within hippocampal neurites and that single granules can contain multiple *β-actin* mRNAs (Lionnet et al., 2011; Park et al., 2014). One disadvantage to the MS2 system is that it labels mRNA with a very large protein complex, depending on the number of MS2-biding sites that are used, that can potentially generate steric hindrance and disruption of proper mRNPs formation required for mRNA transport. Jaffrey and colleagues have recently developed Spinach, a method for labeling mRNA with RNA aptemers that bind and activate exogenous fluorescent dyes (Paige et al., 2011; Strack et al., 2013). While Spinach has not yet been used to visualize mRNA in living neurons, additional development may prove it to be very useful for studying RNA dynamics and regulation within cells.

Live imaging tools for the study axonal mRNAs will provide important spatial and temporal information about localized mRNAs. Using a GFP-tagged mRNA localization motif for *coxIV*, Kaplan and colleagues suggest that *coxIV* mRNA localizes to axonal mitochondria (Aschrafi et al., 2010). This is a common method used to reveal mRNA localization, although the fluorescent signal is a readout of protein, not mRNA. It would be ideal to have a high resolution method for visualizing endogenous mRNAs without incredibly large tags, but the MS2 system is the best method currently available. Future studies using MS2 will be valuable to determine more precisely how neurotrophins regulate *bclw* and *laminb2* within axons and whether the mRNAs are localized to mitochondria prior to translation.

Post-transcriptional mRNA regulation by RNA-binding proteins

A traditional view of gene regulation revolves around transcriptional control, yet we are just beginning to uncover the complexity of factors involved in post-transcriptional regulation. These regulatory steps are the final determinants of which proteins are expressed and where they are synthesized. RNA BPs provide many additional layers of control to ensure precise gene expression and proper cellular responses. Proteins bind RNA directly or indirectly within the nucleus, while additional binding factors may be added or removed during remodeling within the cytoplasm to promote appropriate spatial regulation and translation. Moore and Proudfoot (2009) describe that "mRNP proteins can be thought of as adaptors that add functionality to a transcript by interfacing with a wide variety of cellular machineries." This perfectly captures the versatility and complexity that can occur during post-transcriptional regulation with a variety of mRNP

proteins to promote appropriate interaction with nuclear export, localization, or translation machinery.

While SFPQ was originally identified as a splicing factor, my findings indicate that SFPQ has both nuclear and cytoplasmic roles, and implicate unique function for SFPQ within a neurotrophin-regulated axonal survival pathway. SFPQ is required for nuclear export and axonal localization of *bclw* mRNA, as well as axonal localization of *laminb2* mRNA. In addition to SFPQ, there are likely many other RNA-BPs and associated factors that regulate *bclw* and *laminb2* mRNA within neurons. Localization of a single mRNA may require different BPs to mediate different steps or receive different regulatory cues. For example, studies find that localization of *sensorin* mRNA to neurites requires the 3'UTR, but further concentration at synapses is mediated by an element with the 5'UTR (Meer et al., 2012), strongly suggesting that distinct RNA-BPs interact with each site to mediate a two-step localization process. Several RNA-BPs have been shown to interact with *β-actin* mRNA. Since the identification of ZBP1 for regulation of *β-actin* mRNA localization and translation, it has been shown that additional factors, including HuD and hnRNP-R (Glinka et al., 2010) also bind and regulate *β-actin* mRNA localization. Interestingly, the survival motor neuron (SMN) protein with known roles in snRNP assembly, forms a complex with HuD within axons and is required for mRNA localization in motor axons (Fallini et al., 2011). This suggests that multiple RNA-BPs and associated factors are required for localization of a single mRNA and it will be interesting to identify additional factors that regulation *bclw* and *laminb2* mRNA within cell bodies and axons.

Our studies find that SFPQ binds the 3'UTRs of *bclw* and *laminb2* mRNA. Each of these 3'UTRs contain more than 5 predicted-binding sites for SFPQ. While I have not confirmed that each of these sites mediate SFPQ-binding, I have shown that that SFPQ is able to bind throughout multiple regions of each 3'UTR. Why might an mRNA have multiple binding sites for a single RNA-BP? Since the mRNA regulatory events from the nucleus to the axon are tightly coupled, RNA-BPs and associated factors play multiple functions throughout this process. SFPQ may regulate nuclear mRNA processing, as well as cytoplasmic RNA localization, but mediate distinct roles through different mRNA biding sites. Another attractive model is that the more localization elements an mRNA contains, the more efficiently it will be transported. There is evidence that localization elements increase the number of motor complexes per mRNP (Amrute-Nayak and Bullock, 2012). This would suggest that SFPQ-mediated mRNA localization would be highly effective if the mRNA contain multiple binding-sites, as observed for *bclw* and *laminb2*.

Regulation of mRNA stability and decay is also mediated by an mRNA's interaction with RNA-BPs (Guhaniyogi and Brewer, 2001; Mukherjee et al. 2011) and selective degradation of mRNAs within dendrites establishes proper spatial distribution of mRNA for synaptic plasticity (Farris et. al. 2014). Neurotrophin signaling may promote stabilization of localized mRNAs for axonal survival, possibly through regulation of SFPQ. Our data indicate that neurotrophins do not affect the half-life of *bclw* mRNA (Fig. 2.2E). Thus, neurotrophin-dependent regulation of *bclw* mRNA within axons most likely reflects transport of *bclw* mRNA into axons, rather than selective stabilization of

bclw within axons. It will be interesting to determine whether neurotrophins or SFPQ regulate stabilization of *laminb2* mRNA or other axonally localized mRNAs.

Higher-order mRNP structures are clearly highly dynamic, suggesting that they can be modified at different points or exchange components to promote proper localization of mRNAs. It is as yet unclear how specific components are selected for granule assembly, as well as how many different mRNAs are contained within each complex and the extent of heterogeneity. Work from Kiebler and colleagues (2013) describe the two distinct RNA granules purified from the brain and containing either Staufen2 or Barentz neuronal RNA-BPs. They identify unique, as well as shared components of the two granules and suggest that localized mRNAs are likely to exist within distinct RNP complexes. Several studies using single-molecule FISH have not detected colocalization of distinct mRNAs within neuronal dendrites and conclude that different mRNAs are transported individually (Batish et al., 2012; Farris et al., 2014). In contrast, our studies suggests that distinct axonal mRNAs can be transported within single granules. Additional studies are necessary to determine how specific components are selected and assembled into transport granules.

While mRNAs are bound within transport granules BPs often repress translation activity. Regulation of RNA BPs by post-translational modifications like phosphorylation can regulate BP interaction with mRNA (Huttelmaier et al., 2005). Thus, local cues and signaling pathways can regulate BPs to promote mRNA release, stability or translation, and there is evidence that axonal transmembrane receptors may interact with translational

machinery to regulate protein synthesis at sites of stimulation (Tcherkezian et al., 2010). Regulation of SFPQ activity, binding properties, and subcellular localization is modulated by N-terminal phosphorylation of serine and threonine residues during different cellular states such as mitosis and apoptosis (Shav-Tal et al., 2001). In addition, SFPQ has been identified as a substrate of MAP kinase signal integrating kinases (Mnks), as well as found to localize with cytoplasmic MAPKs, and phosphorylation regulates mRNA-binding ability of SFPQ (Huang et al., 2007; Buxade et al., 2008; Sury et al., 2014). Target-derived neurotrophins activate MAP kinase pathways for both local and long range survival signaling, thus localized phosphorylation events could regulate specific nuclear or cytoplasmic SFPQ activities. Interestingly, our data indicate there is NGF-dependent phosphorylation of SFPQ (data not shown) and that NGF reduces SFPQ binding of *bclw* and *laminb2* mRNAs. One possibility is that local neurotrophin signaling stimulates phosphorylation of SFPQ for release and translation of mRNAs within axonal SFPQ mRNPs. Future studies are needed to identify the specific phosphoresidues of SFPQ that regulate subcellular function.

The RNA regulon model proposes that one or more RNA-binding proteins can coordinate the post-transcriptional processing of functionally related mRNAs, thus allowing a synchronized response in regulated gene expression to specific signaling pathways (Blackinton &Keene 2014) (Keene, 2007). In yeast, the Puf family of RNA BPs each associate with a discrete set of mRNAs which encode functionally related proteins (Gerber et al., 2004) including Puf3p with interacts selectively with nuclear-encoded mRNAs for mitochondrial proteins. These mRNAs are localized to mitochondria where

Puf3 repression is relieved for translation (Garcia-Rodriguez et al., 2007). Several studies find that RNA export or shuttling factors in yeast bind mRNAs enriched for functional classes of protein and suggest links between coordinated transcription and nuclear export (Hieronymus and Silver, 2003; Kim Guisbert et al., 2005). RNA regulons have also been described to function in cell cycle regulation and in response to DNA damage (Blackinton and Keene, 2014). In neurons, the splicing factor Nova is described to bind a subset of mRNAs that encode protein with synaptic function (Ule et al., 2003), suggesting that RNA regulons may be important for neuronal function. The overall extent to which the RNA processing steps are coordinated to generate a cellular response remains unknown, but seems a likely mechanism used across cell types and species to ensure an efficient and precise gene expression.

I propose a novel model for coordinated mRNA regulation of survival factors by SFPQ in neurons to promote health of axons in response to extracellular trophic factors. The data presented here demonstrate SFPQ regulation of *bclw* and *laminb2* mRNA. In addition, there are predicted binding sites for SFPQ within a number of other axonal mRNAs, including *Impa1* mRNA, which is locally translated in axons and prevents degeneration (Andreassi et al., 2010). *Impa1* mRNA contains four SFPQ-binding motifs and we find that SFPQ precipitates *Impa1* mRNA (data not shown), although it is unknown yet whether SFPQ regulates *Impa1* in neurons. A high-throughput method will be extremely useful for identifying the pool of SFPQ interacting mRNAs and additional studies in neurons will be interesting for studying SFPQ regulation of other axonal mRNAs. It

seems likely that SFPQ together with other factors coordinates spatial expression of multiple mRNAs to promote axon health and maintenance.

Functional significance for mRNA localization and translation

While there is tremendous evidence for regulated mRNA localization and translation within neuronal axons, it is challenging to demonstrate functional significance for the localized mRNA and locally synthesized protein. In compartmented cultures, studies use local introduction of siRNA to reduce the axonal pool of a specific mRNA. Local knockdown of *coxIV* mRNA reduces axon elongation (Aschrafi et al., 2010) and this method has been used to study the function of other localized mRNAs including *Creb*, (Cox et al., 2008) R*hoA* (Walker et al., 2012), and *ATP5G1* (Natera-Naranjo et al., 2012). Twiss and colleagues demonstrated that overexpression of the *β-actin* 3'UTR competes with endogenous *β-actin* mRNA for ZBP1 resulting in reduced localization of *β-actin* mRNA (Donnelly et al., 2011). Using this strategy along with the finding that ZBP1 regulates localization of both *β-actin* and *gap-43* mRNA, they selectively reduced the axonal pool of each mRNA and showed that axonal *β-actin* mRNA is required for axon branching, while axonal *gap-43* mRNA is required for axon elongation (Donnelly et al., 2013). Holt and colleagues performed a targeted knockdown *in vivo* to demonstrate that axonal *laminb2* mRNA is required axon maintenance (Yoon et al., 2012).

Rigorous study of localized mRNA function *in vivo* requires removing the localized pool of mRNA genetically. Studies show that *importin β1* mRNA has two 3'UTR isoforms, including a longer 3'UTR that preferentially targets *importin β1* to axons (Perry et al.,

2012). Thus, Fainzilber and colleagues generated a knockout mouse that has had the 3' axonal targeting element removed to selectively deplete axonal *importin β1* mRNA (Perry et al., 2012). Without the long 3'UTR *importin β1* isoform, mice exhibit reduced transcriptional response to injury, as well as delayed nerve regrowth and recovery of walking behavior following injury. This elegant series of studies clearly demonstrate an *in vivo* requirement for localized mRNA and I consider to be a gold standard for studying mRNA localization and translation.

In addition to *in vivo* studies to examine biological significance for localized mRNA, it is also vital to visualize where locally synthesized proteins are made and distributed within the neuron to help understand their function. Therefore, development of novel methods like spaceSTAMP will be critical for future studies of local translation. Selectively labeling locally synthesized protein for fluorescent live imaging will address questions such as: Does locally synthesized Bclw concentrate at particular axonal mitochondria? Or throughout the entire axon? This type of tool will also be helpful to study locally synthesized transcription factors that are suggested to be transported retrogradely and function within the nucleus (Cox et al., 2008; Ben-Yaakov et al., 2012).

In conclusion, these studies demonstrate that target-derived neurotrophins coordinate transcriptional and post-transcriptional regulation of survival factors from the neuronal cell body to the axon to promote axonal survival and maintenance. In contrast to the idea that cue-dependent local translation serves a very localized purpose, like growth cone

guidance or synapse growth, I suggest that post-transcriptional regulation and translation of survival factors throughout axons may provide a mechanism by which sustained neurotrophin signaling promotes the continued maintenance of long axons. Future studies should be aimed at identifying other neurotrophin-regulated RNA-BPs and target mRNAs to understand the breadth of mechanisms employed to support axon health. Finally, the field of mRNA localization and translation will continue to benefit from newly developed tools for visualizing mRNA dynamics and local protein synthesis in living neurons that will hopefully shed light on some of the many unanswered questions.

Appendix I:

Campenot cultures and microfluidics provide complementary platforms for spatial study of dorsal root ganglia neurons

Publication:
Fenstermacher SJ, Pazyra-Murphy MF, Segal RA. Campenot cultures and microfluidics provide complementary platforms for spatial study of dorsal root ganglia neurons. SpringerProtocols *Neuromethods;* Microfluidic and Compartmentalized Platforms for Neurobiological Research. *In Press*.

Contributions:
Sara J. Fenstermacher wrote and prepared manuscript and generated data in Figures A3, A4, & A5.
Maria Pazyra-Murphy wrote Campenot protocol and generated data in Figures A1 & A2.
Rosalind A. Segal edited manuscript.

Acknowledgements:
This work was supported by National Institute of Health Grant RO1 NS050674 to R.A.S. and F31 NS077620 (NINDS) to S.J.F. We thank Katharina Cosker for contributing Campenot figure data and helpful comments, and Sarah Pease for helpful advice with the microfluidics protocol.

Abstract

Dorsal root ganglia (DRG) neurons are a functionally diverse population of sensory neurons with specialized morphology to respond to external stimuli. These pseudo-unipolar neurons extend a single axon that bifurcates to innervate the periphery and spinal cord, allowing sensory information from the environment to be transferred rapidly to the central nervous system. During development, these DRG neurons rely on peripheral target-derived neurotrophins for survival. Due to their unique morphology, DRG neurons exhibit spatially complex signaling and regulated gene expression that are challenging to study *in vivo* or in conventional cultures. The development of compartmented culture systems has been invaluable to the study of neurotrophin signaling, mRNA transport and localization, and local protein synthesis in axons. Here we describe the set up and maintenance of rat DRG neurons in two different compartmented culture platforms: Campenot cultures and microfluidics chambers. These systems are highly complementary and so together can be used for biochemical analysis and for high resolution imaging of neuronal cell bodies and their extensive axons.

Introduction

Sensory neurons of the dorsal root ganglia (DRG) convey information about the environment to the central nervous system including pain, touch, temperature, and body position. To perform these specialized functions, DRG neurons are a heterogeneous population of neurons with diverse molecular and physiological properties. During development, DRG neurons extend long axons towards the periphery to innervate skin, muscle, and other target tissues. These primary sensory neurons rely upon target-derived neurotrophins for their survival (Bibel and Barde, 2000; Segal, 2003). Neurotrophins, including Nerve Growth Factor (NGF) and Brain-derived Neurotrophic Factor (BDNF), bind receptor tyrosine kinases (Trk receptors) at the DRG axon terminal and initiate a retrograde signal to activate a prosurvival response within the neuronal cell body. Thus, DRG neurons that properly innervate their target and receive neurotrophins, survive and maintain appropriate connections within this peripheral sensory circuit. In order to study this highly spatial neurotrophin-dependent signaling in neurons, researchers use unique *in vitro* culture systems to recapitulate the *in vivo* spatial separation of cell bodies and axons of these long and polarized cells.

In 1977, Robert Campenot introduced a compartmented culture known today as the "Campenot chamber" (Campenot, 1977). This three-chambered culture system allows neurons to be plated within a center compartment and neurites to extend outward into the two surrounding axonal compartments (Fig. A.1A), thus retaining the *in vivo* spatial configuration of neurons. In addition, these compartments are fluidically isolated, allowing for local neurotrophin and/or selective experimental treatment of cell bodies or

axons. Finally, cellular material from the individual compartments of Campenot cultures can be collected separately, allowing for analysis of RNA and protein from neuronal cell bodies and distal axons independently. Initially, Campenot cultures were used with sympathetic neurons to study axonal outgrowth and lipid metabolism and transport (Campenot, 1977, 1982; Vance et al., 1991; Campenot, 1994; Karten et al., 2005; Campenot et al., 2009). Since their development, Campenot cultures have been used extensively by many investigators to examine the unique nature of retrograde neurotrophin signaling in both sympathetic and DRG neurons, providing a powerful *in vitro* tool to examine these challenging spatial questions (Riccio et al., 1997; Watson et al., 2001; Ginty and Segal, 2002; MacInnis et al., 2003; Kuruvilla et al., 2004; Mok and Campenot, 2007; Pazyra-Murphy et al., 2009; Harrington et al., 2011; Harrington and Ginty, 2013; Mok et al., 2013).

While Campenot cultures are ideal for RNA isolation and protein biochemistry from subcellular neuronal compartments, they are not well-suited for high-resolution microscopy or for live imaging, since the neurons are grown on plastic tissue cultures dishes. In contrast, microfluidics chambers are superior for imaging as neurons are grown on glass coverslips. Microfluidics are a newer compartmented culture platform which are commercially available or can be fabricated within your lab (Park et al., 2006). DRG neurons are plated in one side of these two-chambered devices and axons grow through a set of microgrooves to extend outward into a distal axon compartment (Fig. A.3A,B). Like Campenot cultures, they provide physical separation between the neuronal cell bodies and distal axons and are fluidically isolated to allow differential treatment of cell

bodies and distal axons (Fig.A.3C). Microfluidic cultures are an ideal system for following RNA and protein live within the distinct cellular compartments, especially within axons and growth cones, and in response to localized extracellular cues. In addition, cultured neurons can be fixed and used for fluorescence *in situ* hybridization (FISH) and immunofluorescence to identify subcellular localization of RNA and protein, respectively, within DRG neurons.

Since the original development and use of compartmented cultures for studying axonal growth and retrograde neurotrophin signaling, they have more recently become an essential tool for studying RNA localization and local protein synthesis within axons (Jung et al., 2012). Hundreds of RNAs have been identified in axons of DRG and other neurons using RNA isolated from compartmented cultures (Willis et al., 2005; Taylor et al., 2009; Andreassi et al., 2010; Gumy et al., 2011) and the cultures provide a way to selectively label and identify locally synthesized proteins by incorporation of non-canonical amino acids (Eng et al., 1999; Dieterich et al., 2006; Kar et al., 2013). In addition, local stimulation within compartmented cultures allows for investigation of cue-induced mRNA localization and translation (Willis et al., 2007; Andreassi et al., 2010; Cosker et al., 2013). Novel methods for labeling and following RNA and newly synthesized protein are being developed regularly and compartmented cultures are powerful tools to use in these experiments and for the continued investigation of spatial regulation of gene expression in neurons (Tyagi, 2009; Dieterich et al., 2010; Chao et al., 2012; Wu et al., 2012).

Here we describe how to set up and maintain DRG neurons in both Campenot and microfluidic cultures and demonstrate the clean spatial separation of cell bodies and distal axons achieved in both culture platforms. In addition, we describe how these complementary culture systems can be used experimentally to explore neurotrophin regulated signaling and gene expression with both neurotrophin stimulation and deprivation paradigms. Together these unique cultures platforms provide a specialized and functional system for investigating spatially localized events within DRG neurons.

Campenot Cultures

Reagents and Materials

Collagen I (BD Biosciences, Cat.# 354249)

P35 tissue culture dish (Fisher Scientific, Cat.# 150318)

Methylcellulose (Xenex, Cat.# E4M)

Trypsin (Worthington, Cat.# LS004452)

Teflon dividers (Tyler Research, CAMP10) (*see* Note 1)

Pin rake (Tyler Research, CAMP-PR)

Grease loader (Tyler Research, Camp-GLSS)

DMEM (Life Technologies, Cat.# 11965118)

NGF (PeproTech Inc., Cat.# 450-01)

BDNF (PeproTech Inc., Cat.# 450-02)

High-vacuum grease (Fisher Scientific, Cat.# 146355D)

Cytosine ß-D-Arabino Furanoside (AraC) (Sigma-Aldrich, Cat.# C-1768)

23 gauge tubing adapter (Fisher Scientific, Cat.# 427565)

90° angle hemostats (Roboz Surgical Instruments Co., Cat.# RS-7035)

Cell culture incubator at 37°C with 7.5% CO_2

Methods

Preparation of reagents:

1. Collagen Coating: Make up collagen to a final concentration of 0.71 mg/mL in 0.001N HCl. Coat p35 tissue culture dish by adding 500 µL to each plate and place in a 37°C oven for 2 days, or until completely dry.

2. Grease Loaders: Fill grease loaders with vacuum grease, wrap with foil, and autoclave.

3. Teflon Dividers: Teflon dividers can be reused following each experiment, but must first be properly cleaned. To clean dividers, wipe off all remaining grease and place in sulfuric acid for 2 days. Remove the dividers from the acid and rinse 3X with water. Boil dividers in water for 20 minutes, let dry, place in a glass p100 petri dish, and autoclave.

4. Methylcellulose: Put 1.5g of methylcellulose into a 500 mL bottle. Add a stir bar and autoclave for 20 minutes on dry (from this point all work must be sterile). Next, add 500 mL of plain DMEM, and stir in a cold room until dissolved. Aliquot into 50 mL conicals and store at -20°C. For working stock, aliquot one of the 50 mL conicals into 1.5 mL tubes and store at -20°C.

5. Media: For culturing DRG neurons in compartmented cultures use DMEM with 5% heat-inactivated horse serum and 1% penicillin streptomycin (*see* Note 2). To make up media with neurotrophins dilute nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) from 1mg/ml stock concentration into the media (*see* Note 3). Thus, to make 100 ng/mL NGF/BDNF media, dilute NGF and BDNF 1:10,000 into

media. To make 10 ng/mL NGF/BDNF media, dilute NGF and BDNF 1:100,000 into media, or dilute the 100 ng/mL NGF/BDNF media (1:10) with media. In addition, Cytosine ß-D-Arabino Furanoside (AraC) should be added to media for a final concentration of 0.3 μM, to limit the growth of Schwann cells and other glia.

Set up of the compartmented chambers (start this process 1-2 days before the dissection):
1. Using the pin rake, scratch across the middle of a collagen-coated p35 dish, from one edge to the opposite edge. Pipette 20 μl of methylcellulose into the middle of the scratch.
2. To prepare the divider, first attach a 23-gauge luer stub adapter to the grease loader. Next, use a pair of 90° angle hemostats to grip the Teflon divider by the thick middle portion and lay flat with the divider facing up under a dissecting microscope (Fig.A.1B). Apply the grease by coating the surface of the divider following the directions within Fig. 1C. Make sure to insert the adapter directly into the grease at each new starting point, creating a continuous line of grease to seal the divider to the culture dish (*see* Note 4). Next, turn one of the prepared p35 dishes upside down and place onto greased-divider with the methylcellulose across the middle of the divider. Use tweezers to press the dish onto the grease making a complete seal (Fig.A.1D). It is important to create a full seal with the dish, but if too much pressure is used, the axons will not cross into the side compartments (*see* Note 5). Lastly, pick up and turn over the hemostats and unclamp the divider. Under the microscope, make a small grease barrier (.25cm) at the front of the middle compartment. The barrier is added to ensure that the cells do not leak out of the middle compartment at the time of cell plating.

Figure A.1

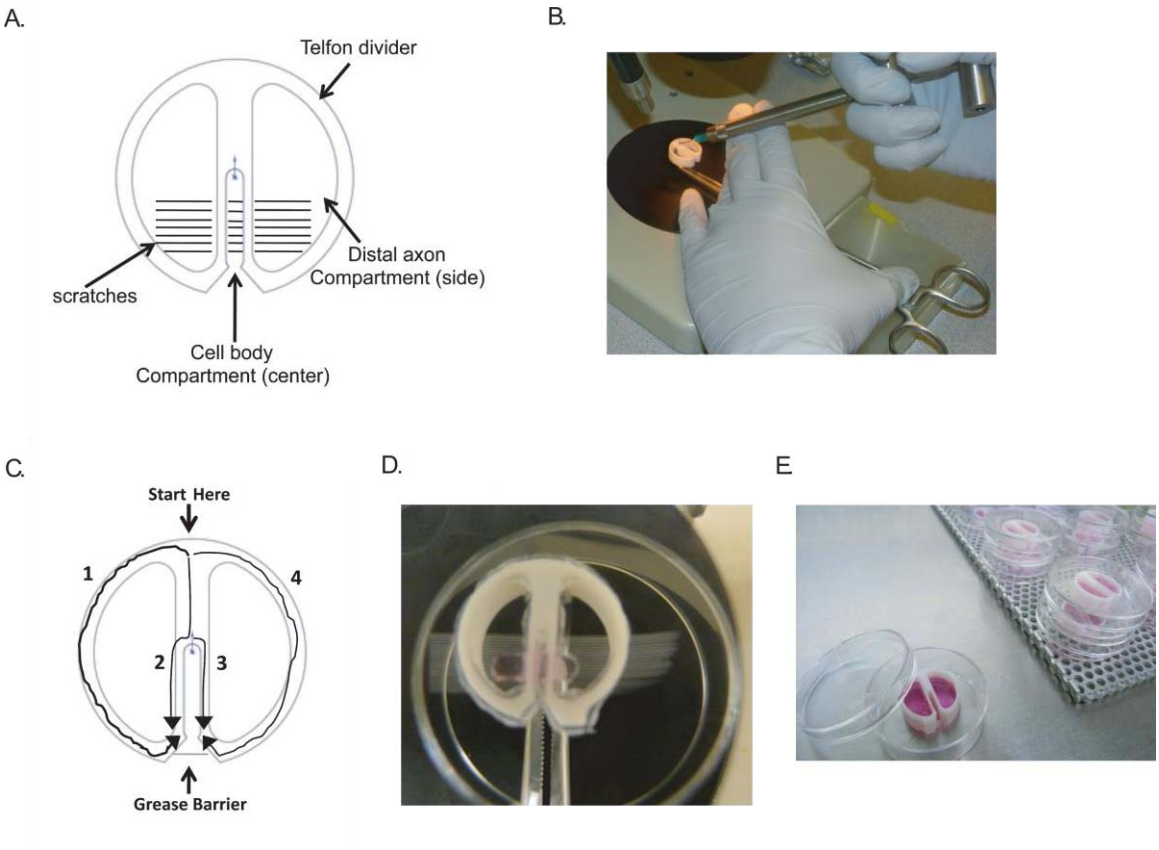


Figure A.1. Assembling Campenot cultures.

A. Labeled schematic of Campenot culture. **B**. Image of hemostat with attached divider and hand placement for adding grease to the divider. **C**. Schematic of grease application. **D**. Image of greased divider attached to p35 dish. E. Image of testing assembled cultures for leaks before use.

3. Add DMEM into each of the side compartments and place in incubator in which the cells will be maintained. Allow the cultures to sit for several hours and then check for leakage into either the middle compartment or surrounding area (Fig.A.1E). Leaky cultures should not be used for plating cells. This is a critical step in the procedure, as it tests each culture for fluidic isolation before use.

DRG dissection:

Compartmented cultures can be used to culture embryonic or adult DRG neurons (Kimpinski et al., 1997) from rat and mouse, as well as iPS-derived neurons. To culture embryonic rat neurons, Sprague-Dawley rats from embryonic day 15 (E15) are used for the DRG dissection. Each dissected spinal cord yields approximately 500,000 cells. Thus, a dissection of 10 rat embryos yield about 5 million cells, providing enough cells to plate about 50 cultures, with 100,000 cells plated per culture. To culture embryonic mouse DRG neurons, mice from E13 are used and each animal generates approximately 200,000 cells. Thus, a dissection of 10 mouse embryos should provide enough cells to plate 20 cultures. Although DRGs contain a heterogeneous population of DRG sensory neurons, DRGs are collected from the entire length of the spinal cord and pooled for trypsinization and plating (*see* Note 3).

1. Remove rat (E15) embryos from the timed pregnant female animal (as seen in (Pacifici and Peruzzi, 2012). In a sterile dissection hood, use sharp forceps to tear open and remove embryos from the amniotic sac. To properly euthanize embryos, use scissors to sever each head from the embryo. Transfer each embryo body to a 50mL conical filled with plain DMEM. Rinse embryos in DMEM and then transfer to a plastic dissecting dish

with fresh DMEM. The next steps should be performed using a dissecting microscope and lamp in a sterile environment.

2. Lay embryo ventral side down, limbs pointing outward. The whitish spinal cord should be visible in this orientation.

3. While holding the embryo still with forceps with one hand, use bent forceps (Roboz, Cat.# RS-5005) to pull away the top layer of skin from atop the spinal cord. Do this by sliding one edge of the bent forceps down from the anterior opening between the skin and spinal cord, and then pull back with pressed forceps to remove skin. This step should be performed several times along the length of the embryo to expose the whole spinal cord and all neighboring DRGs. The DRGs should now be visible as two rows of small spheres ventral and lateral to the spinal cord.

4. Use micro dissecting spring scissors (Roboz, Cat.# RS-5600) to cut directly below the DRGs, therefore cutting the DRGs away from the underlying tissue while they remain attached to the spinal cord. Cut along both sides of the spinal cord, such that the spinal cord can be pulled from the embryo with all of the DRGs attached. Place the spinal cords with DRGs in fresh DMEM on ice. Dissect and collect all spinal cords with DRGs in DMEM before proceeding.

5. To remove DRGs from the spinal cord, take each spinal cord with DRGs and place in a p35 dish with 2 mL Hank's Buffered Salt Solution (HBSS). Use fine-tipped forceps to gently pluck each DRG off of the spinal cord and leave in surrounding HBSS. It is best to grab the DRG by the base where it attached to the spinal cord and pull, rather than squeezing the forceps directly around the DRG. After DRGs have been removed from the spinal cord, discard the spinal cord and move on to the next.

6. Add 1 mL trypsin (1 mg/mL made up in HBSS) to plucked DRGs, swirl gently, and incubate at 37°C for 50 minutes.

7. Add 3 mL DRG media to trypsinized DRGs and spin cells down in centrifuge at 80 rcf (636RPM) for 5 minutes at 4°C. It is important to use the serum-containing DRG media here, as the serum functions to inactivate the trypsin.

8. Carefully aspirate media off pellet and resuspend cells in 100 ng/mL DRG media + AraC for plating.

Plating and maintaining DRG neurons in compartmented cultures:

1. Before plating DRG neurons, replace DMEM in side compartments with 100 ng/mL NGF/BDNF media + AraC. Perform DRG neuron dissection and plate 100,000 cells per compartmented culture into the middle compartment, in a volume of 25-40 µl (cell density is 2.5-4 million cells per ml media). Be sure to plate cells in 100 ng/ml NGF/BDNF media + AraC. See Table A.1 for media conditions.

2. After incubating cells overnight, add 10 ng/ml NGF/BDNF media + AraC to the area surrounding divider until media crosses over grease barrier and exchanges with media in middle compartment.

3. Media should be changed on subsequent days [Table A.1] to slowly reduce NGF and BDNF levels over the week in culture. In addition, we find that pulsing application of AraC substantially limits the number of glial cells. To change media, first aspirate all media from surround and side compartments. Second, add media to side compartments. Third, add media to surround until it exchanges with the middle compartment. One can drip a few drops over the grease barrier to ensure exchange with the middle compartment.

When removing media, it is important to aspirate from the very top of each side compartment so as to avoid the axons. Lastly, do not remove media directly from the middle compartment as that can disturb the cell bodies, instead only remove media from the surround.

4. Axons will grow into the side compartments by around 5 days *in vitro* (DIV) and should have extended well across the side compartment by 8 DIV, when ready for experimental use.

Collecting RNA or protein from compartmented cultures

Since DRG middle and side compartments are fluidically isolated, they can be used to collect cell body and distal axon fractions separately for biochemical analysis (*see* Note 6).

1. To collect RNA from Campenot cultures, carefully remove all media from surrounding the divider, and from both the side and middle compartments. Place cultures on ice and add 10 µl trizol to each compartment. Take a pipette tip in hand and scratch several times perpendicular to the scratches in the side compartments. Use a narrow gel-loading tip to gently scratch the middle compartment. Be careful not to bump or move the divider so as to maintain fluidic isolation and keep cell fractions separate. Collect RNA from side and middle compartments. Pooled RNA from at least 6 compartmented cultures should be used for each experimental condition. Following RNA extraction using trizol, RNA can be analyzed by qRT-PCR (Fig.A.2A).

2. To collect protein from the cultures, repeat the procedure for collecting RNA but instead add 5-10 µl lysis buffer to each compartment. Lysates pooled from 8-12

Figure A.2

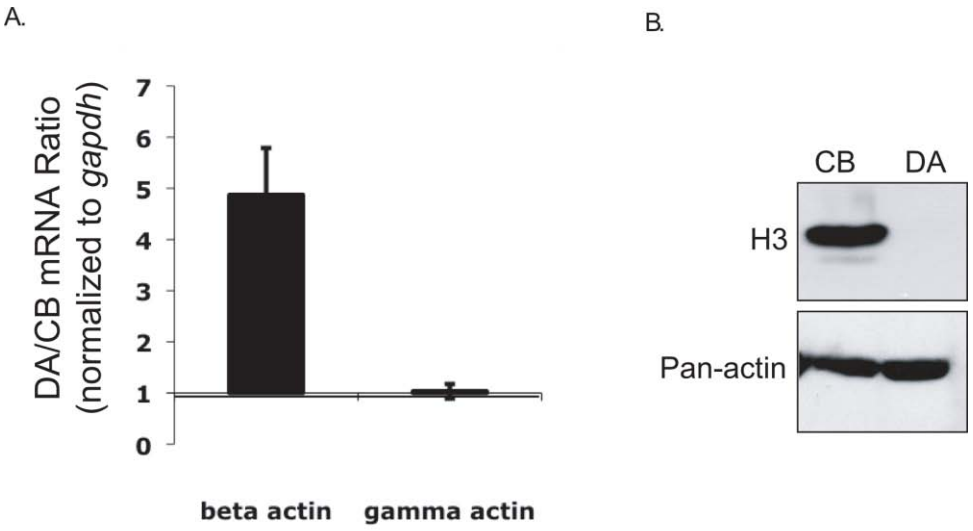


Figure A.2. RNA and protein markers demonstrate fluidic isolation of cell bodies and distal axons in Campenot cultures.

A. mRNA ratio of distal axons to cell bodies (DA:CB) isolated from E15 DRG neurons grown in Campenot cultures, normalized to *gapdh* and analyzed by qRT-PCR. *β -actin* is relatively enriched in DA, while γ *-actin* is not. *β-actin* mRNA localizes to axons, while γ-actin mRNA is restricted to CB, indicating neuronal cell bodies are restricted to the CB compartment and the Campenot cultures are fluidically isolated during RNA extraction. Reproduced from (Cosker et al., 2013). **B**. Protein collected from CB and DA analyzed by western blot shows that histone (H3) is only present in CB, while the loading control, pan-actin, is present in both CB and DA. Thus, cell bodies are restricted to the CB compartment and cultures are fluidically isolated during protein collection.

compartmented cultures should be used for each experimental condition to optimize detection and reproducibility. Lysates can be analyzed by western blot (Fig.A.2B).

Microfluidics

Reagents and Materials

Poly-D-lysine (Sigma, Cat.# P7280)

Laminin (Life Technologies, Cat.# 23017015)

Coverslips, No. 1.5, Size: 40x24mm (Fisher Scientific, Cat.# 12544C)

Hank's Balanced Salt Solution (HBSS) (Invitrogen, Cat.# 14175103)

BD Falcon petri dishes 150x15mm (Fisher Scientific, Cat.# 08-757-148)

Microfluidics (Xona Microfluidics, Cat.# SND450, *see* Notes 7 and 8)

BD Falcon petri dishes 60x15mm (Fisher Scientific, Cat.# 08772B)

Neurobasal (Life Technologies, Cat.# 21103049)

B-27 Supplement (Life Technologies, Cat.# 17504044)

NGF (PeproTech Inc., Cat.# 450-01)

BDNF (PeproTech Inc., Cat.# 450-02)

Cytosine ß-D-Arabino Furanoside (AraC) (Sigma-Aldrich, Cat.# C-1768)

Cell culture incubator at 37°C with 7.5% CO_2

Methods

Preparation of Coverslips: Perform 1 day before cell plating (all in sterile hood).1.

Sterilize Coverslips: Place coverslips in a p150 petri dish and rinse with 70% EtOH. Let

coverslips dry by leaning along edge of plate. Place coverslips in a glass p100 petri dish and autoclave.

2. Coverslip coating: Cut piece of parafilm to fit in p150 petri dish, wipe parafilm with EtOH and press into bottom of petri dish. Place sterilized coverslips on parafilm. Coat coverslips with 0.2mg/mL PDL and leave overnight at room temperature.

Media: For culturing DRG neurons in microfluidics use Neurobasal, 2% B-27, 1% glutamax, 1% penicillin streptomycin, 0.08% Glucose (1:250 dilution of 20% glucose stock solution) (*see* Note 2). To make up media with neurotrophins dilute nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) from 1mg/ml stock concentration into media [Table A.2] (*see* Note 3). Thus, to make 100 ng/mL NGF/BDNF media, dilute NGF and BDNF 1:10,000 into media. To make 50 ng/mL NGF/BDNF media, dilute 100 ng/mL media (1:1) in media. Lastly, AraC should be added to media for a final concentration of 0.3 μM, to limit the growth of Schwann cells. We find that addition of AraC for at least the first 3 days in culture is effective at reducing Schwann cells in microfluidics.

Culture Assembly: Day of DRG dissection (at least 4hr prior to cell plating).

1. Wash Coverslips: Aspirate off PDL and wash coverslips 3X with sterile water. Following last wash, remove water and let coverslips dry in hood.

2. Sterilize Microfluidics: Place microfluidics (*see* Note 9) in a p150 petri dish (grooves facing up) and wash with 70% EtOH. Let dry in hood.

3. Culture Assembly: Place a dry, PDL-coated coverslip face up into a p60 petri dish. Use forceps to place microfluidic chamber (groves down) onto coverslip (Fig.A.3B). To reinforce seal with coverslip, use back end of forceps to gently press down on chamber in center, as well as the four edges and corners (*see* Note 10).

4. Laminin Coating: Dilute 50µl laminin in 5ml HBSS (1:100 dilution) to a final concentration of 10µg/ml. Add laminin to each channel by pipetting 50µl into top two wells with pipette tip pointing directly towards channel. Be sure that laminin flows down to coat the entire channel surface on which cells will grow (culture can be tipped up to encourage flow-through). Add 100µl of laminin to each well. Place cultures in a 37°C incubator for 3 hours.

Plating cells in microfluidics:

1. Following dissection, trypsinization, and cell counting, spin down 500,000 cells at 80 rcf (636RPM) for 5 minutes at 4°C.

2. Carefully aspirate off media, leaving as little media as possible. Resuspend cells in 50µl media (50ng/ml NGF/BDNF + AraC). This step is important for obtaining the proper cell density within the microfluidic.

3. Remove laminin from microfluidics by aspirating off all four wells and placing tip directly towards channel from top and/or bottom to remove all laminin from the channels (*see* Note 11).

4. Plate 40,000 cells in each microfluidic by adding 4µl cells to the top well of cell body side. Pipette tip should point directly into the channel and watch that cells flow down,

Figure A.3

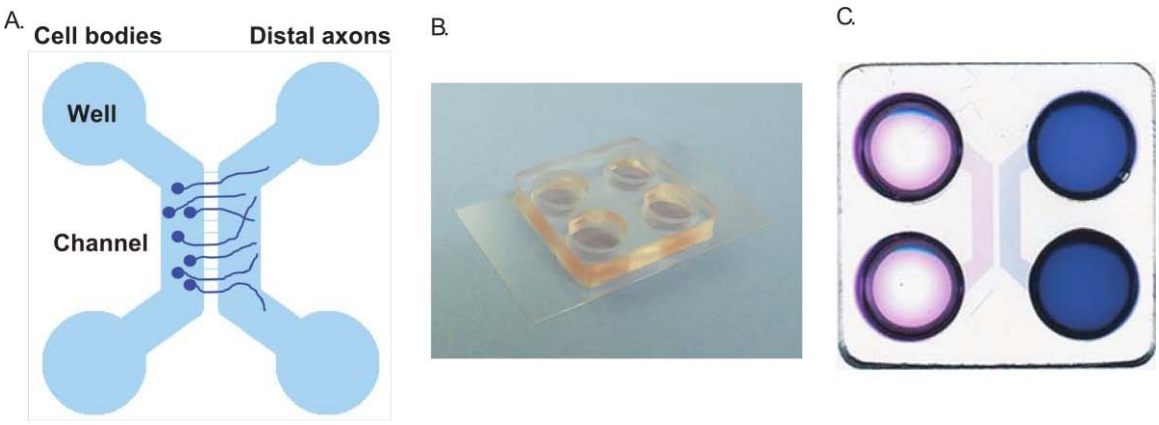


Figure A.3. Microfluidic cultures.

A. Labeled schematic of microfluidic culture. **B**. Assembled microfluidic on glass coverslip. **C**. Microfluidic filled with media on left side and trypan blue in right side is fluidically isolated for 24 hours. Media volume in wells is two times the volume of trypan blue to create isolation by hydrostatic pressure. Blue remains restricted to the right side of the culture after 24 hours.

reaching the base of the channel. If cells do not flow down, apply a slight downward force by aspirating briefly from the bottom of the channel using a 200µl pipette tip.

5. Immediately put cultures into the 37°C incubator to let cells attach to substrate for 5-10 minutes.

6. Add media (100ng/ml NGF/BDNF + AraC) to distal axon compartment channel by pipetting 40µl directly into channel in top right well, and subsequently add media to all four wells: 100µl media (50ng/ml NGF/BDNF + AraC) to both cell body wells and 100ul media (100ng/ml NGF/BDNF + AraC) to both distal axon wells. Return cells to incubator.

Maintenance of microfluidics cultures:

Due to the small volume of media that can be added to microfluidics cultures, media should be changed every day or every other day to minimize effect of evaporation, and ensure optimal health and axon growth. One can adjust the cell feeding schedule depending on the humidification of your incubator, based on changes in the media volumes following feeding. Importantly, media must be changed without disturbing cells. Therefore, it is important that you not remove media directly from the channels themselves (which contain approximately 4ul media). See Table A.2 for schedule of media conditions when feeding cells.

1. When changing media, first aspirate media from all four wells. Keep pipette tip away from entrance to the channel containing the cells.

2. Add 40µl of specified media to top well of each culture. Do not point tip into channel, just add to the well.

3. Tip cultures up to allow media to flow through the channel and into the bottom well. Be sure to see that media begins to fill the bottom well.

4. Add 100µl specified media to each well and return cultures to incubator (*see* Note 12).

5. Axons will grow across microgrooves and enter the distal axon compartment by 1-2 DIV and extend across the compartment by 4-7 DIV. Continue to change media daily or every other day until used for an experiment [Table A.2] (*see* Note 13). Keeping cells in AraC for first 72 hours will significantly limit glia within the cell body compartment and no glia should be present in distal axon compartment.

Fixing microfluidics:

While microfluidics can be used for live imaging of DRG neurons, the cultures can also be fixed for immunofluorescence (IF) or fluorescent in situ hybridization (FISH) (Fig.A.4). Since axons are physically separate from the DRG cell bodies, this allows for high resolution imaging of individual axons and growth cones. This is far superior to imaging in mass cultures where most fields of view contain a mix of cell bodies and axons, making it difficult to select the appropriate exposure time and focal plane for optimizing detection of axonal signal. If using cultures for RNA analysis by FISH, be sure to use RNase-free fixatives.

1. To fix microfluidics, first remove half of the media from each well.

2. Perform a gentle fix by adding 4% PFA to the media in each well (final ratio of media to PFA is 1:1) and leave on cells for 10 minutes at room temperature (*see* Note 14).

Figure A.4

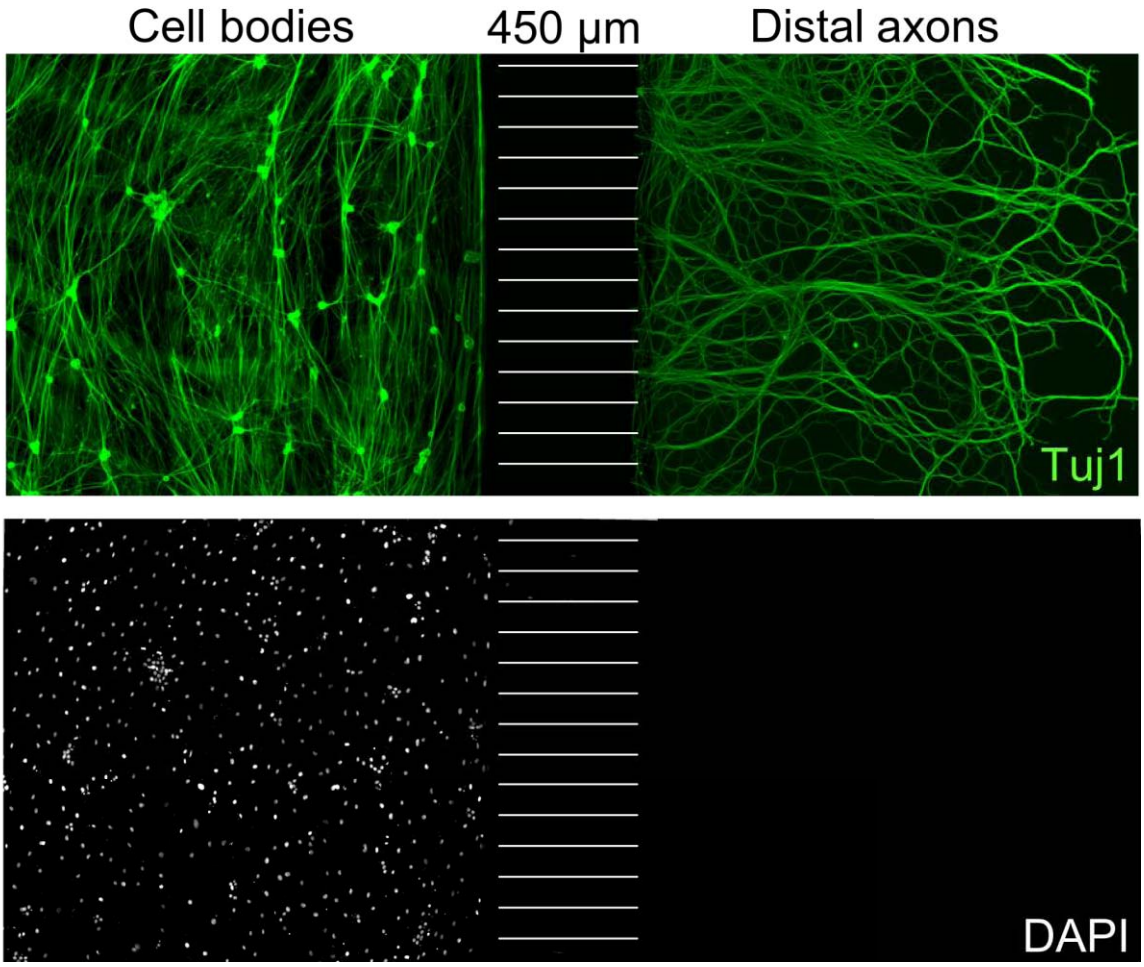


Figure A.4. Immunostaining in microfluidic cultures.

Tuj1 immunostaining of E15 DRG neurons grown in microfluidic culture shows growth of axons into distal axon compartment. DAPI staining demonstrates that cell bodies are restricted to the cell body compartment.

3. Remove all media/PFA from each well and add 50µl fresh 4% PFA to each top well and let flow through to bottom wells. Add additional 4% PFA to each well and fix for 10 minutes.

4. Wash three times with 1X PBS (5 minutes each): Wash microfluidics by adding 50µl 1X PBS to both top wells and tip to allow PBS to flow down through both channels. Then add 100µl to all four wells and wait 5 minutes. Aspirate PBS from all four wells and repeat twice more.

5. Fixed cells can be stored in 1X PBS at 4°C for immunostaining or FISH.

6. Immunostaining and FISH can be performed with the microfluidic chamber still attached by adding reagents just as described previously for media and PBS (Fig.A.4). Mount attached microfluidics by adding 30µl of Fluoromount G to each top well. This is sufficient for imaging cell bodies and distal axons. Alternatively, to image axons within the grooves, one can remove the microfluidic chamber for staining. To do this, outline attached microfluidic chamber with a hydrophobic barrier pen following fixation, remove the microfluidics and proceed with staining, and mount the coverslip onto a slide for imaging.

Neurotrophin Stimulation and Deprivation Experiments

Compartmented cultures can be used to study various aspects of neuronal development, function, and regeneration. Here are two experimental paradigms which can be used to study neurotrophin responses within DRG cell bodies and axons. First, neurotrophin stimulation experiments can be performed alongside a control, vehicle-stimulated condition, to determine how gene expression is regulated both temporally and spatially in

response to neurotrophins. Alternatively, these systems can be used to study changes observed when neurotrophins are removed. These experiments can be performed in either compartment culture system, depending on the analysis method required by your experiment. Lastly, these experiments can be combined with a number of other manipulations, such as knockdown by shRNA, transgene overexpression, or acute injury to investigate the direct role of specific molecules in DRG neuron function and survival (*see* Notes 15 and 16).

Neurotrophin stimulation protocol

1. To perform experiments with neurotrophin stimulation, first remove all media from the cultures. In Campenot cultures this requires aspirating off all of the media from side compartments and the surround. In the microfluidics, this requires aspirating media from the four wells.

2. Add plain media (either DMEM or Neurobasal) to the cultures for 2hr. This brief treatment allows signaling pathways to reset to baseline following growth in neurotrophins. Additionally, blocking antibodies to NGF and/or BDNF can be used during the starvation to rigorously eliminate the effect of any residual neurotrophin.

3. Stimulate the distal axons with 100 ng/ml NGF/BDNF or vehicle control (100 ng/ml BSA) (*see* Note 17). In the Campenots this can be done by making a 1:100 dilution of NGF and BDNF in plain DMEM and adding 5ul of this to each side compartment (assuming side compartments contain about 500µl media). In the microfluidics, plain media with neurotrophins or BSA should be made in advance and added as previously described. Cell bodies are left in plain media during stimulation. This selective

stimulation of distal axons recapitulates the way in which DRG neurons receive neurotrophins from the periphery *in vivo*. To study the differences in the spatially selective response to neurotrophins, one can also compare neurotrophin stimulation of the distal axons with stimulation of the cell bodies, or with combined stimulation of cell bodies and axons (global stimulation similar to conventional mass cultures).

4. The length of neurotrophin stimulation can vary from minutes to hours, or even days, depending on the timing of the biological response. Ideally, one should perform a time course to determine the optimal stimulation time for experimentation and to investigate the temporal properties of the biological response.

5. Following neurotrophin stimulation, collect (Campenots) or fix (microfluidics) cells for subsequent analysis (Fig. A.5A).

Neurotrophin deprivation protocol

1. To selectively deprive distal axons, change axonal compartments to plain DMEM or Neurobasal media and put cell bodies in low neurotrophins (10 ng/ml NGF/BDNF) for 24hr.

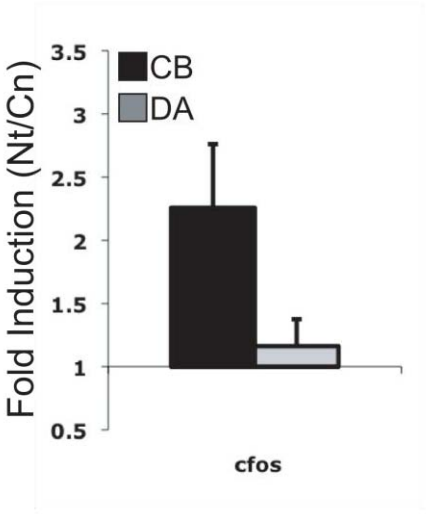
2. Following deprivation, cultures can be fixed and stained for Tuj1 to perform a degeneration assay (Fig. A.5B,C).

Figure A.5. Neurotrophin stimulation and deprivation experimental data.

A. Campenot cultures were stimulated with neurotrophins at distal axons (DA) for 2 hours (Reproduced from (Cosker et al., 2013). Fold induction is compared with control, vehicle-stimulated, neurons. The transcription factor *c-fos*, an immediate early gene, is upregulated in CB, but not DA. **B**. Distal axons (DA) in microfluidic cultures were deprived of neurotrophins for 24 hours, fixed and stained for Tuj1, and compared with control (plus neurotrophin). **C**. Images of DA were binarized and axon fragments were defined by the Analyze Particle (0-200) function in National Institute of Health ImageJ software. Quantified images show that NT-deprived DA exhibit an increase in axon fragmentation by this degeneration index (ratio of fragmented axons to total axon area).

Figure A.5 (continued)

A.



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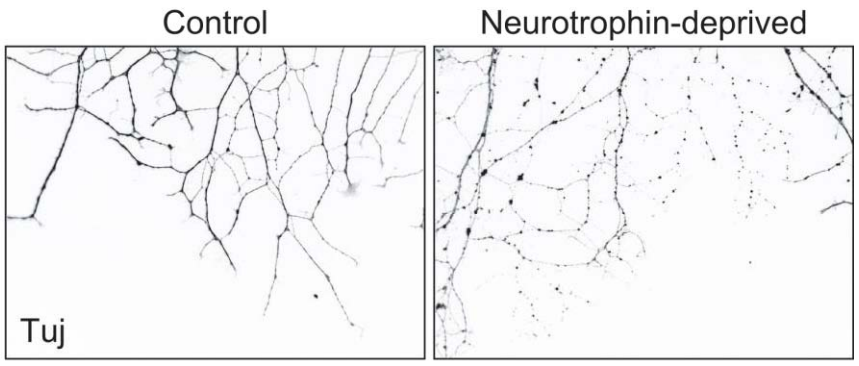
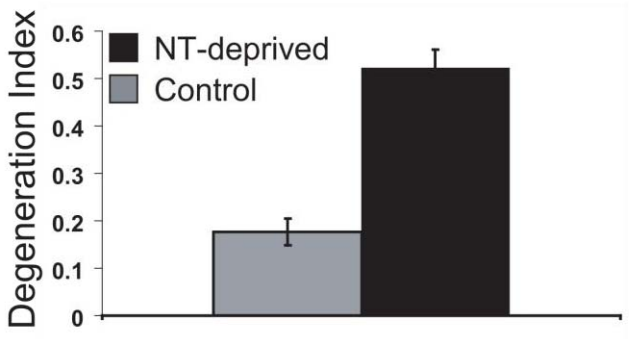


C.



Notes

1. There are many other dividers available from Tyler Research. This protocol is designed especially for the Teflon dividers with one small middle compartment and two larger side compartments (CAMP10-20mm OD), but the compartment set up protocol would be the same with many other shaped dividers.

2. This serum containing media has been used successfully for many years to grow DRGs in compartmented Campenot cultures. As a serum-free alternative, Neurobasal with B-27 can also be used with these cultures. In contrast, we find that growth of DRG neurons in microfluidic chambers are far superior with Neurobasal and B-27. The use of different media for each culture system is based on empirical evidence that the specified media results in optimal growth of DRG neurons in each culture. The difference in effectiveness may be a result of the media interaction with different substrates.

3. DRG neurons are a heterogeneous group of neurons that express different Trk receptors and, in turn, rely on different neurotrophins for their survival during development. These protocols use both NGF and BDNF for culturing DRG neurons, thus supporting the survival and maintenance of TrkA and TrkB expressing neurons which account for the vast majority of embryonic DRG neurons. The TrkC expressing neurons, which are a very small percentage of total DRG neurons, are not maintained in these cultures. Alternatively, the neurons can be grown in NGF alone, yet this will alter the complement of neurons that survive in the cultures.

4. Observing assembly of the cultures can be extremely helpful, especially for proper grease application. To watch this process, see the referenced JoVE video (Pazyra-Murphy and Segal, 2008).

5. The amount of pressure used to seal the p35 dish to the greased Teflon divider will greatly influence the culture outcome. The ideal amount of pressure will seal the compartments and create fluidic isolation, as well as allow the axons to grow through the grease and into the side compartments. In the beginning, it is inevitable that several cultures will be leaky, so be sure to set up more than needed for an experiment.

6. It is possible to collect RNA or protein from microfluidics (Park et al., 2006), although not described here. However, the amount of material is more limited than from the Campenot cultures.

7. This protocol was established using the 450µm width microgroove microfluidics, which seem optimal for DRG neurons. Similar protocols can work for shorter and longer microgrooves. If using shorter microgrooves, there is a greater possibility that fluidic isolation may be compromised.

8. Instead of purchasing pre-made microfluidics, one can also fabricate the chambers using poly dimethylsiloxane (PDMS)(Park et al., 2006). This requires an initial investment to manufacture the master mold.

9. The microfluidic chambers are not readily reusable as are the Campenot dividers, which can be washed and reused for years. Some success with detergent-based cleaners has allowed the microfluidics chambers to be re-used, but optimal growth and fluidic isolation is often not achieved in many of the washed cultures.

10. Following assembly of the culture, the coverslip often sticks to the tissue culture plate. After pressing the chamber onto the coverslip, use the forceps to gently loosen the coverslip away from the plate.

11. If bubbles form within the channels when aspirating laminin or plating cells, aspirate off cells, add 50µl media to the channel and aspirate again to clear the channel. Then re-plate cells into the channel. It is important to examine the channels carefully before adding cells to the culture.

12. While cells are growing, we find it optimal to maintain equal volumes of media on each side of the microfluidics cultures. During an experiment when fluidic isolation is desired, volumes should be kept unequal to create a small hydrostatic pressure difference and ensure that there is no mixing of media between compartments (Fig. A.3C). We have verified that small molecules (>750 Da) in the axonal compartment are not detectable in the cell body compartment if we add 60µl media to the axonal wells, with 200µl media in each cell body well (filled to top). This volume difference maintains fluidic isolation overnight and up to 24hr. Figure A.3C demonstrates that the hydrostatic pressure difference can retain trypan blue to one side of the culture for at least 24hr.

13. When performing experiments with changes in media that will add or remove various treatments (i.e. small molecules, plain media starvation), do two washes through each microfluidic channel with 40µl media and aspirate from wells following each wash, before subsequently adding additional media to the wells. This ensures that cells are cleaned of any prior treatment and are successfully transferred into media of the desired concentration.

14. If you find that axonal morphology is not maintained during PFA fixation, try a methanol pre-treatment to stabilize axonal microtubules. Add 50µl ice cold methanol (kept at -20°C) to the top two wells of each channel and let flow through to bottom wells.

This should be done quickly and only left on cells for 1 minute. Following this, remove methanol from each of the wells and perform a 4% PFA fix for 15 minutes.

15. For transgene overexpression in microfluidics, DRG neurons can be transfected using Amaxa Rat Neuron Nucleofector Kit (Lonza) prior to plating cells. Following transfection of 2 million cells, spin cells down for 3 minutes, resuspend in 50µl media (without Pen-Strep or AraC), and plate 4ul into cell body channel. Variability in cell death during transfection leads to differences in cell number; therefore re-count cells following transfection or adjust media volume slightly after plating first culture to desired density.

16. Recombinant protein can be selectively introduced to a single compartment of the Campenot cultures using the Chariot protein tranfection system (Active Motif) to locally overexpress protein in cell bodies or axons (Cosker et al., 2013).

17. It is important to use a vehicle control (i.e. BSA) alongside the neurotrophin-stimulated cultures for the period of treatment. This controls for the response to new media following starvation with plain media, as well as the mechanical stimulation during the media change.

Table A.1. Media conditions for DRGs in Campenot cultures

DIV (Days *in vitro*)	Middle compartment	Side compartments
Plating cells	100 ng/ml NGF/BDNF + AraC	100 ng/ml NGF/BDNF + AraC
1 DIV	10 ng/ml NGF/BDNF + AraC	Leave as is
2 DIV	10 ng/ml NGF/BDNF	100 ng/ml NGF/BDNF
5 DIV	0 ng/ml NGF/BDNF + AraC	1 ng/ml NGF/BDNF + AraC
8 DIV	Use for experiment	

Table A.2. Media conditions for DRGs in microfluidic cultures

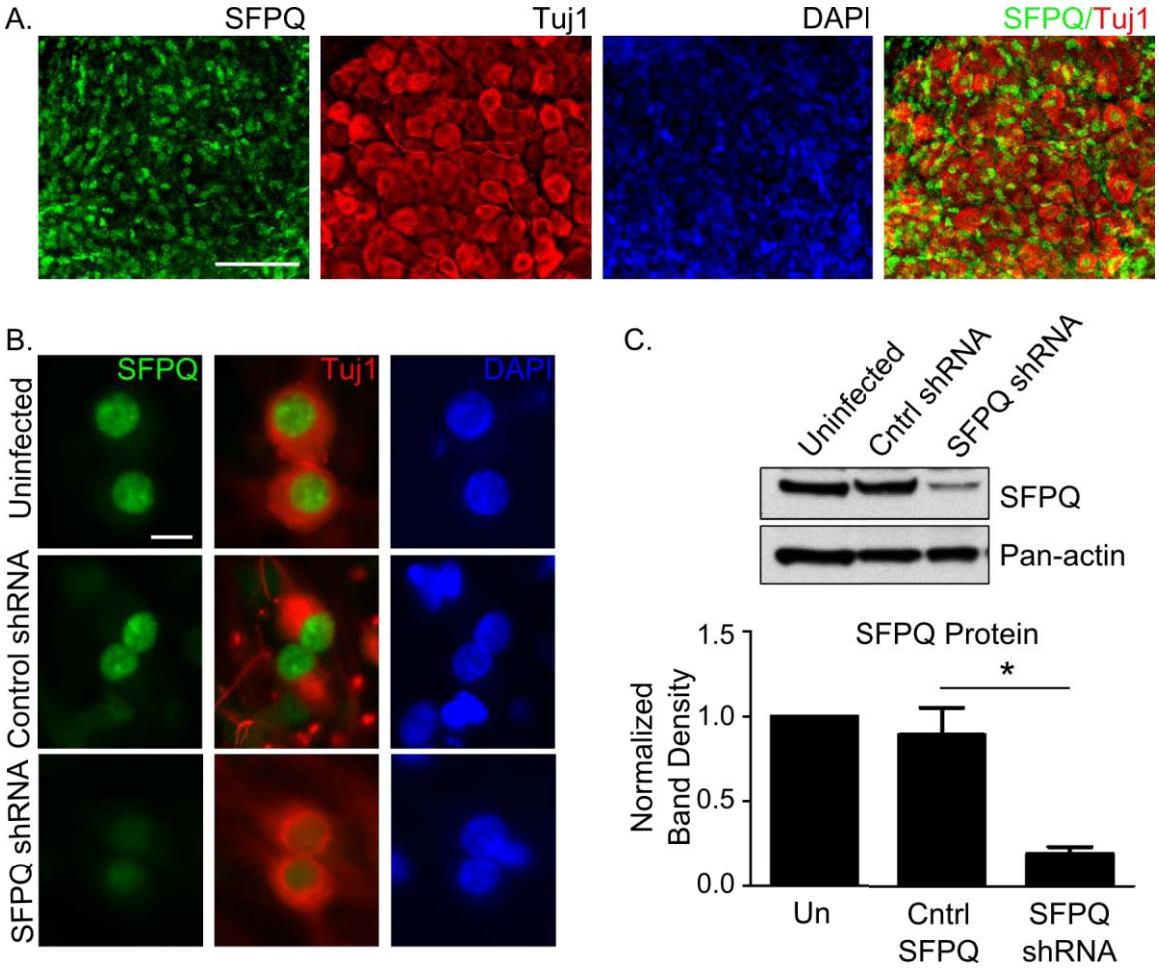
DIV	Cell body compartment	Distal axon compartment
Plating cells	50 ng/ml NGF/BDNF +AraC	100 ng/ml NGF/BDNF + AraC
1 DIV	10 ng/ml NGF/BDNF +AraC	100 ng/ml NGF/BDNF + AraC
2 DIV	1 ng/ml NGF/BDNF +AraC	10 ng/ml NGF/BDNF + AraC
3 DIV	1 ng/ml NGF/BDNF	10 ng/ml NGF/BDNF
4 DIV and longer	1 ng/ml NGF/BDNF Or use for experiment	10 ng/ml NGF/BDNF

Appendix II:

Supplementary Figures

Supplement to Chapter 3

Supplementary Figure S1



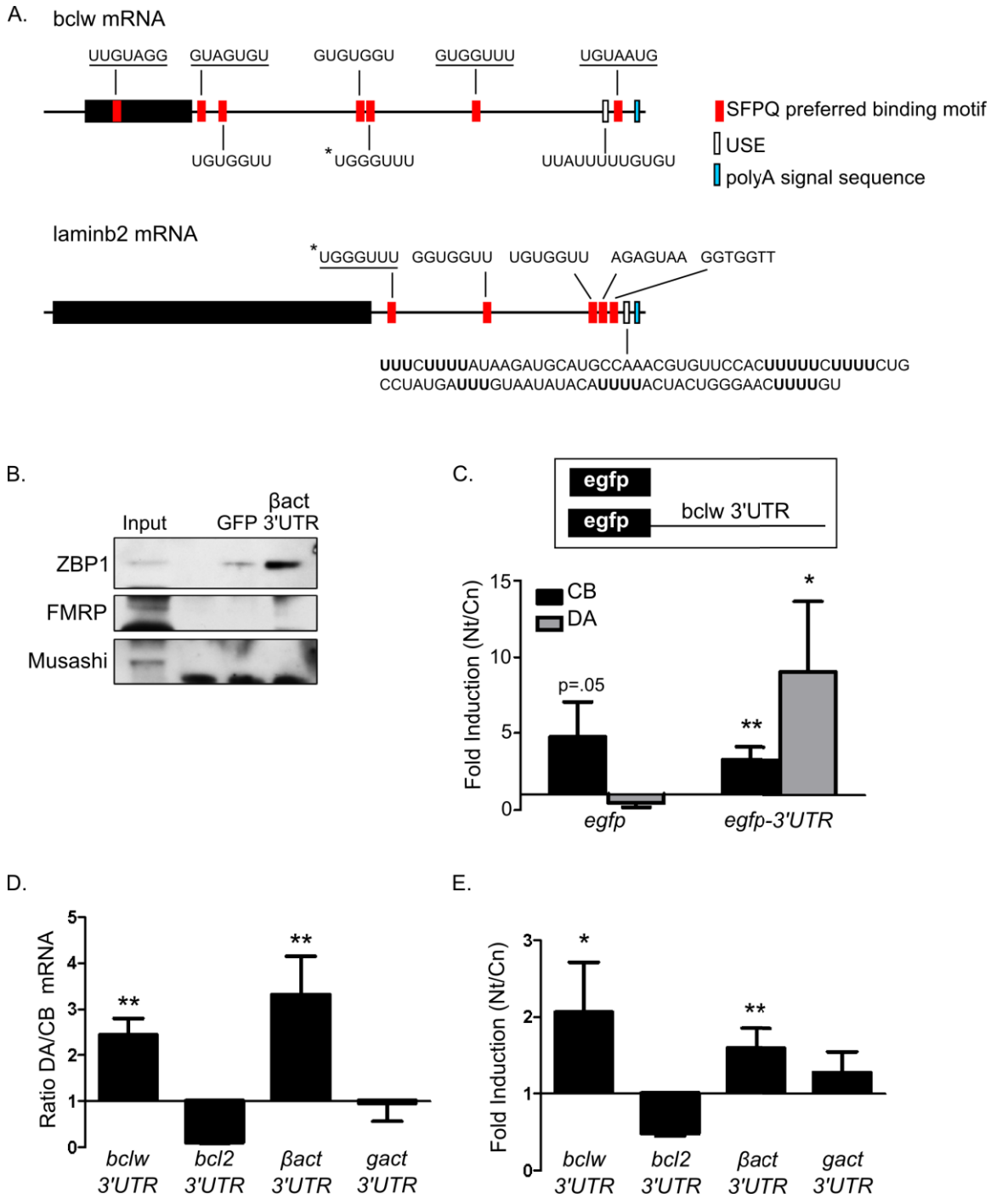
Supplementary Figure S1.

A. SFPQ immunostaining of whole-mount DRG at P0. Scale bar is 100µm. **B**. SFPQ immunostaining of cultured DRG neurons infected with control or SFPQ shRNA. Scale bar is 10µm. **C**. Western blot of cultured DRG neurons infected with control or SFPQ shRNA. Quantification of SFPQ levels normalized to pan-actin loading control ± SEM, t-test, n=3, *p<.05.

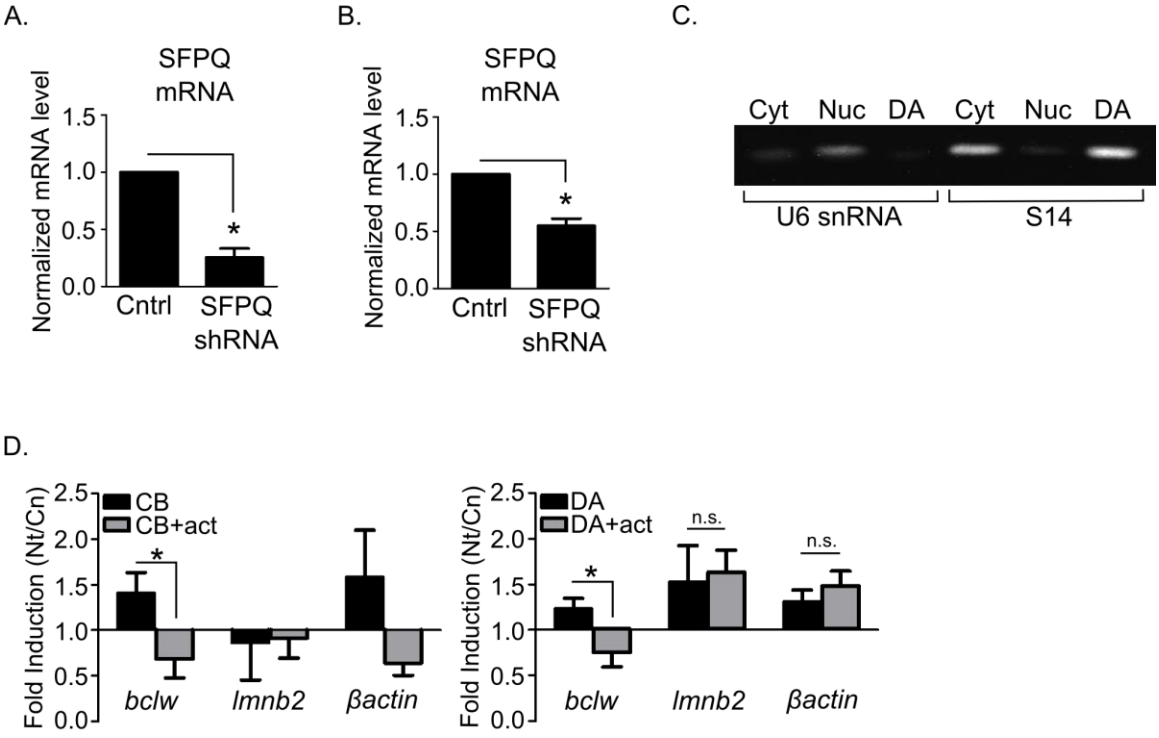
Supplementary Figure S2.

A. Schematic of *bclw* and *laminb2* mRNAs with preferred SFPQ binding sequences (Ray et al.) and USE sequences. Underlined SFPQ sites are conserved in mouse and human. * denotes matching sites. **B**. Biotinylated RNA pulldown of *β-actin* 3'UTR RNA and control *GFP* RNA from DRG lysate. Western blot for ZBP1, FMRP, and Musashi to detect protein pulled-down with RNAs (representative of 2 repeats) **C**. Overexpression of egfp or egfp-bclw3'UTR in compartmented cultures. Egfp RNA levels measured by RT-qPCR following stimulation of DAs with NT or BSA control; Normalized values (Nt/Cn) ± SEM, Z-test comparison with 1, n=6, *p<.05, **p<.01. **D**. DA/CB ratio of endogenous 3'UTR mRNA levels measured by RT-qPCR. Normalized values (DA/CB) ± SEM, Z-test comparison with 1, n=6, **p<.01 **E**. Fold induction of mRNA levels in DA with NT stimulation measure by RT-qPCR. Normalized values (Nt/Cn) ± SEM, Z-test comparison with 1, n=6, *p<.05, **p<.01.

Supplementary Figure S2 (continued)



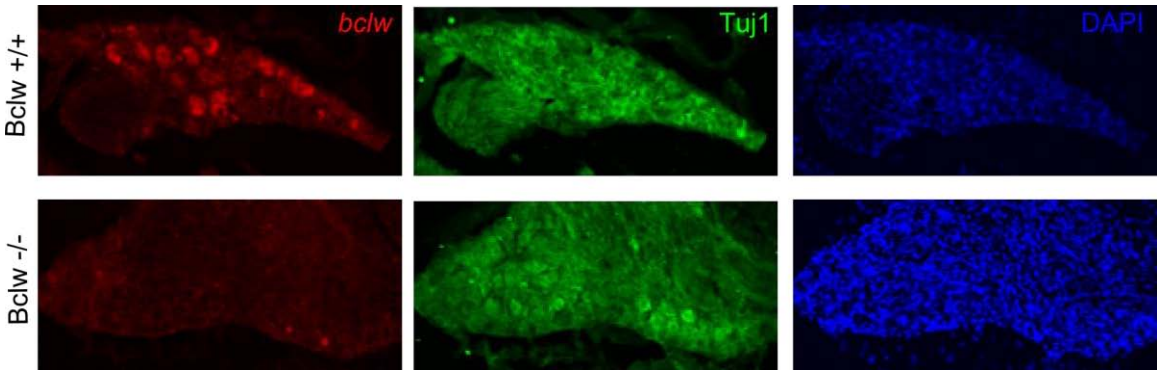
Supplementary Figure S3



Supplementary Figure S3.

A. SFPQ mRNA levels by RT-qPCR in CB of DRG neurons grown in compartmented cultures infected with control or SFPQ shRNA shown in Figure 3.3B. Values normalized to control ± SEM, t-test, n=5, *p<.05. **B**. SFPQ mRNA levels by RT-qPCR in CB of DRG neurons grown in compartmented cultures infected with control or SFPQ shRNA for cell fractionation shown in Figure 3.3C. Values normalized to control ± SEM, t-test, n=8, *p<.05. **C**. U6 snRNA and S14 levels by PCR for Cyt, Nuc, and DA fractions. **D**. Fold induction of mRNA levels in CB (left) and DA (right) with NT stimulation and treatment with transcription inhibitor, actinomycin-D. Fold induction (Nt/Cn) values ± SEM, t-test, n=6, *p<.05.

Supplementary Figure S4



Supplementary Figure S4.

FISH for *bclw* mRNA was performed on frozen sections of DRG (L4) from bclw$^{+/+}$ and bclw$^{-/-}$ mice (6 months) to validate *bclw* probe used for single molecule FISH. Sections stained for Tuj1 and DAPI.

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