MECHANISMS FOR SPATIAL REGULATION OF NGF-SIGNALING IN NEURONS

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Abstract

Neurons are highly polarized cells that require exquisite regulation of signaling to establish functional circuits. Dendrites receive input from synaptic contacts which is relayed along the axon to post-synaptic targets. How these specialized compartments are developed and maintained is poorly understood. In the developing sympathetic nervous system, neurons rely on signaling from the secreted growth factor, Nerve Growth Factor (NGF). NGF binds its TrkA receptors at nerve terminals to locally regulate axon elongation and innervation of post-synaptic targets. NGF-TrkA complexes also undergo retrograde transport back to the cell body to regulate survival, dendritic and synapse development. How NGF signaling mediates multiple diverse processes during neuronal development is an open question. My thesis work identified two mechanisms which operate locally in axons to contribute to NGF-dependent growth.

Local protein synthesis contributes to the development and specialization of neuronal compartments. Neurotrophin-dependent axon growth requires recruitment of diverse classes of mRNA to axons to promote axon growth. While studying Tp53inp2, a transcript that is highly expressed and enriched in sympathetic neuron axons, we unexpectedly discovered that Tp53inp2 mRNA is not translated into a protein. Instead the transcript supports axon growth in a coding-independent manner. Tp53inp2 transcript interacts with the TrkA receptor and regulates both TrkA endocytosis and signaling. Deletion of Tp53inp2 inhibits axon growth in vivo and the defects are rescued by a non-translatable form of the transcript. Thus, Tp53inp2 is an atypical mRNA that regulates the growth of sympathetic axons by enhancing NGF/TrkA signaling in a translation-independent manner.
NGF-TrkA signaling is known to control the activity, sub-cellular localization, and stability of downstream effectors by regulating their post-translational modifications such as phosphorylation or ubiquitination. The post-translational modification of proteins by prenylation, the addition of either a 15-carbon farnesyl or 20-carbon geranylgeranyl lipid to the C-termini of proteins, controls the localization and activity of diverse proteins. It has been assumed that protein prenylation is largely a constitutive mechanism that occurs ubiquitously throughout the cytoplasm and is permissive for cellular functions. In striking contrast, my results reveal that prenylation is under exquisite spatio-temporal control by NGF. NGF signaling acutely regulated geranyl-geranylation of proteins in axons, which is abolished by cycloheximide, a protein translational inhibitor. Inhibition of geranyl-geranylation in axons, but not cell bodies, disrupted NGF-dependent axon growth. I further identified the Rac1 GTPase as an effector that is locally synthesized and prenylated in axons in response to NGF. These results suggest an efficient coupling of local protein synthesis and prenylation in axons in response to NGF. My study elucidates a previously unrecognized mechanism by which neurotrophic factors promote axon growth; NGF regulates the local synthesis of growth-promoting proteins in axons and also ensures their post-translational modification to enable axonal functions.

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Preface

While a thesis summarizes all the successes from years of work, about 99% of a doctorate is harsh lessons in failure and how to confront and surpass it. The completion of my dissertation would never have been possible without the immense support from my advisor, many of the faculty at Johns Hopkins, my friends and family. I generally prefer to show my appreciation to these people through actions, but I would like to provide a public, written acknowledgment of all the time, effort, and help they have provided me through graduate school. For the sake of space, I unfortunately cannot name every single individual and how they have contributed to my growth and maintenance of sanity as this would turn into the longest thesis chapter; so, I sincerely apologize to the vast majority of the people in my life who are about to turn into a generalized statement.

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Chapter 1: INTRODUCTION

A portion of this chapter was previously submitted for publication at Molecular and Cellular Neuroscience: Emily Scott-Solomon and Kuruvilla (2018).

Mechanisms of neurotrophin trafficking via Trk receptors
Neurons are highly specialized cells that receive and transmit information throughout the body. Establishment and maintenance of neuronal circuits requires communication by targets often located millimeters to meters away from the cell body through the secretion of diffusible cues. The family of neurotrophins provide a well-characterized example of these target-derived instructive factors. Neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). Each neurotrophin binds with high-affinity to a specific member of the Trk Receptor Tyrosine Kinase (RTK) family with NGF binding to TrkA, NT4 and BDNF binding to TrkB, and NT3 binding to TrkC. Of the neurotrophins, NGF-TrkA signaling and trafficking is the best characterized. Binding of NGF to TrkA causes dimerization and autophosphorylation to activate canonical signaling pathways MAPK/Erk, PI3-K/AKT, and PLCγ (Huang and Reichardt, 2003). Local NGF-TrkA signaling in axon terminals regulates growth cone motility, axon growth, and branching to innervate target tissues (Bodmer et al., 2011; Scott-Solomon and Kuruvilla, 2018).

However, NGF-TrkA signaling is also retrogradely relayed to the cell body to regulate transcription of survival, growth, and maturation factors (Barford et al., 2017; Scott-Solomon and Kuruvilla, 2018). A key question in the neurotrophin field has been the nature of the signal carried from the axon to the cell body. Currently, several lines of evidence suggest that the retrograde neurotrophin signal is communicated via internalized neurotrophin-Trk receptor complexes in endocytic vesicles, though the identity of these vesicles is still hotly disputed (Barford et al., 2017; Scott-Solomon and Kuruvilla, 2018; Yamashita and Kuruvilla, 2016). Upon
internalization in the axon, a small subset of vesicles undergo long-distance retrograde transport. Actin depolymerization by a Rac1-cofilin signaling module releases NGF-TrkA signaling endosomes from actin meshwork for initiation of the retrograde signal (Harrington et al., 2011). Early ultrastructural analyses revealed active TrkA receptors being retrogradely transported in heterogeneous vesicles of different sizes, both coated and uncoated vesicles, and multi-vesicular bodies (Bhattacharyya et al., 2002). Characterization of NGF-TrkA containing vesicles found Rab5, an early endosome marker (Delcroix et al., 2003), and Rab7, a late endosome marker (Deinhardt et al., 2006) suggesting a dynamic maturation of Trk-positive vesicles from early to late endosomes during transport. Several lines of evidence have also supported the role of multi-vesicular bodies (MVBs) in retrograde transport of Trk receptors (Weible and Hendry, 2004). Delivery of radiolabeled NGF to distal axons in culture or peripheral target tissues found the ligand predominantly in MVBs in neuronal cell bodies (Bhattacharyya et al., 2002; Claude et al., 1982; Sandow et al., 2000). These early studies were strengthened by more recent evidence using sympathetic neurons isolated from a FLAG-TrkA knock-in mouse line, where the majority (>70%) of retrogradely transported TrkA organelles in axons were found to be MVBs that are positive for Rab7 (Ye et al., 2018). Intriguingly, in cortical and hippocampal neurons, Rab7-positive MVBs have been postulated to be the retrograde carriers for active TrkB receptors (Kononenko et al., 2017). The observations of heterogenous Trk-containing vesicular structures raise the possibility of functionally distinct classes of Trk-signaling endosomes that may be specialized to mediate different biological outcomes though the
mechanisms regulating the formation and sorting of these distinct classes remains unresolved.

Upon arrival in the cell body, one of the fates of retrogradely transported active Trk receptors is to undergo exocytosis to the soma surface (Suo et al., 2014; Yamashita et al., 2017), where these receptors interact with naïve Trk receptors to influence their anterograde transcytosis to axons (Yamashita et al., 2017). Trk signaling endosomes are also capable of persistent signaling in cell bodies to activate transcriptional changes necessary for survival and growth partially through local recycling of axon-derived Trk receptors between the soma surface and interior (Suo et al., 2014). A further subset of axon-derived Trk endosomes are also transported to dendritic compartments to regulate synapse formation and maintenance (Lehigh et al., 2017; Sharma et al., 2010). The spatial context of NGF signaling likely requires recruitment and maintenance of distinct proteins to mediate compartment specific outcomes. Additionally, synapses, dendrites, and axonal growth cones require highly restricted signaling as they continually undergo cycles of growth, maintenance, and pruning. However, the mechanisms responsible for spatial regulation of NGF-TrkA signaling in distinct cellular compartments are still being elucidated.

Local translation in neuronal sub-domains permits high temporal resolution and spatially restricted gene expression for acute responses to extrinsic stimuli (Martin and Ephrussi, 2009). It is appreciated that neuronal processes that continually undergo cycles of growth and pruning in response to cellular contacts are heavily reliant on local protein synthesis for development, maintenance, and
function with shifts in local gene expression based on compartmental maturation (Gumy et al., 2011; Rangaraju et al., 2017). This is highlighted by elegant studies which characterized mRNA transcripts in individual neuronal compartments including recent work that identified over 6,000 unique genes in developing sensory axons (Andreassi et al., 2010; Cajigas et al., 2012; Gumy et al., 2011; Kar et al., 2018; Riccio, 2018). Together these studies found enrichment of specific mRNA populations encoding for signaling proteins, scaffolds, and metabolic enzymes in various compartments, suggesting that active sorting and delivery of specific mRNAs contribute to the functional specialization of neuronal compartments (Riccio, 2018). The identities of these transcripts and how their expression is spatially and temporally regulated in the axon to drive directed growth and target innervation remains poorly understood. Further, how specific mRNAs are recruited to axons in response to extrinsic signals is an active area of research.

In addition to local protein synthesis, one mechanism that could contribute to the compartmentalized signaling in neurons is protein lipidation, the attachment of lipid groups to proteins. In non-neuronal cells, lipid modifications are known to regulate protein membrane localization, trafficking, stability, activity, and formation of protein complexes (Resh, 2013). Lipidation presents an underappreciated mechanism by which neurons could restrict protein localization and signaling to distinct sub-cellular domains. Over a 1,000 proteins are predicted to be lipid modified (Resh, 2013), many of which have key roles in signaling (Casey et al., 1989; Kilpatrick et al., 2016; Oku et al., 2013; Resh, 2006; Thomas et al., 2012),
cytoskeletal remodeling and stability (Fukata et al., 2004; Kinsella et al., 1991; Levy et al., 2011; Nishimura and Linder, 2013), cell adhesion (Little et al., 1998), and protein and membrane trafficking (Gomes et al., 2003) in neuronal development, function, and maintenance (Fukata and Fukata, 2010; Kilpatrick et al., 2016; Matsuura et al., 2004; Ng et al., 2002; Thomas et al., 2012). These modifications include the addition of fatty acyl groups (palmitoyl and myristoyl), prenyl groups (farnesyl and geranylgeranyl), sterols, phospholipids, and glycosylphosphatidylinositol (GPI) anchors (Resh, 2013). Each lipid confers unique qualities to the modified protein to specialize its function (Resh, 2013). In neurons, fatty acylation and prenylation are the most prevalent with misregulation of these modifications associated with neurodegenerative diseases (Fukata and Fukata, 2010; Jeong et al., 2018). Perturbations in lipidation have been linked to neurological disorders such as schizophrenia (Mukai et al., 2008), and neurodegenerative diseases including Huntingtin’s disease (HD) (Singaraja et al., 2011; Sutton et al., 2013), Alzheimer’s disease (AD) (Cho and Park, 2016; Hottman and Li, 2014), and amyotrophic lateral sclerosis (ALS) (Li et al., 2016). However, much of the work on protein lipidation in neurons so far has focused on palmitoylation. To date, little is known about the regulation and functions of protein prenylation in neurons.

Prenylation involves the addition of either a 15-carbon farnesyl group catalyzed by cytosolic farnesyltransferase (FTase) or 20-carbon geranylgeranyl group catalyzed by cytosolic geranylgeranyltransferase I (GGTase I) to a cysteine residue. The modified cysteine is located in a “CaaX” box (Cys-aliphatic-aliphatic-
X) at the C-terminus of the protein with the “X” amino acid biasing the prenyl-group added (Resh, 2013). Over 2% of all expressed genes are identified or predicted to undergo prenylation. They include key signaling proteins, cytoskeletal regulators, vesicular trafficking proteins, and growth and survival factors (Wang and Casey, 2016). The best studied group of prenylated proteins belong to the Ras superfamily of small GTPases which act as molecular switches in regulating signaling, trafficking and cytoskeletal stability (Bar-Sagi and Hall, 2000; Pereira-Leal and Seabra, 2000). The Rab small GTPases, which regulate cellular trafficking, are dual geranylgeranylated at a four or five amino acid box in the C-terminus containing a “CC” or “CXC” motif by geranylgeranyltransferase II (GGTase II) (Kinsella and Maltese, 1992; Pereira-Leal and Seabra, 2000). Prenylated proteins containing the “CaaX” motif undergo secondary modifications including cleavage of the last three amino acids in the C-terminal by the ER-resident protease Rce1 and methylation of the free C-terminal carboxylate by Icmt making prenylation irreversible (Resh, 2013). Interestingly, a small handful of proteins either do not require or undergo proteolytic processing following prenylation including some isoforms of RalA, and RalB, and Rho small GTPases (Cdc42, Rac1, and RhoA) with the composition of the CaaX domain likely serving as a signal against proteolytic processing (Michaelson et al., 2005; Nishimura and Linder, 2013). A shunt pathway was even identified in yeast that prevents cleavage of prenylated Hsp40 family member Ydj1p by Rce1 or Ste24 which is detrimental to its function (Hildebrandt et al., 2016) suggesting prenylation is an actively regulated process with factors responsible for the sorting and delivery of substrates.
Because many lipidated proteins have key cellular functions, lipidation of protein is considered a constitutive “house-keeping” process with regulation dependent on the availability of lipid substrates (Chen et al., 2018). However, emerging evidence suggests that lipidation is a multi-layered, highly regulated process. Extrinsic signals regulate the activities of many of the lipid transferases responsible for protein modification including both FTase and GGTase I (Chamberlain and Shipston, 2015; Goalstone et al., 1997; Luo et al., 2003; Noritake et al., 2009; Ponimaskin et al., 2008; Zhou et al., 2008). Additionally, it has become clear prenylation does not involve merely a passive encounter of prenyltransferase with the protein substrate, but is an active process mediated by chaperones. The SmgGDS splice variant, SmgGDS-607, recognizes un-prenylated Ras and Rho GTPases to promote their association with the prenyltransferases (Berg et al., 2010). Chaperones including SmgGDS-558 (Berg et al., 2010), Arl2, Arl3 (Ismael et al., 2011), RhoGDI, and PDEδ (Chandra et al., 2011) contain a hydrophobic domain for binding the prenylation motif and facilitate trafficking of prenylated proteins to different membrane compartments. Further characterization of prenylation binding chaperones and enzymes will be required to understand the regulatory mechanisms.

The chemical properties of each prenyl-modification instill decisive roles for the protein substrate in the regulation of signaling and downstream cellular functions. The functional consequences of each prenyl-modification can best be exemplified by RhoB, which can be farnesylated or geranylgeranylated by GGTase I (Armstrong et al., 1995). Farnesylated RhoB promotes trafficking of the EGF
receptor to lysosomes for signal termination. In contrast, geranylgeranylated RhoB promotes the retention of EGF receptors in multi-vesicular bodies (Wherlock et al., 2004), organelles thought to function in sustained signaling (Dobrowolski and De Robertis, 2011; Ye et al., 2018) and recycling to the plasma membrane (Wherlock et al., 2004). Further work to elucidate the chemical mechanisms regulating localization of prenylated proteins and their interactions at these locations will provide insight into how prenylated proteins regulate diverse processes.

Despite the ubiquitous expression of FTase and GGTase I, there appears to be differences in the extent of prenyl-modifications in a cell-and-tissue specific manner. The nervous system appears to be more reliant on geranylgeranylation, which is ten times more prevalent in the brain than farnesylation (Epstein et al., 1991). Haplo-deficiency or deletion of GGTase I, but not FTase, reduced spine density of cortical neurons and decreased LTP in hippocampal neurons (Hottman et al., 2018). Interestingly, GGTase I activity was also shown to be required for the formation and maintenance of neuromuscular synapses (Luo et al., 2003). Agrin-mediated activation of the receptor tyrosine kinase, MusK, promoted activation and recruitment of GGTase I to the receptor. Localization of GGTase I activity to active MusK was required for clustering of acetylcholine receptors (AChR) at the neuromuscular synapses (Luo et al., 2003). Additionally, in hippocampal neurons, neuronal activity and BDNF recruits GGTase I to the TrkB receptor to promote dendritic morphogenesis (Zhou et al., 2008). This suggests a mechanism by which extrinsic signals localize the prenylation machinery at specific neuronal sites to
regulate signaling and trafficking for the formation and maintenance of neuronal connections.

Asymmetric distribution of biological molecules is essential for the specialization of neuronal compartments. Our understanding of the processes responsible for sorting, delivery, and retention of biological material to these different compartments is still in its infancy. Intra-axonal protein synthesis promotes axon elongation and branching in response to neurotrophins during development (Scott-Solomon and Kuruvilla, 2018; Terenzio et al., 2017). The gene composition of these neurotrophin responsive mRNA pools is still under investigation. A screen to identify genes enriched in distal axons identified \textit{Tp53inp2} (tumor protein p53-inducible nuclear protein 2) to be one of the most abundant mRNAs in axons (Andreassi et al., 2010). \textit{Tp53inp2} codes for a protein that promotes the formation and maturation of autophagosomes for basal autophagy (Liu and Klionsky, 2015; Nowak et al., 2009), a key process in axon homeostasis (Maday and Holzbaur, 2016). In collaboration with the Riccio laboratory, we identified a non-coding function for \textit{Tp53inp2} in NGF-directed axon growth and neuronal development. Deletion of \textit{Tp53inp2} attenuated TrkA signaling and internalization in response to NGF. Interestingly, NGF induced the recruitment of \textit{Tp53inp2} to TrkA suggesting the mRNA may act as a scaffold to localize downstream effectors to promote NGF-dependent development. This work identifies a novel role for non-coding mRNA locally in axons for neurotrophin dependent development.
While local protein synthesis and protein prenylation can both contribute to compartmentalized signaling, if and how these processes might be linked has never been addressed. Intriguingly, many mRNAs encoding for proteins known to undergo prenylation, including the GTPases, RhoA, Rac1, RhoB, and Rab11, have been identified in mRNA screens in axons (Gumy et al., 2011; Holt and Schuman, 2013; Rangaraju et al., 2017; Wu et al., 2005). It is reasonable to assume that proteins that are locally translated in axons must also undergo post-translational modifications in the same compartment to allow local growth events. However, this assumption has never been tested directly. We reveal an efficient coupling of local protein synthesis and prenylation in axons in response to NGF. In contrast to the belief prenylation is a constitutive process, we found NGF acutely regulates the activity of GGTase I to promote local geranylgeranylation of proteins in axons. Local synthesis and geranylgeranylation of Rac1, an actin-regulatory small GTPase, in axons is necessary for axon growth in response to NGF. This work presents a previously unrecognized mechanism by which NGF regulates the local synthesis and post-translational modification of growth-promoting proteins in axons during development.
CHAPTER 2. REGULATION OF NGF SIGNALING
BY AN AXONAL NON-CODING mRNA

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Hamish Crerar, Emily Scott-Solomon, Chantal Bodkin-Clarke, Antonella Riccio and Rejji Kuruvilla conceived the work. Hamish Crerar, Emily Scott-Solomon, Chantal Bodkin-Clark, Catia Andreassi, Maria Hazbon, Emilie Logie, Marife Cano-Jaimez and Marco Gaspari performed the experiments. Hamish Crerar cloned and created $Tp53inp2$ viruses, performed RNA immunoprecipitation assays, performed RIP-assays, and polysomal fractionation. Emily Scott-Solomon performed in vitro survival assays, endocytic assays, and analyses of TrkA phosphorylation and signaling. Chantal Bodkin-Clarke generated $Tp53inp2^{tm1a}$ mice, performed smFISH, in vivo analyses of axon innervation and axon growth in compartmentalized cultures. Catia Andreassi performed 3’end RNAseq. Marco
Gaspari performed the Mass Spectrometry analysis. Maria Hazbon quantified *in vivo* axon growth and neuron survival data. Antonella Riccio and Rejji Kuruvilla wrote the manuscript with input from all authors.
INTRODUCTION

Neurons are highly morphologically complex cells that require the expression of a large number of genes encoding proteins that support growth, branching and synaptic functions in dendrites, and growth cone migration, extension, and regeneration in axons (Andreassi et al., 2018; Terenzio et al., 2017). Neuronal cells rely on asymmetric localization of mRNAs to compartmentalize gene expression, a mechanism shared with most eukaryotic cells (Martin and Ephrussi, 2009). Transcripts are transported to dendrites and axons, where they can be rapidly translated in response to extrinsic signals, such as synaptic activity, neurotrophic factors, guidance cues, and injury (Andreassi et al., 2018; Andreassi et al., 2010; Cajigas et al., 2012; Gumy et al., 2011; Sambandan et al., 2017; Terenzio et al., 2018). Genome-wide screens performed on RNA isolated from either dendrites or axons revealed that thousands of transcripts are asymmetrically localized in neurons (Andreassi et al., 2019; Andreassi et al., 2010; Cajigas et al., 2012; Gumy et al., 2011; Shigeoka et al., 2016). However, how peripherally localized transcripts influence axon growth and whether they may function independently of their translational capacity remains largely unknown.

Here we show that Tp53inp2, one of the most abundant and enriched mRNA transcripts in axons, is not translated in sympathetic neurons and regulates axon growth in a coding-independent manner. The unusually long 3’ UTR of Tp53inp2 maintains the transcript in a translationally repressed state, possibly conferring to the transcript unique, neuron-specific roles. Importantly, we
demonstrate that $Tp53inp2$ interacts with the nerve growth factor (NGF) receptor TrkA, promoting receptor trafficking and intracellular signaling. Analysis of transgenic mice lacking $Tp53inp2$ demonstrated that the gene is required for axon growth and sympathetic target innervation. Noticeably, the defects were rescued by a translation-deficient $Tp53inp2$ transcript, indicating that, at least in sympathetic neurons, $Tp53inp2$ functions independently of translation. Thus, our study reveals the essential role of the $Tp53inp2$ transcript in regulating sympathetic neuron growth and innervation and represents the first evidence of an axonal mRNA capable of directly modulating NGF-TrkA signaling.
RESULTS

The *Tp53inp2* Transcript Is Highly Expressed, but Not Translated, in Sympathetic Neuron Axons

Eukaryotic mRNAs include a coding sequence (CDS) encoding the protein and flanking UTRs of variable length, called 5' and 3' UTRs, that harbor regulatory elements that determine transcript localization, stability, and translation (Andreassi and Riccio, 2009; Lianoglou et al., 2013). To obtain a comprehensive characterization of the 3' UTR transcript isoforms expressed in sympathetic neuron axons, we performed 3' end RNA sequencing (RNA-seq) on mRNA isolated from either axons or cell bodies of rat sympathetic neurons cultured in compartmentalized chambers (Andreassi et al., 2019). In this model system, NGF is added only to the lateral axonal compartment, creating experimental conditions that closely resemble the release of neurotrophins from target tissues (Kuruvilla et al., 2000; Riccio et al., 1997). mRNA was subjected to two rounds of linear poly(A) amplification before sequencing to enrich for 3' UTR (Andreassi et al., 2019; Andreassi et al., 2010). *Tp53inp2* was the most abundant transcript in axons, accounting for almost one-third of the reads (Figures 2-1A and 2-1B). The transcript is unusual in that the 3' UTR is over 3,000 nt long (3,121 nt) accounting for nearly 80% of the transcript length, whereas the open reading frame (ORF) is 666 nt long, encoding a small protein of predicted low complexity. Although the Tp53inp2 protein has been implicated in the regulation of autophagy in skeletal muscle fibers and other mammalian cell lines (Nowak et al., 2009; Sala et al., 2014), extensive attempts to detect the endogenous Tp53inp2 protein in PC12
cells and sympathetic neurons using either homemade, commercial, or previously published antibodies were unsuccessful. Western blotting of PC12 cells transfected with a vector expressing the CDS of $Tp53inp2$ showed that, under these conditions, the transcript was translated and easily detected (Figure 2-2A; Figures 2-1C to D). Co-transfection with a small interfering RNA (siRNA) that efficiently inhibited $Tp53inp2$ expression completely abolished the signal (Figure 2-2A), indicating the specificity of the antibodies. Importantly, we tested several cell types and confirmed that endogenous $Tp53inp2$ was expressed in HeLa cells (Xu et al., 2016) and that the protein was stable, with a half-life of at least 4 h (Figure 2-2B; Figure 2-1E).

To further investigate whether $Tp53inp2$ mRNA was translated in sympathetic neurons, we performed polysome fractionation (Johannes and Sarnow, 1998). Although not devoid of limitations, the technique allows an accurate prediction of transcript translation rates. qRT-PCR of the $Tp53inp2$ transcript associated with either polyribosomes or monosomes revealed that, in sympathetic neurons, a large fraction of $Tp53inp2$ mRNA co sedimented with the monosomal fraction, whereas the efficiently translated transcript $b\text{-}actin$ was mostly associated with polysomes (Figure 2-2C; Figure 2-1F). Because monosomes in yeast can translate transcripts containing an ORF of up to 600 nt (Heyer and Moore, 2016), we further investigated, using mass spectrometry, whether $Tp53inp2$ protein was expressed in sympathetic neurons. Three proteotypic peptides identified by a data-dependent analysis of immunoprecipitated $Tp53inp2$ were selected for high-sensitivity detection in
targeted mode (Table 2-1). Targeted mass spectrometry performed either on severed axons or cell bodies of sympathetic ganglia explants (superior cervical ganglia [SCG]) confirmed that Tp53inp2 peptides were not present in axons (Figure 2-2D to F; Figure 2-1G). Very small signals corresponding to ALHHAAPMoxPAR and HQGSIYQPCQR fragment ions were detected at the limit of the signal-to-noise ratio only in cell bodies (Figure 2-2F; Figure 2-1G). It should be noted however, that SCG explants contain few non-neuronal cells that may account for the Tp53inp2 peptides detected in the cell body samples. Thus, although we cannot exclude the possibility that the Tp53inp2 transcript is translated at extremely low levels in cell bodies of sympathetic neurons, Tp53inp2 protein was undetectable in axons using three distinct sensitive technical approaches.

The unexpected finding that, despite its abundance, Tp53inp2 mRNA was not synthesized into a protein prompted us to investigate the mechanisms that maintain the transcript in a translationally silent state in sympathetic neurons. Because vectors expressing only the coding region of Tp53inp2 were translated (Figure 2-2A; Figures 2-1C to D), we reasoned that the Tp53inp2 3' UTR may harbor regulatory elements that inhibit translation. To this end, we generated expression vectors carrying the 5' UTR of Tp53inp2 upstream of the coding region of GFP and followed by either the full-length (~3,100 nt) or truncated forms (~2,200 nt and ~1,200 nt, respectively) of the Tp53inp2 3' UTR. Although there were some differences in the expression levels of one vector (Figure S-1I), when relative mRNA expression was taken into account, shortening of the 3' UTR correlated with substantial relief of translation inhibition (Figure 2-2G; Figure 2-1H). A possible
implication of these results is that cells expressing Tp53inp2 protein also express a Tp53inp2 transcript harboring a shorter 3’ UTR. Indeed, qRT-PCR performed on RNA isolated from either sympathetic neurons or HeLa cells revealed that an isoform bearing a shorter 3’ UTR was predominantly expressed in HeLa cells (Figure 2-1J). These findings indicate that elements contained within the longer 3’ UTR may be responsible for maintaining the transcript translationally silent in sympathetic neurons.

**The Tp53inp2 Transcript Interacts with the TrkA Receptor to Mediate NGF Signaling**

Recent studies have shown that 3’ UTRs may have a more flexible role in regulating gene expression than previously thought (Andreassi et al., 2018; Mayr, 2017). In hippocampal neurons, for example, an E3 ubiquitin ligase isoform expressing a short 3’ UTR (Ube3a1) inhibits dendritogenesis in a coding-independent manner by competing with endogenous Ube3a for miR-134 binding (Valluy et al., 2015). Moreover, in cancer cell lines, the long 3’ UTR of the CD47 transcript acts as a scaffold for RNA binding proteins (RBPs) and determines the translocation of the CD47 protein to the plasma membrane (Berkovits and Mayr, 2015).

In axons, NGF binds to its cognate receptor TrkA and, following receptor dimerization and autophosphorylation, ligand-receptor complexes are internalized within endosomes and retrogradely transported to the cell bodies (Harrington et al., 2011; Yamashita and Kuruvilla, 2016). Signaling endosomes regulate axon growth and are transported long-distance to somato-dendritic compartments to modulate
synapse assembly and activate transcription (Lehigh et al., 2017; Scott-Solomon and Kuruvilla, 2018). Given the lack of translation, we reasoned that in sympathetic neurons, the *Tp53inp2* transcript could influence NGF signaling in a coding-independent manner. We first investigated whether *Tp53inp2* transcripts interacted with the TrkA receptor in axons by performing RNA immunoprecipitation (RIP) on sympathetic neurons. Remarkably, the pan-Trk antibody immunoprecipitated the *Tp53inp2* transcript, and the interaction was greatly increased in response to NGF stimulation (**Figure 2-3A; Figure 2-4A**). The cognate receptor TrkB also interacted with *Tp53inp2* in mouse cortical neurons (**Figure 2-3B**), whereas, in sympathetic neurons, the highly abundant axonal transcript *IMPA1-L* was not immunoprecipitated (Andreassi et al., 2010)**(Figure 2-4A**). Both transcripts were immunoprecipitated by the neuron-specific RBP HuD (**Figure 2-4B to C**), and endogenous HuD and TrkA co-immunoprecipitated in sympathetic neurons (**Figure 2-4D**), suggesting that HuD may mediate TrkA binding to the *Tp53inp2* transcript. As a control, we performed RIP of *Tp53inp2* with another transmembrane protein, NCAM, and found no significant interaction (**Figure 2-4E to F**).

Next, we asked whether the *Tp53inp2* transcript influences NGF signaling in sympathetic neurons. To this end, we generated transgenic mice carrying a floxed allele of *Tp53inp2*, where exons 2 and 3 are flanked by *loxP* sites (*Tp53inp2*fl/fl mice) (**Figure 2-4G**). To assess the effects of *Tp53inp2* loss on NGF signaling in axons, SCG explants isolated from *Tp53inp2*fl/fl mice on postnatal day 0.5 (P0.5) were infected with either an adenoviral vector expressing the Cre
recombinase to acutely delete $Tp53inp2$ (Figure 2-4H) or a LacZ virus as a control. Cell bodies were mechanically severed, and the isolated axons were stimulated with NGF. Axon protein lysate was either immunoblotted for the canonical signaling effectors Erk1-2 and Akt or subjected to immunoprecipitation with a phosphotyrosine antibody, and the pellet was immunoblotted using antibodies for TrkA. As expected, NGF stimulation increased pTrkA, pAkt, and pErk1-2 levels in axons under control conditions, whereas NGF-mediated phosphorylation of TrkA and downstream effectors was inhibited in axons lacking $Tp53inp2$ (Figure 2-3C to D), indicating that $Tp53inp2$ interaction with TrkA receptors mediates TrkA signaling in axons. Moreover, immunofluorescence analysis showed that $Tp53inp2$ depletion significantly decreased the retrograde appearance of phosphorylated TrkA receptors in cell bodies of cultured sympathetic neurons lacking $Tp53inp2$ (Figure 2-3E).

A key event that mediates NGF-Trk signaling is the ligand induced endocytosis of TrkA receptors. The inhibition of local and retrograde NGF signaling in $Tp53inp2$-deficient neurons prompted us to investigate whether $Tp53inp2$ could influence TrkA internalization. To this end, we performed a live-cell anti body-feeding assay (Yamashita et al., 2017) on $Tp53inp2^{fl/fl}$ sympathetic neurons co-infected with adenoviruses expressing either LacZ or Cre and FLAG-TrkA. Robust receptor internalization was observed in both cell bodies and axons of control neurons in response to NGF stimulation (Figure 2-3F to G). In contrast, internalization was markedly attenuated in neurons lacking $Tp53inp2$. Similar results were obtained when internalization of endogenous TrkA receptors was
probed using a cell surface biotinylation assay (Houtz et al., 2016). NGF induced TrkA internalization was significantly reduced in neurons lacking Tp53inp2 compared with control conditions (Figure 2-5A). Loss of Tp53inp2 had no effect on basal levels of surface TrkA (Figure 2-5B to C) or on total TrkA protein expression in sympathetic neurons (Figure 2-5D). Together, these results indicate that Tp53inp2 regulates NGF-dependent TrkA internalization.

**Tp53inp2 Is a Translationally Repressed mRNA that Affects Cell Survival and Axon Growth**

The findings that the Tp53inp2 transcript interacts with the TrkA receptor and regulates NGF signaling in sympathetic neurons prompted us to investigate whether it affected NGF-dependent axon growth and cell survival. Tp53inp2fl/fl sympathetic neurons were grown in compartmentalized chambers with NGF added only to distal axons and infected with either Cre or LacZ adeno viruses (Figure 2-5E). Neurons expressing LacZ showed robust growth in the presence of NGF (Figure 2-6A to B). In contrast, NGF-mediated growth was remarkably stunted in neurons lacking Tp53inp2 (Figure 2-6A; Figure 2-5B). To determine whether the Tp53inp2 transcript rescued the growth defects induced by Tp53inp2 loss, adenoviral vectors expressing either full-length wild-type (WT) Tp53inp2 or a form bearing mutations of all putative translational start sites within the CDS (ATGNull) were generated (Figure 2-5F). Western blotting and qRT-PCR confirmed that the WT and ATGNull Tp53inp2 transcripts were not translated despite being transcribed efficiently in PC12 cells (Figure 2-5F; Figure 2-7A to B). When Tp53inp2-deficient neurons were infected with either one of the two adenoviral
vectors, we observed that the WT and ATGNull Tp53inp2 were equally efficient in rescuing axon growth defects induced by the loss of Tp53inp2 (Figure 2-6C to D). Together, these findings indicate that Tp53inp2 translation is not required for promoting sympathetic neuron axon growth and support the hypothesis that it may function outside of its coding capacity.

Next, we asked whether Tp53inp2 loss would compromise the ability of axonally applied NGF to retrogradely support neuronal survival. The survival of Tp53inp2^{fl/fl} neurons was studied in the absence or presence of NGF on distal axons. When NGF was withdrawn, 80% of neurons in both LacZ- and Cre-infected neurons underwent apoptotic cell death (Figure 2-6E). NGF added to the distal axons was sufficient to promote survival of the majority of LacZ-infected neurons, whereas neurons lacking Tp53inp2 continued to exhibit significant levels of apoptosis (Figure 2-6E; Figure 2-7C). In line with evidence showing that NGF applied directly to cell bodies promotes neuronal survival by an endocytosis-independent mechanism (Riccio et al., 1997; Ye et al., 2003), we found that, under these conditions, loss of Tp53inp2 did not increase apoptosis (Figure 2-6E; Figure 2-7C). Thus, Tp53inp2 supports neuronal survival by regulating endocytosis and retrograde trafficking of NGF-TrkA receptor complexes.

The Tp53inp2 Transcript Is Necessary for Sympathetic Neuron Target Innervation

Next, we investigated whether the loss of Tp53inp2 affected sympathetic neuron survival and growth in vivo. First, in situ hybridization confirmed that Tp53inp2 transcript is expressed in sympathetic neuron ganglia at high levels from
embryonic day 14.5 (E14.5) until early postnatal stages, a time when they are highly dependent on NGF for cell survival and axon growth (Figure 2-8A). Fluorescence in situ hybridization (FISH) of the 3' UTR of the Tp53inp2 transcript showed punctate staining in both axons and cell bodies of sympathetic neurons (Figure 2-8B). The FISH signal was lost when the assay was performed on Tp53inp2<sup>fl/fl</sup> neurons infected with an adenoviral vector expressing the Cre recombinase, confirming the specificity of the FISH signal.

To generate mice lacking Tp53inp2 in sympathetic neurons, Tp53inp2<sup>fl/fl</sup> animals were crossed with transgenic mice expressing the Cre recombinase under the control of the tyrosine hydroxylase promoter (Gong et al., 2007) (Th-Cre;Tp53inp2<sup>fl/fl</sup> mice; Figure 2-4G), which resulted in efficient deletion (Figure 2-7D). Analyses of the SCGs revealed normal numbers of sympathetic neurons in Th-Cre;Tp53inp2<sup>fl/fl</sup> mice at E16.5 but significant cell loss at birth (P0.5) and 3 weeks after birth (P21) compared with control littermates (Figure 2-8C). Despite the absence of neuronal loss at E16.5, sympathetic innervation of the heart was decreased in the mutant embryos, with a significant reduction in both density of innervation and axonal branching in Th-Cre;Tp53inp2<sup>fl/fl</sup> embryos compared with control littermates (Figure 2-8D to E). Taken together, these findings indicate that Tp53inp2 is required for the development of sympathetic neurons when they are most dependent on NGF for cell survival and axon growth.
NGF is secreted by the target tissues and acts locally in axons to support growth. The signal generated by the interaction of NGF with TrkA receptors is also retrogradely transported to the cell bodies, where it initiates nuclear events necessary for cell survival and axon growth (Ascano et al., 2012; Harrington et al., 2011). We observed that the defects of target innervation preceded neuronal loss, suggesting that lack of \textit{Tp53inp2} primarily affects axon growth \textit{in vivo}. Thus, in mice lacking \textit{Tp53inp2}, neuronal loss was likely due to the axons failing to reach the target tissues and gain access to adequate levels of NGF. We cannot exclude, however, that impaired retrograde NGF signaling from distal axons to the cell bodies may also play a role.
DISCUSSION

Crosstalk between NGF Signaling and mRNA Transcripts in Axons

Neurotrophic signaling is essential for the development of the sympathetic nervous system. The binding of neurotrophins to Trk receptors induces a variety of cellular processes required for survival, axon growth, dendritogenesis, and synaptogenesis (Scott-Solomon and Kuruvilla, 2018), which are principally mediated by transcriptional activation and extensive RNA localization in dendrites and axons (Andreassi et al., 2019; Andreassi et al., 2010; Cosker et al., 2016). Although it is well known that neurotrophic signaling affects gene transcription, asymmetric localization of RNA, and local translation, whether the opposite event may occur was unknown.

Our findings add to the small but growing list of mRNA transcripts that, in addition to being translated into proteins, harbor coding-independent functions (Nam et al., 2016), such as acting as a sponge for miRNA (Valluy et al., 2015) or as a scaffold for the formation of protein complexes (Berkovits and Mayr, 2015). Importantly, the finding that Tp53inp2 mRNA acts in a coding-independent manner in neurons but is translated in other cell types adds a further layer of complexity to RNA regulation and provides evidence for a new mechanism that exploits the 3' UTR to confer cell-type-specific functions to ubiquitously expressed transcripts.
A

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B

C

Myc-Tp53inp2  
5’-3’ assay  
5’-3’ cell bodies

- α-Tp53inp2
- α-myc
- α-p85

D

Ty3inp2 (ENRNOT000000155310)

α-Tp53inp2
α-Myc
α-alpha tubulin

E

myc Tp53inp2CDS
Tp53inp2 siRNA

- α-Tp53inp2
- α-myc
- α-HSP90

F

β-Actin polyosomal profile

G

IP: myc-Tp53inp2CDS
axons  
cell bodies

H

3.1 Kb  
2.2 Kb  
1.2 Kb  

I

Relative GFP mRNA levels
(normalized to mCherry)

J

Sympathetic Neurons  
HeLa Cells

Proximal  
Distal
Figure 2-1: *Tp53inp2* translation is repressed in sympathetic neurons.

**Related to Figure 2-2.** (A) Top ten most abundant axonal transcripts as identified by 3’end RNAseq screen performed on axons and cell bodies of sympathetic neurons. (B) (upper) 3’end Seq tracks of *Tp53inp2* mRNA from cell body and axonal mRNA and (lower) schematic of Ensembl transcript ID ENSRNOT00000055310, red arrowhead represent the positions of AATAAA PAS hexamer, black arrows represent the position of proximal (P) and distal (D) primers used for analysis in Figure 2-1J. (C) Western blotting analysis of Tp53inp2, Myc and PI3K subunit of p85 on PC12 lysates transfected with Myc-Tp53inp2 CDS, and axonal and cell body lysates of sympathetic neurons. See also (Figure 2-2 A,B). (D) Western blot analysis of PC12 cells transfected with mycTp53inp2, PC12 cells ±NGF stimulation, SCG sympathetic neurons and Nnr5 PC12 cell line. (E) Western blot analysis of HeLa lysates co-transfected with mycTp53inp2CDS and Tp53inp2 siRNA, as indicated (n=3). (See also Figure 2-2B). (F) Polysomal fractionation profile of β-actin mRNA from sympathetic neurons. Values are expressed as percentage of total β-actin mRNA (n=3). (See also Figure 2-2C). (G) Pseudo-Selected Reaction Monitoring traces for the detection of a Tp53inp2 tryptic peptide in cultured sympathetic neuron axon or cell body samples, and in immunoprecipitated mycTp53inp2 control. The 5 traces represent the 5 most abundant fragments of the Tp53inp2 peptide HQGSFIYQPCQR (m/z 507.6). Arrows indicate where at least three transitions are detected at the same retention time. Top value on trace=retention value, bottom value=m/z. (See also Figure 2-2D-F). (H)
Densitometry of GFP protein levels normalized by mCherry levels and expressed as percentage of the mean GFP protein amount of the 1.2 Kb construct. Data presented as average ±s.e.m.; Ordinary one-way ANOVA Tukey’s multiple comparisons (n=5, ****P<0.0001). See also (Figure 2-2G).

(I) Relative GFP mRNA expression in PC12 cells transfected with GFP constructs carrying Tp53inp2 3’UTR of indicated length (Kb). mRNA levels were normalized to levels of mCherry mRNA and expressed as percentage of the mean GFP amount of the 1.2 Kb construct. Data are presented as averages ±s.e.m., ordinary one-way Tukey’s multiple comparisons ANOVA (n=5,*P<0.05 **P<0.01 n.s. = not statistically significant). (See also Figure 2-2G).

(J) RT-qPCR analysis of Tp53inp2 mRNA expression in rat sympathetic neurons and HeLa cell lysate. Primers proximal and distal of the internal polyA site in rat Tp53inp2 3’UTR (see Figure 2-1B) were used for analysis and values are normalized to percentage expression of the respective proximal primer set. Data are presented as average ±s.e.m. multiple t-test (n=6, ****P<0.0001 n.s. = not statistically significant).
**Figure 2-2. Tp53inp2 Translation Is Repressed in Sympathetic Neurons.**

(A) Western blot of PC12 lysates transfected with Tp53inp2CDS-2xFLAG and Tp53inp2 siRNA, as indicated (n = 3). (B) Western blot of lysates of HeLa cells treated with cycloheximide (CHX) for the indicated time (n = 3). (C) qRT-PCR of Tp53inp2 and β-actin in polysomal fractions from sympathetic neurons lysates; paired two-tailed t test (n = 3, **p < 0.01). (D–F) Pseudo-selected reaction monitoring traces for the detection of a Tp53inp2 tryptic peptide in cultured sympathetic neuron axon (E) or cell body (F) samples and in an immunoprecipitated myc-Tp53inp2 control (D). The four traces represent the 4 most abundant fragments of the Tp53inp2 peptide ALHHAAPMoxPAR. Arrows indicate where at least three transitions are detected at the same retention time, indicating peptide presence. Top value on trace, retention value; bottom value, mass to charge ratio (m/z). (G) Left: western blot of PC12 cells co-transfected with GFP fusion constructs containing Tp53inp2 3’ UTR 3.1, 2.2, or 1.2 kb and an mCherry control vector. Right: densitometry of GFP protein levels was normalized by mCherry levels and then further normalized by levels of GFP mRNA. Values are expressed as percentage of the mean GFP protein amount of the 1.2-kb construct. Ordinary one-way ANOVA, Tukey’s multiple comparisons test (n = 5, *p < 0.05, ***p < 0.001). Data are presented as average ± SEM. See also Figure 2-1
Tp53inp2 tryptic peptides identified by data-dependent nanoLC-MS/MS analysis on a Tp53inp2 immunoprecipitate. Experimental m/z, mass error, chromatographic retention time, peptide score according to the Sequest search engine and suitable fragments for pseudo-SRM targeted analysis (based on both ion abundance and specificity) are reported.

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Figure 2-3. *Tp53inp2* mRNA Interacts with the TrkA Complex to Regulate NGF Signaling and Endocytosis in Sympathetic Neurons. (A) RNA immunoprecipitation (RIP) performed on sympathetic neuron lysates using antibodies for pan-Trk (sympathetic neurons grown under NGF stimulation predominantly express TrkA) or IgG. The levels of *Tp53inp2* were analyzed by qRT-PCR. Ordinary one-way ANOVA, Tukey’s multiple comparisons test (n = 5; **p < 0.01). (B) RIP performed on mouse cortical neuron lysates using antibodies for pan-Trk (cortical neurons predominantly express TrkB) or IgG. The levels of *Tp53inp2* mRNA were analyzed by qRT-PCR. Unpaired two-tailed t test (n = 3, *p < 0.05). (C) Western blot of axonal lysates from *Tp53inp2* fl/fl SCG explants infected with an adenovirus expressing either Cre or LacZ. Isolated axons were stimulated with NGF or left untreated before immunoprecipitation with a phosphotyrosine (PY20) antibody, followed by immunoblotting for TrkA (pellet). Supernatants (Sup.) were immunoblotted as indicated. (D) Densitometry analysis of data shown in (C). Values are normalized relative to the no neurotrophin condition for LacZ-expressing neurons. Ordinary two-way ANOVA, Tukey’s multiple comparisons test (n = 3–4, *p < 0.05, **p < 0.01). (E) Left: representative images of pTrkA and tyrosine hydroxylase (TH) immunostaining in cell bodies of *Tp53inp2* fl/fl sympathetic neurons infected with an adenovirus expressing either Cre or LacZ. Distal axons were stimulated with NGF or left unstimulated. Scale bar, 5 mm. Right: quantification of pTrkA puncta per neuron. Ordinary two-way ANOVA, Tukey’s multiple comparisons test (n = 4; each data point represents the average of 20–30 neurons per condition per experiment; ***p < 0.001, **p < 0.01). (F)
Representative images of FLAG-TrkA immunostaining in cell bodies (left) and axons (right) of Tp53inp2fl/fl neurons infected with an adenovirus expressing LacZ or Cre and co-infected with a FLAG-TrkA adenovirus. Neurons were live-labeled with FLAG antibody under non-permeabilizing conditions and NGF-treated as indicated. Arrows indicate internalized FLAG-TrkA receptors in axons. Scale bar, 5 mm. (G) Quantification of the data in (F). Average fluorescence density was determined per square micrometer for each cell body or per micrometer for each axon. Values are expressed relative to no NGF condition in LacZ-expressing neurons. Data are presented as average fluorescence density ± SEM. Two-way ANOVA (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001). At least 30–50 cell bodies and 20–30 axons were analyzed per condition. Data are presented as average ± SEM. See also Figure 2-4
Figure 2-4: *Tp53inp2* mRNA interacts with the TrkA complex to regulate its function. Related to Figure 2-3. (A) RNA immunoprecipitation performed on sympathetic neuron lysates using antibodies for pan-Trk, or normal mouse IgG. Levels of *Tp53inp2* were analysed by RT-qPCR, normalised as fraction of total input and expressed as fold over IgG. Data are presented as average ±s.e.m. multiple t-test (n=4, **P<0.01 n.s. = not statistically significant). (See also Figure 2-3A). (B) RNA immunoprecipitation performed on sympathetic
neuron lysates using antibodies for HuD or normal mouse IgG. Levels of *Tp53inp2* or *Impa-L* mRNAs were analysed by RT-qPCR, normalised as fraction of total input and expressed as fold of control IgG. Data are presented as average ±s.e.m. multiple t-test (*n*=4, ****P<0.0001). (C) Western blot analysis of HuD immunoprecipitation in sympathetic neurons. (D) Western blot analysis of Trk co-immunoprecipitation experiments in PC12 cell lysates. HuD co-immuno-precipitates with Trk. Images separated by vertical lines are taken from the same exposure. Low exposure of HuD in input sample shows presence of duplet (*n*=3). € RNA immunoprecipitation performed on sympathetic neuron lysates using antibodies for NCAM or normal mouse IgG. Levels of *Tp53inp2* were analysed by RT-qPCR and expressed as fold over IgG. Data are presented as average ±s.e.m. Unpaired two tailed *T*-test with Welch’s correction (*n*=4, n.s. = not statistically significant). (F) Western blot analysis of NCAM immunoprecipitation in two representative sympathetic neuron lysates. (G) Schematic for the generation of the transgenic *Tp53inp2*\(^{fl/fl}\) and *Th-Cre;Tp53inp2*\(^{fl/fl}\) mice. *Tp53inp2*\(^{flm1a}\) mice were first crossed with the ubiquitously-expressing Flippase 129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J to excise the LacZ/neo cassette, and then with *Th-Cre* transgenic mice to generate mice lacking *Tp53inp2* in sympathetic neurons (*Th-Cre;Tp53inp2*\(^{fl/fl}\) mice). Exons are indicated by yellow bars, with UTRs as projecting thin yellow bars. Location of primers used for genotyping indicated as F and R labelled black arrows. (H) *Tp53inp2* mRNA levels in axons of *Tp53inp2*\(^{fl/fl}\) SCG explants infected with adenovirus expressing LacZ.
or Cre. Values are normalised by levels of 18S rRNA and expressed as percentage of LacZ infected neurons values. Data are presented as average ±s.e.m. one-sample t-test (n=3 ***P<0.001). (See also Figure 2-3 and Figure 2-6).
Figure 2-5: Tp53inp2 function in axon growth is translation independent. Related to Figure 2-3 and 2-6. (A) Membrane proteins in mass cultures of Tp53inp2fl/fl neurons, infected with either LacZ or Cre, were subjected to cell surface biotinylation. After NGF stimulation for 30 minutes, surface biotin was stripped off, and internalized TrkA receptors were detected by neutravidin precipitation and TrkA immunoblotting (Left). Supernatants were probed for p85 as a loading control. Densitometry analysis (Right) of internalized biotinylated TrkA levels, normalised by p85. Values expressed as relative to LacZ-expressing neurons. Data presented as average ± s.e.m., unpaired two-tailed t-test (**P<0.01 n=3). See also (Figure 2-3F, G). (B) Representative images of surface Flag-TrkA immunostaining in Tp53inp2fl/fl sympathetic neurons infected with adenovirus expressing Cre or LacZ and co-infected with adenovirus expressing Flag-TrkA. Neurons were live-labelled with Flag antibody in the absence of NGF at 4°C. (C) Surface Flag-TrkA receptor distribution in Tp53inp2fl/fl sympathetic neurons infected with adenovirus expressing LacZ or Cre was analysed by measuring the integrated fluorescence values along the longest axis of the cell body using line-plot in ImageJ and normalized to the total cell body fluorescence intensity. Data are presented as averages ± s.e.m. Mann-Whitney two-tailed test (P=0.1089, n=3 with at least 15-20 cells analysed per condition per experiment). (D) Western blot analysis (Left) of endogenous TrkA expression in Tp53inp2fl/fl neurons infected with LacZ and
Cre adenoviruses, with p85 as a loading control. Densitometry analyses (Right) of TrkA protein levels normalized to p85. Data presented as average ± s.e.m. Unpaired two-tailed t-test (n.s.= not significant, n=3). (E) Schematic representation of compartmentalised chambers. Neurons are plated in central compartment and axons project into the lateral compartments. For adenoviral infections, adenovirus is supplied to the central compartment after axons start projecting into the lateral compartments. See also (Figure 2-3 and 2-6). (F) Nucleotide sequence of ATGNull CDS mutant sequence of Tp53inp2 rat mRNA. Mutations are indicated by red underlined characters; deletion of adenosine from initial translational start codon and substitution of adenosine to cytosine in subsequent in-frame ATG codons. See also (Figure 2-6 C, D). (G) Western blotting on lysates of PC12 cells transfected with Tp53inp2CDS-1xFlag, ATGNullCDS-1xFlag or pcDNA3.1 as control. Cells were also transfected with siRNA for Tp53inp2 mRNA or non-targeting control siRNA (n=3). See also (Figure 2-6 C, D)
**Figure 2-6.** *Tp53inp2* Function in Axon Growth Is Translation Independent. (A) Compartmentalized cultures of *Tp53inp2*fl/fl sympathetic neurons infected with an adenovirus expressing LacZ or Cre were either maintained with NGF in axons or deprived of NGF. Shown are representative images of axons immunostained with anti-b-III tubulin 48 h after starting the treatments. Scale bar, 100 mm. (B) Average growth rate of axons measured at 24-h intervals for 72 h. Ordinary two-way ANOVA, Tukey’s multiple comparisons test (n = 3, each data point represents the average at least 70 axons traced per condition, **p < 0.01, *p < 0.05). (C) *Tp53inp2*fl/fl sympathetic neurons cultured in compartmentalized chambers were infected with adenoviruses as indicated. Shown are representative images of axons immunostained with anti-b-III tubulin 48 h after infection. Scale bar, 100 mm. (D) Average growth rate of axons at 24 h intervals for a total of 72 h. Ordinary two-way ANOVA, Dunnett’s multiple comparisons test (n = 3, each data point represents the average of at least 50 axons traced per condition; hinges correspond to the first and third quartiles, the center line corresponds to the median, and the maxima and minima correspond to 5–95 percentiles; ****p < 0.0001). (E) Left: distal axons of *Tp53inp2*fl/fl neurons infected with an adenovirus expressing LacZ or Cre were stimulated with NGF or deprived of NGF. Neurons that had projected to axonal chambers were identified through the uptake of inert fluorescent beads (red) supplied to the axon chambers. Neuronal apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining. Scale bar, 5 mm. Right: quantification of neuronal cell death. Ordinary two-way ANOVA, Tukey’s multiple comparisons
test (n = 3, each data point represents the average of at least 30 cells per condition per experiment, ****p < 0.0001). Data are presented as average ± SEM. See also Figure 2-5 and Figure 2-7
Figure 2-7: *Tp53inp2* is essential for the growth of NGF-responsive sympathetic neurons. Related to Figure 2-6 and 2-8. (A) Western blotting analysis of PC12 cell lysates transfected with vectors expressing mycTp53inp2CDS, WildType *Tp53inp2* mRNA, full length ATGNull *Tp53inp2* or empty vector. (n=3). See also (Figure 2-6C, D). (B) Expression levels of *Tp53inp2* mRNA in PC12 cells transfected with vectors expressing full length WildType *Tp53inp2* mRNA, full length ATGNull *Tp53inp2* or empty vector, as a control. Levels of *Tp53inp2* mRNA were analyzed by RT-qPCR, normalized to levels of β-actin mRNA and expressed as fold over control *Tp53inp2* mRNA levels. Data are presented as average ±s.e.m. Ordinary one-way ANOVA (n=4, ****P<0.0001). (See Figure 2-6C, D). (C) (Left) Cell bodies of compartmentalized cultures of *Tp53inp2* fl/fl neurons infected with adenovirus expressing LacZ or Cre were treated with NGF (25 ng/ml) or anti-NGF antibody for 48 hours. Neurons that had projected to axonal chambers were identified through the uptake of fluorescent beads (red) supplied to the axon chambers. Neuronal apoptosis was detected using TUNEL staining. Scale bar 5 µm. (Right) Quantification of neuronal cell death in *Tp53inp2* 2/2 sympathetic neurons expressing Cre or LacZ and NGF supplied to the cell bodies (cb). Data are presented as averages ± s.e.m Ordinary two-way ANOVA Tukey’s multiple comparisons, (n=3, each data point represents the average of at least 30 cells per condition per experiment, ****P<0.0001). See also (Figure 2-6E). (D) SCGs were harvested from P0.5 *Th-Cre;Tp53inp2* fl/fl mice and *Tp53inp2* 2/2 littermates. Levels of *Tp53inp2* mRNA were analyzed by RT-qPCR,
normalized by GAPDH and calculated as percentage of the Tp53inp2\textsuperscript{fl/fl} values. Data are presented as average ±s.e.m. unpaired two-tailed t-test (n=4 mice per genotype ****P<0.0001). (See also Figure 2-8)
Figure 2-8. *Tp53inp2* Is Essential for the Growth of NGF-Responsive Sympathetic Neurons. (A) *In situ* hybridization of *Tp53inp2* mRNA in the developing mouse SCG at the indicated developmental stages. Hybridization of the control sense probe is shown at P0.5. Scale bar, 100 mm. (B) Fluorescence *in situ* hybridization (FISH) of the Tp53inp2 30 UTR in cell bodies (top) and axons (bottom) of adenovirus-infected *Tp53inp2*fl/fl sympathetic neurons. Scale bars, cell bodies, 10 mm; axons, 20 mm. (C) TH immunohistochemistry of SCGs from *Th-Cre;Tp53inp2*fl/fl and control *Tp53inp2*fl/fl mice at the indicated developmental stages (left). Counts of cell number were performed on Nissl-stained tissue sections (right). Scale bar, 200 mm. Unpaired t test (n = 3 mice per genotype, *p < 0.05, **p < 0.01). (D) Whole-mount TH immunostaining of the heart in E16.5 *Th-Cre;Tp53inp2*fl/fl mice and *Tp53inp2*fl/fl control littermates. Higher magnification images of the boxed area are shown at the bottom. Scale bars, 100 mm (top) and 400 mm (bottom). (E) Quantitative analysis of the data shown in (D). Total innervation was measured as the area covered by TH-positive axon fibers (top, n = 5 and 6 per *Tp53inp2*fl/fl and *Th-Cre;Tp53inp2*fl/fl). The branchpoints were counted as the number of axon terminal endpoints (bottom, n = 9 and 8 per *Tp53inp2*fl/fl and *Th-Cre;Tp53inp2*fl/fl). Unpaired two-tailed t test (*p < 0.05, **p < 0.01). Data are presented as average ± SEM. See also Figure 2-7.
### METHODS

#### KEY RESOURCES TABLE

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**Critical Commercial Assays**

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**Deposited Data**

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**Experimental Models: Organisms/Strains**

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**Oligonucleotides**

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| **Primer for genotyping: Reverse Tp53inp2LoxP3R GCACCTGCCAGCTA** | This paper | N/A |
| **Primers for cloning, see Table 2-2** | This paper | N/A |
| **Primers for qRT-PCR, see Table 2-2** | This paper | N/A |
| **siRNA:ON-TARGET plus Smartpool Rat Tp53inp2 CTAAAGTGTTGCAACGGCA** | Dharmacon | J-093056-09 |
| **siRNA:ON-TARGET plus Smartpool Rat Tp53inp2 GATCAGGACCTCAGCGATG** | Dharmacon | J-093056-10 |
| **siRNA:ON-TARGET plus Smartpool Rat Tp53inp2 GTCACCTGCCAGCTA** | Dharmacon | J-093056-11 |
| **siRNA:ON-TARGET plus Smartpool Rat Tp53inp2 GAGCACCTGCCAGCTA** | Dharmacon | J-093056-12 |

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**Software and Algorithms**

| **Prism** | GraphPad | https://www.graphpad.com/ scientific- |
Generation of Tp53inp2^{floxed} mutant mice

ES cells containing the Tp53inp2^{floxed}(KOMP)Mbp targeting vector were obtained from the trans-NIH Knock-Out Mouse Project (KOMP) Repository (https://www.komp.org/) and used by transgenic core facility at Johns Hopkins University to generate chimeric mice carrying the Tp53inp2^{floxed} allele. Tp53inp2^{floxed} chimeric male mice were mated to wild-type albino mice (Jackson Laboratory Stock No: 000058). Tp53inp2^{floxed} mice carry a knockout-first allele, in which a promoter less cassette including LacZ and neo genes were inserted in intron 1 of the Tp53inp2 gene. For the sympathetic neuron-specific conditional knockout mice, Tp53inp2^{floxed} mice were crossed with a ubiquitously-expressing Flippase line 129S4/SvJaeSer-Gt(ROSA)26Sortm1(FLP1)Dym/J (Jackson Lab) to excise the LacZ/neo cassette. These mice were then crossed with TH-Cre transgenic mice (gifted by Dr. C. Gerfen NIH) to generate mice lacking Tp53inp2 in sympathetic neurons. The offspring of these mice were then backcrossed for 2-3 generations to C57/BL6 mice to obtain offspring of several genotypes including Th-Cre;Tp53inp2^{floxed} mice and control Tp53inp2^{floxed} littermates.

Primers Tp53inp2Loxp3F and Tp53inp2Loxp3R which span the third loxP site, were used to genotype the Tp53inp2 allele by PCR, with expected band sizes being 240 bp for wild-type, and 310 bp for the floxed exon. See Table 2-2 and

Key Resources Table for primer sequences.

Mouse husbandry
Mice were housed in a standard 12:12 light-dark cycle. Both sexes were used for analyses. The ages of mice are indicated in the figure legends or methods. All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines, or the Institutional Animal Care and Use Committee at University College London.

**Primary cultures**

Superior cervical ganglia (SCG) were dissected from postnatal day 0 or 1 (P0 or P1) Sprague Dawley rats or *Tp53inp2*fl/fl mice and immediately plated as explant tissue or enzymatically dissociated and cultured either in compartmentalized chambers or as mass cultures. Rats of both sexes were used for analyses. For immunocytochemistry, cells were plated on poly-D-lysine-coated (1 μg/ml) coverslips. Campenot compartmentalized cultures were established as described previously (Riccio et al., 1997). Neurons were cultured in high-glucose DMEM supplemented with 10% FBS, 2 mM glutamine and 100 ng/ml NGF. The antimitotic inhibitor cytosine arabinoside (10 μM) was provided to minimize non-neuronal, contaminating cell types. When indicated, the cell body cluster was removed surgically using a scalpel. To withdraw NGF before any stimulation experiments, neurons were placed in DMEM containing 0.5% FBS with 1:1000 anti-NGF antibody (N6655, Sigma) and BAF (50 μM; MP Biomedical) for 48 hours. For restimulations, neurons were treated with the indicated concentration of NGF for variable amounts of time as indicated.

**Mouse cortical neurons**

Cortical neurons were dissected from E15.5 C57BL/6J mouse embryos and
enzymatically dissociated. Embryos of both sexes were used for analyses. Neurons were plated on Nunc dishes coated with 40 mg/ml poly-D-lysine and 2 mg/ml Laminin and cultured in DMEM supplemented with 10% FBS and 5% Horse serum. After 6-24 h, culture medium was replaced with Neurobasal medium supplemented with B27, 1 mM glutamine, penicillin-streptomycin and 10 μM 5-Fluoro-2’deoxyuridine (FdU, Merck). Cells were cultured at 37°C, 5% CO₂ and one day before the experiment, 2/3 plating medium was replaced with medium without B27 (serum starve conditions).

**PC12 cell line**

PC12 cell lines were purchased from ATCC. PC12 cells were cultured in DMEM containing 10% FBS, 5% Horse Serum (HS), 2mM glutamine and grown at 37°C 10% CO₂. Cells were transfected with Lipofectamine 2000 in OptiMEM according to the manufacturers’ guidelines. For NGF stimulation, cells were cultured in DMEM containing 0.5% FBS, 0.25% HS, 2 mM glutamine and 50 ng/ml NGF for 4 days under standard growth conditions. Cells were not cultured past 20 passages and sensitivity to NGF stimulation routinely tested. The Nnr5 PC12 subclone cell line was a kind gift from David Ginty’s Laboratory. Nnr5 cells were cultured in DMEM containing 10% FBS, 5% Horse Serum (HS), 2 mM glutamine and grown at 37°C 10% CO₂. Cells were transfected with Lipofectamine 2000 in OptiMEM according to the manufacturers’ guidelines.

**HEK293 cell line**

The HEK293 cell line was purchased from ATCC and not further authenticated. HEK293 cells were cultured in DMEM containing 5% FBS,
penicillin/streptomycin, 2mM glutamine and grown at 37°C 5% CO₂. Cells were transfected with Lipofectamine 2000 in OptiMEM according to the manufacturers’ guidelines. Cells were not cultured past 20 passages.

**HeLa cell line**

The HeLa cell line was purchased from ATCC and not further authenticated. HeLa cells were cultured in MEM containing 5% FBS, penicillin/streptomycin, 2mM glutamine and grown at 37°C 5% CO₂. Cells were transfected with Lipofectamine 2000 in OptiMEM according to the manufacturers’ guidelines. For cyclohexamide analysis, cyclohexamide was added directly to the growth media at a final concentration of 20 μg/ml and cells were maintained in culture for times indicated. Cells were not cultured past 20 passages.

**Adenoviral vectors and Plasmids**

Rat Tp53inp2CDS was amplified from SCG cDNA and cloned into pCMV-Myc (Clontech) using SalI and BglII to generate Myc-Tp53inp2CDS plasmid. Tp53inp2CDS-2xFlag was PCR amplified from Myc-Tp53inp2CDS plasmid, using primers that encode KpnI/XbaI and C terminus 2xFlag tag. Tp53inp2 3' UTR 1.2, 2.2 and 3.1 Kb and 5' UTR were PCR amplified from RACE clones (C.A. and A.R., unpublished data) and following NotI/XhoI and BamHI/Nhel digestion respectively, were used to replace the IMPA UTRs in myrdEGFP-IMPA1-L (Andreassi et al., 2010). Mutation of the ATG codons to generate ATGNullCDS was performed on Myc-Tp53inp2CDS using the QuikChange Site-Directed Mutagenesis kit (Agilent) according to manufacturer’s instructions. The ATGNullTp53inp2 CDS was then PCR amplified and used to replace the
myrdEGFP sequence in the myrdEGFP-Tp53inp2 3.1 Kb UTR vector to create ATGNull Tp53inp2. Rat Tp53inp2 5′UTR+CDS was amplified from SCG cDNA to include endogenous Kozack sequence, and cloned in place of the ATGNull CDS to generate WT Tp53inp2 vector. To generate adenoviral vectors, WT Tp53inp2 and ATGNull Tp53inp2 were amplified using primers containing KpnI and EcoRV and inserted into pCMV-Shuttle plasmid (Agilent). Adenoviral vectors were generated using the AdEasy Adenoviral Vector system (Agilent) according to manufacturer’s instructions. Adenoviral vectors were transfected into HEK293 cells and high-titer viral stocks were prepared using a CsCl gradient. Tp53inp2CDS-1xFlag and ATGNullCDS-1xFlag were PCR amplified from Myc-Tp53inp2CDS and ATGNullCDS Tp53inp2 respectively and following BamHI/NotI digestion were cloned into pcDNA3.1. Primers used for cloning are listed in Table 2-2.

RNA isolation and RT-qPCR

Samples were washed with PBS and RNA was extracted using Trizol reagent following standard manufacturer’s protocol. RNA pellet was resuspended in water and contaminating genomic and plasmid DNA removed by DNase digestion with TURBO DNase. PCR was performed to amplify Actin gDNA or plasmid DNA prior to reverse transcription to confirm DNase digestion. For cell bodies excised from explants of sympathetic neurons used in the axonal TrkA signaling assays, RNA was isolated using RNAquesous-Micro Total RNA Isolation Kit (ThermoFisher) per manufacturer’s protocol with recommended DNase treatment. Total RNA was reverse transcribed using random hexamers
and SuperscriptIII reverse transcriptase. RT-qPCR was performed using 20 μL reactions using MESA Blue (Eurogentec), SYBR green or SYBR Select master mix in the Mastercycler realplex qPCR machine (Eppendorf) or CFX Connect RT-PCR machine (BIORAD). Reactions were performed in triplicate and a no template control. For absolute quantification, a standard curve was included, generated through serial dilutions of known concentration of the DNA amplicon for each primer set. For relative quantification, the power(2,-Ct) of the experimental gene was normalized by the power(2,-Ct) of a house-keeping gene and then expressed as fold change relative to a control. Following 40 cycles, a dissociation curve was performed to assess melting temperature of amplicons. The primers used for RT-qPCR in this study are listed in Table S2.

**In situ hybridization**

**In situ** hybridization was performed using a digoxigenin-labeled probe spanning a 450-bp region within exons 2-3 of mouse Tp53inp2. Embryos or torsos at various developmental stages were fresh frozen in OCT and serially sectioned (12 μm) using a cryostat. Tissue sections from different developmental stages were collected and processed simultaneously. Sections were post-fixed in 4% PFA-PBS, washed in PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine with 0.9% NaCl. After hybridization with the labeled RNA probe (2 μg/ml) at 68 °C o/n, sections were washed with 0.2x SSC buffer at 65 °C, blocked with TBS containing 1% normal goat serum and then incubated with alkaline phosphatase-labeled anti-DIG antibody (1:5,000; Roche) o/n at 4 °C. The alkaline phosphatase reaction was visualized with NBT/BCIP, rinsed in PBS, fixed
in 4% PFA-PBS and mounted in AquaMount (EMD Chemicals).

**smFISH**

smFISH was performed as described (Chen et al., 2015; Raj et al., 2008) with minor modifications. Sympathetic neurons were cultured 3-7 days *in vitro* on glass coverslips, washed with PBS at room temperature (RT) and fixed using 3.7% PFA-PBS RT 10 min. Cells were permeabilized with 70% EtOH at 4°C for 3 h. Coverslips were prehybridized in 2xSSC 10% formamide for 5 min RT then incubated at 37°C o/n with Cy3 labeled probe sets designed against *Tp53inp2* 3′UTR (Stellaris probes, Biosearch technologies) in hybridization buffer (10% formamide, 2x SSC, 10% dextran sulfate and 2 mM vanadyl ribonucleoside). Cells were rinsed in warm 2x SSC 10% formamide and then 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) (Thermo Fischer, D16), a reactive dye that labels amines in proteins, was added at 1:10,000 (stock 10 mg/ml) in GLOX buffer for 10 min as a counterstain. Cells were then rinsed in 2x SSC and mounted using Aquamount containing 1:1000 DAPI.

**Neuronal counts**

The torsos of *Tp53inp2flo/flo* or *Th-Cre;Tp53inp2flo/flo* E16.5, P0.5 and P21 mice were immersion fixed in 4% PFA-PBS for 4 h (E16.5, P0.5), or overnight (P21) respectively. The heads were then cryoprotected overnight (for E16.5 and P0.5) or 2 days (P21) in 30% sucrose-PBS, frozen in OCT and then serially sectioned (12 µm). Tissue sections were stained with a solution containing 0.5% cresyl violet (Nissl). Cells with characteristic neuronal morphology and visible nucleoli were counted in every fifth Nissl-stained section. For representative images,
immunohistochemistry was performed on tissue sections. Sections were first blocked in a PBS buffer containing 5% goat serum and 0.1% triton and then incubated overnight in rabbit anti-TH (1:200; Millipore AB152). Following PBS washes; sections were incubated with anti-rabbit Alexa 488 secondary antibodies (1:200; Life Technologies). Sections were then washed in PBS and mounted in VectaShield (Vector Laboratories). For practical reasons, analyses were done in a semi-blinded manner, with the investigator knowing the genotypes prior to the experiment, yet performed the staining and quantification without knowing the genotype of the sample.

Whole-mount diaminobenzidine-TH immunohistochemistry

E16.5 embryonic organs fixed in 4% PFA-PBS were dehydrated by methanol series (50%–80%) and incubated overnight in 20% dimethylsulfoxide (DMSO)/80% methanol solution containing 3% H$_2$O$_2$ to quench endogenous peroxidase activity. Tissues were then re-hydrated, blocked o/n in blocking solution (4% BSA/1% Triton X-100 in PBS) using a rabbit anti-TH (Millipore, AB152) diluted at 1:200 in blocking solution and incubated for 72 h at 4°C. Detection was performed with horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare) at 1:200 in blocking solution and incubated o/n at 4°C. Visualization was accomplished with diaminobenzidine. Tissues were re-fixed in 4% paraformaldehyde-PBS, dehydrated by methanol series and cleared with a 2:1 mixture of benzyl benzoate/benzyl alcohol to allow visualization of staining inside the tissue followed by clearing. For practical reasons, analyses were done in a semi-blinded manner, with the investigator knowing the genotypes prior to the
experiment, yet performed the staining and quantification without knowing the genotype of the sample.

**Neuronal survival**

SCG neurons isolated from P0.5-P2 *Tp53inp2fl/fl* were grown in Campenot chambers for 9 days *in vitro*. NGF was removed and neurons were infected for 48-72 h with either LacZ or Cre adenovirus. FluoSpheres (ThermoFisher: F8789) were added to axonal compartments to label projecting neurons. Neurons were starved of NGF and restimulated with 25 ng/ml NGF for 48 h or left deprived. To detect neuronal death, neurons were fixed in 4% PFA-PBS for 30 min at room temperature followed by permeabilization with 0.1% Triton/PBS for 10 min. After extensive PBS washes, dying neurons were visualized using TUNEL staining (Roche: 11684795910) and mounted in Fluoromount with DAPI (1:1000). Neuronal apoptosis was calculated by determining the percentage of neurons that had extended to distal compartments that were also TUNEL positive. Between 30-50 neurons were counted per condition in each experiment.

**Axon growth**

Compartmentalized neuronal cultures from P0.5 *Tp53inp2fl/fl* neurons were infected with LacZ or Cre adenovirus after 7-10 days *in vitro* after axons had extended into the side compartments. Neurons were either deprived or treated with NGF (100 ng/ml) added solely to the axonal compartments, and axon growth was measured by capturing phase contrast images of the distal axons over consecutive 24 h intervals for 3 days, using a Zeiss Axiovert 200 microscope with a Retiga EXi camera. The rate of axonal growth (μm/day) was
quantified using Openlab 4.04. For representative images, neurons were
immunostained with b-III-tubulin (1:200; Sigma-Aldrich T8660) 48 h after
treatment.

Polysomal Fractionation
SCG neurons were cultured for 7 days under NGF stimulation, then ribosomes
were immobilized with 0.1 mg/ml cyclohexamide added directly to growth media
for 3 min at 37°C. Cells were washed 2x on ice with cold PBS + 0.1 mg/ml
cyclohexamide and lysed in 10 mM Tris pH 8, 150 mM NaCl, 5 mM MgCl2, 1% NP40,
10 mM VRC, 1% sodium deoxycholate, 40 mM DTT, 500 U/ml RNaseOUT. Nuclei
were removed by a brief centrifugation and supernatants were supplemented with
0.1 M Tris pH 7.5, 150 mM NaCl, 75 mg/ml cyclohexamide, 1:100 protease inhibitor
cocktail, followed by further centrifugation to remove any insoluble material.
Lysates were loaded on top of 15%–40% sucrose gradients, prepared in 10 mM
Tris pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, 10 mM DTT, 0.1 mg/ml cyclohexamide,
and subjected to ultracentrifugation in Beckman SW41Ti rotor 2 h 4°C 25000xg.
Nineteen fractions were collected and digested with proteinase K (100 mg
proteinase K, 0.1% SDS, 10 mM EDTA) for 30 min at 37°C. To normalize for RNA
loss during extraction, 2.5 ng of in vitro transcribed RNA was spiked into each
fraction prior to phenol:chloroform extraction. Twenty-five per cent of sample was
precipitated for RT-qPCR, and the remainder was precipitated for denaturing
agarose gel electrophoresis to assess quality of the fractionation. RNA was run on
1% agarose MOPS gel under denaturing conditions and the gel was stained with
SYBRGold prior to imaging.
Mass Spectrometry

PC12 cells transfected with a plasmid expressing Myc-Tp53inp2 were washed with cold PBS and lysed in cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 1:100 Protease inhibitor cocktail). Lysates were sheared through a 25 G needle and protein content was quantified by BCA assay according to manufacturer's instructions (Pierce). 2 µg Myc 9E10 antibody (Ab32, Abcam) was conjugated to protein G-Sepharose (GE Healthcare Life Science) o/n rotating at 4°C. Bound antibody was then cross-linked to the beads with 2x30 min RT rotation with 6.5 mg/ml DMP (Thermo Fisher Scientific) in 0.2 M pH8.2 triethanolamine, followed by 30 min RT blocking in 100 mM pH8.2 ethanolamine. Beads were washed extensively with PBS and the unbound antibody eluted with 0.1 M Glycine 10 min RT. Following further PBS washes of beads, 300 mg of protein lysate was added and incubated o/n rotating at 4°C. Beads were washed 3 times 10 min RT in wash buffer (10 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA pH8, 1 mM EGTA pH8, 0.1% Triton X-100) followed by a final PBS wash. Samples were then subjected to on-bead digestion using 200 ng sequencing grade trypsin in 300 µL 100 mM Tris pH8 15 min 37°C. Beads were pelleted by centrifugation and supernatant collected and analyzed by mass-spectrometry. Lyophilized samples were reconstituted in HPLC-grade water and reduced with 10 mM DTT 1h at 37°C, followed by cysteine alkylation (25 mM iodoacetamide, 1 h at 37°C in the dark); excess iodoacetamide was quenched by 1 µL of 100 mM DTT. Full tryptic digestion was achieved by an additional overnight incubation in presence of 200 ng of trypsin proteomics grade. Peptides
were purified by strong cation exchange (SCX) extraction tips (Rappsilber et al., 2007), and eluted in 7 μL of 500 mM ammonium acetate containing 20% acetonitrile v/v. A 10% aliquot of the IP digest was injected for nanoLC-MS/MS analysis in data-dependent mode.

SCG explants were cultured under NGF stimulation for 7 days and axons surgically dissected from cell body. Axon and cell body samples were then lysed in cold RIPA buffer, quantified by BCA and analyzed by mass-spectrometry. Proteins were precipitated by adding four volumes of cold acetone, and by incubating the solution at −20°C for 1 h. After a centrifugation step carried out at 12,000xg for 20 min 4°C, the supernatant was discarded, and the pellet was resuspended in buffer containing 8 M urea, 100 mM Tris pH 8.5 and 0.2% w/v SDS. Protein reduction and alkylation was performed as described above. The protein solution was brought to a final volume of 400 μL by adding HPLC-grade water to reduce urea and SDS concentrations. Finally, 2 μg of trypsin proteomics grade was added to each sample and incubated o/n at 37°C with agitation. The digests were diluted 8-fold in 80% acetonitrile/0.5% formic acid before being loaded onto SCX extraction tips. For targeted analysis, 30 μL of the initial digest solutions were loaded on SCX extraction tips and then eluted in 7 μL of 500 mM ammonium acetate containing 20% acetonitrile (v/v); the eluate was evaporated to dryness and reconstituted in 20 μL of mobile phase A (0.1% formic acid, 2% acetonitrile); a 3 μL aliquot was then injected for nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) analysis.

NanoLC-MS/MS analysis
Proteomic analysis by nanoLC-MS/MS was essentially performed as described in (Tavera et al., 2017). Briefly, peptides were loaded directly on-column at 500 nL/min and separated at 300 nL/min via a 60-min gradient ramped from 5% to 35% mobile phase B (80% acetonitrile, 0.1% formic acid); mobile phase A was 2% acetonitrile, 0.1% formic acid. The analytical column, a pulled 75 μm i.d capillary in-house packed to a length of 12 cm, was connected to a liquid chromatography system (EasyLC 1000, Thermo Fisher Scientific) and directly interfaced to a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific) via a nanoelectrospray interface operating in positive ion mode. Data-dependent acquisition was performed using a top-12 method. Full scan parameters were: resolution 70,000, m/z range 350-1800. Tandem mass spectrometry parameters were: resolution 17,500, isolation window 1.6 m/z, maximum injection time 60 ms, AGC target 100,000, normalized collision energy 25, dynamic exclusion 30 s. Data were processed using Proteome Discoverer 1.3 (Thermo Fisher Scientific). Tandem mass spectrometry data were searched against the Rattus norvegicus Uniprot database (downloaded on 03/2015) merged with a list of common contaminating proteins (albumin, trypsin, human keratins). Sequest search parameters were: MS tolerance 20 ppm; MS/MS tolerance 0.02 Da; oxidized methionine (variable); carbamidomethyl cysteine (static); enzyme trypsin; maximum missed cleavages 2. Search results were filtered by Percolator (Spivak et al., 2009), integrated in Proteome Discoverer, using default parameters. Protein hits based on two high-confidence peptide identifications (q-value < 0.01) were considered valid.
For targeted mode of analysis, the Orbitrap analyzer was operated in targeted MS/MS mode: a single full MS scan (17,500 resolution, 500,000 target ions) was followed by three targeted MS/MS events on precursors at 420.2 (ALHAAAAPMoxPAR, z = 3), 507.6 (HQGSFIYQPCQR, z = 3), 414.9 (ALHAAAAPMPAR, z = 3) m/z. MS/MS conditions were, in all cases, the following: MS resolution 70,000, maximum injection time 250 ms, isolation window 2.0 m/z, AGC target 200,000, normalized collision energy 25.

RNA Immunoprecipitation

Four μg Trk antibody (Santa Cruz sc7268), HuD antibody (Santa Cruz sc28299), NCAM-L1 antibody (Santa Cruz sc514360) or Mouse IgG antibody (Santa Cruz sc2027) were incubated with prewashed protein A/G agarose beads (Santa Cruz) in a PBS buffer containing 1 mg/ml heparin and 1% BSA for 2 h 4°C, and then washed with wash buffer (50 mM Tris pH8, 150 mM NaCl, 1% Triton X-100). Rat SCG neurons or mouse cortical neurons were cultured 5-7 days in vitro and then, for SCG cultures, deprived of NGF for 24 h before stimulating for 30 min with NGF or leaving unstimulated. Cultures were washed with PBS and lysed in Buffer A (50 mM Tris pH8, 150 mM NaCl, 1% Triton X-100, 1:100 Protease inhibitor cocktail, 500 U/ml RNaseOUT) 10 min 4°C. Insoluble material was removed by centrifugation, a 10% fraction saved for total input, and lysates were incubated with antibody conjugated beads for 1 h at 4°C in wash buffer containing 0.2 mg/ml heparin. Beads were washed 3 times with wash buffer 10 min 4°C followed by elution of RNA in extraction buffer (0.2 M NaAcetate, 1 mM EDTA, 0.2% SDS) for 5 min at 70°C. RNA was purified from inputs and immunocomplexes using
PureLink® RNA Micro Scale Kit according to manufacturer’s instructions and analyzed by qRT-PCR.

Immunoprecipitation and immunoblotting analyses

SCGs were isolated from P0-P3 Tp53inp2fl/fl mice and grown as explant cultures. After 7-8 days in vitro, NGF was removed and neurons infected for 48 h with either LacZ or Cre adenovirus. Cell bodies were surgically removed and the axons were stimulated with 50 ng/ml NGF for 30 min or treated with anti-NGF antibody. Three to seven explants per condition were lysed in RIPA buffer with 2.5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate and protease inhibitors. To detect P-TrkA, axon lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibody (PY-20; 4 µl; Sigma-Aldrich) and incubated with Protein G-Plus (Santa Cruz: sc-2002) agarose beads. Immunoprecipitates were then immunoblotted for TrkA (1:1000 Millipore; 06-574).

The supernatants from the immunoprecipitations were subjected to immunoblotting with pAkt (1:1000 Cell Signaling; 9271), pErk1/2 (1:1000 Cell signaling; 9106) and p85 antibodies (1:1000 Upstate Biotechnology, 06-195). All immunoblots were visualized with ECL Plus Detection Reagent (GE Healthcare) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare).

For Trk and HuD immunoprecipitations, PC12 cells or SCG neurons were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, Protease Inhibitors Cocktail) for 10 min on ice, and then insoluble material was removed by centrifugation. Lysates were pre-cleared and 0.5-1 mg of lysate was incubated o/n at 4°C with 2 µg of indicated
antibody. Immuno-complexes were precipitated by the addition of protein A-agarose beads at 4°C for 2 h, and then beads washed extensively with RIPA buffer. Samples were eluted from beads by boiling at 95°C in 1x LDS buffer + 10% β-mercaptoethanol and resolved on polyacrylamide gels.

In all other western blots, cell lysates were prepared in 1x NuPAGE LDS buffer + 10% β-mercaptoethanol, mechanically disrupted by syringing and boiled at 95°C for 5 min before loading. The primary antibodies used were goat anti-Tp53inp2 (1:250 Santa Cruz sc85972), rabbit anti-Tp53inp2 (1:200 generated by Riccio Laboratory) rabbit anti-Tp53inp2 (1:500 a kind gift from Zorzano Laboratory; (Baumgartner et al., 2007)), rabbit anti-Tp53inp2 (1:1000 Sigma AB4502917) mouse anti-Flag (1:1000 Sigma F3165), rabbit anti-Flag (1:1000 Sigma F7425), goat anti-Hsp90 (1:1000 Santa Cruz sc1055), mouse anti-Myc (1:2000 Upstate 05-724), rabbit anti-p85 PI3K (1:1000 Upstate 06-497), mouse anti-a-Tubulin (1:10000 Sigma T9026), chicken anti-GFP (1:5000 Abcam ab13970) mouse anti-mCherry (1:4000 Abcam ab125096), mouse anti-HuD (1:1000 Santa Cruz sc28299), mouse anti-Trk (1:1000 Santa Cruz sc7268) mouse anti-NCAM (1:1000 Santa cruz sc514360). The HRP-conjugated secondary antibodies used were anti-mouse (GE Healthcare life sciences) anti-rabbit (GE Healthcare life sciences), anti-goat (Sigma), anti-chicken (Sigma). Signal was detected using ECL or ECL Prime (GE Healthcare Life Science) and exposing membrane to Amersham Hyperfilm (GE Healthcare Life Science).

pTrkA Immunostaining

SCG neurons isolated from P0-P3 Tp53inp2<sup>fl/fl</sup> mice were grown in Campenot
chambers for 8-10 days in vitro. NGF was removed and neurons infected for 48 h with either LacZ or Cre adenovirus. Fluorespheres were added to axonal compartment to label projected neurons. Distal axons were treated for 4 h with either 50 ng/ml NGF or anti-NGF antibody. To detect pTrkA in the cell bodies induced by retrograde NGF signal, neurons were fixed in 4% PFA-PBS for 30 min at room temperature and blocked in a PBS buffer containing 5% Goat Serum and 0.1% TritonX for 1 h. Neurons were incubated with rabbit anti-pTrkA (1:1000; Cell Signaling Technology, 4168S) and mouse anti-TH antibodies (1:1000, Sigma, T2928) in a PBS buffer containing 1% Goat Serum and 0.1% Triton, o/n at 4°C. Following extensive washes with PBS, neurons were incubated with fluorescently conjugated anti-rabbit (ThermoFisher, A11008) and anti-mouse antibodies (Thermo Fisher, A21240) secondary antibodies at 1:1000 dilution, 37°C for 2 h. Slides were mounted in Fluoromount (Sigma) with DAPI. Images representing 0.8 μm slices were acquired using a Zeiss LSM 700 confocal scanning microscope. The same confocal settings were used to acquire all images taken from a single experiment. For practical reasons, analyses were done in a semi-blinded manner, with the investigator knowing the genotypes prior to the experiment, yet performed the staining and quantification without knowing the genotype of the sample. Total pTrkA positive punctae were quantified using Particle Analyses for 20-30 neurons per condition.

**Analyses of surface TrkA, receptor endocytosis, and total TrkA**

Live-cell antibody feeding to monitor surface TrkA and receptor endocytosis were performed as previously described (Yamashita et al., 2017). Cultured
sympathetic neurons isolated from \textit{Tp53inp2}^{fl/fl} mice were infected o/n with a doxycycline-inducible Flag-TrkA adenovirus, and either LacZ or Cre adenovirus. Neurons were then treated with doxycycline (200 ng/mL, 18 hr) to induce Flag-TrkA expression. Surface Flag-TrkA was labeled by incubating neurons with anti-Flag antibody (Sigma, F7425, 1:500) in PBS for 30 mins at 4\(^\circ\)C, in the absence of NGF. Excess antibody was washed off with ice-cold PBS, and neurons either immediately fixed with 4\% PFA/PBS for 30 mins to assess surface Flag-TrkA distribution, or treated with anti-NGF or NGF (50 ng/mL) for another 30 min to assess receptor internalization. Following treatment with NGF or anti-NGF, neurons were returned to 4\(^\circ\)C and quickly washed in ice-cold acidic buffer (0.2 M acetic acid, 0.5 M NaCl, pH 3.0) to strip surface-bound Flag antibodies and fixed. Neurons were permeabilized with 0.1\% Triton X-100/5\%Normal Goat Serum/PBS and receptors visualized by incubation with anti-rabbit-Alexa 488 secondary antibody. Neuronal morphology was visualized by co-staining with anti-\(\beta\)-tubulin III antibody (Sigma-Aldrich, T8660, 1:1000). Images representing 0.8 \(\mu\)m slices were acquired using a Zeiss LSM 700 confocal scanning microscope. The same confocal settings were used to acquire all images taken from a single experiment. For practical reasons, analyses were done in a semi-blinded manner, with the investigator knowing the genotypes prior to the experiment, yet performed the staining and quantification without knowing the genotype of the sample. Surface Flag-TrkA receptor distribution was analyzed by measuring the integrated fluorescence values along the longest axis of the cell body using line-plot in ImageJ and normalized to the total cell body fluorescence.
Intensity. Intracellular accumulation of Flag-TrkA receptors in cell bodies were quantified as the number of FLAG-immunopositive punctae per neuron. Cell bodies were visualized using the GFP signal and FLAG signals overlapping with GFP fluorescence were defined as internalized soma surface-derived receptors. To assess Flag-TrkA internalization, cell bodies or axons were outlined using b-tubulin III immunostaining and internalized receptors calculated as integrated density of Alexa 488 pixels per μm² (cell bodies) or Alexa 488 pixels per μm (axons).

Cell surface biotinylation was performed on Tp53inp2<sup>fl/fl</sup> neurons as previously described (Houtz et al., 2016). Briefly, Tp53inp2<sup>fl/fl</sup> neurons, infected with either LacZ or Cre adenoviruses, were deprived of NGF in the media after a culture period of 8-10 d.i.v. and biotinylated at 4°C with a reversible membrane-impermeable form of biotin (EZ-Link NHS-SS-Biotin, 1.5 mg/ml in PBS, ThermoFisher Scientific, Cat# 21441) for 30 min. Cells were washed briefly with PBS containing 50 mM glycine (Sigma) to remove remaining unconjugated biotin, and then moved to 37°C and stimulated with NGF (50 ng/mL) for 30 min to promote internalization. Cells were returned to 4°C, the remaining biotinylated surface receptors were stripped of their biotin tag with 50 mM glutathione (Sigma), followed by two washes with 50 mM iodoacetamide (Sigma) to quench excess glutathione. Cells were lysed with 500 μl of RIPA buffer (50mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate), and supernatants subjected to precipitation with 40 μl immobilized neutravidin agarose beads (ThermoFisher Scientific) and immunoblotted for TrkA.
Quantification and Statistical Analyses

Data are expressed as average ± SEM. One-way ANOVA, two-way ANOVA, Mann-Whitney two-tailed test, or t test were used as indicated in the figure legends to test for statistical significance using GraphPad Prism. n values are indicated in the corresponding figure legends. Significance was placed at p < 0.05 unless otherwise noted in the figure legends. Statistical methods were not used to determine sample size, but sample size was selected based on similar studies within the field.

Data and Software Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.
Table S2: List of primers used in this study

<table>
<thead>
<tr>
<th>Application</th>
<th>Primer Name</th>
<th>Primer Sequences</th>
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CHAPTER 3. COUPLED LOCAL PROTEIN PRENYLATION AND TRANSLATION DIRECTS AXON GROWTH
INTRODUCTION

Neurons are polarized cells with dendrites receiving input from synaptic contacts and a single long axon relaying the signal to innervated targets. The establishment and maintenance of neuronal connectivity relies on exquisite regulation of signaling events in distinct neuronal compartments by external cues. The family of neurotrophins regulate many aspects of neuronal development, function, and maintenance through signaling activated by receptor tyrosine kinases of the Trk family. The prototypic neurotrophin, nerve growth factor (NGF) binds its receptor TrkA in the growth cones of developing sympathetic neurons to activate canonical signaling pathways MAPK/Erk, PLCγ, and PI3K/Akt to recruit effectors necessary for NGF-TrkA internalization (Bodmer et al., 2011; Harrington et al., 2011; Huang and Reichardt, 2003). Active NGF-TrkA signaling can remain in the axon to promote growth events or be retrogradely transported to the cell body to activate transcription of survival and growth genes (Barford et al., 2017; Yamashita and Kuruvilla, 2016; Ye et al., 2018). Retrogradely transported NGF-TrkA can be further trafficked to the dendrites to regulate synapse formation and maintenance (Lehigh et al., 2017; Sharma et al., 2010). Thus, trafficking and localization of NGF-TrkA signaling in specific compartments is fundamental for regulation of its wide array of functions.

Although it is postulated that NGF-TrkA signaling relies on distinct protein effectors in different subcellular domains to promote different cellular outcomes, the mechanisms by which these effectors are localized in neuronal compartments is not well understood. Lipid modifications of proteins presents an attractive
mechanism for spatial regulation of neurotrophin signaling due to their roles in regulating protein localization, trafficking, and formation of protein complexes (Resh, 2013; Wang and Casey, 2016). In fact, several hundreds of signaling, cytoskeletal and trafficking regulators in neurons are predicted to be lipid modified (Aicart-Ramos et al., 2011; Fukata and Fukata, 2010; Hayashi and Titani, 2010; Moutinho et al., 2017). These include known NGF signaling effectors belonging to the Ras small GTPase super family (Ginty et al., 1994), Rap1 (Delcroix et al., 2003), Rac1 (Harrington et al., 2011; Kinsella et al., 1991; Nusser et al., 2002) and Cdc42 (Aoki et al., 2004; Hottman and Li, 2014). How lipid modifications contribute to the functions of each protein is unclear. Additionally, the enzymes responsible for catalyzing the addition of lipid groups to proteins are traditionally thought to be constitutively active, which has led to incomplete understanding of their regulation and spatiotemporal requirements (Chen et al., 2018). However, emerging studies suggest that aberrant activity of lipid transferases contributes to the progression of neurodegeneration (Chen et al., 2018; Cho and Park, 2016; Hottman and Li, 2014). Perturbation in prenylation is associated with several neurodegenerative diseases including Alzheimer’s disease (Hottman and Li, 2014; Li et al., 2016; Taheri et al., 2018).

Prenylation is the addition of either a 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoid by farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) respectively, to a cysteine residue located in a “CAAX” recognition motif at the C-terminus of proteins. The amino acid in the “X” position biases the protein for either farnesylation or geranylgeranylation.
(Resh, 2013). Interestingly, the development and maintenance of specialized cellular compartments may require localization of prenyltransferases (FTase and GGTase I) to active signaling sites. Recruitment of GGTase I to active MusK receptors promotes clustering of acetylcholine receptors necessary for formation and maintenance of the neuromuscular junction (Luo et al., 2003). Additionally, TrkB-dependent dendritic morphogenesis required localization of GGTase I to active receptors (Zhou et al., 2008). Based in these limited examples, prenylation-mediated targeting of proteins could contribute to the development and function of distinct neuronal compartments. However, little is known about the regulation of prenylation and how it affects protein function in long and morphologically specialized neurons.

Here, we report a compartment-specific requirement for protein geranylgeranylation in axons of sympathetic neurons to direct NGF-dependent growth. NGF acutely stimulates the activity of GGTase-I to promote geranylgeranylation of several proteins, including the Rac1 GTPase, in axons. Contrary to the belief that prenylation is a ubiquitous process in cells, our studies provide evidence of a spatiotemporal regulation of prenylation by an extrinsic cue in sympathetic axons. Excitingly, the proteins that are being locally prenylated in response to NGF are also locally synthesized in axons. These findings elucidate a previously unrecognized mechanism by which neurotrophic factors promote axon growth; NGF regulates the local synthesis of growth-promoting proteins in axons and also ensures their post-translational modification to enable axonal functions.
RESULTS

Geranylgeranylation is required locally in axons for NGF-dependent growth

Isoprenoids are intermediate metabolic products of cholesterol metabolism. Previous studies have shown that inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol and isoprenoid biosynthesis, results in defects in neuron survival (Michikawa and Yanagisawa, 1999), dendritic and axonal growth (Kim et al., 2009; Sato-Suzuki and Murota, 1996), and synaptic function and maintenance (Maggo and Ashton, 2014). HMG-CoA reductase inhibition affects not only cholesterol production, but also the availability of metabolites required for glycosylation, prenylation, and squalene synthesis (Moutinho et al., 2017). The previous studies did not address which branch of cholesterol metabolism is required in specific neuronal processes. Since the nervous system contains the highest levels of isoprenoids and prenylated proteins, it was of specific interest to elucidate the role of prenylation in neuronal development and maintenance (Epstein et al., 1991).

As an initial step to define the functions of prenylation in NGF-dependent development, we assessed expression of the prenyltransferases in sympathetic neurons during a period of reliance on NGF. Prenyltransferases are heterodimers with a common α-subunit (GGTase1-α) and unique β-subunits (Wang and Casey, 2016). Immunohistochemistry with an antibody against the common GGTase1-α showed robust expression in the cell bodies (Figure 3-1A-B) and axonal compartments of sympathetic neurons in mice at 5 days of age (Figure 3-1C-D). Consistent with findings in tissues, immunostaining of isolated neurons in culture
revealed prenyltransferase localization in cell bodies and axons of sympathetic neurons, and even in growth cones of isolated neurons (Figure 3-1E-F). Furthermore, immunoblotting for GGTase1-α in lysates isolated from cell bodies or axons of sympathetic neurons, grown in compartmental cultures where a Teflon-grease diffusion barrier separates the cell bodies from the axons, showed a protein of the predicted size (44 kDa) in both compartments (Figure 3-1G) (Campenot, 1977). Protein lysate isolated from PC12 cells transfected with a vector expressing shRNA against GGTase1-α showed a reduction in intensity of the predicted protein band demonstrating antibody specificity (Figure 3-1H). Together, these results indicate that the molecular machinery required for prenylation is present throughout sympathetic neurons. To visualize sub-cellular sites of protein prenylation in neurons, we performed a live feeding assay using a cell-permeant alkyne isoprenoid reporter added to either the cell body or axons of neurons in compartmental cultures. Both FTase and GGTase I use the isoprenyl analog as a substrate to prenylate endogenous proteins (DeGraw et al., 2010). Newly labeled proteins were then identified by conjugation with biotin using Click-It chemistry and biotin-streptavidin immunostaining. Prenylated proteins were present in both the cell bodies (Figure 3-2A), and axons (Figure 3-2B-C) following addition of the isoprenyl reporter to the cell body or axon compartments. Together, these results indicate that prenylation of proteins occurs in the cell bodies and axons of sympathetic neurons. These findings led to the question of the role of prenylation in distinct neuronal compartments.
NGF-mediated axon growth requires activation of signaling, trafficking, and cytoskeleton remodeling enzymes to promote axon elongation and branching events (Barford et al., 2017; Yamashita and Kuruvilla, 2016). Many of these signaling effectors are known to undergo prenylation, although the functional requirement for these modifications is unknown (Scott-Solomon and Kuruvilla, 2018; Wang and Casey, 2016). To determine if prenylation was required for NGF-mediated axon growth, we investigated the specific requirement of farnesylation and geranylgeranylation in distinct neuronal compartments. Cell bodies or axons of neurons grown in compartmentalized cultures were treated with competitive inhibitors specifically against FTase (FTI-277) or GGTase I (GGTI-2133) in conjunction with NGF treatment to distal axons (Figure 1D). NGF treatment resulted in robust growth of sympathetic axons (76.75 ± 4.26 μm/day) (Figure 3-2E-F, J-K). In contrast, FTase inhibitor treatment at either the cell body or the axon (Figure 3-2G-H) attenuated NGF dependent growth (1.19 ± 8.64 μm/day and 4.42 ± 7.35 μm/day respectively). Treatment of axons with the GGTase I inhibitor (GGTI) also diminished growth (-8.54 ± 5.735 μm/day) (Figure 1N-O), but surprisingly, inhibition of GGTase I activity in cell bodies of sympathetic neurons had no effect on axon growth (104.7 ± 2.14 μm/day for control versus 90.9 ± 8.87 μm/day) (Figure 3-2J and L). These results suggest a unique requirement for geranylgeranylation in axons of sympathetic neurons for NGF-dependent growth.

NGF acutely regulates geranylgeranylation in axons
The unexpected result that localized GGTase I activity is required for axon growth led us to focus on protein geranylgeranylation. The global requirement for farnesylation in axon growth suggested that it may have more permissive functions in neuronal morphogenesis. The axon-specific requirement for GGTase I activity in NGF-directed axon growth raised the possibility it is regulated by NGF-TrkA signaling. To investigate NGF regulation of GGTase I, we used a fluorescent enzymatic assay to measure GGTase I enzymatic activity after acute treatment of distal axons with NGF for 30 minutes (Figure 3-3A). NGF treatment triggered a 4-fold increase in GGTase I activity locally in sympathetic axons. In contrast, GGTase I activity in cell bodies was not affected by NGF stimulation within this time period (Figure 3-3B). To visualize axonal prenylation in response to NGF, we fed the isoprenyl-analog to sympathetic neurons in the presence or absence of NGF for 4 hours. Excess analog was washed from neurons and prenylated proteins visualized by conjugation of a biotin tag and streptavidin immunostaining (Figure 3-3C-N). NGF increased prenylation of protein locally in axons (Figure 3-3I) with less prenylation occurring in the absence of the growth factor (60.31 ± 0.05% of NGF treated condition) (Figure 3-3F). Treatment of neurons with GGTI-2133 (Figure 3-3L) attenuated incorporation of isoprenyl-analog in NGF treated neurons (Figure 3-3C) (Figure 3-3O). Thus, these results indicate that the majority of prenylation in axons represents NGF-induced geranylgeranylation.

Which proteins are locally modified in sympathetic axons in response to NGF? The Rac1 GTPase is a known GGTase I substrate (Kinsella et al., 1991). Rac1 is a key effector of TrkA trafficking and trophic signaling (Harrington et al.,
Therefore, we reasoned Rac1 is an attractive substrate for NGF-regulated modification in axons. To detect local Rac1 geranylgeranylation in axons independent of anterograde transport from cell bodies, we employed an explant culture system that permits the mechanical removal of cell bodies leaving the axons in isolation (Figure 3-4A-B). Isolated axons were incubated with the isoprenyl-analog in the absence or presence of NGF. Protein lysates from multiple explants were then pooled and Rac1 immunoprecipitated. Modified Rac1 was labeled with a TAMRA-tag and detected by immunoblotting for the TAMRA label (Figure 3-4C). We found that NGF promotes robust prenylation of Rac1 (2.97±0.39-fold) in isolated sympathetic axons compared to un-stimulated axons. Treatment of axons with GGTI-2133 attenuated Rac1 geranylgeranylation without affecting total Rac1 levels (Figure 3-4D). Thus, NGF acutely promotes Rac1 geranylgeranylation locally in axons.

Local protein synthesis underlies requirement for local prenylation in axons

The ability of NGF to promote geranylgeranylation of protein in axons raises the interesting question as to the source of these nascent proteins. Proteins are immediately prenylated following synthesis (Wang and Casey, 2016); therefore, it is unlikely that proteins synthesized in the cell bodies are transported to axons in an un-prenylated state. This raised the intriguing possibility that the protein being geranyl-geranylated in axons in response to NGF are locally translated. Previously, screens of axonal mRNA have identified many transcripts that are known to encode for prenylated proteins (Gumy et al., 2011). To determine the contribution
of intra-axonal protein synthesis to local geranylgeranylation in response to NGF, we measured the incorporation of the isoprenyl-analog in NGF-treated axons in the presence or absence of the translation inhibitor, cycloheximide (CHX). As before, NGF enhanced prenylation of proteins isolated axons, which was completely abolished by CHX (Figure 3-5A-M).

We next asked if prenylation of Rac1 in axons was dependent on its local synthesis. First, RT-PCR of mRNA isolated from axons of sympathetic neurons demonstrated the presence of Rac1 mRNA in axons (Figure 3-5N). To determine if Rac1 geranylgeranylation was dependent on its local synthesis, isolated axons were incubated with the isoprenyl analog and stimulated with NGF in the presence or absence of CHX. Geranylgeranylated Rac1 was detected by Click-iT labeling and immunoblotting. Treatment of isolated axons with CHX reduced both geranylgeranylated Rac1 and total Rac1 in axons (Figure 3-5O-P). Together, these results indicate that Rac1 geranylgeranylation in axons is dependent on local translation.

**Local synthesis and prenylation of Rac1 is required for NGF dependent growth.**

Asymmetric subcellular localization of mRNA depends on localization elements commonly located within the 3'UTR. These include short "zip codes" sequences (Andreassi et al., 2010; Kim et al., 2015), retained intron isoforms (Sharangdhar et al., 2017), and intriguingly, possibly, remodeling of the 3'UTR (Andreassi et al., 2017). To study the intra-axonal synthesis of Rac1 and to
potentially identify element(s) responsible for the trafficking of Rac1 mRNA to axons, we performed rapid amplification of 3’cDNA ends (3’RACE) on mRNA isolated from either cell bodies or distal axons (Figure 3-6A). Interestingly, 3’RACE revealed two isoforms of the Rac1 3’UTR, a long 3’UTR (~1500 bp) and short 3’UTR (~250 bp) (Figure 3-6B-C). The sequence of the short isoform aligned to the first 250 bps of the long isoform upstream of a poly-adenylation site. Although both isoforms were found in the cell bodies of sympathetic axons, only the long isoform was detected in the axonal compartment.

We hypothesized the Rac1 long 3’UTR isoform contributed to the locally synthesized pool of Rac1 protein in axons. To determine which Rac1 isoform was necessary for axon growth, we generated adenoviruses that express Rac1 mRNA fused to either the long (Rac1\textsuperscript{Long}) or short (Rac1\textsuperscript{Short}) 3’UTR (Figure 3-7A). These constructs were used to infect neurons homozygous for a Rac1 floxed allele (Rac1\textsuperscript{f/f}). Endogenous Rac1 expression was acutely deleted by co-infection with adenovirus expressing Cre (Figure 3-7B). Following deletion of Rac1, we found that expression of Rac1\textsuperscript{Long}, but not Rac1\textsuperscript{Short} rescued NGF-dependent axon growth (Figure 3-7C-H). These results suggest local Rac1 synthesis in axons is essential for its role in NGF-mediated development.

Prenylation is postulated to be essential for the localization and function of proteins to maintain compartmental identity (Wang and Casey, 2016; Zhou et al., 2008). We then addressed the essential role of Rac1 prenylation in NGF-mediated functions. To prevent modification of Rac1 isoform present in axons, the Cys residue in the CaaX prenylation motif on the Rac1\textsuperscript{Long} isoform was mutated to a
serine (Rac1\textsuperscript{C189S}) (Figure 3-7A). Rac1 knockout neurons were infected with Rac1\textsuperscript{Long} or Rac1\textsuperscript{C189S} adenoviruses. While expression of Rac1\textsuperscript{Long} fully restored growth in knockout neurons, Rac1\textsuperscript{C189S} was unable to do so (Figure 3-7G, H). Together, these results suggest that intra-axonal synthesis coupled to geranylgeranylation of Rac1 is required for its function in NGF-dependent growth.
DISCUSSION

NGF-TrkA signaling requires diverse effectors in specific compartments in order to regulate a wide array of neuronal events. The mechanisms responsible for regulating the localization of effectors to distinct compartments remain poorly understood. Protein prenylation regulates protein localization and protein-protein interactions (Resh, 2013; Wang and Casey, 2016). Thus, prenylation may have a role in the recruitment and retention of proteins to form and maintain specialized neuronal domains in response to extrinsic neurotrophic cues. Here we found a unique requirement for protein geranylgeranylation locally in axons for NGF-mediated growth. NGF acutely regulated the activity of GGTase I locally in axons. Increased GGTase I activity was necessary for the modification of axonally synthesized proteins, including Rac1, to promote NGF-dependent axon growth (Figure 3-8).

Prenylation is traditionally viewed as having a ubiquitous requirement throughout the cell (Wang and Casey, 2016). However, our finding of a unique dependence on geranylgeranylation locally in axons for growth in contrast to the global requirement of farnesylation in neurons indicate these modifications have specific roles in cellular compartments. The unique dependence for each prenyl-modification between neuronal compartments is likely due to a difference in the function or requirement for the different prenylated proteins. Axon navigation requires the activity of many proteins including cytoskeletal remodeling enzymes Rac1, Cdc42, and RhoA which are known to be geranylgeranylated (Kinsella et al., 1991; Reddy et al., 2015; Samuel and Hynds, 2010; Wang and Casey, 2016).
Axon development requires exquisite spatial regulation of local Rac1 and Cdc42, which promote axon elongation, and RhoA, which promotes axon retraction, in response to extrinsic guidance cues (Hall and Lalli, 2010). Therefore, the high demand for geranylgeranylated proteins leads to an exquisite dependence on local geranylgeranylation in the axons. The global requirement for farnesylation could be due to distinct actions of farnesylated proteins in the cell body and axon regulating the same developmental processes despite differences in location (Fukada et al., 1990; Lerner et al., 1995; Wedegaertner et al., 1995). NGF-TrkA signaling activates MAPK signaling through farnesylated Ras in the axon to promote neurite growth; in the cell body, the activated Ras-MAPK pathway regulates transcription factors for the synthesis of genes necessary for growth (Howe et al., 2001; Obara et al., 2004; Scott-Solomon and Kuruvilla, 2018; Xing et al., 1998). Thus, Ras has distinct functions in two different neuronal compartments that culminates in the regulation of axon growth. Interestingly, axonal Ras can also be retrogradely carried in NGF-TrkA signaling endosomes to the cell body (Howe et al., 2001). The trafficking of Ras between neuronal compartments could mean there is more cross talk between farnesylated proteins derived from the axon and cell body compartment. Further work to elucidate the spatial requirements of prenylation will provide insight into unique roles of each prenyl modification in regulating cellular functions.

The ability of NGF to acutely control the activity of GGTase I demonstrates prenylation is a highly regulated process. Prenylation has long been considered a constitutive process, with prenylation dependent on the availability of lipid
substrates and passive encounters with unmodified proteins (Chen et al., 2018). However, when the concentration of unmodified protein substrates increases, it makes sense the activity of the prenyltransferases would similarly have to rise to ensure continued production of functional proteins. Neurotrophin signaling induces translation of diverse mRNAs involved in cytoskeletal regulation, metabolism, and signaling necessary for axon viability and neurotrophic function (Andreassi et al., 2010; Willis et al., 2007). Many mRNA transcripts present in the axon encode for known prenylated proteins (Gumy et al., 2011; Wu et al., 2005). NGF-directed prenylation in axons depended on the local synthesis of proteins including the known NGF effector, Rac1. Thus, coupling the regulation of protein synthesis and subsequent post-translational modification in axons by the same signaling factor ensures the formation of functional proteins for axonal responses. How NGF signaling regulates GGTase-I activity is still a mystery. Activity of GGTase I could be regulated through phosphorylation of tyrosine residues (Luo et al., 2003; Zhou et al., 2008). Tyrosine phosphorylation of GGTase-I could serve to recruit it to activated TrkA receptors, as previously reported for MuSK (Zhou et al., 2008). Future work to identify chaperones and binding partners of prenyltransferases will provide insight into regulation of prenylation.

A major question in the neurotrophin field is how NGF recruits protein effectors to distinct neuronal compartments to mediate diverse neuronal processes. The coupling of local protein synthesis with local prenylation presents a spatial mechanism for the recruitment and maintenance of proteins for specialized NGF-TrkA signaling in distinct neuronal domains. The cell body and
axons of neurons have unique enrichments of specific mRNAs (Andreassi et al., 2010). RNA binding proteins (RBPs) sort mRNA based on cis-regulatory elements commonly found in the 5’ and 3’UTR to RNA granules (Andreassi et al., 2017; Andreassi et al., 2010; Cosker et al., 2016; Kim et al., 2015; Lepelletier et al., 2017; Sharangdhar et al., 2017). Activation of RBPs in these granules promotes anterograde trafficking to the axon in response to extrinsic signals (Cosker et al., 2016). Similarly, NGF signaling could activate distinct RBPs in the axon to transport RNA granules containing mRNA encoding for different proteins effectors to sites of NGF-TrkA signaling (Riccio, 2018; Willis et al., 2007). Following NGF-directed local protein synthesis, these effectors would be retained at these distinct compartments by subsequent local prenylation (Wang and Casey, 2016). Asymmetrical mRNA localization and local translation of proteins is thought to underlie the establishment and function of specialized neuronal compartments (Riccio, 2018). Profiling of protein translated and prenylated in response to NGF in different subcellular domains will provide insight into not only the regulation of NGF effectors, but how these compartments are established and maintained. This knowledge could have applications in understanding of the progression of neurological diseases as perturbations in RNA metabolism and prenylation are associated with many neurodegenerative diseases (Jeong et al., 2018; Li et al., 2016; Riccio, 2018; Taheri et al., 2018). Uncoupling of local protein synthesis and post-translational modifications would fail to produce functional proteins necessary for the specialization of neuronal compartments which could lead to neuronal dysfunction.
Figure 3-1. The GGTase-1 α-subunit is localized in both cell bodies and axons of sympathetic neurons. A-F GGTase1-α is expressed in the cell bodies and axons of sympathetic neurons. Immunofluorescence staining for the prenyltransferase subunit GGTase1-α on sections of superior cervical ganglia (SCG) (A-B) and their innervation target, the salivary gland (C-D), from early postnatal (P5) Th-Cre;Rosa26tm14(CAG-tdTomato) mouse. Immunostaining of isolated rat sympathetic neurons for GGTase1-α similarly shows presence in the cell bodies (E) and axons of sympathetic (F) neurons. Counterstaining with a total protein stain, DTAF, was used to show neuronal morphology (E-F). (G) Isolation of protein from exclusively cell bodies and axons using a compartmental culture device demonstrates transferase is detected in both cell bodies and axons by a western blot. (H) Immunoblot of PC12 cell lysate transfected with empty vector or shRNA against GGTase1-α confirmed antibody specificity. Scale bars 25 μm (A, B) and 10 μm (C, D). Insets represent area magnified in image panels.
Figure 3-2. Geranylgeranylation is required locally in axons while farnesylation is required globally for NGF-mediated growth. A-C Prenylation occurs in both the cell body and axons of neurons. Sympathetic neurons were stimulated for 4 hours only on axons with 50 ng/mL NGF. (A) Cell bodies or (B-C) axons were exclusively fed an isoprenyl-analog to label prenylated proteins in the specific compartments. D Utilization of compartmental chambers allows exclusive treatment of cell bodies or axons of sympathetic neurons with either the FTase specific inhibitor FTI-277 (100nM) (Lerner et al., 1995) or GGTase I inhibitor GGTI-2133 (75 nM) (Johnson et al., 2004). E-I Farnesylation is required in both neuronal soma and axons to mediate NGF-dependent growth. J-N Geranylgeranylation is required locally in axons for NGF-mediated growth. Sympathetic neuron cell bodies or axons in compartmentalized cultures were locally treated with either (G-H) FTI-298 or (L-M) GGTI-2133. Only axons treated with 50 ng/mL NGF and growth rate measured in 24-hour increments over 72 hours following FTI treatment or GGTI treatment. ****: p<0.001 One-way ANOVA, n=3 independent experiments (at least 20 axons per condition), Scale bars 10 μm (A-C) and 100 μm (E-H, J-M).
Figure 3-3. NGF stimulates geranylgeranyl transferase activity in axons. A Fluorescent enzymatic activity assay to measure response of endogenous GGTase1 activity in cell body and axon protein lysates following local NGF treatment of only axons (50 ng/ml, 30 minutes). Cell body or axon lysates were incubated with a fluorescent dye-tagged CaaX peptide that serves as a GGTase I substrate. GGTase I catalyzed addition of geranylgeranyl pyrophosphate to the CaaX peptide is measured by an increase in fluorescence at 460 nm. B NGF treatment acutely stimulates GGTase1 activity in sympathetic axons. Transferase activity was normalized to total protein amounts, n=5 independent experiments. C-N Enhancement of prenylation in axons following treatment with (I) NGF (50 ng/mL) for 4 hours was blocked through the addition of (L) GGTL-2133 (75 nM). (C, F, I, L) Prenylated proteins labeled with prenyl-analog conjugated to biotin-streptavidin 488. (D, G, J, M) Growth cone morphology shown using phalloidin to stain actin and immunohistochemistry for βIII-Tubulin. (E, H, K, N) Merged images. O Quantification of prenyl-analog fluorescent intensity normalized to actin (20 growth cones/experiment, n=5 different experiments). Scale bars 10 μm (C-N). *:p<0.05 T-test (B) and ***:p<0.001, ****:p<0.0001 One-way ANOVA on average of each experiment (O).
Figure 3-4. NGF-dependent geranylgeranylation of axonal Rac1.  

A-B Surgical removal of cell bodies leaves axons in isolation permitting labeling of only axonal resident proteins by prenyl-analog.  

C Rac1 prenylated locally in axons in response to NGF is blocked upon cotreatment with GGTI-2133 (75 nM). Isolated axons in presence of prenyl-analog treated for 8 hours with anti-NGF, NGF or NGF with GGTI-2133. Prenylated Rac1 detected following immunoprecipitation of Rac1 through conjugation of TAMRA-tag to prenyl-analog and immunoblotting.  

D NGF increases Rac1 prenylation in axons and is blocked by GGTI-2133.  

E Inhibition of Rac1 prenylation does not lead to diminishment of total Rac1 levels. Prenylated and total Rac1 from protein lysate input normalized to p85. *:p<0.05 One-way ANOVA, n=3 (50-70 explants pooled for each condition per experiment). Scale bar equals 500 μm.
Figure 3-5. NGF-dependent geranylgeranylation of axonal Rac1 is dependent on local translation. A-L Prenylation is required locally in axons for the modification of proteins newly synthetized in axons. (D, G) NGF promoted prenylation of protein in isolated axons is blocked when cotreated with the translational inhibitor (J), cycloheximide (CHX, 25 μM) for 6 hours. (A, D, G, J) Prenylated proteins labeled via conjugation to biotin-streptavidin 488 and (B, E, H, K) axons stained with βIII-Tubulin. (C, F, I, L) Merged image. M Quantification of protein prenylation normalized to βIII-Tubulin. N Rac1 mRNA present in the axons of sympathetic neurons. RT-PCR for known axonal mRNA β-actin and cell body specific transcript Phox2b. O NGF-dependent prenylation of Rac1 requires local
translation. Prenylated Rac1 detected as previously described. P Absence of NGF or treatment with CHX significantly reduced prenylated Rac1. Q Local translation contributes to axonal Rac1 protein levels. Prenylated and total Rac1 normalized to p85*: p<0.05, **: p<0.01, ****: p<0.001, ANOVA, (M) n=13 explants (no analog) and 17-20 explants (10 independent experiments), (P-Q) n=3 (50-70 explants pooled for each condition per experiment).
Figure 3-6. A long isoform of Rac1 3’UTR is enriched in sympathetic axons.

**A** 3’UTR-RACE was performed on RNA isolated from cell bodies or axons of rat sympathetic neurons for Rac1 using two unique primer sets specific for *Rac1*. **B-C** A short isoform and a long isoform were detected as the two major isoforms for the Rac1 3’UTR in cell bodies while only the long form was detected in axons using two different primer sets.
Figure 3-7. Local translation and prenylation of Rac1 is required for NGF-dependent growth. A Rescue viral constructs expressing wild-type Rac1 with short and long 3’UTR isoform, and prenylation deficient (C189S) Rac1. B Acute knockdown of endogenous Rac1 in Rac1f/f sympathetic neurons in vitro following infection with Cre virus. C-H Neurons require local translation and prenylation of Rac1 for NGF-mediated growth. Axon growth assays performed on neurons infected with (C) control viruses LacZ and GFP, (D) knockdown with Cre and rescue with control GFP, knockdown of Rac1 with Cre adenovirus and rescue with (E) wild-type Rac1 with long 3’UTR, (F) wild-type Rac1 with short 3’UTR, or (G) C189S Rac1 with long 3’UTR over 72-hour period taking measurements in 24-hour increments. Representative axons stained with βIII-Tubulin. H Quantification of axon growth rate. (B) n=3, **:p<0.01 t-test. (H) n=3, 40-60 axons measured per condition, ****:p<0.0001 One-way ANOVA.
Figure 3-8. NGF-dependent growth requires geranylgeranylation of proteins newly synthesized locally in axons. Model. (A) Neurons traffic transcripts encoding for prenylated proteins to axon terminal where, (B) in response to NGF, proteins synthesized from these transcripts (C) undergo prenylation. Local prenylation is required locally in axons to promote neurotrophic functions, specifically axon growth.
METHODS

Animals

All procedures relating to animal care and treatment conformed to The Johns Hopkins University Animal Care and Use Committee (ACUC) and NIH guidelines. Animals were group housed in a standard 12:12 light-dark cycle. Postnatal day P0.5-P6 pups of both sexes were used for analyses. The following mouse lines were used in this study; \textit{Rac1}^{\text{fl/fl}} (\textit{Rac1}^{\text{tm1Djk}/J}) mice (Jackson Laboratory, stock no. 005550), \textit{Rosa-26}^{\text{tm9(CAG-tdTomato)}} (Jackson Laboratory, stock no. 007909), and \textit{TH-Cre} mice (Gong et al., 2007) by Dr. Charles Gerfen (NIH).

Pregnant Sprague Dawley rats were purchased from Charles River or Taconic Biosciences. Dissociated or explant cultures of sympathetic neurons were established from superior cervical ganglia (SCG) dissected from P0.5 rat pups as previously described (Zareen and Greene, 2009).

Antibodies and reagents

The following antibodies were used in this study: mouse anti-Rac1 (Millipore, 05-389, immunoprecipitation, immunoblotting), rabbit anti-p85 (Millipore, 06-195, immunoblotting), mouse anti-\(\beta\)-III-tubulin (Sigma, T8660, immunocytochemistry), sheep anti-NGF (Cedarlane Labs, CLM/CNET-031, NGF neutralization), rabbit anti-GGTase I-\(\alpha\) (Santa Cruz, sc-136, immunoblotting, immunohistochemistry, and immunocytochemistry), mouse anti-\(\alpha\)-tubulin (Sigma-Aldrich, T6074, immunoblotting), and mouse anti-TAMRA (Abcam, ab171120, immunoblotting). Alexa Fluor conjugated secondary antibodies were obtained from
ThermoFisher. Prenyl transferase inhibitors, GGTI-2133 (G5294) and FTI-277 (F9803), were from Sigma. The following reagents were used for Click-iT labeling; propargyl-farnesol (isoprenyl analog; Echelon, S-0160), Biotin-TEG azide (Berry & Associates, BT 1085), TAMRA-PEG azide (Sigma-Aldrich, 760757), dansyl-GCVLL (synthesized at Johns Hopkins Synthesis and Sequencing Facility), and geranylgeranyl diphosphate (GGPP) (Echelon, I-0200). Additional reagents included Streptavidin-Alexa-488 (ThermoFisher, S11223), Phalloidin-Alexa-546 (ThermoFisher, A22283), 5-4,6-Dichlorotriazinyl Aminofluorescein (5-DTAF, ThermoFisher, D16) for total protein staining, ProSignal Dura ECL Reagent (Genesee Scientific, 20-301, immunoblotting), Signal Enhancer HIKARI 250 (Nacalai, NU00102, immunoblotting), and OneBlock™ Western-CL Blocking Buffer (Genesee Scientific, 20-313, immunoblotting).

**Adenoviral and plasmid constructs**

Human Myc-tagged Rac1 coding sequence CDS, a gift from Dr. Derek Prosser, was fused to the Rac1 short or long 3’UTR which were PCR amplified from RACE clones from compartmentalized cultures of rat sympathetic neurons. The CaaX motif mutation (C189S) was introduced by PCR-based site-directed mutagenesis. Plasmids were verified by DNA sequencing. Rac1 constructs were sub-cloned into pAdTrack-CMV (Addgene #16405) using SalI and NotI restriction sites. Recombinant adenoviral constructs for Myc-tagged Rac1-long 3’UTR (Rac1-L) or Rac1-short 3’UTR (Rac1-S) were generated using AdEasy adenoviral system (Stratagene), and recombinants were transfected into HEK 293 cells using
Lipofectamine 3000 (ThermoFisher, L3000). Adenovirus for Myc-Rac1-L-C189S was generated by ViGene Biosciences, Inc. based on recombinant plasmid generated by E.S. High titer virus stocks were obtained using Vivapure® AdenoPACK™ (Sartorius). Cre adenovirus was a gift from Dr. Lois Greene (NIH) and LacZ adenovirus was a gift from Dr. Jeffrey Pessin (Albert Einstein College of Medicine).

To generate shRNA for GGTase I-α, a custom oligonucleotide with the sequence 5’-GTC GAC CCG CAC CAT AGG AGA GTA TTA GTT TCA AGA GAA CTA ATA CTC TCC TAT GGT GCT TTT TGA ATT C-3’, directed against the GGTase I CDS, was digested with SalI and EcoRI, and then ligated into N1 Venus pA H1 backbone (gift from Dr. Chih-Ming Chen), which had been linearized with XhoI and EcoRI. The shuttle vector was derived from pEGFP-N1 (Clontech 6085-1). Vector was digested with XhoI and EcoRI. PC12 cells (American Type Culture Collection, CRL-1721) were electroporated with shRNA plasmid using Cell Line Optimization 4D-Nucleofector™ X-kit (Lonza, V4XC-9064) per manufacturer protocol with empty vector as control. Protein lysates were collected 48 hr later, and GGTase I-α expression assessed by immunoblotting with anti-GGTase I-α. Immunoblots were stripped and re-probed for α-tubulin as loading control.

**RT-PCR and qRT-PCR analyses**

Cell body and axonal RNA was isolated from rat sympathetic neurons grown in compartmentalized cultures using Trizol (ThermoFisher, 15596018). cDNA was
generated using Ambion RETROscript kit (AM1710) and transcripts analyzed by PCR using gene specific primers (see Table S1 for primer sequences).

For Rac1 deletion, sympathetic neuron cultures established from P0.5 \textit{Rac1^{fl/fl}} mice were infected with adenoviruses for Cre or LacZ. mRNA was extracted 48 hr post-infection using RNAqueous Micro Total RNA Isolation Kit (ThermoFisher, AM1931). cDNA was prepared using Superscript IV First Strand Synthesis System (ThermoFisher, 18091050). Real-time qPCR analysis was performed using TaqMan probes (ThermoFisher) for Rac1 (Mm01201653) in a StepOnePlus™ Real-Time PCR Systems (ThermoFisher). Each sample was analyzed in triplicate reactions. Fold change in transcript levels were calculated using the $2^{-(\Delta\Delta Ct)}$ method, normalizing to 18s rRNA transcript (ThermoFisher, Mm04277571_s1).

3’ RACE analysis

Total RNA was isolated from cell bodies or axons of compartmentalized sympathetic neuron cultures and cDNA generated as described above. cDNA generation was done using a hybrid primer containing an oligo-(dT) to enrich for the 3’-end of mRNA and a unique 35-nucleotide sequence, as previously described (Scotto-Lavino et al., 2006). Rac1 3’UTR was then amplified using a primer against the 35-nucleotide sequence, and a \textit{Rac1} CDS specific primer. Non-specific products were removed through a second amplification step using two different \textit{Rac1} CDS specific primers (See Table S1: Primer Sequences). PCR products were separated on an agarose gel, and bands purified using NucleoSpin® Gel
Clean-Up (Macherey-Nagel, 740609). Purified bands were cloned into the TOPO TA Cloning System (ThermoFisher, 450640) and sequenced in both orientations.

**Immunostaining**

P0.5 mouse sections (12 μm) from TH-Cre;TdTomato reporter mice were permeabilized with 0.5% Triton X-100 in Phosphate Buffered Saline (PBS) and blocked using 3%BSA/PBS/0.5% Triton X-100. Sections were then incubated with rabbit anti-GGTase I-α antibody (1:200) overnight. Following PBS washes, sections were incubated with anti-rabbit Alexa-488 secondary antibody (1:200). Sections were then washed in PBS and mounted in Fluoromount Aqueous Mounting Medium containing 100 μg/ml DAPI. Images representing 0.8 μm optical slices were acquired using a Zeiss LSM 700 confocal scanning microscope.

P0.5 rat SCGs were grown as explants or dissociated sympathetic neuron cultures on poly-d-lysine and laminin-coated glass coverslips. Neurons were fixed for 30 min. at room temperature in 4% Paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS). Coverslips were washed with PBS and blocked in 3%BSA/0.1%Triton X-100/PBS or 5%Goat Serum/1%BSA/0.1% Triton X-100/PBS for 1 hr. Explants or dissociated neurons were incubated overnight with β-III-tubulin (1:500) or GGTase I-α (1:200). After washes with PBS, neurons were incubated with anti-rabbit-488 or anti-mouse-546 secondary antibodies. In immunostaining for GGTase I-α, DTAF, a reactive dye that labels amines in proteins, was added at 1:10,000 (stock 10mg/ml) for 1 hr as a counterstain, prior to adding the primary antibody. Neurons were then mounted in Aqueous Mounting
Medium containing 100 μg/ml DAPI. Images representing 0.8 μm optical slices were acquired using a Zeiss LSM 700 confocal scanning microscope.

Neuronal cultures

Sympathetic neurons were harvested from P0.5 Sprague-Dawley rats or Rac1fl/fl mice and were grown as explant cultures, or dissociated for mass or compartmentalized cultures. Neurons were maintained with high-glucose DMEM media supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (1U/ml), and NGF (100 ng/ml). For immunocytochemistry, cells were grown on coverslips coated with poly-d-lysine (1 μg/ml; Sigma-Aldrich) and laminin (10 μg/ml; Sigma-Aldrich). NGF deprivation was performed in high-glucose DMEM supplemented with 1% FBS with anti-NGF (1:1000) and the caspase inhibitor, boc-aspartyl(O-methyl)-fluoromethylketone (BAF, 50 μM) for 36 hr. For adenovirus infections, neurons were infected with high-titer viruses for 36-48 hr.

Axon growth

For assessing axon growth, neurons isolated from P0.5 rats were grown in Campenot chambers for 7-9 days. Neurons were either completely deprived of NGF or NGF (50 ng/ml) was added only to distal axons. BAF (50 μM) was also included to allow assessment of axon growth without the complications of cell death. For compartmentalized inhibition of prenyltransferases, rat sympathetic neurons in compartmentalized cultures were treated with GGTLI-2133 (75 nM) or FTI-277 (100nM) added either exclusively to cell body or distal axon
compartments. Phase contrast images of axons were captured using a Retiga EXi camera in 24-hr intervals for 3 days on a Zeiss Axiovert 200 microscope. Axon growth rate was measured using Openlab 4.0.4 for an average of 30-60 axons per condition. Axons were fixed in 4% PFA and stained with β-III-tubulin for representative images following experiments.

Compartmentalized cultures from P0.5-P6 Rac1fl/fl mice were infected with adenoviruses expressing GFP, Cre, Cre + Rac1-L, Cre + Rac1-S, or Cre + Rac1-L-C189S after axons had extended into the side compartments (7-10 days in vitro). Axon growth was then assessed in response to axon-applied NGF (50 ng/ml) in 24 hr intervals for 48 hr, as described above.

**Visualization of protein prenylation**

Visualization of protein prenylation in sympathetic neurons was performed as previously described (Gao and Hannoush, 2014), with a few modifications. Briefly, sympathetic neurons grown in mass cultures or compartmentalized cultures were deprived of NGF for 36 hr, and then treated with propargyl-farnesol (isoprenoid analog, 25 μM) in the presence of NGF (50 ng/ml), NGF + GGTI-2133 (75 nM) or anti-NGF (1:1000) for 4 hr. After treatments, cells were washed with ice-cold PBS and fixed at room temperature for 10 minutes in 4% PFA/ in Cytoskeleton Buffer supplemented with Sucrose (CBS; 10mM MES pH 6.1, 138 mM KCl, 3mM MgCl, 2mM EGTA, 0.32 M sucrose). After PBS washes, neurons were permeabilized with 0.1% Triton in PBS for 2 min followed by extensive PBS washes to remove detergent. A Click-IT cocktail containing 1 mM CuSO₄, 250 μM
Biotin-Azide, and 1 mM TCEP (Tris(2-carboxyethyl) phosphine hydrochloride)) in 100 mM phosphate buffer pH 7.2 (PB) was added to each coverslip and incubated for 1 hr in the dark. Coverslips were thoroughly washed and blocked in 1%BSA/5% goat serum/0.1% Triton X-100 in PBS for 1 hr and then incubated overnight with anti-β-III-tubulin at 4°C. Cells were washed with PBS and incubated for 1.5 hr with Streptavidin-Alexa-488 and secondary antibody in blocking solution at room temperature. To visualize axonal growth cones, Phalloidin-Alexa-546 labeling (1:50) was done during secondary antibody incubation. Neurons were then mounted in Aqueous Mounting Medium containing 100 μg/ml DAPI. Images were acquired using a Zeiss LSM 700 confocal scanning microscope.

Explant cultures were grown for 4-5 days in culture, and cell bodies surgically removed using a scalpel. Isolated axons were treated with propargyl-farnesol (isoprenoid analog, 25 μM) in the presence of NGF (50 ng/ml), NGF + cycloheximide (CHX, 25 μM) or anti-NGF (1:1000) for 6 hr. Axons were then fixed, permeabilized, and newly prenylated proteins visualized as described above. β-III-tubulin immunostaining was performed to visualize axons. An area corresponding to 1 mm² of explants was imaged using an LSM 700 confocal microscope. Images represent z-projections that were 1.8 μm thick; all images were taken at the same intensity.

Biochemical assay for prenylated Rac1

SCG explant cultures were grown for 4-5 days, after which cell bodies were excised, and isolated axons incubated with isoprenoid analog (25 μM) in the
presence of NGF (50 ng/ml), NGF + GGTI-2133 (75 nM) or NGF + CHX (25 μM) or anti-NGF (1:1000) for 6 hr. Axons were lysed in 0.1% CHAPS/150 mM KCl/50 mM HEPES buffer and sonicated on ice. Lysates were concentrated using a 10 kDa Amicon Ultra Filter (Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane). Rac1 was immunoprecipitated using mouse anti-Rac1 (1μg) for 4 hr at 4°C. Protein G-agarose beads (Santa-Cruz, sc-2002, 40 μl) were added and sample incubated for 4 hr at 4°C. After washes with lysis buffer, beads were resuspended in PB and Click-IT reaction performed at 4°C for 1 hr to conjugate TAMRA-PEG azide to the isoprenoid group, as previously described (Nishimura and Linder, 2013). Immunoprecipitates and supernatants were immunoblotted using TAMRA and Rac1 antibodies, respectively. Rac1 immunoblots were stripped and re-probed for p85 for protein normalization. All immunoblots were visualized with ProSignal Dura ECL Reagent (Genesee Scientific, 20-301) and scanned with a Typhoon 9410 Variable Mode Imager (GE Healthcare).

GGTase I enzymatic assay

Compartmentalized sympathetic neurons were stimulated with NGF (50 ng/ml) applied to distal axons for 30 min, after 48 hr of NGF deprivation. Cell body and axon lysates prepared in 0.2% octyl-β-D-glucopyranoside, 50 mM Tris-HCl (pH 7.5) were incubated with 50 μM ZnCl₂, 5 mM MgCl₂, 20 mM KCl, 10 μM dansyl-GCVLL peptide (synthesized by the Johns Hopkins Synthesis and Sequencing Facility) and 10 μM geranylgeranyl pyrophosphate (GGPP, Echelon Biosciences). GGTase I activity was measured by an increase in fluorescence at 460 nm using
a Tecan Infinite 200 plate reader (Pickett et al., 1995). GGTase I activity was normalized to total protein using a Pierce™ BCA Protein Assay Kit (ThermoFisher, 23225).

**Statistical analyses**

Sample sizes were similar to those reported in previous publications (Bodmer et al., 2011; Yamashita et al., 2017). Data were collected randomly. All Student’s t tests were performed assuming Gaussian distribution, two-tailed, unpaired, and a confidence interval of 95%. One-way or two-way ANOVA analyses with post hoc Tukey test were performed when more than two groups were compared. Statistical analyses were based on at least 3 independent experiments, and described in the figure legends. All error bars represent the standard error of the mean (s.e.m).
### Table 3-1 Primer Sequences:

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CHAPTER 4: IDENTIFYING THE NGF-REGULATED PRENYLOME IN SYMPATHETIC NEURONS
INTRODUCTION

We found that NGF acutely regulates prenylation of newly synthesized Rac1 GTPase in sympathetic axons. Further, Rac1 in axons is required for NGF-mediated axon growth. Potentially, NGF-promoted prenylation presents a mechanism for spatially restricting signaling adaptors to axons to promote local growth events such as axon extension, navigation, branching, and pruning. While approximately 1% of the mammalian proteome is predicted to undergo prenylation based on bioinformatic analyses, only a small number of proteins have been biochemically characterized (Resh, 2013; Wang and Casey, 2016).

Historically, identification of prenylated and other lipid modified proteins relied on feeding of [H3+]-fatty acid precursors to label endogenous substrates. However, issues with radiolabel sensitivity and stability due to long exposure times made proteomic screens difficult (Gao and Hannoush, 2018). Recent advances in biorthogonal labeling of proteins has revolutionized the ability to investigate protein lipidation. The development of fatty acid analogs for use in copper (I) catalyzed Click-iT chemistry has permitted the identification of many proteins that undergo the lipid modifications palmitoylation and myristylation (DeGraw et al., 2010; Gao and Hannoush, 2018; Thinon et al., 2014). Click-iT reactions involve Cu^{1+} catalyzed formation of an amide bond between an alkyne and azide containing molecules with the lipid analog preferentially an alkyne and the recoverable tag an azide to reduce background (Gao and Hannoush, 2018; Hannoush and Sun, 2010). A recent study developed a cell-permeable alkyne isoprenyl analog that could act as a substrate for both FTase and GGTase I to label endogenous
proteins (DeGraw et al., 2010). To biochemically identify GGTase I substrates modified in response to NGF, we performed metabolic labeling with the isoprenoid analog in live sympathetic neurons, followed by conjugation of labeled prenylated proteins to an affinity tag for purification using click chemistry for mass spectrometry. As proof-of-principle, we show labeled Rac1 can be recovered following Click-iT conjugation to photocleavable agarose beads. Together, this approach provides a means to characterize the neuronal prenylome that is responsive to NGF.
RESULTS

NGF promotes prenylation of a wide diversity of proteins in neurons.

To identify NGF-induced prenylated proteins, we sought to optimize a Click-It labeling assay in neurons given the limiting starting material in neurons compared to previous studies done in cell lines (Gao and Hannoush, 2018). Sympathetic neuron cultures containing ~250,000 cells were incubated with a published isoprenyl-analog and acutely treated with NGF for 30 minutes. The isoprenyl-analog is a 15-carbon alkyne that acts as a dual substrate for the prenyltransferases (DeGraw et al., 2010; Hosokawa et al., 2007). A substitution of a hydroxyl group for the terminal diphosphate makes the isoprenyl analog cell permeant (DeGraw et al., 2010). Neurons were lysed in 0.1% CHAPS buffer and protein concentrated to $\geq 0.5$ mg/mL followed by biorthogonal labeling with a TAMRA fluorophore for in gel analysis of prenylation by fluorescence. Prenylation was detected in the absence of NGF demonstrating that a basal level of lipidation occurs (Figure 4-1A). However, NGF treatment enhanced prenylation of several proteins in sympathetic neurons, which was blocked by treatment with the GGTase I inhibitor, GGThI-2133 (Johnson et al., 2004). Intriguingly, most of NGF-induced prenylation was detected in the 20-37 kDA weight range, corresponding to the expected sizes of small GTPases (Figure 4-1A). This data suggests the majority of NGF-induced prenylation represents geranylgeranylation of small molecular weight proteins.

For recovery of prenylated proteins, we opted to conjugate alkyne-labeled proteins to a photocleavable azide-resin (agarose-azide beads) via click chemistry
The use of cleavable agarose-azide resin is preferable to the more classical biotin-streptavidin affinity purification since the former allows for cleavage of intact labeled proteins from the resin under milder conditions (irradiation with UV light at 365 nm) (Sibbersen et al., 2014). This method also has less contamination from endogenous biotinylated proteins and is substantially cheaper (Sibbersen et al., 2014; Szychowski et al., 2010). To test recovery, we incubated PC12 cells, an NGF-responsive cell line, with the isoprenyl-analog followed by NGF stimulation for 1 hour. We then performed the Click-iT reaction with the azide agarose beads. After extensive washes, prenylated proteins was recovered by exposure to UV light for 20 minutes (Figure 4-1B). Immunoblotting of eluted proteins with an anti-Rac1 antibody demonstrates that we can effectively label and recover prenylated Rac1 after 20 minutes of UV irradiation (Figure 4-1C). These results provide proof-of-principle that we have a working method for isoprenoid affinity tagging and recovery of prenylated proteins. This can be used to identify NGF-mediated prenylation in neurons in future studies.
DISCUSSION

Localized signaling is essential for the development, function, and maintenance of the nervous system. Spatial regulation of signaling pathways contributes to NGF-mediated axon growth and branching, synaptic establishment and maintenance, and survival (Barford et al., 2017; Yamashita and Kuruvilla, 2016). How NGF is able to recruit unique proteins to specific neuronal domains remains unclear. Local protein prenylation presents an attractive candidate for spatial regulation of NGF-signaling as it mediates protein localization and protein-protein interactions of several signaling, trafficking, and cytoskeletal molecules. However, the identity of proteins undergoing NGF-directed prenylation remain to be determined.

Here we described a methodology based on biorthogonal labeling using Click-iT, chemistry to label prenylated proteins in sympathetic neurons grown in vitro. Many of the proteins prenylated in response to NGF are in the 20-37 kDa size range. Further, NGF-induced prenylation is inhibited by GGTI-2133, suggesting the majority of prenylation in response to NGF is geranylgeranylation. These findings emphasize the central notion that geranylgeranylation is highly regulated by extrinsic signals (Luo et al., 2003; Zhou et al., 2008). Additionally, we demonstrate successful recovery of geranylgeranylated Rac1 using a photocleavable bead system. This methodology can be used for an unbiased screen to characterize the prenylome of proteins modified in response to NGF, which may provide further insight into mechanisms acting downstream as well as identify novel neuronal prenylated proteins. These studies will guide future studies to
investigate the prenylation of individual proteins in the development, maintenance, and regeneration of the nervous system. As many neurological diseases are associated with deregulation of lipid modification pathways, characterization of the neuronal prenylome could additional identify novel therapeutic targets for treatment of neurological disorders. With innovations in Click-iT chemistry making it possible to study lipid modifications in whole organisms, the information generated from future work could be applied to studying the prenylation of novel therapeutic targets in neurodegenerative disease models (Berry et al., 2010; Chang et al., 2010).
**Figure 4-1 Screen for identification of NGF-regulated prenylation.** A NGF acutely promotes prenylation of proteins. Mass cultures of sympathetic neurons were acutely treated with 50 ng/mL NGF for 30 minutes following withdrawal in the presence of an isoprenyl-analog. Newly modified protein identified through conjugation of a TAMRA fluorophore and in gel fluorescence following SDS-PAGE. Incubation with GGTI-2133 reduced NGF-induced prenylation (n=3). B Schematic for prenylation screen in neurons using Click-IT mediated conjugation to agarose beads. C Recovery of Rac1 following Click-iT catalyzed conjugations to agarose
beads. PC12 cells acutely stimulated with 50 ng/mL NGF for 30 minutes in presence or absence of isoprenyl-analog were conjugated to agarose and irradiated with 365 nm UV light to release protein from beads. Analyzed by SDS-Page and immunoblotting for Rac1 from input lysate and pulldown.
METHODS:

Animals:
All procedures relating to animal care and treatment conformed to Johns Hopkins University Animal Care and Use Committee and NIH guidelines. Animals were housed in a standard 12:12 light-dark cycle. Postnatal day P0.5 – 6 (P0.5-P6) pups of both sexes were used for analyses. Pregnant Sprague Dawley rats were obtained from Charles River or Taconic.

Neuronal Cultures:
Sympathetic neurons were harvested from P0.5 Sprague-Dawley rats or Rac1<sup>fl/fl</sup> and grown as a dissociated for mass culture as previously described (Bodmer et al., 2011). Neurons were maintained with high-glucose DMEM media supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (1U/mL), and NGF (100 ng/mL). NGF was prepared from male mouse submaxillary glands as previously described (Mobley et al., 1976). For immunochemistry, cells were grown on coverslips coated with poly-d-lysine (1 µg per mL; Sigma-Aldrich) and laminin (10 µg per mL; Sigma-Aldrich). Deprivation of NGF was performed in high-glucose DMEM supplemented with 1% FBS with 1:1000 anti-NGF and 50 µM boc-aspartyl(O-methyl)-fluoromethylketone (Baf) for 36 hours.

Labeling of prenylated proteins for Western Blot and Mass Spec Analysis.
Cells were incubated overnight in 25 µM GGOH in 1%FBS plus Baf, or for axonal analysis, for 2 hours in low serum. Cells were stimulated for 8 hours with 50 ng/mL NGF (with or without 75 nM GGTL-2133) or anti-NGF in the presence of 25 µM GGOH. Protein was isolated from neurons, axons, or PC12 cells in .1% CHAPS/
150 mM KCl/ 50 mM HEPES buffer through sonification. Protein was used at a concentration of 1 mg/mL. For identification of proteins through mass spectrometry, protein lysate was incubated in a Click-iT cocktail containing 1 mM CuSO$_4$, 250 μM Agarose-Azide (Szychowski et al. 2010; Sibberson et al. 2014), 100 μM THPTA and 1 mM TCEP in 100 mM PB buffer at 4°C on undulating rocker for 1-2 hours (Nishimura and Linder, 2013). Resin was washed 3 times with lysis buffer and proteins released from beads using 365 nM UV light for 20 minutes. Protein was separated on 12% acrylamide gel and detected through Coomassie staining or Western Blot using Rac1 (1:2000) and alpha-tubulin (1:10,000) as a loading control. For in gel fluoresce, 200 μM TAMRA-Azide (Sigma) and protein recovered using ProteoExtract before being loaded onto gel. TAMRA signal was normalized to total protein signal as detected by SYPRO-staining.

Statistical analyses

Data are expressed as average ± SEM. One-way ANOVA was used as indicated to test for statistical significance using GraphPad Prism. Significance was placed at P<0.05 unless otherwise noted.
Chapter 5: CONCLUSIONS
Concluding Remarks:

Neurons require exquisitely regulated spatial signaling for the establishment, maintenance, and function of neuronal compartments. Mechanisms responsible for the localization and retention of proteins to form these specialized cellular compartments remain poorly characterized. Asymmetric distribution of mRNA in neurons is proposed to underlie specialization of neuronal compartments through local protein expression (Riccio, 2018; Terenzio et al., 2017). Here we report a non-coding function for the axon-enriched mRNA, \(\text{Tp53inp2}\) in promoting NGF-dependent axon growth. \(\text{Tp53inp2}\) is essential for NGF-mediated axon growth and branching during development. Loss of \(\text{Tp53inp2}\) attenuates NGF signaling and endocytosis of TrkA locally in axons. Interestingly, NGF induced the recruitment of \(\text{Tp53inp2}\) to TrkA receptors, suggesting that the mRNA may function as a scaffold to recruit TrkA signaling and endocytic effectors at the cell surface.

Prenylation regulates protein localization and formation of protein-protein complexes to create specialized signaling domains in cells (Wang and Casey, 2016). While prenylation is traditionally thought of as a ubiquitous house-keeping mechanism, we find a compartment-specific requirement for geranylgeranylation in axons for NGF-directed growth. Further, NGF acutely enhances the activity of GGТase I in axons demonstrating prenylation is an acutely regulated process. The increase in GGТase I activity is required for modification of locally synthesized proteins, including Rac1, to promote axon development. Many other transcripts encoding for known prenylated proteins are present in the axons (Gumy et al.,
We developed a methodology for a proteomic screen to identify NGF-induced prenylated proteins. Together, these two studies demonstrate NGF mediates diverse mechanisms of spatial signaling to promote axonal growth during neuronal development.

**Mechanisms of *Tp53inp2* regulation of NGF-signaling**

Neurotrophin signaling regulates the recruitment of diverse mRNAs to axons for growth, branching and signaling (Terenzio et al., 2017; Willis et al., 2007). Axon guidance in response to navigational cues requires spatial and temporal control over the distribution of the proteins produced by intra-axonal synthesis for axon elongation and branching (Leung et al., 2006; Shigeoka et al., 2013). The mechanisms responsible for establishing asymmetric protein distribution locally in axons are still under investigation. It was recently discovered the 3'UTR of mRNA can act as a scaffold to facilitate protein complex formations immediately following local protein synthesis to promote trafficking of newly synthesized proteins to specific cellular domains (Berkovits and Mayr, 2015). The long 3'UTR of the transmembrane protein CD47 interacts with the RNA binding protein, HuR, and SET. Recruitment of SET to active translational sites permits immediate interactions with newly translated CD47 to facilitate its transport to the plasma membrane (Berkovits and Mayr, 2015). *Tp53inp2* interacts with TrkA receptors and the RNA binding protein, HuD. Therefore, *Tp53inp2* may similarly function as a scaffold to facilitate the assembly of protein complexes between TrkA and locally synthesized effectors necessary for TrkA signaling and trafficking.
Interestingly, HuD also binds and stabilizes $\beta$-actin (Kim et al., 2015), whose local synthesis at the leading edge of growth cones promotes turning in response to attractive navigational cues (Donnelly et al., 2013; Leung et al., 2006; Zhang et al., 1999). Potentially, $Tp53inp2$ is in RNA granules containing mRNAs that encode for factors required for axon growth. Interaction between $Tp53inp2$ with TrkA would recruit these mRNA granules to subcellular domains of active NGF-signaling for local protein synthesis of transcripts regulating axon elongation and navigation.

Another potential function for $Tp53inp2$ is it could act as a “molecular sponge” for the regulation of axonal micro-RNAs. Initial work in zebrafish demonstrated an essential role for Dicer-mediated maturation of microRNA in axonal pathfinding (Hancock et al., 2014). Screens to characterize axon localized miRNA led to the identification of over 100 miRNAs, many of which regulated genes involved in energy metabolism, axon elongation and branching (Wang and Bao, 2017). Recently, the long non-coding RNA, BC048612, was found to bind miR-203 to regulate expression of the neurite promoting gene neuronal growth regulator 1 (Kaur et al., 2016). It will be interesting to determine whether $Tp53inp2$ contains binding domains for known axon miRNAs and identify the genes regulated downstream. Additionally, employment of proximity ligation assays would provide insight into RNA and protein interaction partners of $Tp53inp2$ to further elucidate its role in NGF-TrkA dependent signaling.

**GGTase I activity as a spatial regulator of receptor tyrosine kinase signaling.**
NGF-directed axon guidance and branching requires recruitment of Rho-GTPases to distinct microdomains for growth cone navigation (Buck and Zheng, 2002). One major question is how proteins are restricted to subcellular sites to promote directional growth in response to extrinsic signals. GGTase I activity in axons modified the known NGF-effector, Rac1, to promote growth in response to NGF. Additionally, NGF signaling acutely regulated the activity of GGTase I. Since prenylation regulates the localization and function of proteins (Afshordel et al., 2014; Reddy et al., 2015; Wang and Casey, 2016), these observations suggest a positive-feedback mechanism by which NGF actively promotes the modification and recruitment of downstream effectors for propagation of NGF signaling. Sustained NGF signaling would maintain elevated activity of GGTase I. Given the significance of spatial regulation in NGF-mediated processes, it is interesting to speculate that activated GGTase I likely localizes to NGF-TrkA complexes to spatially restrict the production of newly modified proteins.

How could NGF-TrkA signaling direct the recruitment of GGTase I to active TrkA receptors? One explanation is that phosphorylation of GGTase I, downstream of NGF-signaling, promotes its association with TrkA receptors. Phosphorylation of tyrosine residues located in GGTase1-α was observed downstream of MuSK-Agrin signaling (Luo et al., 2003). Interestingly, the tyrosine residue Y200 in GGTase1-α was required for GGTase I recruitment to MuSK receptors. Localization of GGTase I promoted Agrin-MuSK directed clustering of acetylcholine receptors to form the neuromuscular junction (Luo et al., 2003). Similarly, phosphorylation of Y166 on GGTase1-α promoted localization of
GGTase I to TrkB receptors (Zhou et al., 2008). Together, these studies imply phosphorylation of tyrosine residues promote the recruitment of GGTase I to active receptors. The residues Y166 and Y200 are also known to regulate the activity and substrate binding of the prenyltransferase (Wu et al., 1999). These specific tyrosine residues in GGTase1-α are predicted phosphorylation sites for receptor tyrosine kinases or the downstream tyrosine kinase Src (Blom et al., 1999); therefore, these may also be the sites by which NGF-TrkA signaling regulates GGTase I activity. Phosphorylation has also been reported on serine and threonine residues in GGTase1-α downstream of the MAPK-pathway (Goalstone et al., 1997). Characterization of the function of these phosphorylation sites will provide insight into how these residues regulate prenyltransferase activity downstream of extrinsic signals. Additionally, these studies identified phosphorylation on the common alpha-subunit that is shared by FTase and GGTase, raising the question as to whether extrinsic signaling independently regulates each prenyltransferase. If so, this would further suggest an unappreciated regulation of the prenylation machinery in cells.

**Prenylation as a sorting mechanism for TrkA endosomes?**

Upon NGF activation, TrkA signaling recruits effectors for internalization of the NGF-TrkA complex to form signaling endosomes in the axon (Harrington et al., 2011; Yamashita and Kuruvilla, 2016). NGF-TrkA signaling endosomes are highly heterogenous comprising of coated and un-coated vesicles, and multi-vesicular bodies (Bhattacharyya et al., 2002; Claude et al., 1982; Deinhardt et al., 2006;
Delcroix et al., 2003). Only a small percentage of NGF-TrkA endosomes are retrogradely trafficked to the cell body; but, it is not known what designates axonal NGF-TrkA endosomes for retrograde transport. The identity of the prenyl motif on recruited NGF-effectors may direct the sorting of TrkA signaling endosomes. GGTase I can efficiently promote the farnesylation or geranylgeranylation of RhoB (Armstrong et al., 1995). Farnesylated RhoB directs internalized epidermal growth factor (EGF) receptor to the lysosome for degradation. In contrast, geranylgeranylated RhoB promotes the retention of EGF receptors in multi-vesicular vesicles for recycling back to the membrane (Wherlock et al., 2004). Interestingly, multi-vesicular bodies comprise the majority of retrogradely trafficked Trk-signaling endosomes (Claude et al., 1982; Kononenko et al., 2017; Wang et al., 2016; Ye et al., 2018). Whether RhoB or another protein that can undergo alternative prenylation act to sort NGF-TrkA into distinct vesicles is an open question (Benetka et al., 2006; Berndt et al., 2011; Kinsella et al., 1991).

**Functions of the CaaX motif**

Prenylation is essential for the maturation and function of its modified proteins (Resh, 2013). As we observed when we mutated the CaaX motif of Rac1, mutations to prevent protein prenylation phenocopy loss of function of the modified protein (Jung et al., 2013; Mulligan et al., 2010). How prenyl motifs contribute to the function of modified proteins is poorly understood. Failure to localize to the plasma membrane or internal membrane compartments is often used to explain why prenylation mutants fail to elicit normal cellular functions. However, this infers
membrane targeting alone is sufficient for protein activity. Modification of proteins by either farnesylation or geranylgeranylation drives localization of proteins to distinct membrane compartments due to the unique lipid affinities of the 15-carbon farnesyl and 20-carbon geranylgeranyl (Wherlock et al., 2004; Zhou et al., 2017). The difference in membrane location can alter the cellular processes regulated by the prenylated protein (Wherlock et al., 2004). Protein localization in different membrane compartments could promote different protein-protein interactions based on protein proximity and/or direct interactions with the prenyl-motif (Berg et al., 2010; Chandra et al., 2011; Ismail et al., 2011). The composition of these protein complexes would then regulate the activity, stability, or trafficking of the prenylated proteins to promote distinct cellular events. Interestingly, many of the small GTPase paralogs, such as HRas, KRas, and NRas, share high protein sequence homology except at the C-terminus. The hypervariable C-terminus contains different prenylation motifs and secondary modifications that can alter membrane localization (Baier et al., 2014; Prior and Hancock, 2012; Sun et al., 2007). This suggests changes in post-translational modifications helped evolutionarily drive the functional specialization of these proteins. Rigorous characterization of how an individual protein’s prenyl-modification contributes to the localization and protein complex formation will contribute to the understanding of how protein function is specialized and regulated.
References


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