Insight into the Brain-Specific Alpha Isoform of the Scaffold Protein SH2B1 and its Rare Obesity-Associated Variants

By

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Abstract

Obesity poses a major health problem since it increases the risk for type 2 diabetes, metabolic syndrome, heart disease, and cancer. Mutations in SH2B1 have been identified in patients exhibiting severe childhood obesity and insulin resistance. Mice deficient in SH2B1 exhibit a similar phenotype to the patients, suggesting an important role for SH2B1 in regulating energy homeostasis. SH2B1 is alternatively spliced, leading to four isoforms (alpha, beta, gamma, and delta) that share an N-terminal 631 amino acids but have unique C-terminal tails. The findings that all SH2B1 isoforms are expressed in the brain and neuronal expression of SH2B1 beta rescues the obese phenotype in SH2B1-deficient mice suggest that SH2B1 plays an important function in neurons. In further support of a neuronal function for SH2B1, SH2B1 enhances neurotrophic factor-induced neurite outgrowth and the human obesity-associated mutations impair the ability of SH2B1 to promote neurite outgrowth. To gain insight into critical functions of SH2B1, its isoforms, and the human obesity-associated mutations, the work in this thesis characterizes the ability of rare SH2B1 mutations associated with human obesity both shared by all isoforms and those specific to SH2B1 alpha to affect cellular actions of SH2B1. It further examines how the unique C-terminal tails of SH2B1 regulate the actions of the shared 631 N-terminal amino acids of SH2B1.

Our collaborator I. S. Farooqi identified variants in *SH2B1* encoding for R227C, G238C, R270W, E299G, or T546A and the SH2B1 alpha-specific mutations A663V,

V695M, or A723V in individuals with severe-early onset obesity and insulin resistance. We determined that like SH2B1 beta, SH2B1 alpha enhanced growth hormone-stimulated motility of macrophages and the mutations T546A, A663V and A723V impaired that enhancement. Unlike SH2B1 beta, SH2B1 alpha was unable to enhance NGF-mediated neurite outgrowth and the mutations in SH2B1 alpha had no impact. However, mutations G238C, R270W, E299G and T546A impaired SH2B1 beta-enhancement of NGF-induced neurite outgrowth. This led us to conclude that variants can disrupt specific isoform function, suggesting novel regulatory roles in SH2B1 isoform function. The discrepancy between SH2B1 alpha and beta regulation of neurite outgrowth directed us to ask how the unique C-terminal tails of the alpha and beta isoforms affect the ability of SH2B1 to regulate NGF-induced neurite outgrowth. By comparing the actions of SH2B1 alpha and beta to those of the N-terminal 631 amino acids shared by all isoforms of SH2B1, we found that the alpha tail prevents: 1) the ability of SH2B1 to cycle through the nucleus, and 2) SH2B1 enhancement of NGF-induced neurite outgrowth, gene expression, and phosphorylation of NGF-receptor, TrkA, and downstream signaling proteins Akt and PLC gamma. These functions were restored when Tyr753 in the alpha tail was mutated to Phe, suggesting that phosphorylation of Tyr753 regulates SH2B1 alpha function. We provide evidence that TrkA phosphorylates Tyr55 and Tyr439 in both SH2B1 alpha and SH2B1 beta, and Tyr753 in SH2B1 alpha. Finally, co-expression of SH2B1 alpha inhibits the ability of SH2B1 beta to enhance NGF-induced neurite outgrowth. These results suggest that the C-terminal tails of SH2B1 isoforms are key determinants of their cellular roles and are regulated by phosphorylation. By providing valuable information about how

seemingly minor differences between isoforms can have a profound impact on the function of that protein, my studies also provide important insight into the impact of differential splicing on neuron function.

Chapter 1

Introduction

Chromosomal deletions and variants within *SH2B1* promote obesity

Obesity affects an ever-growing and alarming percentage of adults in developed countries. The prevalence of obesity in the United States is high, with 35% of adults and 17% of youth (ages 2 through 19) considered obese (1). Obesity poses a major health problem since it increases the risk for such diseases as type 2 diabetes, metabolic syndrome, heart disease, and cancer. Obesity has been described as an imbalance of energy intake to energy output. According to the Centers for Disease Control, this imbalance results from many factors including environmental, social, behavioral, and physiological factors, mental and physical disabilities, drugs and genetic predisposition. Thus, insight into novel factors that regulate human obesity are of obvious health relevance. The gene encoding the scaffolding protein SH2B1 (SH2-B; PSM) is one such novel factor.

SH2B1 encodes for the scaffold protein SH2B1, a member of a family of SH2 domain-containing scaffold proteins along with SH2B2 (APS) and SH2B3 (LNK), all of which bind to phosphotyrosines in specific receptor tyrosine kinases (RTKs) and cytokine receptor-associated JAK kinases. *SH2B1* is composed of 10 exons; four SH2B1 isoforms (α, β, γ, δ) are generated through alternative splicing by utilization of

two splice sites in exon 8 and exon skipping or inclusion of exon 9. The four isoforms share an N-terminal region but have unique C-terminal tails that diverge after the SH2 domain and differ in both amino acid sequence and length (Figure 1.1) (2-5). The isoforms have been shown to be expressed in pairs of α and δ or β and γ, in both rodents and human (2,6). All four isoforms of SH2B1 are expressed in the human brain; the ubiquitously expressed β and γ isoforms are also expressed in non-neuronal tissues (6). All four isoforms of SH2B1 contain several proline-rich domains, a phenylalanine zipper dimerization domain, a nuclear localization signal and nuclear export signal, a pleckstrin homology (PH) domain and a Src homology 2 (SH2) domain (Figure 1.1).

Variants at loci in or near *SH2B1* have been associated with obesity based on genomic wide association studies (GWAS) (7-9). These studies using a cohort with obese individuals from primarily European descent identified variants at or near a region containing the *SH2B1* locus and other genes. *SH2B1* and the other genes are expressed in the brain, including the hypothalamus (7). In the central nervous system (CNS), the hypothalamus regulates food intake and energy homeostasis. Several more GWASs have recapitulated these results in non-European populations to further establish that these variants at or near loci containing *SH2B1* are associated with obesity (10,11). Individuals with deletions of a region at chromosome 16p11.2 that encompass the *SH2B1* locus exhibit severe early-onset obesity (12-15). They also exhibit severe insulin resistance that is disproportionate for the degree of obesity, and developmental delay (12-15). Our collaborator, I. Sadaf Farooqi (University of Cambridge) has now identified more than 20 patients with mutations in *SH2B1* in a cohort of patients from the Genetics of Obesity Study (GOOS) that exhibit severe early-

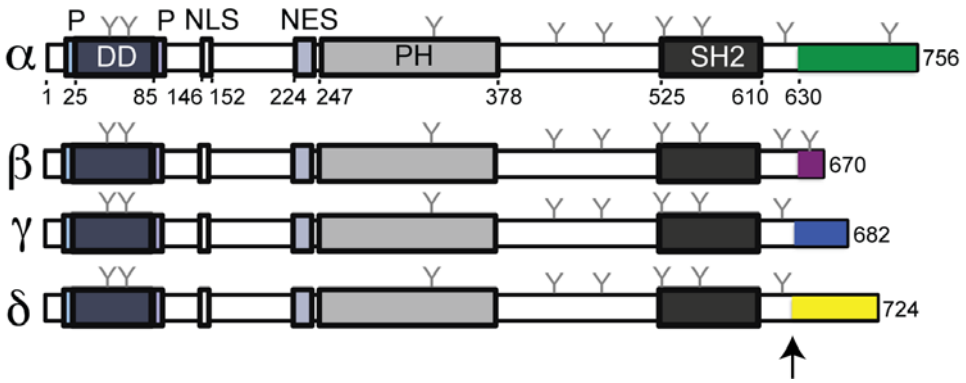

Figure 1.1. Schematic of the four human SH2B1 isoforms. Shown are the proline-rich regions (**P**), dimerization domain (**DD**), nuclear localization signal (**NLS**), nuclear export signal (**NES**), pleckstrin homology domain (**PH**), Src-homology 2 domain (**SH2**), and tyrosine sites (**Y**). Unique C-terminal tails are depicted in color.

onset childhood obesity and insulin resistance (6).

The GOOS was established in 1997 to determine how individuals at an early age (<10 years old) gain weight. Since most children are not pathologically obese prior to 10 years of age, studying obesity at an early age enhances the ability to identify individuals with a genetic predisposition to obesity. As of 2002, the consortium had recruited over 4300 severely obese children to the obesity study and discovered mutations that disrupt the leptin neuro-endocrine signaling pathway in 8% of their cohort (16). To determine whether *SH2B1* variants associate with severe-early onset obesity, our collaborator, I. Sadaaf Farooqi selected from the GOOS, a cohort of 300 obese children (≤ 10 years old) with a phenotype similar to that of the 16p11.2 gene deletion patients (BMI SDS of ≥ 3, and disproportionate degree of insulin resistance as defined by the presence of type 2 diabetes and/or markedly elevated plasma insulin levels) and screened them for mutations in *SH2B1*. Four non-synonymous mutations were identified in *SH2B1* codons encoding for amino acids P90, T175, P322, and F344 (6). Three of the mutations caused single amino acid changes (P90H, T175N, P322S) and the fourth mutation caused a frame shift and premature termination before the SH2 domain (F344LfsX20) (6). Carriers were all hyperphagic, and when both phenotype and genotype information of family members were available, mutations were usually found to be inherited from overweight/obese parents. Surprisingly, these identified patients, but not the parents, exhibited maladaptive behavior (social isolation, aggression). Studies using a different cohort of obese patients (17), identified a rare variant associated with obesity in children and adolescents in exon 9 of SH2B1, an exon only present in the β and γ isoforms of SH2B1 (Figure 1.2). This non-synonymous mutation changed Thr656 to Ile in SH2B1β

and Pro674 to Ser in SH2B1γ (17). Taken together, the GWAS, gene deletion and rare variant data suggest that SH2B1 helps protect against obesity. As described in Chapter 2, additional mutations have been identified in the unique C-terminal tails of *SH2B1* (Figure 1.2).

Mice lacking SH2B1 (*Sh2b1*$^{-/-}$) have a similar phenotype to that seen in humans with mutations in *SH2B1* or chromosomal deletions encompassing *SH2B1*. *Sh2b1*$^{-/-}$ mice, on either a 129Sv/C57BL/6 or C57BL/6 background, weighed significantly more than their wild-type (WT) littermate controls after 10 weeks of age when fed a 9% fat standard chow (18). *Sh2b1*$^{-/-}$ mice exhibited hyperphagia, hyperleptinemia, insulin resistance and hyperglycemia (18,19). However, using a different group of *Sh2b1*$^{-/-}$ mice on a 129sv/C57BL/6 background Ohtsuka, et al. (20) reported no obesity in their *Sh2b1*$^{-/-}$ mice assessed at 4- and 6- weeks of age. This apparent discrepancy may be due to the age at which the Ohtsuka group assessed body weight, since the Rui lab found that *Sh2b1*$^{-/-}$ mice has normal weight at less than 6 weeks of age and further analysis revealed weight gain after 10 weeks of age in their *Sh2b1*$^{-/-}$ mice. These studies, in mice, show that SH2B1 is an important regulator of body weight in mice.

In addition to obesity, *Sh2b1*$^{-/-}$ mice exhibit behavioral and reproductive phenotypes. Like humans with mutations in *SH2B1,* male *Sh2b1*$^{-/-}$ mice are aggressive toward their littermates and animal handlers (L. Rui, University of Michigan; personal communication). Both male and female *Sh2b1*$^{-/-}$ mice also have impaired fertility exhibited by the lower than normal Mendelian ratio of *Sh2b1*$^{-/-}$ intercrosses (19,20). The impaired fertility in *Sh2b1*$^{-/-}$ females was attributed to small ovaries with reduces follicle numbers and small testes with low sperm number in *Sh2b1*$^{-/-}$ males (20). These

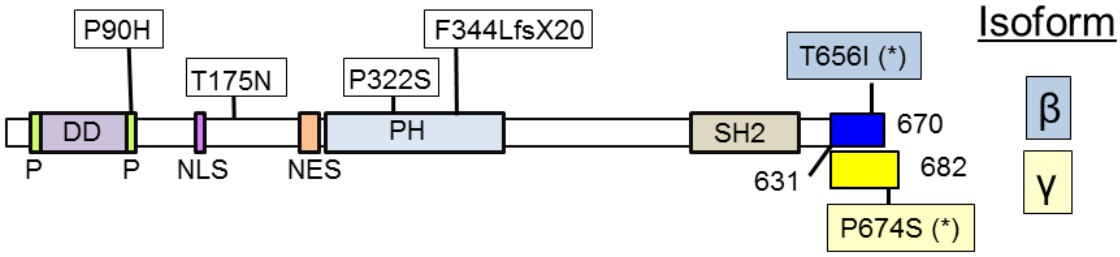

Figure 1.2. Schematic of SH2B1 isoforms and obesity-associated mutations identified in humans. P= Proline-rich region, DD = Dimerization Domain, NLS = Nuclear Localization Sequence, NES = Nuclear Export Sequence. Numbers = amino acids. The obesity-associated mutations are indicated. The T656I (*) mutations in the SH2B1β isoform and the P674S mutation in the SH2B1γ isoform originate from the same nucleotide variant.

additional phenotypes suggest that the role of SH2B1 extends beyond the regulation of energy homeostasis.

Collectively, these results in humans and mice suggest that SH2B1 is an important regulator of energy balance, insulin and leptin sensitivity.

The scaffold protein SH2B1 is implicated in metabolic and neuronal cell signaling

Cells respond to an environmental stimulus through cellular signaling pathways to attain a precise and suitable response. An extracellular signaling factor (e.g. hormone, growth factor) mediates signaling outputs within the cell by interacting with its receptor located in the cell's plasma membrane. These receptors include ligand-gated ion channels, G-protein coupled receptors, and protein kinase receptors, including receptor tyrosine kinases. In the case of receptor tyrosine kinases, signaling events are initiated by ligand to binding its receptor tyrosine kinase resulting in a conformational change which activates the kinase, thereby causing the kinase to auto-phosphorylate. The resulting phosphorylated tyrosines recruit proteins, including scaffold proteins, that contain SH2 or other phosphotyrosine binding domains to create a signaling complex that mediates downstream signaling. These signaling complexes mediate a variety of cellular responses, including an appropriate transcriptional response (21). Scaffold proteins, despite lacking enzymatic activity, can serve to recruit a unique multiprotein complex, therefore helping to create a diverse and individual response for a specific signal.

SH2B1 interacts with activated receptor tyrosine kinases through its SH2 domain to modulate signaling. Isoforms of SH2B1 have been found to interact with phosphorylated tyrosines in receptors for insulin (3,22), insulin-like growth factor (IGF-1) (23), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) (24), nerve

growth factor (NGF) (25,26), brain-derived neurotrophic factor (BDNF) (25,27), and glial-derived neurotrophic factor (GDNF) (28). In addition, SH2B1β was found to interact with multiple members of the Jak family of tyrosine kinases (Jaks 1, 2, and 3) (4,29,30). Jak kinases associate with members of the cytokine family of receptors and are activated by ligand binding to these receptors. Like SH2B1β, several of these receptors play critical roles in metabolism and energy expenditure, suggesting the importance of understanding how SH2B1 impacts their function.

The role of SH2B1 in leptin signaling

Leptin is an adipose tissue-derived hormone that regulates energy homeostasis by interacting with leptin receptor-containing neurons within the arcuate nucleus of the hypothalamus to decrease food intake (31,32). Humans with mutations in leptin and leptin-deficient mice are extremely obese due to their hyperphagia (33,34). In mice, mutations in the long form of the leptin receptor (LepRb) or deletion of the leptin receptor also result in severe obesity and hyperphagia (35,36). These phenotypes are similar to those found in humans with haploinsufficiency of, or point mutation, in SH2B1 and *Sh2b1*$^{-/-}$ mice. as previously discussed in section I of the introduction. SH2B1 is thought to enhance leptin signaling through its interaction with the leptin receptor-associated kinase, Jak2 (18,37-39). Leptin binding to LepRb activates Jak2, which phosphorylates LepRb on tyrosines 985, 1107, and 1138 (39). PhosphoTyr985 on LepRb has been implicated as a binding site for SHP2 (40) and/or SOCS3. PhosphoTyr1138 on LepRb recruits the transcription factor STAT3 (signal transducer

and activator of transcription 3) (39,40). In response to leptin, Jak2 autophosphorylation of Tyr1007 activates Jak2 (41). Phosphorylation of Tyr317 (42) and Tyr570 (43) inhibit Jak2 activity and Tyr637 stimulates Jak2 activity (42). In addition, Tyr221 on Jak2 is phosphorylated in response to leptin but a function for Tyr221 in leptin function has yet to be determined (44). Auto-phosphorylation of Jak2-Tyr813 has been reported to recruit SH2B1 via its SH2 domain, and enhance the activity of Jak2 (37). In addition, insulin receptor substrates (IRS) 1 and 2 are recruited to the LepRb-Jak2-SH2B1 complex where they are phosphorylated by Jak2 and in turn recruit and activate phosphatidylinositol 3-kinase (PI3K), leading to activation of signaling pathways downstream of PI3K (37,45). Consistent with these in vitro results, *Sh2b1*$^{-/-}$ mice are leptin resistant (18) and when wild-type and *Sh2b1*$^{-/-}$ mice are administered leptin, *Sh2b1*$^{-/-}$ mice display decreased tyrosine phosphorylation of hypothalamic IRS1 (18). These data suggest that SH2B1 is a critical signaling molecule for and positive regulator of leptin signaling.

The role of SH2B1 in insulin signaling

In addition to leptin signaling, SH2B1 has been shown to play a role in insulin signaling. Mouse models lacking *Sh2b1* have shown impaired insulin receptor activation and signaling in liver, skeletal muscle, and adipose tissue (19,46). This impaired signaling is thought to contribute to the hyperinsulinemia, hyperglycemia, and glucose intolerance seen in the *Sh2b1*$^{-/-}$ mice (19,46). SH2B1α and β isoforms have been shown to associate through their SH2 domain with phosphotyrosines within the kinase activation loop (Tyr1158, Tyr1162, Tyr1163) of the insulin receptor (3,47). In 293 cells,

co-expression of SH2B1β with insulin receptor enhances tyrosine phosphorylation of IRS1 and IRS2. While co-expression of SH2B1β R555E, which has a defective SH2 domain, with insulin receptor, shows reduced tyrosine phosphorylation of IRS1 and IRS2 (45). In addition, decreased insulin-induced Akt phosphorylation in liver and muscle from *Sh2b1*$^{-/-}$ mice and reduced insulin-stimulated phosphorylation of ERKs 1 and 2 in brown fat from *Sh2b1*$^{-/-}$ mice suggested that deletion of SH2B1 impairs the ability of insulin to stimulate phosphorylation of downstream signaling proteins (19). In addition, SH2B1 protects IRS1 from dephosphorylation by the tyrosine phosphatase PTP1B, suggesting SH2B1 may block the ability of IRS1 to interact with tyrosine phosphatases and thereby enhance and prolong tyrosyl-phosphorylation of IRS1 (48). Together these data suggest SH2B1 plays a significant role in insulin receptor activation and signaling important for glucose homeostasis.

Despite these studies, the role of SH2B1 in insulin signaling in the brain has yet to be explored. Several studies have shown insulin receptor expression in the hypothalamus, cerebral cortex, cerebellum, and hippocampus, which are regions of the brain associated with energy homeostasis and behavior (49,50). Administration of higher than physiological levels of insulin directly to the brain results in an inhibition of food intake and decreased body weight, whereas inhibition of insulin signaling increases food intake, resulting in higher body weight (51-53). These data suggest insulin signaling in the brain has a unique role in regulating energy homeostasis. Restoration SH2B1β expression in neurons in transgenic *Sh2b1*$^{-/-}$ mouse models largely rescued the insulin resistance, glucose intolerance and reduced tissue adiposity in *Sh2b1*$^{-/-}$ mice

(54), suggesting SH2B1 plays a critical role in neuronal insulin signaling regulating glucose homeostasis.

Effects of human obesity-associated mutations in SH2B1 on leptin and insulin signaling

It has yet to be determined how the human obesity-associated mutations in SH2B1 affect leptin and insulin signaling. The Carter-Su laboratory has found that introducing mutations associated with human obesity (P90H, A175N, and P322S) into SH2B1β did not alter SH2B1β enhancement of Jak2 activity when both were transiently expressed in 293T cells; nor did the mutations alter SH2B1β enhancement of leptin-induced phosphorylation of IRS2 in 293 cells stably expressing LepRb and transiently expressing IRS2 and SH2B1β (WT or mutant) (6). Similar results were reported for insulin-induced phosphorylation of IRS2 in 293 cells transiently overexpressing the various forms of SH2B1 and IRS2 (6). However, the artificially high level of expression of SH2B1 and IRS2 in the transfected 293 cells may have concealed any impairments induced by SH2B1 human obesity-associated mutations enhancement. Volckmar, et al. (17) similarly determined that the obesity variants T656I in SH2B1β and P674S in SH2B1γ had no effect on the ability of the respective SH2B1 isoforms to stimulate leptin-mediated activation of STAT3. Collectively, these data suggest that SH2B1 is a positive regulator of leptin signaling and that subtle impairments by SH2B1 human obesity-associated variants need to be further examined under more physiological conditions or in different assays.

The role of SH2B1 in neurotrophic factor signaling

In addition to its role in leptin and insulin signaling, the role of SH2B1 in mediating signal transduction pathways for several neurotrophic factors has been investigated. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) bind to their receptor tyrosine kinases TrkA, TrkB, and TrkC, respectively (55,56). Neurotrophic factors are critical for the differentiation and survival of developing neurons in the central and peripheral nervous systems (57). NGF binding to TrkA activates TrkA and causes TrkA auto-phosphorylation of tyrosines in both the kinase (Tyr670, Tyr674, and Tyr675) and non-kinase (Tyr490 and Tyr785) domains (58-60). Most studies looking at the effects of SH2B1 on neurotrophic factor signaling have been carried out in the rat adrenal pheochromocytoma cells (PC12), which have endogenous expression of TrkA and extend neurites in response to NGF (61,62). In response to NGF, SH2B1 has been shown to interact via its SH2 domain with phosphorylated tyrosines within the kinase domain of TrkA (25). The exact tyrosine responsible for recruiting SH2B1 remains to be determined. Overexpression of SH2B1α enhances NGF-mediated tyrosine phosphorylation of Tyr490 in TrkA (63). Activation of TrkA in PC12 cells also results in phosphorylation of SH2B1 on tyrosines as well as serines and threonines suggesting that kinases, like ERK1 and ERK2, or other downstream kinases are important for NGF-stimulated neurite extension (64). Prolonged phosphorylation of ERK1 and ERK2, is critical for NGF-mediated neurite outgrowth (65-67). Overexpression of SH2B1α(25) or SH2B1β (26) enhances NGF-induced neurite outgrowth in PC12 cells suggesting that SH2B1 may enhance energy

balance at least in part by enhancing formation of the neuronal circuits involved in feeding behavior.

NGF-mediated activation of TrkA and recruitment of SH2B1 presumably promotes the recruitment of signaling molecules that elicit or regulate downstream signaling pathways that regulate neurite extension. Induction of NGF-mediated neurites in PC12 cells requires prolonged phosphorylation of TrkA and its downstream signaling proteins, especially ERK1 and 2 (65-67). Qian et al showed that the adapter protein, Grb2, interacts with SHB21α under non-NGF and NGF conditions. Grb2 is implicated in the regulation of ERK 1 and ERK2 (68). In 293 cells, transient co-expression of TrkA and truncated versions of SH2B1α identified a Grb2 binding domain within a proline-rich region between amino acids 384-504 (25). To determine whether the proline-rich region of SH2B1α is necessary for enhancing MAPK activity, 293 cells transiently expressing TrkA, HA-ERK1, and SH2B1α WT or SH2B1α without the Grb2 interacting region were tested for NGF-mediated ERK1 activity. NGF-mediated ERK1 activity was increased by SH2B1α WT and to a lesser extent by the truncated SH2B1α suggesting that constitutive binding of SH2B1α with Grb2 enhances ERK1 activity (25). These data suggest SH2B1 interacts with at least one other adaptor protein (i.e. Grb2) to enhance neurotrophic signaling necessary for neurite extension. However, not all research labs have found that SH2B1 isoforms that enhance neurite extension enhance ERK1 and ERK2 activity.

The Carter-Su lab has not found SH2B1β to reproducibly enhance NGF-activation of ERK1 and ERK2 (26,69). The Carter-Su lab has, however, identified other NGF signaling pathways that may contribute to SH2B1-enhancement of NGF-induced

neurite extension. NGF-induced phosphorylation of ERK1 and ERK2 was not enhanced in PC12 cells overexpressing SH2B1β, or decreased in cells either overexpressing the dominant negative SH2B1β R555E, or in which SH2B1 levels were reduced using shRNA (26,69). They found that stable overexpression of SH2B1β, but not SH2B1β R555E, in PC12 cells enhances and prolongs NGF-dependent phosphorylation of Akt, GSK3α/β, and FOXO3 in an NGF-dependent manner (70). The PI3K/Akt has been shown to be required for NGF-mediated sympathetic neuron survival (71). However, surprisingly, reducing endogenous levels of SH2B1 by 50-70% using shRNA did not reduce NGF-induced phosphorylation of Akt (69). Taken together, these data suggest that SH2B1β and SH2B1α can modulate NGF signaling pathways implicated in neuronal differentiation and survival. However, it is unclear how the dominant negative SH2B1 R555E or knockdown of SH2B1 impairs enhanced NGF-mediated neurite outgrowth without altering NGF-induced activation of ERK1 and ERK2 suggesting SH2B1 may impact NGF-signaling pathways yet to be identified.

The interaction of SH2B1 with the BDNF receptor tyrosine kinase, TrkB, has also been studied. Like *Sh2b1*$^{-/-}$ mice, disruption of BDNF or TrkB in mice has been shown to induce hyperphagia and obesity (72-74) suggesting that the TrkB-SH2B1 interaction may be important for the effects of SH2B1 on energy balance. SH2B1 was found to interact with TrkB in a yeast-two hybrid screen used to identify TrkB substrates in dorsal root ganglia (25). Shih et al. provided further validation of the BDNF-mediated interaction between SH2B1β and TrkB in a 293T cell overexpression system (27). Like TrkA, TrkB tyrosyl phosphorylates SH2B1 isoforms in vitro and in cultured cortical neurons (25,27). Transient overexpression of SH2B1β in cultured primary hippocampal

neurons enhanced BDNF-induced neurite length and branching (27). In addition, Shih et al. showed reduced BDNF-induced neurite length and branching using shRNA to knockdown SH2B1 in cultured primary cortical neurons (27). In PC12 cells stably overexpressing TrkB and SH2B1β, BDNF-mediated neurite-outgrowth and phosphorylation of downstream signaling proteins PLCγ, STAT3, ERK1/2, and Akt was enhanced and prolonged compared to TrkB expressing cells (75). Collectively, these ex vivo and in vitro experiments suggest SH2B1 is an important mediator of BDNF signaling leading to BDNF-induced neurite extension.

Together, the demonstration that SH2B1 enhances neurotrophic factor induced neurite outgrowths and signaling pathways indicate that it is critical to understand the function of SH2B1 in neurons. In support of this, SH2B1 has been implicated in neurotrophin-induced neurite outgrowth of several different neurons yet the mechanism by which SH2B1 regulates neurite outgrowth and branching remains unclear. Also, it is unclear whether SH2B1 enhanced neurite outgrowth is critical for maintaining energy homeostasis.

Characterizing the cellular and molecular functions of SH2B1

Subcellular localization of SH2B1

In this section, I will summarize critical data performed by our laboratory and others concerning the subcellular localization of SH2B1. Domains and post-translational modifications in unique regions of SH2B1 play a critical role in regulating the cellular localization of SH2B1. Some of the roles of SH2B1β in response to NGF are illustrated in the model shown in Figure 1.3. The dimerization, NLS, NES, and SH2 domains and post-translational modifications within the N-terminal 630 amino acids dictate where SH2B1 localizes within the cell. N-terminal GFP-tagged SH2B1β has been shown to localize on the plasma membrane and the cytoplasm (26,69,76). GFP-tagged SH2B1β is not visible in the nucleus by confocal microscopy under steady state conditions in 293T, COS-7, and PC12 cells (69,76). Truncation mutants of GFP-tagged SH2B1 that lack the NES (GERWTHRFERL$_{231}$RL$_{233}$SR), mutation of the NES, or inhibition of the nuclear exportin protein, Crm1, with leptomycin B (LMB) leads to an accumulation of SH2B1β in the nucleus (76) indicating that SH2B1β cycles in and out of the nucleus. The localization of the α, γ, and δ SH2B1 isoforms had yet to be determined at the start of this thesis. A putative NLS (KLK$_{150}$KR) in SH2B1β was identified by sequence analysis (69) and point mutations within the NLS of SH2B1β prevented SH2B1β from accumulating in the nucleus even in the presence of LMB (69)

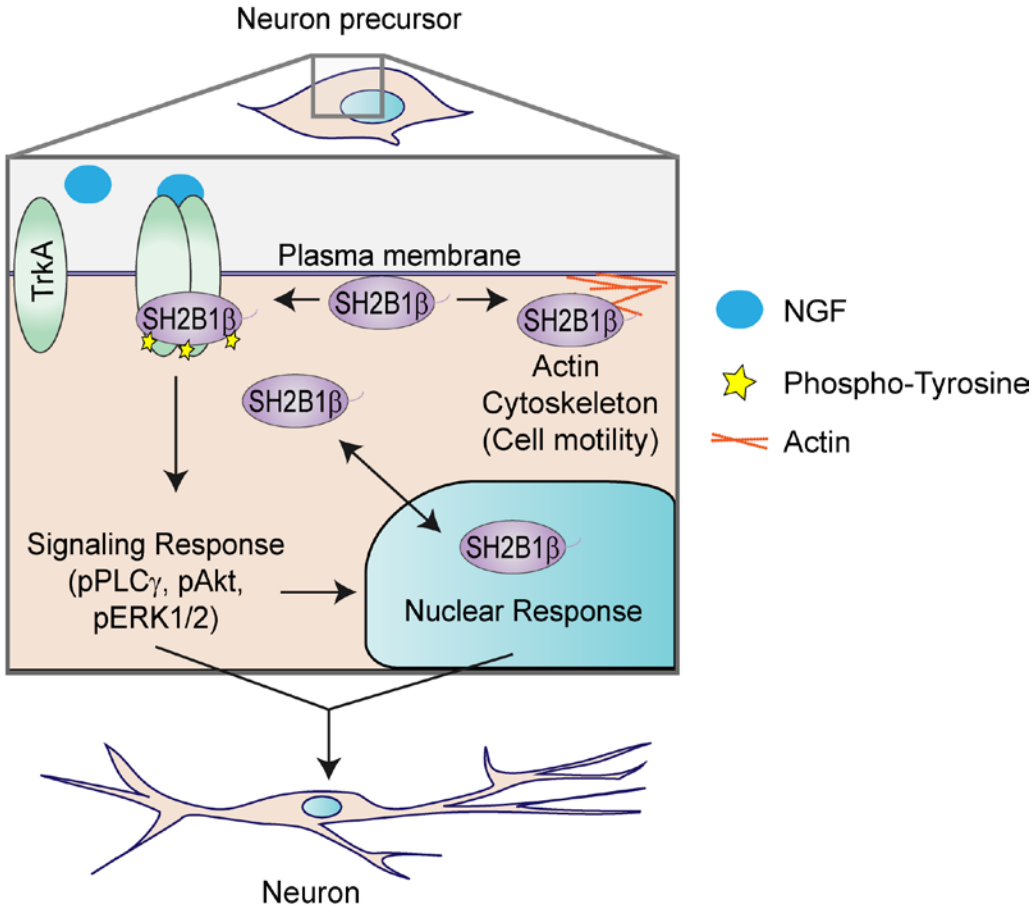

Figure 1.3. Model illustrating some of the many roles of SH2B1β in response to NGF. SH2B1β is localized in the cytoplasm and on the plasma membrane and shuttles between the cytoplasm and nucleus. TrkA dimerizes in response to NGF stimulation to recruit SH2B1β and phosphorylate SH2B1β. The signaling complex mediates phosphorylation of downstream signaling proteins to elicit a nuclear response necessary neuronal differentiation. In addition, SH2B1β binds to the actin and mediates cytoskeleton rearrangement necessary for neurite outgrowths and motility.

verified that this region functions as an NLS. SH2B1β was unable to enter the nucleus under ATP-depleted conditions, suggesting that nuclear localization of SH2B1β is energy dependent. Intact NLS and NES motifs are required for SH2B1β to enhance NGF-mediated neurite outgrowth in PC12 cells (69,76). Mutations within the NLS or NES of SH2B1 which blocked nuclear entry or exit, respectively, had no effect on NGF-mediated phosphorylation of TrkA or ERKs 1 and 2 (69,76). Furthermore, the dominant negative SH2B1 R555E mutant with a defective SH2 domain, which inhibits NGF-induced neurite outgrowth, also fails to translocate to the nucleus but does not impair NGF-mediated ERK 1 and ERK2 phosphorylation (26) (69). These data suggest that nuclear translocation may be necessary for SH2B1 to enhance NGF-mediated neurite outgrowth. However, the role SH2B1 plays in the nucleus remains unknown.

In addition to being observed in the cytoplasm and nucleus, SH2B1β has been observed at the plasma membrane. Some PH domains bring proteins to the plasma membrane through phosphatidylinositide interactions (77). Subcellular fractionation of cells expressing SH2B1 truncation mutants implicated the region encompassing the NLS and NES and not the PH domain for SH2B1β localization at the plasma membrane (78). To confirm the results of the subcellular fractionation assay, Maures et al. used confocal microscopy of live PC12 cells expressing GFP-tagged SH2B1β lacking amino acids 147-198 which encompasses the NLS to show a loss of SH2B1β on the plasma membrane (78). Mutating the polybasic NLS itself also prevented SH2B1β from localizing at the plasma membrane suggesting that the NLS localizes SH2B1β to the nucleus and the plasma membrane through electrostatic interactions (78). To determine why the NLS is needed to recruit SH2B1β to both the nucleus and the plasma

membrane, Maures et al. (78) mutated all the serine residues adjacent to the polybasic NLS to alanines (13SA) and found that SH2B1β localized primarily on the plasma membrane. By reducing the number of serine mutations in SH2B1β from 13SA to 2 serine mutations adjacent to the NLS, it was determined that Ser161 and Ser165 were responsible for enhanced localization of SH2B1β at the plasma membrane in 293T and PC12 cells (78). Ser161 of SH2B1β was found to be phosphorylated by mass spectroscopy of phorbal ester treated PC12 cells, suggesting serines may be phosphorylated by protein kinase C (PKC) or another serine/threonine kinase (78). In support of this, activation of PKC using phorbol esters caused SH2B1β to leave the plasma membrane and accumulate in the nucleus (78). However, while NLS was found to be necessary for plasma membrane localization of SH2B1b, it was not sufficient. In addition, the dimerization domain was found to have a role in localizing SH2B1β to the plasma membrane (78). Mutating Ser154, Ser157, Ser161, and Ser165 to prevent their phosphorylation also reduced the ability of SH2B1β to enhance NGF-mediated expression of the early-response gene, *Plaur* (78). Collectively, these data concerning the plasma membrane localization of SH2B1 suggest that ligands that stimulate PKC or a PKC-like kinase cause Ser161 and Ser165 adjacent to the polybasic region of SH2B1β to be phosphorylated, thereby releasing SH2B1β from the plasma membrane and enabling it to enter the nucleus. It unknown whether serine phosphorylation on α, γ, or δ SH2B1 isoforms similarly mediate the localization of SH2B1 to the plasma membrane.

SH2B1 enhances transcriptional responses to NGF

To identify NGF- and SH2B1β-responsive genes that might play a role in SH2B1β promotion of NGF-induced neurite outgrowth, the Carter-Su laboratory performed microarray experiments using PC12 cells, or PC12 cells stably overexpressing SH2B1β or the dominant-negative SH2B1β R555E. Cells were treated for 6 hours with or without 100 ng/ml NGF (79). Subsequent microarray analysis revealed multiple NGF- and SH2B1β-regulated genes including *plasminogen activator urokinase receptor (Plaur),* and *matrix metalloproteinases 3 and 10 (Mmp3, Mmp10)* whose expression was substantially increased in cells expressing SH2B1β or decreased in cells expressing SH2B1β R555E, respectively*. Plaur* encodes urokinase plasminogen activator receptor, uPAR, which is located in the plasma membrane (79) and is required for NGF-mediated neurite outgrowth (80). uPAR has also been implicated in such diverse cellular functions as cell migration and modulation of cyclic AMP(cAMP)/diacylglycerol (DAG)/calcium/inositol phosphate levels, which in turn affect many signaling molecules including PKC (81-83). uPAR, MMP3 and MMP10 are primary components of extracellular matrix degradation (ECM) pathways, which are necessary for cellular differentiation (i.e. pre-neuronal to neuronal) or motility (80,84-86). Inactive pro-urokinase plasminogen activator (pro-UPA) binds to uPAR and is activated by cathepsin cleavage. The activated uPA can then cleave inactive plasminogen to form active plasmin enzyme which in turn cleaves pro-MMPs to form active MMPs (87). Northern blot analysis of *Plaur* transcripts from PC12 cells treated with epidermal growth factor (EGF) or NGF indicate that *Plaur* is induced to a much greater extent by NGF (80). Knockdown of uPAR using antisense oligonucleotide to *Plaur* mRNA impeded PC12 NGF-mediated neurite differentiation (80). These results suggest that

nuclear SH2B1β may promote the expression of genes necessary for degrading the extracellular matrix to enable neurite extension. However, the mechanism by which SH2B1β promotes transcriptional changes in response to NGF is unknown. In addition, it is unknown whether SH2B1α, SH2B1γ, and SH2B1δ promote NGF-mediated enhancement of the same extracellular matrix degradation transcriptome as SH2B1β.

The role of SH2B1 in cellular cytoskeleton rearrangement

Neurite extension requires changes in the actin cytoskeleton. Therefore, it is not surprising that SH2B1β interacts with the cytoskeleton to help mediate ligand-induced changes to the cellular architecture (i.e. actin rearrangement). SH2B1β colocalizes with actin, and induces GH- and PDGF-mediated membrane ruffling and lamellipodia formation in 3T3-F442A fibroblasts (88). Overexpression of SH2B1β WT enhanced, whereas the dominant-negative SH2B1β R555E impaired, GH-mediated membrane ruffling (88). SH2B1β was shown to binds to the small GTPase, Rac, in a GH-mediated fashion that requires amino acids 85-106 in SH2B1β (89). Membrane ruffles and lamellipodia formation have been shown to involve activated Rac (90). Mutation studies revealed that post-translational modifications regulate SH2B1 functions critical for neurite extension and cellular motility. Mutation to Phe of SH2B1 Tyr439 and Tyr494, both YXXL sites shown to be substrates of GH-activated Jak2 (91,92), inhibited membrane ruffling in 3T3-F442A cells (91) and RAW macrophages (92) indicating tyrosine phosphorylation of SH2B1β regulates its ability to promote membrane ruffles. SH2B1β also localizes to focal adhesions (93). Focal adhesions are integrin-based complexes that mediate the attachment interface between cells and the extracellular

matrix, mediate signaling between the extracellular matrix and cells as well as coordinate cell anchorage and motility (94). Phorbal esters stimulated release of SH2B1β from focal adhesions in fibroblasts (93). Mutation of Ser161 and Ser165, which prevents SH2B1β from dissociating from the plasma membrane, also blocked SH2B1 release from focal adhesions (93). Mutating the NLS or introducing phosphomimetics to Ser161 and Ser165 in SH2B1β to reduce the ability of SH2B1 to bind the plasma membrane also enhanced GH-induced motility of RAW macrophages (92). These results show that SH2B1β is involved with actin rearrangement and focal adhesion dynamics that modulate the cellular architecture. The ability of SH2B1α, γ, and δ isoforms to modulate cellular ruffling and motility has yet to be examined.

In this thesis, I aimed to characterize the ability of the neuronal-specific isoform SH2B1α to shuttle through the nucleus, localize to the plasma membrane, modify NGF-induced gene expression and promote both cell migration and neurite outgrowth.

Effects of human obesity-associated mutations in SH2B1 on cellular motility and neurite extension

The Carter-Su laboratory has characterized the effect of human obesity-associated mutations in SH2B1 on cellular functions of SH2B1β. The human obesity-associated mutations P90H, A175N, P322S, and F322LfsX20 impaired the ability of SH2B1β to shuttle through the nucleus (6). The F344LfsX20 mutation causes a frame shift resulting in a stop codon prior to the SH2 domain of SH2B1. Only the F322SLfsX20 mutation in SH2B1β altered the plasma membrane localization of SH2B1 in 293T cells

(6). The loss of the SH2 domain due to the F344LfsX20 mutation, resulted in the loss of the ability of SH2B1 to enhance NGF-dependent PC12 neurite outgrowth and enhance GH-induced RAW macrophage mobility presumably due to an inability of SH2B1 to bind to NGF-receptor, TrkA, and GH receptor-associated kinase, Jak2 (6). Introducing the point mutations P90H, A175N, and P322S reduced the ability of SH2B1β to enhance both GH-mediated motility of macrophages and NGF-mediated neurite outgrowth in PC12 cells (6). In addition, the SH2B1 P322S mutation enhanced dimerization between SH2B1β P322S and SH2B1β WT (6). Because the human mutations are associated with obesity, these results suggest that the human mutations in SH2B1 might hinder the formation of the correct neuronal projections of neurons which regulate energy balance.

Alternative splicing of SH2B1 generates isoforms with unique C-terminal tails, enabling diversity in protein-protein interactions

Changes in cellular morphology induced by growth factors require an appropriate cell signaling response that can elicit changes in the transcriptome and proteome. Sequencing of the human genome revealed 20,000 – 25,000 protein-coding genes (95). Approximately 92-95% of multi-exon genes have since been identified to undergo alternative splicing (96). Variation and regulation of transcript splicing events is more prevalent between tissues than amongst individuals (96). These data suggest that alternative splicing of the human genome contributes to proteomic diversity and may facilitate the vast number of processes demanded by the phenotypic complexity of mammals. The vast majority of spliced transcripts have been assocaited with key cell functions that include developmental processes, cell communication, signal transduction, regulation of metabolism, cell proliferation, and cell survival (96-98). Approximately 40% of genes expressed in the human brain are alternatively spliced, making the brain the tissue with the most alternative spliced events followed by the liver and testis (99-102). The expression of all four isoforms (α, β, γ, δ) of SH2B1 in the human brain (6) follow this trend. Therefore, the alternatively spliced transcripts of SH2B1 may function to modulate key complex cellular processes. Here I present background on the current research on alternative splicing of SH2B1 and the known roles of the unique C-terminal tails of SH2B1.

SH2B1 isoforms (α, β, γ, δ) are the products of a combination of exon skipping and alternative donor and acceptor splice sites (Figure 1.4). The first 7 exons are common to all four isoforms. The divergence of isoforms starts within exon 8 where an alternative splice donor site results in a 53-base pair insertion unique to γ and δ isoforms. Inclusion of exon 8 results in a 100 base-pair insertion unique to β and γ isoforms. These different insertions generate reading frameshifts leading to termination codons and C-terminal amino acid sequences unique to each isoform. (2).

The C-terminal tails of SH2B1 have unique motifs that affect their cellular localization, protein-protein interactions, and post-translational modification. SH2B1δ contains a unique nucleolus localization motif that resembles a basic nuclear localization sequence (K/R)(K/R)X(K/R) and a tryptophan-rich region but the function of SH2B1δ has yet to be determined. The C-terminal tail of SH2B1β contains an actin binding site that facilitates cross-linking of actin filaments and is necessary for growth hormone-induced cellular ruffling (103). The C-terminal tails of SH2B1β and α each contain a single tyrosine residue, but their ability to be phosphorylated remains unclear. The SH2B1α C-terminal tail also contains a potential PDZ domain, S_{754}-F-V(104,105). In addition, Tyr753 in SH2B1α is located within a potential phosphotyrosine-binding motif, N-X-X-Y, which resembles N-P-X-Y motifs that recruit adapter signaling proteins like Shc (106). The C-terminal Tyr753 in SH2B1α is in a motif similar to that surrounding the equivalent tyrosine in other SH2B family members, Tyr618 in SH2B2 and in Tyr536 in SH2B3. Tyr618 in SH2B2 has been shown to be a substrate for PDGF and insulin receptors (107-110). Tyr536 in SH2B3 is a substrate for stem cell factor the receptor tyrosine kinase c-Kit (111). Phosphorylation of Tyr536 has been implicated in the

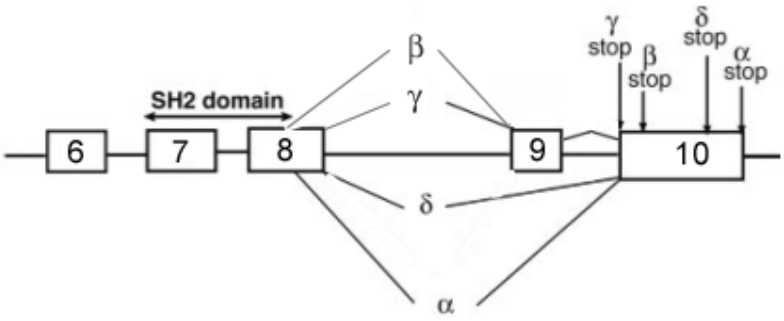

Figure 1.4. Schematic illustrating the alternative splicing of the four SH2B1 isoforms. Exons are denoted by boxes. Arrows denote where stop codons reside for the 4 defined isoforms.

impairment of proliferation of bone marrow mast cells (112). These data collectively suggest that phosphorylation of Tyr753 in the C-terminal tail of SH2B1α may regulate the function of SH2B1α through recruitment of proteins that modify signal transduction. Furthermore, these data suggest unique functional roles for each isoform of SH2B1, which would enhance proteomic diversity.

In this thesis, I further expand our current knowledge of the neuron-specific SH2B1 isoform, SH2B1α, by revealing the ability of the unique C-terminal tail to impair SH2B1 signal transduction pathways for NGF-mediated neurite outgrowth in PC12 cells and NGF-mediated gene transcription. I further reveal that TrkA can phosphorylate Tyr753 in SH2B1α and that mutation of Ty753 restores the ability of SH2B1α to enhance NGF signaling pathways as well as NGF-mediated neurite extension and gene expression.

PC12 cells as a model system for delineating cellular actions of SH2B1 in neurotrophic factor signaling

The PC12 cell line has been a popular cell line to use for studying NGF signal transduction mechanisms leading to sympathetic neurite outgrowth. PC12 cells were first established in 1976 from a rat adrenal pheochromocytoma (62). PC12 cells respond to NGF-treatment by ceasing proliferation and differentiating into sympathetic-like neurons (61). After ceasing proliferation, an NGF-mediated signaling response facilitates changes to the transcriptome and proteome resulting in expression of neuron-specific proteins (113). In addition, after NGF-induced differentiation, PC12 cells become electrically excitable (62,114) and have been reported to form synapses with primary neurons from rat cerebral cortex in culture (115). These data support the use and relevance of PC12 cells as an effective cell line for elucidating molecular neuronal differentiation mechanisms.

PC12 cells have been used to determine NGF signaling pathways, including those that regulate neuronal differentiation. NGF binds to the NGF-receptor, TrkA, with high-affinity to activate TrkA leading to, autophosphorylation on multiple tyrosines and recruitment of signaling molecules including members of the PI3K/Akt, PLCγ, Shc/Grb2/SOS/Ras/Raf/MEK/ERK, Jun N-terminal kinase, p38 kinase and atypical protein kinase C pathways (55,116,117). Like NGF, EGF activates the EGF receptor to activate ERK1 and ERK2 (118). However, the transient activation of ERK1 and ERK2 by EGF promotes cellular proliferation while prolonged ERK1 and ERK2 activation in

response to NGF promotes differentiation (119). The difference in response between EGF and NGF may be due to the recruitment of different adaptor proteins to the receptor signaling complex. Unlike activated EGF receptor, activated NGF-receptor, TrkA, recruits a multitude of adaptor proteins (e.g. FRS2 and Crk) to promote prolonged phosphorylation of ERK1 and ERK2 (120,121). These data suggest growth factors use similar signaling pathways for different physiological responses and that the signaling pathways are modified by adapter proteins like SH2B1.

NGF-mediated ERK phosphorylation occurs through multiple NGF pathways that synergize to maximize the cellular response. Shc adaptor protein is recruited to phosphoTyr490 in TrkA, activating ERK1 and ERK2 via a Shc/Grb2/SOS/Ras/Raf/MEK pathway (122). Additionally, ERK1 and ERK2 activation can be induced through NGF-mediated phosphorylation of Tyr785 on TrkA, which recruits PLCγ (123). Activated PLCγ hydrolyzes phosphatidyl inositides yielding inositol tris-phosphate and diacylglycerol (DAG) (124) to promote PKCδ which in turn activates ERK1 and ERK2. Determining how SH2B1 amplifies signal transduction cascades to promote a physiological cellular response from a growth factor is essential for discovering potential therapeutic targets.

The goal of the work in this thesis was to provide insight into how human mutations associated with human obesity affect cellular actions of SH2B1 (Chapter 2 and 4) and how the unique C-terminal tails of the four isoforms affect the function of SH2B1 (Chapter 2 and 3).

Chapter 2

Functional Characterization of Obesity-Associated Variants Involving the α and β Isoforms of Human SH2B1

Abstract

We have previously reported rare variants in Src homology 2 (SH2) B adaptor protein 1 (*SH2B1*) in individuals with obesity, insulin resistance and maladaptive behavior. Here, we identify four additional *SH2B1* variants by sequencing 500 individuals with severe early-onset obesity. SH2B1 has four alternatively spliced isoforms. One variant (T546A) lies within the N-terminal region common to all isoforms. As shown for past variants in this region, T546A impairs SH2B1β enhancement of nerve growth factor (NGF)-induced neurite outgrowth and the individual with the T546A variant exhibits mild developmental delay. The other three variants (A663V, V695M and A723V) lie in the C-terminal tail of SH2B1α. SH2B1α variant carriers were hyperinsulinemic but did not exhibit the behavioral phenotype observed in individuals with SH2B1 variants that disrupt all isoforms. In in vitro assays, SH2B1α, like SH2B1β, enhances insulin- and leptin-induced IRS2 phosphorylation and growth hormone (GH)-induced cell motility. None of the variants affect SH2B1α enhancement of insulin- and leptin-induced IRS2 phosphorylation. However, T546A, A663V and A723V all impair the ability of SH2B1α

to enhance GH-induced cell motility. In contrast to SH2B1β, SH2B1α does not enhance NGF-induced neurite outgrowth. These studies suggest that genetic variants that disrupt isoforms other than SH2B1β may be functionally significant. Further studies are needed to understand the mechanism by which the individual isoforms regulate energy homeostasis and behavior.

Introduction

Src homology 2 (SH2) B adaptor protein 1 (SH2B1) is a member of a family of scaffold proteins implicated in signaling downstream of a variety of receptor tyrosine kinases and cytokine receptors that bind to JAKs. These include receptors for leptin, insulin, growth hormone (GH), IGF-1, nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) (125) .In mice, targeted deletion of *Sh2b1* results in marked leptin resistance, increased food intake, severe obesity and insulin resistance. An intermediate obesity phenotype is seen in heterozygous null mice fed a high fat diet (18,19), suggesting that the obesity phenotype is dosage-dependent.

Given the large number of receptor tyrosine kinases and cytokine receptor/JAK complexes that bind to SH2B1 (125), dissecting the molecular mechanisms by which SH2B1 regulates energy balance and glucose homeostasis has proved challenging. SH2B1 is alternatively spliced to yield four isoforms (α,β,δ,γ) that vary in length from 671 to 756 amino acids. All isoforms share a phenylalanine (Phe) zipper dimerization domain, nuclear localization sequence (NLS), nuclear export sequence (NES), Pleckstrin homology (PH) domain and SH2 domain but exhibit unique C-termini that vary in length from 40 (SH2B1β) to 125 (SH2B1α) amino acids (Figure 2.1) (2). The human SH2B1 isoforms have distinct expression patterns. While the β and γ isoforms are widely expressed, the α and δ isoforms are restricted to brain regions (6). Although very little is known about the physiological relevance of the different SH2B1 isoforms,

neuron-specific restoration of the β isoform in *Sh2b1* null mice rescues the obese phenotype (54).

We previously reported rare genetic variants in *SH2B1* (P90H, T175N, P322S, F344Lfs*20) that are located in the N-terminal 631 amino acids shared by all four isoforms (1-631 region). Individuals carrying these variants exhibit severe early-onset obesity and insulin resistance, and a neurobehavioral phenotype characterized by delayed speech and language development, and maladaptive behavior (6). These variants disrupted SH2B1 cellular function in in vitro assays that measured GH-induced cell motility and NGF-induced neurite outgrowth. An additional SH2B1 variant (g.9483C/T), which affects only the beta (T656I) and gamma (P674S) isoforms, was also recently identified in obese subjects (17). This variant had no functional effect in the one assay tested (SH2B1 enhancement of leptin stimulation of STAT3 activity).

Here we describe four additional *SH2B1* variants identified by sequencing a further 500 unrelated severely obese individuals from the Genetics of Obesity Study (GOOS) cohort. We performed a series of functional studies of these new variants and those previously identified by us (P90H, T175N, P322S, F344Lfs*20) within the context of SH2B1α. There is evidence to support not only the role of rare variants in *SH2B1* in severe obesity but also of common variants with a broader role in the regulation of body mass index (BMI). As such, we also studied a common coding variant (rs7498665; A484T) that has been strongly associated with BMI in genome wide association studies (7,126).

Materials and Methods

SH2B1 variant analysis

500 individuals with severe early-onset obesity (defined as a BMI sds>3; onset< 10 years) were randomly selected from the Genetics of Obesity Study (GOOS) cohort study. Primers were designed to cover the coding sequence (NM015503) and splice junctions of *SH2B1*. Variant screening was performed using PCR, followed by direct sequencing using BigDye terminator chemistry (Applied Biosystems, UK) and analysis on an ABI 3730 automated sequencer (Applied Biosystems, UK).

Methods for functional studies are similar to those described previously (6).

Expression plasmids

Unless designated otherwise, the cDNA encoding human SH2B1β(NM_001145812.1) (30) had the T484 SNP whereas the cDNA encoding human SH2B1α (NM_001145795.1) (30) had the A484 SNP. Standard PCR methods were used to subclone these cDNAs into pEGFPC2 (N-terminal GFP tag) or pCDNA3.1(+) (N-terminal flag tag) vector. Mutagenesis was performed using QuikChange site-directed mutagenesis kit (Stratagene). cDNAs encoding flag-tagged rat JAK2 (127); flag-tagged IRS2 and GFP- and flag-tagged rat SH2B1β(NM_001048180) (6) were described previously.

Antibodies and hormones

Anti-phosphotyrosine (P-Tyr-100, #9411) and anti-phospho-JAK2 [pY1007/1008] (#3776) were from Cell Signaling Technology and were used at a dilution of 1:1000. Anti-flag M2 (#F1804) (1:1000) was from Sigma, and anti-GFP (#11-814-460-001) (1:5000) was from Roche. The secondary antibodies (anti-mouse and anti-rabbit) were from Dako and used at 1:2500 dilution. Recombinant human insulin was from Novo Nordisk; recombinant human leptin was from Calbiochem, recombinant human GH was a gift from Eli Lilly & Co. and mouse NGF was from BD Biosciences.

Cell culture and transfection

Q293A cells stably expressing the long form of the mouse leptin receptor (293^{LRb}) (45), 293 cells and 293T cells were transiently transfected using the polyethylenimine (PEI) method (Figures. 2.2A-C, 3.3A-D) or calcium phosphate (Figure. 2.2E). PC12 cells (stock from ATCC) were cultured and transfected as described previously (6). RAW264.7 cells (from J. Swanson, University of Michigan) were cultured in DMEM (Invitrogen) supplemented with 8% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals), 1 mM L-glutamine and 1 mM antibiotic-antimycotic (Invitrogen) at 37°C in 5% CO_2. RAW264.7 cells were transiently transfected using Amaxa nucleofector (Lonza) using solution V and setting D32.

Neurite outgrowth of PC12 cells

Transiently transfected PC12 cells were plated on collagen-coated 60-mm dishes. 24 h post transfection, 25 ng/ml NGF in DMEM containing 2% horse serum, 1% FBS was added. The GFP+ cells were visualized by fluorescence microscopy (Nikon Eclipse

TE200). The percent of GFP+ cells that had neurite outgrowths >2 times the length of the cell body was determined.

Cell imaging

Transfected 293T cells plated on 35-mm, poly-d-lysine-coated glass-bottom culture dishes (MatTek Corp., Ashland, MA) were incubated for 6h in growth medium supplemented with 20nM leptomycin B (Sigma) to inhibit Crm1-dependent nuclear export. Live GFP+ cells were visualized with an Olympus FluoView 500 laser scanning confocal microscope using a 60x water-immersion objective and FluoView version 5.0 software. Linescan profiles were obtained using MetaVue Software (Universal Imaging, Sunnyvale, CA).

GH-induced migration of RAW264.7 macrophages

RAW264.7 cells (2 x 10^5 cells/well) transiently expressing GFP-tagged human SH2B1α WT or variant were incubated in growth medium for 24h, then in serum-free medium (1% BSA) overnight. Transfected cells suspended in serum-free medium were added to the upper chamber of a transwell (Costar) unit with 5-µm pore size; the lower chamber contained serum-free medium ± 500ng/ml human GH. Transwell units were incubated at 37°C in 5% CO_2 for 18 h, fixed in methanol and air-dried. Cells in the upper chamber were removed with a cotton swab. Filter membranes were stained with hematoxylin and eosin (H&E) (1:10 dilution) each for 1 h and then washed with ddH_2O. Cells that had migrated were counted (≥3 independent fields per condition) under a light microscope.

Statistics

Statistics were calculated using Prism 6.0c.

Study subjects

The Genetics of Obesity Study (GOOS) cohort consists of 5000 probands with severe obesity (Body Mass Index Standard Deviation Score (BMI SDS) >3) before 10 years of age. BMI SDS values were calculated using UK reference data (128). We randomly selected five hundred individuals from this cohort; mean (±SD) BMI SDS was 4.5 ± 1.8. All studies were approved by the Anglia and Oxford multiregional ethics committee. Each subject, or parent (for children< 16 years), provided written informed consent (oral consent was obtained from the minors themselves). Clinical studies were conducted in accordance with the principles of the Declaration of Helsinki. In adults, overweight was defined as BMI 25–29.9 kg/m^2, obesity as BMI > 30 kg/m^2 according to WHO criteria. In children (<18y), we used definitions proposed by the International Obesity Task Force: overweight defined as >91st and obesity as >99th percentile for age-adjusted BMI.

Publicly available database used to determine minor allele frequency

The publicly available ESP6500 exome database is composed of exome data from several richly phenotyped cohorts, each consisting of controls and the extremes associated with heart, lung, or blood disorders (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL:http://evs.gs.washington.edu/EVS/). ESP6500 incudes 2203 African-Americans and 4300 European-Americans, totaling 6503 sample of unrelated individuals. Phenotype information about the individuals in this cohort is not available for release from EVS.

Results

Identification of novel SH2B1 variants in severely obese individuals.

We previously identified four variants in *SH2B1* (P90H, T175N, P322S, F344Lfs*20) in individuals with severe early-onset obesity from the Genetics of Obesity (GOOS) cohort (6). In the present study, we sequenced *SH2B1* in 500 additional individuals from this cohort. In addition to another individual carrying the T175N variant, we found three novel heterozygous variants in unrelated severely obese individuals: T546A (n=1), A663V (n=14) and A723V (n=1) (Table 2.1). One individual was homozygous for V695M. As with the previously reported variants, the T546A variant is present in all four SH2B1 isoforms. However, the three other variants (A663V, V695M and A723V) affect the unique C-terminal tail of SH2B1α (Figure 2.1). We sequenced *SH2B1* in 28 available family members of severely obese probands (Table 2.1). A663V variants did not co-segregate with obesity in families in a classical Mendelian manner, suggesting that *SH2B1* variants may predispose to obesity against a background of other genetic and environmental factors. There were an equal number of male and female mutation carriers (Table 2.1). Adult variant carriers were hyperinsulinemic (mean fasting plasma insulin 128 $\pm$ 32 pmol/l; reference range 0-60 pmol/l), but euglycemic; liver function tests, lipid profiles and final height were in the normal range. The individual with the T546A variant had mild developmental delay (Table 2.1). However, no

neurobehavioral abnormalities were reported in individuals carrying the A663V, V695M or A723V variants.

Differences in cellular signaling mediated by human SH2B1α and β isoforms.

We next explored the molecular mechanisms by which these variants might disrupt SH2B1 function. We first studied the ability of human SH2B1α to mediate signaling in response to a number of ligands. As a point of reference, we compared these findings to those obtained using human SH2B1β. Both SH2B1α and SH2B1β bind to JAK2 and enhance JAK2 autophosphorylation to a similar degree (Figure 2.2A), consistent with results of Nishi et al. (30). SH2B1β is reported to bind to IRS proteins and promote their tyrosyl phosphorylation in response to insulin and leptin (45,48). Like SH2B1β, SH2B1α enhances both leptin-stimulated (Figure 2.2B) and insulin-stimulated (Figure 2.2C) tyrosyl phosphorylation of IRS2. Next, we sought to determine whether SH2B1α is involved in mediating the effects of neurotrophins such as NGF. Surprisingly, while SH2B1β enhances NGF-induced neurite outgrowth of PC12 cells (26), SH2B1α does not (Figure 2.2D). SH2B1β shuttles between the nucleus and the cytoplasm (76). Shuttling is thought to be necessary for SH2B1β to enhance transcription of NGF-responsive genes such as uPAR, MMP3 and MMP10 (69,79), which are implicated in neurite outgrowth of PC12 cells (69). When 293T cells expressing either SH2B1α or SH2B1β are treated with the nuclear export inhibitor, leptomycin B, only the beta isoform is retained in the nucleus (Figure 2.2E). These results indicate that SH2B1α and SH2B1β share the ability to mediate signaling downstream of insulin, leptin and GH.

Table 2.1. Variants in *SH2B1* identified in severely obese individuals and the prevalence of these variants in the publicly available databases.

		Body Mass Index					Prevalence of variant in publicly available databases	
Variant	**No. of patients**	**Patient BMI (SDS)**	**Hetero-zygous family members (BMI)**	**Homo-zygous family members (BMI)**	**Wild-type family members (BMI)**	**Patient neuro-behavioral phenotype**	**dbSNP ID**	**NHLBI exomes MAF (%)**
T546A (c.1636A>G)	1	29(4.7)#	33#; 30#	-	28	Mild developmental delay	0	0
A663V (c.1988C>T)	14	Hetero-zygous 34(4.1)#; 36(3.4); 22(3.4); 28(3.6)#; 35(3.5); 47(4.0); 26(4.3)#; 43(4.2)#; 26(3.1)#; 39(3.6); 29(5.1)#; 27(4.5); 41(3.7); 33(6.2);	38#; 34; 54; 28; 27#; 21; 52; 30; 34#; 27; 32;	50#; 52	32; 40; 27#; 23; 21#; 29; 33#; 36; 26#; 42; 39; 37	--- --- --- --- --- --- --- --- --- --- --- --- --- ---	rs190981290	0.8103
V695M (c.2101G>A)	1	Homo-zygous 31(5.3)	N/A	N/A	N/A	---	rs375992097	0.0219
A723V (c.2168C>T)	1	Hetero-zygous 42	N/A	N/A	N/A	---	0	0

Abbreviations: **dbSNP** (SNP database dbSNP138); ***MAF*** *(minor-allele frequency),* ***NHLBI*** *(NIH Heart, Lung, Blood Institute) exomes (http://evs.gs.washington.edu/EVS);* ***N/A****, not available*. # denotes males.

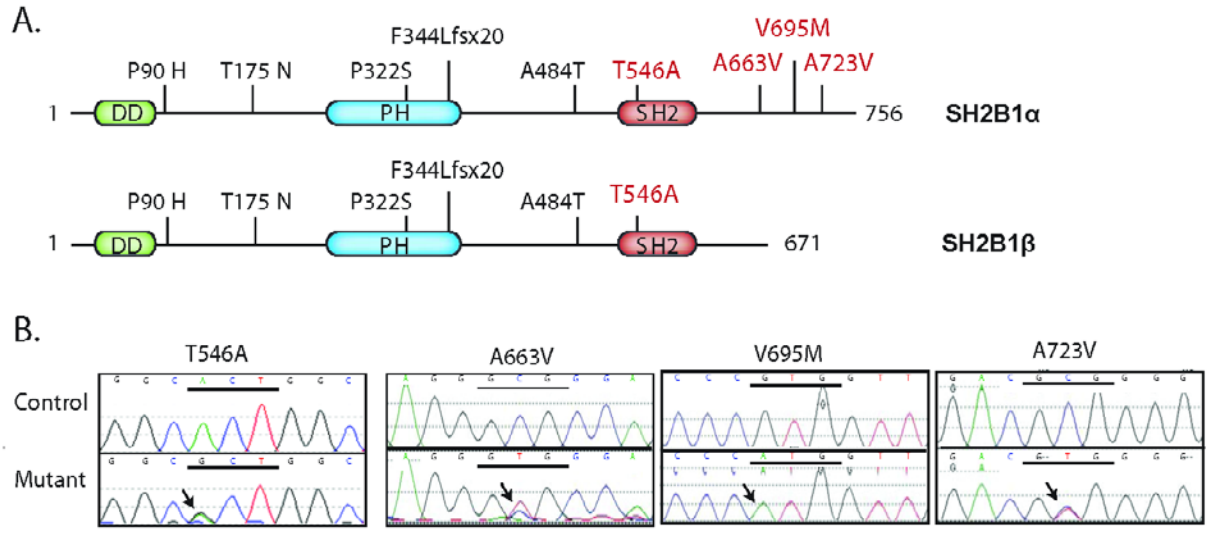

Figure 2.1. Identification of novel variants in *SH2B1.* A) Schematic showing the location of variants identified in *SH2B1* identified in individuals with severe obesity. The novel variants identified in this study are shown in red. Variants reported previously and the common SNP (A484T) are shown in black. DD = dimerization domain, PH = Pleckstrin homology domain, SH2 = SH2 domain. **B)** Sequence traces of the novel variants in this study.

Functional characterization of variants affecting SH2B1α.

We next investigated the functional consequences of variants when expressed in the SH2B1α isoform. The distribution of SH2B1α between the plasma membrane and the cytoplasm is not altered However, only the beta isoform translocates to the nucleus and promotes NGF-induced neurite outgrowth.by any of the variants (Figure 2.3A). However, compared to SH2B1β, the intensity of SH2B1α in the plasma membrane relative to the cytoplasm is diminished (Figure 2.2E, 2.3A). Except for the frameshift mutant F344Lfs*20 that lacks the SH2 domain, none of the variants affect the ability of SH2B1α to enhance JAK2 autophosphorylation, or leptin- or insulin-induced tyrosyl phosphorylation of IRS2 (Figures 2.3B-D). As reported previously for SH2B1β (6), the P90H and P332S variants reduce the ability of SH2B1α to stimulate GH-induced cell migration. The T546A, A663V and A723V variants also reduce GH-induced cell migration (Figure 2.3E). T175N and V695M and the common SNP (A484T) have no impact. Finally, we tested the effect of the variants on SH2B1 enhancement of NGF-induced neurite outgrowth. Like wild-type SH2B1α (Figure 2.2D), SH2B1α A663V, V695M and A723V do not enhance NGF-induced neurite outgrowth (data not shown). However, like the previously described human variants in the 1-631 region, the T546A variant impairs the ability of SH2B1β to enhance NGF-induced neurite outgrowth. The A484T SNP has no effect on SH2B1β enhancement of NGF-induced neurite outgrowth (Figure 2.3F).

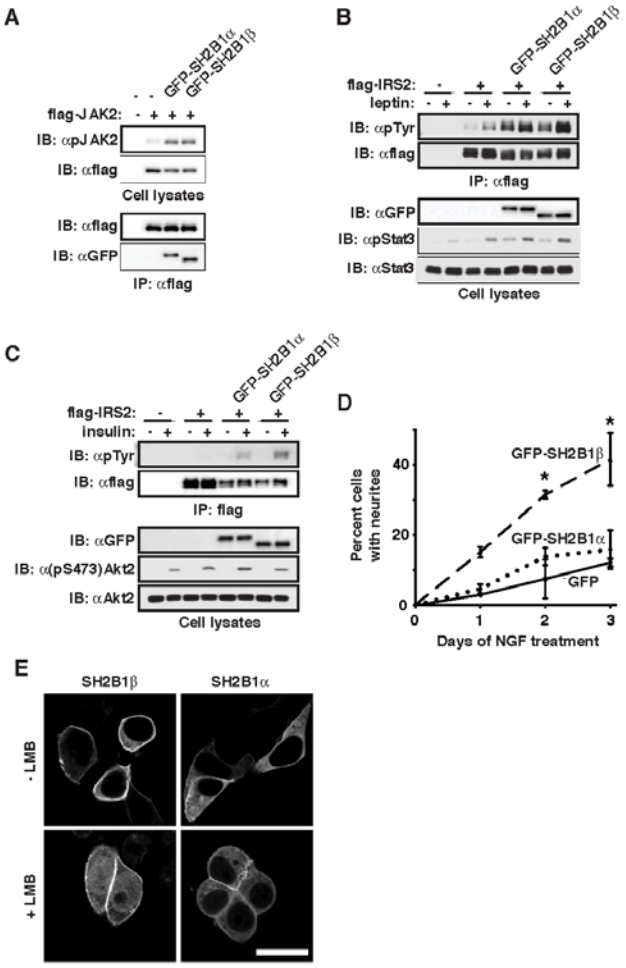

Figure 2.2. Comparison of SH2B1α and SH2B1β *in vitro.* A) 293 cells were transfected with the indicated constructs. Proteins in lysates were immunoprecipitated with anti-flag agarose. Immunoblotting was performed using the indicated antibodies. Results are representative of 3 experiments. **B)** 293^{LRb} cells were transfected as shown, serum-starved, and then treated with 100ng/ml leptin for 5 min. Results are representative of 4 experiments. **C)** 293 cells were transfected with the indicated constructs, serum-starved and then stimulated with 100nM insulin for 5min. Results are representative of 4 experiments. **D)** PC12 cells were transiently transfected with the indicated constructs and treated with 25ng/ml NGF to induce neurite outgrowth. Results show the % of GFP+ cells with neurites greater than twice cell body length. Means ± range, n = 2 different experiments with 300 cells counted per condition per day of NGF treatment. *P<0.05 compared to GFP+ cells at the same time-point. Statistical significance was assessed using two-way ANOVA and Bonferroni's Multiple Comparisons post-test. **E)** Live 293T cells transiently expressing GFP-tagged human SH2B1β or SH2B1α were incubated ± leptomycin-B (LMB, 20 nM) for 4h and imaged using fluorescent confocal microscopy. Each image is representative of 50-60 cells visualized in 2 separate experiments. Scale bar = 20μm.

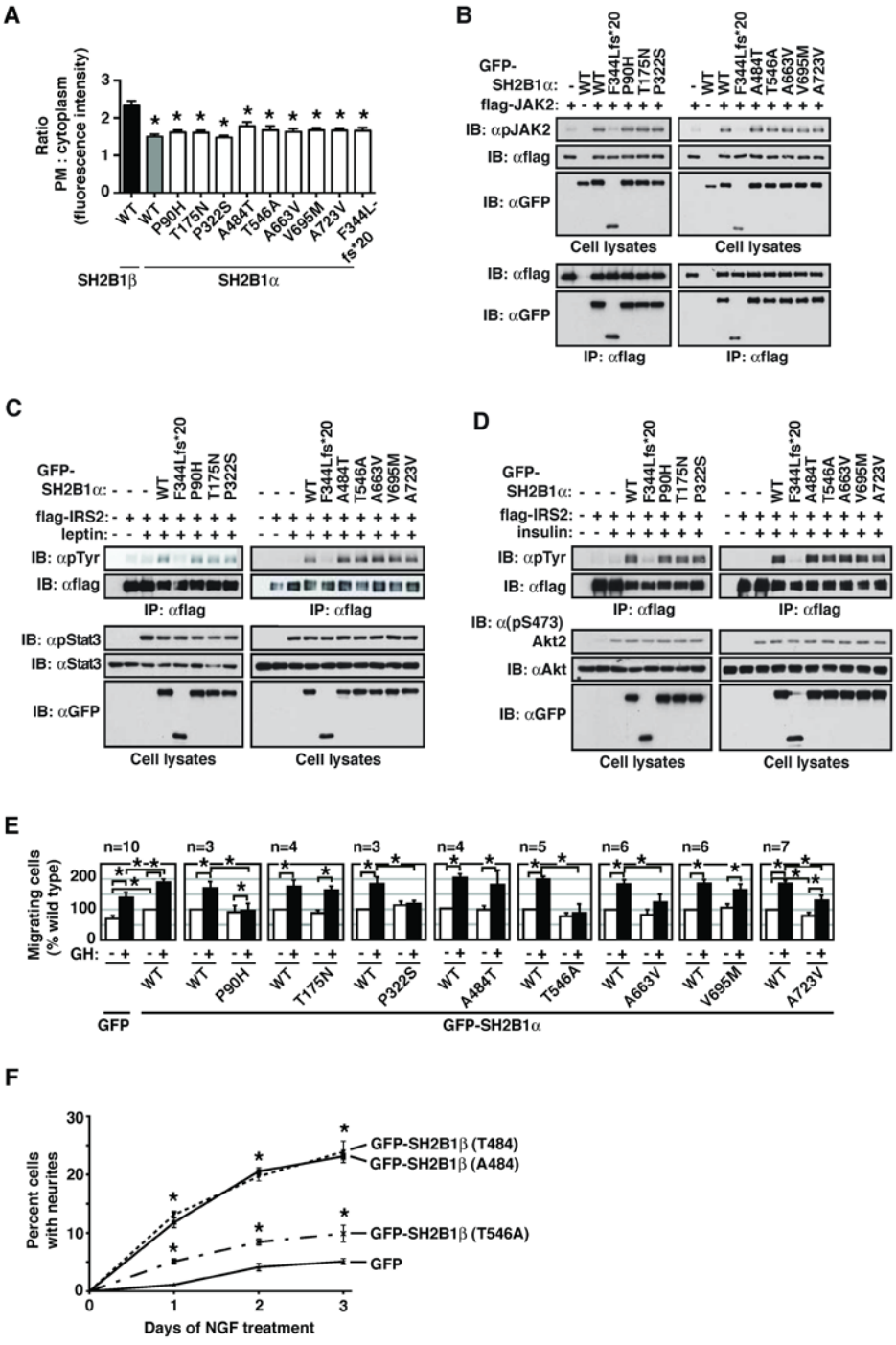

Figure 2.3. Characterization of novel human variants in SH2B1. A) Live 293T cells transiently expressing GFP-tagged rat SH2B1β, human SH2B1α WT or human SH2B1α mutants were stained with the plasma membrane marker wheat germ agglutinin Alexa Fluor 594 and imaged by confocal microscopy. Green and red signal intensity across each cell were determined using line scan analysis (MetaVue). The ratio of the plasma membrane: cytoplasmic signal intensity is shown (mean ±SEM). * = p< 0.0001 by two-

tailed, unpaired Student's *t*-test compared to the ratio for rat SH2B1β WT, n = 13-19 cells/condition. **B)** 293 cells were transfected with the indicated constructs and resulting lysates subjected to immunoprecipitation with anti-flag agarose. Immunoblotting was performed using the indicated antibodies. Results are representative of 3 experiments. **C)** 293^{LRb} cells were transfected as shown, serum-starved, and treated with 100ng/ml leptin for 5 min. Results are representative of 4 experiments. **D)** 293 cells were transfected with the indicated constructs, serum-starved and stimulated with 100nM insulin for 5min. Results are representative of 4 experiments. **E)** RAW264.7 cells (2 x 10^5 cells/well) were transiently transfected as indicated. Migration was analyzed using a transwell migration assay with or without GH (500 ng/ml) in the lower chamber for 18 h. Values are normalized to the non-hGH treated GFP-SH2B1α cells. Mean ± SEM from 3-10 independent experiments. * = p< 0.05 by one-tailed paired Student's t-test. F) PC12 cells transiently expressing GFP, or GFP-tagged human SH2B1β T484, A484, or T546A, were treated with NGF (25 ng/ml) to induce neurite outgrowth. Results show the % of GFP+ cells with neurites greater than twice cell body length were counted. Means ± SEM, n = 3 different experiments with 300 cells counted per condition per day of NGF treatment. * P < 0.05 compared to GFP cells at the same time-point. GFP-SH2B1β T546A cells exhibited a statistically lower (P < 0.05) number of neurite outgrowths at days 1-3 compared to GFP-SH2B1β A484 and T484. Statistical significance was assessed using two-way ANOVA and Bonferroni's Multiple Comparisons post-test.

Discussion

Here we describe the identification of four novel variants in *SH2B1* that are present in individuals with obesity and insulin resistance. Some of the variants we found in severely obese individuals are also found in publicly available exomes (Table 1). However, as BMI and additional phenotypic information for individuals in these datasets are not available, the precise contribution of these variants to obesity remains to be established.

These findings suggest that *SH2B1* contains a spectrum of common and rare alleles which contribute to BMI and obesity predisposition with a broad range of penetrance, from low to more highly penetrant rare alleles. One variant, A663V, was identified in 14 severely obese individuals in the GOOS cohort as well as in many publicly available exomes. In cells, A663V affected the ability of SH2B1 to enhance cell motility in response to GH, therefore, it is possible that this variant may contribute to the phenotype of variant carriers. Additional genetic studies will be needed to determine whether this variant is significantly enriched in obese cohorts compared to controls. The nucleotide change that causes the A663V variant in SH2B1α also causes an amino acid change (R680C) in the δ isoform of SH2B1. Mutation of this residue in SH2B1δ impairs the ability of SH2B1δ to enhance NGF-induced neurite outgrowth (data not shown), a finding that requires further investigation.

We also studied the common coding SNP, A484T. A484T did not impact upon SH2B1α or SH2B1β in the functional assays employed here. This is consistent with a previous study (17), which was unable to demonstrate any functional consequence of the A484T SNP. It is possible that this SNP affects cellular functions of SH2B1 other than those tested or it may have subtle functional consequences that cannot be detected in the cell systems and assays we employed.

We previously showed that individuals carrying heterozygous variants in the 1-631 region of SH2B1 were hyperphagic, with a reduced final height, elevated plasma insulin levels that are disproportionate to the degree of obesity, and surprisingly, maladaptive behavior (6). Consistent with these findings, the individual carrying the T546A variant had a markedly elevated fasting plasma insulin of 123 pmol/l at age 6 years and mild developmental delay. Whilst individuals carrying the A663V, V695M and A723V variants were hyperinsulinemic, none of them were reported to display any of the behavioral characteristics reported previously. Given the small number of individuals studied, these observations need to be replicated in additional studies. We also need to determine, at the mechanistic level, whether the behavioral phenotype results from disruption of a specific SH2B1 isoform (e.g.SH2B1β) or a function emanating from the 1-631 region shared by all 4 isoforms.

Three of the variants identified in this study are present within the unique C-terminal tail of SH2B1α but not SH2B1β. A limited number of studies have compared the actions of the various isoforms in vitro. All four SH2B1 isoforms enhanced mitogenesis and cell proliferation in response to IGF-1, insulin and platelet-derived growth factor (PDGF) stimulation (2,129). Stable expression of each isoform in NIH3T3

fibroblasts led to enhanced insulin receptor autophosphorylation and phosphorylation of IRS1 (130). In 3T3-L1 cells, all four isoforms enhance insulin-stimulated glucose and amino acid transport, glycogen synthesis, lipogenesis, Akt activity, and p70 S6 kinase activity (130). In these assays, SH2B1α was as effective as, or more effective than, SH2B1β. Thus, it was surprising to observe the inability of SH2B1α to enhance NGF-induced neurite outgrowth. The finding that the γ and δ isoforms of SH2B1 resemble SH2B1β in their ability to enhance NGF-induced neurite outgrowth (data not shown) suggest that the unique C-terminal tail of SH2B1α inhibits at least some functions mediated by the region of SH2B1 between amino acids 1-631. In contrast to its inability to promote NGF-induced neurite outgrowth, SH2B1α, like SH2B1β, was found to enhance GH-induced macrophage motility. Exactly how SH2B1 stimulates motility is not known. However, one of the proline-rich regions present in all isoforms has been shown to bind Rac (89), a protein known to be involved in motility. Ligand-dependent phosphorylation of tyrosines within SH2B1 appears to be critical for SH2B1 enhancement of GH-dependent cell motility (92) suggesting that these phosphorylated tyrosines may recruit critical proteins to SH2B1 complexes. SH2B1β has also been shown to increase NGF-induced migration of PC12 cells in a wounding assay, perhaps by a protein kinase C-dependent process (131). The finding that most of the human variants impair the ability of SH2B1α and SH2B1β to enhance motility raises the possibility that regulation of the actin cytoskeleton and/or motility of cells is an important and vital component of SH2B1 function that plays a critical role in the ability of SH2B1 to regulate energy balance and the response to insulin.

SH2B1 is among a small number of adaptor proteins that undergo

nucleocytoplasmic shuttling (76) although its exact role within the nucleus is not yet clear. Our previous studies suggest that neurite outgrowth requires nuclear SH2B1 (69). Human mutations such as P90H, T175N, P322S and F344Lfs*20 that reside in the 1-631 region of SH2B1 impair both nuclear accumulation in the presence of LMB and enhancement of NGF-induced neurite outgrowth (6). Our finding here that SH2B1α neither accumulates in the nucleus nor enhances NGF-induced neurite outgrowth is consistent with SH2B1β enhancement of neurite outgrowth requiring nuclear SH2B1β. In contrast to neurite outgrowth, SH2B1β enhancement of GH-induced macrophage motility does not require its nuclear localization (92), a finding consistent with our observation here that SH2B1α retains the ability to enhance macrophage motility despite its inability to enter the nucleus. It is possible that the unique C-terminal tail of SH2B1α interferes with the region of SH2B1 that is required for nuclear localization. Masking of the region around the NLS in SH2B1α might explain why altering amino acid 175, which lies near the NLS, impairs the ability of SH2B1β but not SH2B1α to enhance GH-induced macrophage migration.

In summary, we have identified additional SH2B1 variants in individuals with obesity and that implicate SH2B1 isoforms besides SH2B1β as important for the regulation of body weight. Further studies will be needed to understand how the distinct C-terminal tails of the α, β, γ and δ isoforms influence SH2B1 function and their precise roles in vivo.

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Chapter 3

The C-terminal Tail of the α Isoform of SH2B1 Inhibits the Ability of SH2B1 to Enhance NGF Functions and Alters SH2B1 Subcellular Localization, Both by a Phosphorylation-Dependent Mechanism.

Abstract

The scaffold protein SH2B1, implicated as a major regulator of body weight, is recruited to the receptors of multiple cytokines and growth factors, including nerve growth factor (NGF). We found that the β isoform, but not the α isoform of SH2B1, greatly enhances NGF-dependent neurite outgrowth of PC12 cells, leading us to ask how the unique C-terminal tails of the α and β isoforms affect the ability of SH2B1 to regulate NGF function. We first compared the actions of SH2B1α and SH2B1β to those of SH2B1 1-631, consisting of the N-terminal region of SH2B1 shared by all isoforms. These results indicated that in contrast to the β tail, the α tail inhibits the ability of SH2B1 to cycle through the nucleus and enhance NGF-mediated neurite outgrowth, gene expression, phosphorylation of Akt and PLCγ and autophosphorylation of the NGF receptor TrkA. These functions were all restored when Tyr753 in the α tail was mutated to Phe. We provide evidence that TrkA can phosphorylate Tyr753 in SH2B1α, as well as Tyr439 and Tyr55 in both SH2B1α and SH2B1β. Finally, co-expression of SH2B1α, but not SH2β1α Y753F, inhibited the ability of SH2B1β to enhance neurite outgrowth. These

results suggest that the C-terminal tails of SH2B1 isoforms are key determinants of their cellular roles, which are regulated by phosphorylation.

Introduction

Proteomic complexity arising from alternative splicing has provided avenues for cells to optimize their response to a given stimulus. Receptors for growth factors and ligands are often alternatively spliced to produce optimized cellular signaling events for a single ligand, which enable distinct changes necessary for cellular homeostasis (97,132). The scaffold protein SH2B1 has the ability to interact with receptor tyrosine kinases via an SH2 domain to further modulate ligand signaling (4,23-26,125,133). To add further to the signaling complexity, SH2B1 is comprised of four isoforms (α, β, γ, δ) (2) that share their first 631 amino acids which contain a dimerization, pleckstrin homology (PH), and SH2 domain, nuclear localization and export sequences, and several proline-rich regions (134). Each isoform contains a unique C-terminal tail of 39-125 amino acids due to alternative splicing involving exon skipping and/or alternative 5' splice donor sites (2,3). In humans, SH2B1β and γ are ubiquitously expressed whereas SH2B1α and γ are expressed primarily in the brain (6). Studies using SH2B1$^{-/-}$ mice and patients with mutations in SH2B1 implicate SH2B1 in a number of physiological and biological functions, including control of body weight and energy expenditure (6,13,17,135), cardiac hypertrophy(136), aggressive behavior and developmental delay (6,135), and glucose homeostasis (18).

The most pronounced phenotype in mice and humans lacking SH2B1 is their obesity and impaired regulation of energy balance. *Sh2b1*$^{-/-}$ mice are also hyperphagic,

leptin-resistant, and insulin-resistant and the males are aggressive (6,18,19). In humans, SH2B1 has also been implicated as a potent regulator of energy balance. GWAS studies have identified SH2B1 as a gene associated with body weight (7,137). Individuals with gene deletions within chromosome 16p11.2 that include the SH2B1 gene exhibit early-onset obesity and greater than expected insulin resistance (12,14). Most recently, non-synonymous mutations have been identified in individuals that exhibit severe early-onset childhood obesity (GOOS cohort). These individuals also exhibit hyperphagia and greater than expected insulin resistance; some also appear to be aggressive and have learning issues (6,135). Restoration of the ubiquitously expressed SH2B1β isoform to SH2B1$^{-/-}$ mice using the neuron-targeted enolase driver largely restores the lean phenotype (54), consistent with SH2B1 in neurons being critical to the physiological functions of SH2B1.

SH2B1 has been implicated as an important regulator of cellular signaling for receptors for multiple growth factors and cytokines that are critical for the differentiation and/or function of various subsets of nerves. These include receptors for nerve growth factor (NGF), brain-derived growth factor (BDNF), glial-derived neurotrophic factor (GDNF), fibroblast growth factor, leptin, and insulin (3,22,26,27,37,133,138). The cellular role of SH2B1 has been most studied in the context of signaling by the endogenous NGF receptor TrkA in PC12 cells, a cell type that sends out neurite outgrowths and expresses neuronal-specific genes in response to NGF (26,62,79). In response to NGF, SH2B1 is recruited, via its SH2 domain, to the NGF receptor TrkA, which in turn appears to phosphorylate SH2B1 (25,26). Overexpression and knock-down studies showed that SH2B1 is required for maximal NGF-stimulated neurite

outgrowth and expression of a subset of NGF-sensitive genes (25,26,69,79). Live cell confocal microscopy of GFP-SH2B1β revealed that SH2B1β localizes primarily at the plasma membrane and in the cytoplasm in PC12, COS-7, 293T cells, and primary hippocampal neurons (78,139). However, SH2B1 has both a nuclear localization sequence (NLS) and nuclear export signal (NES), and mutating the NES or adding an inhibitor of nuclear export, leptomycin B (LMB), enables SH2B1β to accumulate in the nucleus (69,76), indicating that SH2B1 cycles through the nucleus. Blocking the ability of SH2B1 to cycle through the nucleus also blocks the ability of SH2B1 to stimulate NGF-induced neurite outgrowth and gene expression, suggesting that cycling through the nucleus may be required for at least a subset of SH2B1 functions important for NGF signaling.

The brain specific tissue expression of the α and δ isoforms of SH2B1 suggests that these two isoforms may play particularly important role in neurons. Curiously, however, while we found that the β isoform of SH2B1 potently enhances neurite outgrowth of PC12 cells, the α isoform had little to no effect nor did the α isoform appear to cycle through the nucleus (135). These findings suggest that the α isoform may have different functions than the β isoform and that the unique C-terminal tail of SH2B1α and/or β is a potent regulator of SH2B1 function. In this study, we wished to gain insight into how relatively small variations in scaffold proteins due to differential splicing might regulate the function of those proteins. We first asked whether the unique α or β tail inhibits or promotes the actions of SH2B1, respectively. We then examined how the isoform specific α and β tails might regulate the function of SH2B1 and which steps in NGF signaling are affected. Our results provide strong evidence that

the unique C-terminal tails of SH2B1 isoforms are key determinants of their cellular roles, which can be regulated by phosphorylation and dephosphorylation of critical amino acids.

Materials and methods

Antibodies

Rabbit polyclonal antibody to rat SH2B1 that recognizes all isoforms of SHB21 (αSH2B1, 211) (kind gift of L. Rui, University of Michigan) was raised against glutathione S-transferase SH2B1β fusion protein and used at a dilution of 1:1000 for immunoblotting (IB) (45). Mouse monoclonal antibody to Myc (αMyc) (9E10; IB 1:2000) and rabbit polyclonal antibodies to Myc (A14; IB 1:1000; immunoprecipitation (IP) 1:50) or Trk (αTrk; C-14, sc-11; 1:500) were from Santa Cruz. Mouse monoclonal antibodies to Akt (αAkt; 2920S; 1:2000) and Erks 1 and 2 (αErk 1/2; 4696S; 1:2000); rabbit monoclonal antibodies to phospho-Akt (Ser473) (αpAkt-Ser473; 4058L; 1:1000), phospho-TrkA (Y490) (αpTrkA-Y490; 4619S; 1:500), phospho-TrkA (Y674/5) (αpTrkA-Y674/5; 4621P; 1:500), and phospho-TrkA (Y785) (αpTrkA-Y785; 4168S; 1:500); and rabbit polyclonal antibodies to doubly phosphorylated Erks 1 and 2 (Thr202/Tyr204) (αpErk 1/2; 9101S; 1:1000), PLCγ (αPLCγ; 2822S; 1:1000) and phospho-PLCγ (Tyr 783) (αpPLCγ-Tyr 783; 2821S; 1:500) were from Cell Signaling. Rabbit polyclonal antibody to Myc (αC-Myc (A190-105A); IP 1:400) came from Bethyl laboratories. Mouse monoclonal antibody to phosphotyrosine (αPY; 4G10; 1:1000) came from Millipore. Rabbit polyclonal antibody to GFP (αGFP; #632592; 1:2000) came from Clontech. IRDye800-conjugated, affinity purified anti-goat GFP (αGFP (600-132-215); IB:

1:20,000) came from Rockland Immunochemicals and IRDye 800- (827-11081) and IRDye 700- (926-32211) conjugated affinity-purified anti-mouse IgG and anti-rabbit IgG were from Li-cor Odyssey.

Plasmids

cDNA encoding mouse SH2B1α was a kind gift of H. Riedel (2). cDNAs encoding GFP- and Myc- tagged rat SH2B1β have been described previously (26). The sequence encoding mouse SH2B1α (AF421138) was subcloned into pEGFPC1 (Clontech) and Prk5 vector containing an N-terminal GFP and myc tag, respectively. Additional mutations were introduced into SH2B1β or SH2B1α using Quickchange II Site-Directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Multiple mutation sites were introduced into SH2B1β or SH2B1α in successive steps using the same primer pairs as indicated in Table S1. Vectors encoding Td-tomato rat SH2B1β and mouse SH2B1α were generated by restriction digest of 5' AgeI and 3' BsrGI sites flanking the region encoding the respective GFP-tagged isoform and swapped with the Td-tomato sequence vectors encoding N-terminal tagged Td-tomato SH2B1 isoforms.

Cell culture and transfection

PC12 cells (CRL-1721; ATCC) were plated on rat tail type I collagen (BD Biosciences) coated dishes and maintained at 37°C with humidified air at 5% CO_2 in normal growth medium (RPMI-1640 medium [Life Technologies] 10% horse serum [HS; Atlanta], 5% fetal bovine serum [FBS; Life Technologies]). PC12 cells were transiently transfected using Lipofectamine LTX (Life Technologies) per manufacturer's instructions for 24-72

h. PC12 cell lines stably overexpressing GFP or GFP tagged SH2B1β, SH2B1α, SH2B1α Y753F or SH2B1 1-631 were generated by transfecting cells with the indicated plasmid using Lipofectamine LTX for 72 h and then selecting in G418 medium (normal growth medium containing 0.5 mg/ml G418 [Cellco; Corning]) in normal growth medium for 5 days. Cells were expanded and maintained for 30 days. The top 60% of GFP-positive cells were gated and sorted from non-GFP expressing cells by fluorescence-activated cell sorting (FACs [Beckman Coulter MoFlo Astrios, University of Michigan Flow Cytometry Core]). Sorted cells were maintained in G418 medium. Prior to experimental use, PC12 cells were incubated overnight in deprivation medium (RPMI-1640 containing 1% bovine serum albumin (BSA; Proliant Biologicals)).

HEK293T cells were maintained at 37°C with humidified air at 5% CO_2 in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 1 mM L-glutamine, 0.25 μg/ml amphotericin, 100 U/ml penicillin, 100 μg/ml streptomycin (DMEM culture medium), and 8% calf serum (CS; Life Technologies). HEK293T cells were transiently transfected using calcium phosphate precipitation (140) or polyethyleneimine (PEI; Polysciences Cat # 23966). Briefly, PEI (1 μg/μL) was mixed with DNA at a 4:1(w/v) ratio in Opti-Mem (Gibco by Life Technologies; 31985-070) and incubated for 10 min at room temperature. Cells were used after 24 or 48 h.

Neurite outgrowth

PC12 cells or stably transfected PC12 cells were plated in 6-well dishes and transiently transfected with the indicated construct. Cells were treated with 25 ng/ml 2.5S mouse NGF (BD BioSciences, 356004; or Harlan, BT.5025) in RPMI-1640 medium containing

2% HS and 1% FBS. GFP- or Td-tomato positive cells were visualized by fluorescence microscopy (20X or 40X objective, Nikon Eclipse TE200) after 1, 2, or 3 days. 100 GFP- or Td-tomato-positive cells were scored in three different areas of each plate for the presence of neurites 2 times the length of the cell body (total 300 cells per condition per experiment). The experiment was repeated 3 times (total 900 cells per condition). The percentage of cells with neurites was determined by dividing the number of cells with neurites by the total number of GFP-, Td-tomato, or GFP/Td-tomato positive cells counted.

Immunoblotting and immunoprecipitation

Stably transfected PC12 cells were seeded at $10x10^6$ cells per 10-cm collagen-coated dish in normal growth medium. After 24 h, cells were incubated overnight in deprivation medium. Cells were incubated at 37°C with NGF (25 or 100 ng/ml) as indicated, placed on ice and washed two times with phosphate buffered saline (10 mM $NaPO_4$, 140 mM NaCl, pH 7.4; PBS) containing 1 mM Na_3VO_4 pH 7.3 (PBSV). Cells were then lysed on ice for 10 min with ice-cold 20mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin), pH 7.5. Cell lysates were centrifuged at ~15,000 x g for 10 min at 4°C and protein concentrations of the supernatant were determined using DC protein assay (Bio-Rad). Proteins were resolved by SDS-PAGE using 9%, 10%, or 5-12% gradient acrylamide gels.

Transfected HEK293T cells were maintained in normal growth medium and incubated at 37°C, placed on ice and washed 2x with PBSV. Cells were then lysed on ice for 5-10 min with ice-cold L-RIPA buffer (50mM Tris, 150 mM NaCl, 0.1% Triton X-100, 2 mM EGTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.2. Cell lysates were centrifuged at 8800 x g for 10 min at 4°C.

For immunoprecipitation, antibodies to Myc ((αMyc; A14; 1:80) or (αMyc; C-Myc; 1:450)) were pre-conjugated to protein A beads (Repligen) overnight at 4°C, and then added to cell lysates containing equal amounts of protein for 2-3 h at 4°C under constant rotation. Bound proteins were eluted and resolved on 9% acrylamide SDS-PAGE gels. Proteins were transferred onto nitrocellulose (Bio-Rad) or low-fluorescence PVDF (Bio-Rad) membranes. Blots were incubated with antibodies in 10 mM Tris, 150 mM NaCl, pH 7.4 and 0.1% tween-20 containing either 3-5% BSA (or 1% chicken egg albumin for blots probed with αpTyr) overnight at 4°C, followed by secondary antibody for 1 h at room temperature. Bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences) and quantified using Image Studio Lite 4.0.

Cell imaging

GFP or indicated GFP-SH2B1α or β was transiently expressed in HEK 293T cells seeded on 10mm glass-bottom dishes (MatTek) in normal growth medium. Cells were imaged in Ringer's buffer (10 mM HEPES, 155 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM NaH_2PO_4 and 10 mM glucose, pH 7.2) using a 60x water-immersion objective on an Olympus FV500 laser scanning confocal microscope and FluoView version 5.0 software. A multi-line argon blue laser was used to excite GFP fluorescence

at 488 nm. Relative levels of GFP-SH2B1 in the plasma membrane (PM), cytoplasm, and nucleus were determined using line scans and MetaVue 6.0 (Universal Imaging, Sunnyvale, CA) from 16-bit images. Image planes were chosen to allow maximal visualization of the plasma membrane and nucleus. GFP intensity was quantified using a 15-pixel wide linescan in MetaVue that was drawn perpendicular to the plasma or nuclear membrane and into the cytoplasm in Figures. 1E and 3A. Pixel intensity averages for each linescan in the PM, nucleus or cytoplasm were used to generate PM/cytoplasm or nuclear/cytoplasm ratios. To avoid bias, an individual blinded to the data generated line scans of the micrographs. Cells were monitored after 4-6 h with 20 nM Leptomycin B (LMB; Sigma), and imaged when the nuclear to cytoplasmic ratio of GFP–SH2B1β was ≥ 1. Two or more individual experiments were conducted with each form of SH2B1 and 16-80 cells were quantified by line scan. Generally, 90% of the cells showed the same subcellular distribution for individual forms of GFP–SH2B1 at a medium expression level. The outliers were either clearly dying or dead cells, or the highest expressing cells in which the ratio of cytoplasmic to PM signal was abnormally high, presumably due to saturation of a limited number of binding sites in the PM.

Quantitative polymerase chain reaction (qPCR)

PC12 cells stably overexpressing GFP or the indicated GFP-SH2B1 were incubated at 37°C in deprivation medium overnight. Cells were treated with or without 100 ng/ml NGF (Harlan) for 6 h. Total RNA was extracted using Trizol (Ambion by Life Technologies) according to the manufacturer's instructions and RNA quality confirmed by OD via Nanodrop spectrophotomer. cDNA was generated from the RNA using Taqman Reverse Transcription Reagent Kit (Applied Bio-Systems; N808-0234) per

manufacturer's instructions. Transcript levels of *Plaur, Mmp10, Mmp3, FosL1, Hprt (141),* and *Cyclophilin A* (142) were determined in triplicate per gene by qPCR using the TaqMan kit (Invitrogen), SYBR green I (Life Technologies) and an Eppendorf Realplex2 (using Mastercycler ® software). Cycle threshold values were normalized to the geometric mean of the cycle thresholds for *HPRT* and *Cyclophilin A* whose expression did not differ between the different cell lines or with NGF treatment (143). Results were then normalized to the GFP-SH2B1β with NGF treatment in Figure. 3.4.

Statistics

Statistics were performed using GraphPad Prism 7.01. For neurite outgrowth and western blot quantification, time course curves were converted to area under the curve (AUC). For qPCR experiments, relative transcript expression levels were normalized to GFP-SH2B1β NGF-treated values. To account for large differences in experimental variability, data were transformed by taking the log of the relative expression. One-way ANOVA with repeated measures and multiple comparison analysis with Dunnett's post-test (compared to GFP control cells) were used to test for statistical significance. Data shows the mean ± S.E.M for three independent experiments (N=3) except for Figure. 3.8A, in which the mean ± range is depicted (N=2). * indicates p-values ≤ 0.05, which were considered significant.

Results

The unique C-terminal tail of the α isoform of SH2B1 inhibits the ability of SH2B1 to enhance NGF-mediated neurite outgrowth and affects its subcellular localization.

Previously, we showed that, unlike SH2B1β, SH2B1α does not enhance the ability of NGF to promote neurite outgrowth (135). To determine whether the unique β tail enables SH2B1β to enhance neurite outgrowth or if the unique α tail prevents SH2B1α from enhancing neurite outgrowth, we truncated the C-terminus of SH2B1 at the site of alternative splicing, leaving only the N-terminal 631 amino acids shared by both isoforms (Figure. 3.1A). We transiently expressed GFP, or GFP-tagged SH2B1α, β, or 1-631 in PC12 cells and assessed the percentage of transfected cells exhibiting neurites after 1, 2 and 3 days of NGF treatment (Figure. 3.1B, 3.1C). As reported previously (26,135), SH2B1β greatly enhanced, while SH2B1α had no effect on, neurite outgrowth. SH2B1 1-631 enhanced neurite outgrowth to an extent similar to that seen with SH2B1β, suggesting that the unique C-terminal tail of SH2B1α prevents the N-terminal 631 amino acids of SH2B1 from enhancing neurite outgrowth. Figure. 3.1D shows that these observed differences in SH2B1's ability to enhance neurite outgrowth cannot be attributed to differences in levels of expression of the various forms of GFP-SH2B1.

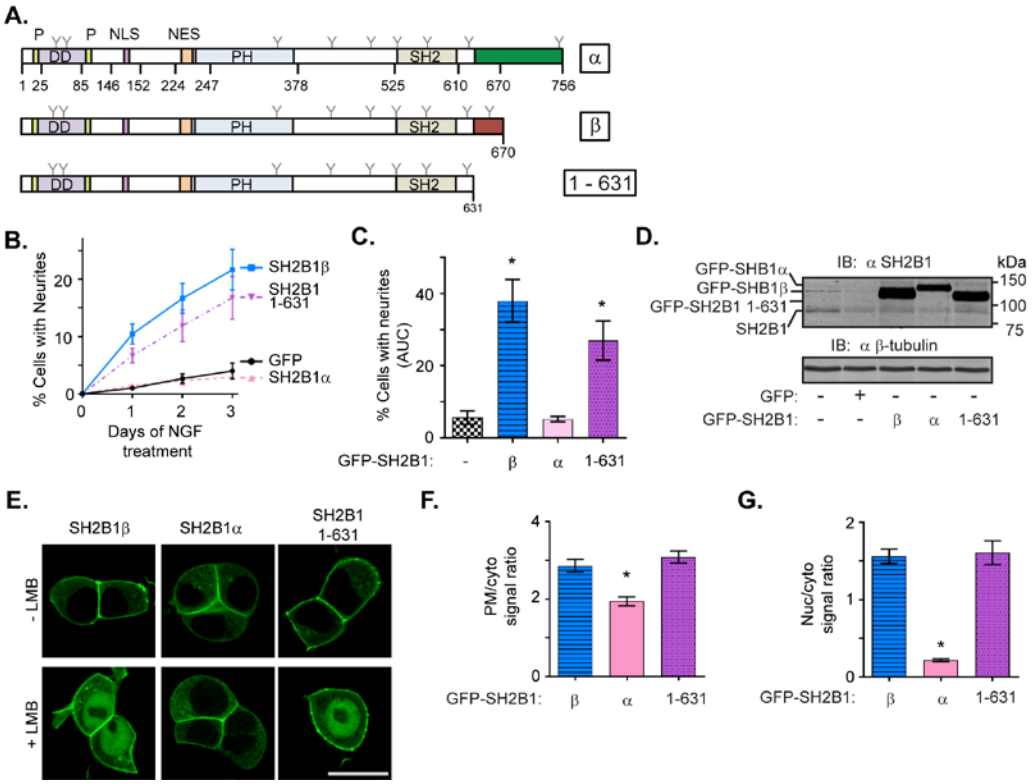

Figure 3.1. SH2B1α's C-terminal tail regulates SH2B1's ability to enhance NGF-mediated neurite outgrowth and translocate to the nucleus. A) Schematic of SH2B1α, β and 1-631. DD, dimerization domain (a.a. 25–85); NLS, nuclear localization sequence (a.a. 146–152); NES, nuclear export sequence (a.a. 224–233); PH, pleckstrin homology domain (a.a. 247–378); SH2 domain (a.a. 525–610); P, proline-rich domains (a.a. 16-24; 85-106); Y indicates tyrosine location. Different color tails represent unique isoform sequences. Numbers indicate a.a. in rat and mouse sequences. **B)** PC12 cells transiently expressing GFP or GFP-tagged SH2B1β, SH2B1α, or 1-631 were incubated with 25 ng/ml NGF for the indicated number of days. The percentage of GFP-expressing PC12 cells with neurite outgrowths at least twice the length of the cell body was determined on the indicated days. Results show means + s.e.m., n = 3 experiments. **C)** Area under the curve (AUC) was determined from the data in panel B. * p ≤ 0.05 compared to cells expressing GFP. **D)** PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 treated with NGF (25 ng/mL) for 2 days. Proteins were separated by a 10% acrylamide SDS-PAGE gel and immunoblotted with αSH2B1 or αβ-tubulin (control). Migration of Mw standards and the various GFP-SH2B1 are shown. **E)** 293T cells transiently expressing the indicated GFP-SH2B1 were treated with or without 20 nM LMB for 6 h. Live cells were imaged by confocal microscopy. Scale bar = 20 μM. **F, G)** Plasma membrane (PM) to cytoplasmic (cyto) (**F**) or nuclear (nuc) to cyto (**G**) fluorescence ratios of cells treated as in Panel **E** were measured by linescan using MetaVue. Means ± s.e.m. of 47-80 cells from 3-4 independent experiments are shown. * p≤0.05 compared to GFP-SH2B1β.

Mutating the NLS in SH2B1β abrogates the ability of SH2B1β to both cycle through the nucleus and enhance NGF-mediated neurite outgrowth (69,76), suggesting that SH2B1 might need to enter the nucleus to promote neurite outgrowth. Since the α tail prevents SH2B1α from enhancing NGF-induced neurite outgrowth, we next examined whether the α tail also prevents SH2B1α from cycling through the nucleus. Using live cell confocal microscopy, we visualized and quantified the subcellular localization of transiently expressed GFP tagged SH2B1α, β and 1-631 in 293T cells before and after treatment with the nuclear export inhibitor leptomycin B (LMB) (Figure. 3.1E). As reported previously (135), in the steady state (absence of LMB), GFP-SH2B1α and β both localize primarily at the plasma membrane and in the cytoplasm (Figure. 3.1F). By preventing its export from the nucleus, LMB enables GFP-SH2B1β, but not SH2B1α, to accumulate in the nucleus (Figures. 3.1E, 3.1G), consistent with SH2B1β, but not SH2B1α, being able to cycle through the nucleus. Like GFP-SH2B1α and β, GFP-SH2B1 1-631 localizes primarily at the plasma membrane and in the cytoplasm (Figures. 3.1E, 3.1F). In the presence of LMB, SH2B1 1-631 accumulates in the nucleus like SH2B1β and unlike SH2B1α (Figures. 3.1E, 3.1G), consistent with the C-terminal tail of SH2B1α preventing SH2B1α from entering the nucleus.

Confocal microscopy also confirmed our earlier observation (135) that SH2B1α localizes to the plasma membrane to a lower extent than SH2B1β and further revealed that SH2B1 1-631 localizes to the plasma membrane to a similar extent to that of SH2B1β (Figures. 3.1E, 3.1F). These results suggest that the unique C-terminal tail of SH2B1α interferes with the ability of the N-terminal 631 amino acids of SH2B1 to

localize to the plasma membrane.

Mutating Tyr753 in the unique C-terminal tail of SH2B1α enables SH2B1α to enhance NGF-mediated neurite outgrowth and cycle through the nucleus.

The results shown in Figures. 3.1 raise the possibility that some function of the unique C-terminal tail (e.g protein-protein interaction) prevents SH2B1α from enhancing neurite outgrowth and cycling through the nucleus. We therefore analyzed the amino acid sequence of the α tail for a functional domain. We found that the α tail contains a potential PDZ binding domain (N_{751}QYSFV) (105) not present in any of the other SH2B1 isoforms. To gain insight into whether binding of the α tail to a PDZ domain-containing protein might contribute to the decreased ability of SH2B1α to enhance neurite outgrowth, enter the nucleus, and/or localize to the plasma membrane, we mutated key residues in this potential PDZ binding domain. Mutating Ser754 to Ala (S754A) and Val756 to Asp (V756D) did not enable GFP-SH2B1α to enhance NGF-mediated neurite outgrowth to a statistically significant extent (Figures. 3.2A, 3.2B) or to accumulate in the nucleus of LMB treated cells (Figures. 3.3A, 3.3B). However, mutating Tyr753 to Phe (Y753F) enabled SH2B1α to enhance neurite outgrowth to almost the same extent as SH2B1β (Figures. 3.2A, 3.2B) and to accumulate in the nucleus to a significantly greater extent than SH2B1α, although not to the same extent as SH2B1β (Figures. 3.3B). In contrast, mutating Tyr753 to the phosphomimetic glutamic acid (Y753E) did not enable SH2B1α to enhance NGF-mediated neurite outgrowth or accumulate in the nucleus. These observed differences in SH2B1 ability to enhance neurite outgrowth could not be attributed to differences in levels of expression of the various forms of

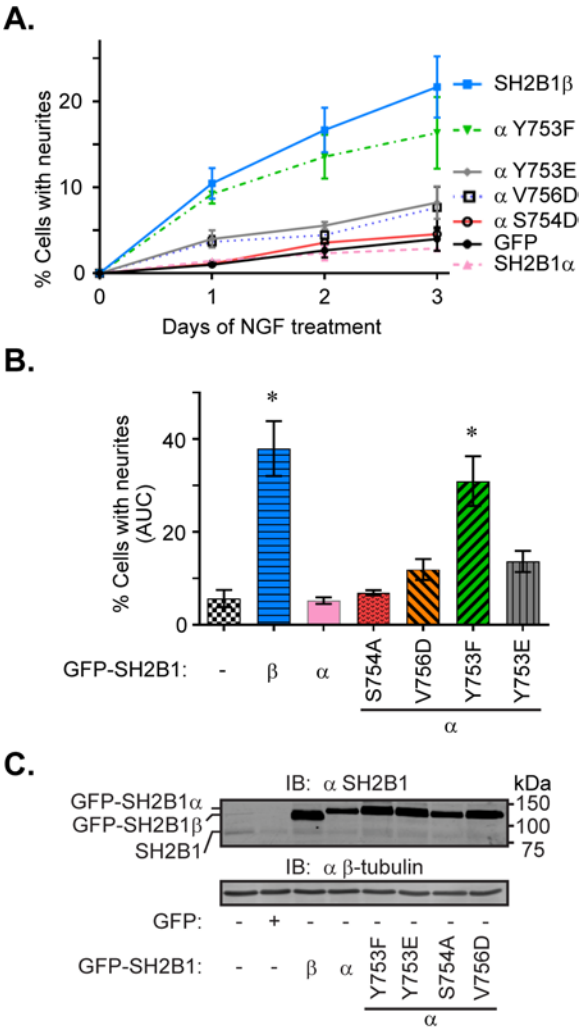

Figure 3.2. Mutating Tyr753 in SH2B1α's C-terminal tail enables SH2B1α to enhance NGF-mediated neurite outgrowth. A) Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the indicated days. **B)** AUC was determined from the data in Panel **A**. **C)** PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 treated with NGF (25 ng/mL) for 2 days. Proteins were separated by a 10% acrylamide SDS-PAGE gel and immunoblotted with αSH2B1 or αtubulin (control). Migration of Mw standards and the various GFP-SH2B1 are shown. * $p \leq 0.05$ compared to GFP expressing cells.

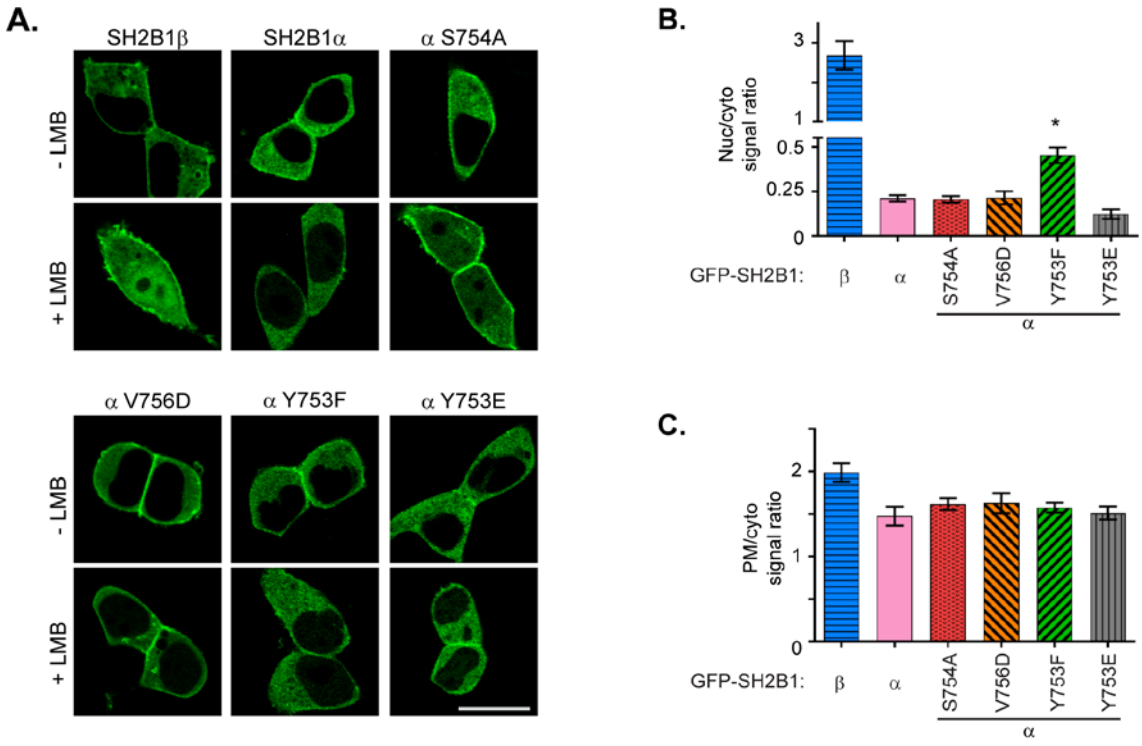

Figure 3.3. Tyr753 in SH2B1α regulates the ability of SH2B1α to translocate to the nucleus. A) 293T cells expressing GFP-SH2B1β or GFP-SH2B1α were treated with or without 20 nM LMB for 6 h. Live cells were imaged by confocal microscopy. Scale bar = 20 μM. **B, C)** Nuclear (nuc) to cytoplasmic (cyto) (**B**) or plasma membrane (PM) to cyto (**C**) fluorescence ratios of cells treated as in Panel A were measured by linescan using MetaVue. Means ± s.e.m. of 16-55 cells from 2-4 independent experiments are shown. * p≤0.05 compared to GFP-SH2B1β.

GFP-SH2B1 (Figures. 3.2C). None of the mutations (S754A, V756D, Y753F, Y753E) affected the plasma membrane localization of SH2B1α (Figures. 3.3C). These data suggest that the C-terminal tail of SH2B1α does not block the effects of SH2B1 by binding directly to a PDZ-domain containing protein. Rather, phosphorylation of Tyr753 may prevent SH2B1α from enhancing NGF-induced neurite outgrowth and entering the nucleus.

SH2B1α's C-terminal tail prevents it from enhancing expression of NGF-responsive genes.

Previous studies showed that SH2B1β enhances the expression of a subset of NGF responsive genes, including *Plaur, Mmp3, Mmp10,* and *FosL1* (79). *Plaur* is an early response gene that encodes urokinase plasminogen activator receptor (UPAR). UPAR is located on the plasma membrane where it binds to and activates plasminogen activator (uPA). UPAR has been shown to be necessary for NGF-mediated neurite-outgrowth in PC12 cells (80,86). The matrix metalloproteases MMP3 and MMP10, along with uPA, are critical for degradation of the extracellular matrix to promote neuronal axon and dendrite extension (144). Fos-like antigen 1 (FosL1, Fra1*)* is a member of the Fos family of early-response genes (145). As seen with the neurite outgrowth assays, overexpression of SH2B1β and 1-631 both significantly enhanced NGF-mediated expression of *Plaur, Mmp10,* and *FosL1* whereas SH2B1α had no effect (Figure. 3.4). Mutating Tyr753 to Phe enabled SH2B1α to enhance expression of *Plaur*, *Mmp10*, and *FosL1*. A similar trend was seen with *Mmp3*, although the differences did not achieve statistical significance. These data provide further evidence that the α tail negatively

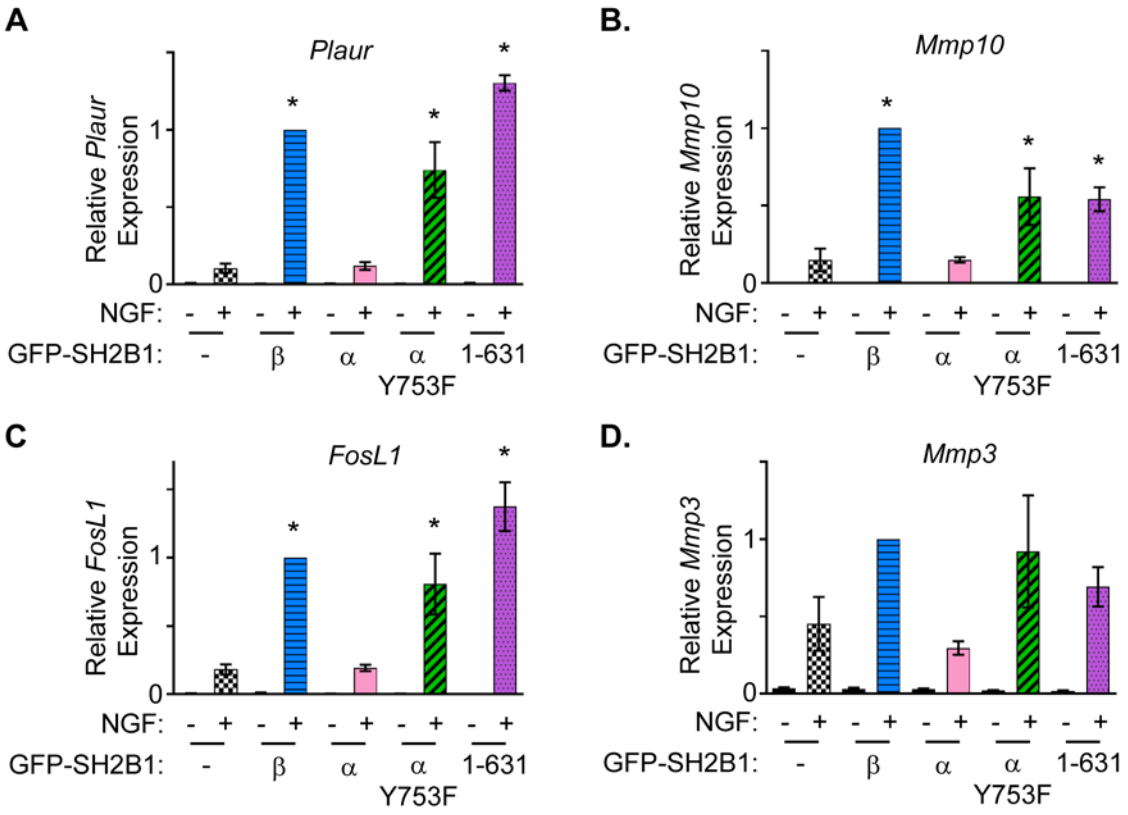

Figure 3.4. Loss of SH2B1α's C-terminal tail or mutation of Tyr753 in the tail enables SH2B1α to enhance NGF-mediated gene transcription. PC12 cells stably overexpressing GFP (-) or the indicated GFP-tagged SH2B1 were treated with 100 ng/mL NGF for 6 h. mRNA was collected and levels for *Plaur* (**A**.), *Mmp10* (**B**), *FosL1* (**C**), *Mmp3* (**D**) were determined by qPCR. Relative mRNA abundance was normalized to the geometric mean of the levels of *Hprt* and *Cyclophilin A* mRNA. Data were normalized to values obtained by GFP-SH2B1β cells treated with NGF. Means ± s.e.m. are shown for 3 independent experiments. mRNA levels for untreated samples are plotted but were usually too low to be visible on the graph. * p≤0.05 compared to GFP with NGF.

regulates the ability of the N-terminus of SH2B1 to enhance NGF-mediated functions by a mechanism requiring phosphorylation of Tyr753.

The C-terminal tail of SH2B1α inhibits SH2B1 enhancement of NGF signaling pathways; mutation of Tyr753 reverses that inhibition.

To gain insight into the mechanism by which the α tail inhibits the ability of SH2B1α to enhance NGF-induced neurite outgrowth and gene expression, we next examined the effect of the α tail on the ability of SH2B1 to affect NGF signaling pathways. NGF-mediated neurite outgrowth in PC12 cells is initiated by NGF binding to and activating the receptor tyrosine kinase TrkA (55). The resulting auto-phosphorylation sites in TrkA recruit signaling proteins via their SH2 and/or PTB domains, which initiates multiple signaling pathways including those leading to rapid and prolonged activation of Erk1 and Erk2, Akt, and PLCγ (55). Our lab and others have previously shown that overexpression of SH2B1β in PC12 cells enhances and prolongs NGF-induced phosphorylation of activating amino acids in Akt (Ser473) and PLCγ (Tyr783) (70,146). Enhancement of phosphorylation of activating amino acids (Thr202, Tyr204) of Erks 1 and 2 has been observed in some (63,146) but not all (69,146) studies. Consistent with these previous results, overexpression of SH2B1β in PC12 cells enhanced and prolonged phosphorylation of Tyr783 in PLCγ (Figures. 3.5A, top panel, 3.5B) and Ser473 in Akt (Figures. 3.5A, 3rd panel, 3.5C) but did not have a statistically significant effect on phosphorylation of Thr202/Tyr204 in Erks 1 and 2 (Figures. 3.5A, 5th panel, 3.5D). In contrast, overexpression of SH2B1α had no effect

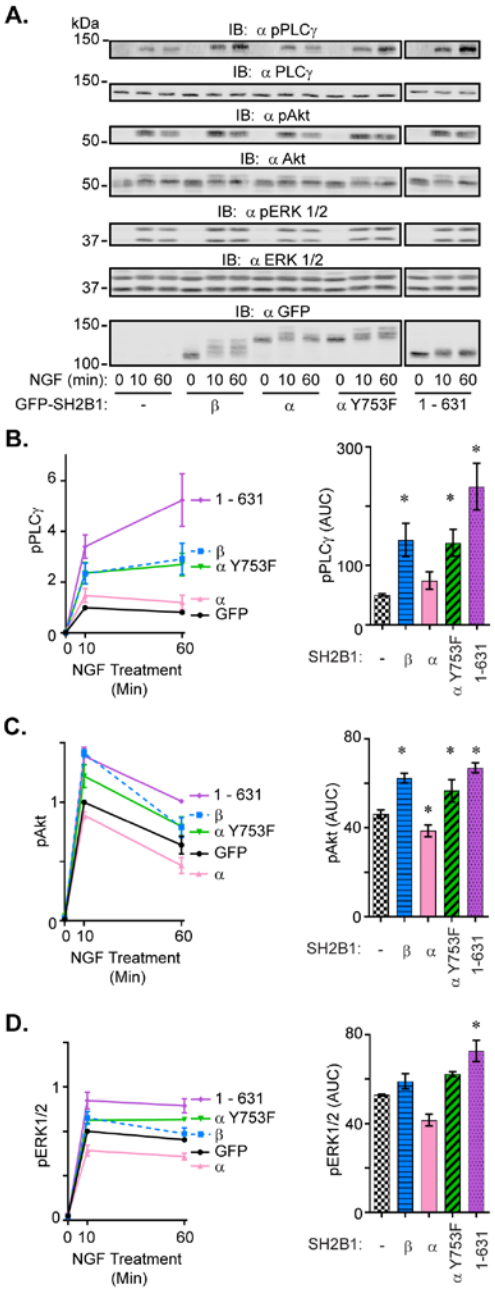

Figure 3.5. SH2B1's C-terminal tails modulate NGF-dependent phosphorylation of NGF signaling proteins. A) Serum-starved (15h) PC12 cells stably overexpressing GFP (-) or the indicated GFP-tagged SH2B1 were treated with 25 ng/mL NGF for 10 or 60 min. Proteins in cell lysates were separated by 5-12% acrylamide gradient SDS-PAGE gels and immunoblotted with the indicated antibodies. The 3 SH2B1 1-631 lanes were run on the same gel. **B)** The relative signal intensities of the bands & AUC determined from the relative signal intensities corresponding to pPLCγ, **C)** pAkt, and **D)** pERK1/2 were determined and normalized to the GFP signal at 10 minutes. Means ± s.e.m. are shown from 3 independent experiments. Mean ± s.e.m. is shown. * $p \leq 0.05$ compared to GFP**.**

on PLCγ phosphorylation (Figures 3.5A, 3.5B) and instead inhibited NGF stimulation of phosphorylation of Akt (Figures 3.5A, 3.5C). Overexpression of SH2B1 1-631 (Figure 3.5) showed enhanced and prolonged phosphorylation of PLCγ, Akt and Erks 1 and 2 (Figures. 3.5A-D), indicating that the α tail of SH2B1α inhibits the ability of SH2B1 to enhance NGF signaling. Further mutating Tyr753 to Phe in the C-terminal tail of SH2B1 overcomes the inhibitory effect of the α tail on SH2B1 enhancement of NGF-induced phosphorylation of PLCγ, Akt, and Erk1/2 phosphorylation. The SH2B1 Tyr753 results suggest that phosphorylation of Tyr753 in the α tail prevents SH2B1 from enhancing NGF signaling pathways, and for some pathways (e.g. Akt, Erks1/2), may even inhibit them, which could contribute to the inability of SH2B1α to enhance NGF-induced neurite outgrowth and gene expression.

Immunoblots of the various forms of GFP-SH2B1 stably expressed in PC12 cells reveal that NGF causes a broadening and an upward shift in their migration in SDS PAGE-gels. The upward shift in SH2B1β has been shown previously to be due primarily to Ser/Thr phosphorylation (64). Phosphorylation on at least some of these Ser residues (Ser 161, 165) appears to be important for SH2B1 to leave the plasma membrane, enter the nucleus and enhance both NGF-induced neurite outgrowth and gene expression (78). As reported previously, GFP-SH2B1β showed a robust decrease and spread in migration after 10 and 60 min of NGF treatment. (Figure. 3.5A, bottom panel). GFP-SH2B1α also exhibited a robust but more transient decrease and spread in migration. Mutating Tyr753 enhanced and prolonged the NGF-dependent upward shift in GFP-SH2B1α resulting in a banding pattern that more closely resembled the migration profile of GFP-SH2B1β. Surprisingly, GFP-SH2B1 1-631 showed a much more modest change

in migration in response to NGF than either GFP-SH2B1α or β. These data are consistent with the C-terminal tails of both α and β increasing the ability of SH2B1 to become phosphorylated on Ser/Thr in response to NGF. Phosphorylation of Tyr753 in the α tail appears to accelerate dephosphorylation of at least some of those Ser/Thr residues.

The C-terminal tail of SH2B1α inhibits SH2B1 enhancement of NGF induced autophosphorylation of TrkA; mutation of Tyr753 reverses that inhibition.

Some studies have suggested that SH2B1 isoforms, which are recruited to TrkA via their shared SH2 domain (25,26), are capable of activating TrkA, based on their ability to enhance NGF-induced phosphorylation of Tyr490 in TrkA (63). Differences in the ability of the different forms of SH2B1 to enhance TrkA activity or phosphorylation of specific Tyr in TrkA might therefore contribute to the differences in their ability to enhance downstream phosphorylation of NGF signaling proteins. We used phosphospecific antibodies and our stably transfected PC12 cell lines to examine the impact of SH2B1α, β, and 1-631 on the ability of NGF (100 ng/mL) to stimulate phosphorylation of Tyr490, 674/675 and 785 in TrkA after 10 and 60 min (Figure. 3.6). Tyrosines 674/675 are the activating tyrosines of TrkA and their phosphorylation correlates with TrkA activity (58). pTyr490 recruits both Shc and FRS2, both of which are thought to lead to activation of Erks 1 and 2 - Shc via the Shc-Grb2-SOS-Ras-Raf-MEK-Erk1/2 pathway and FRS2 via a Crk-C3G-Rap1-B-Raf-Erks 1/2 pathway (116,117). pTyr785 recruits PLCγ which catalyzes the formation of inositol 1,4,5-trisphosphate and diacylglycerol, leading to, among a variety of things, release of Ca^{+2}

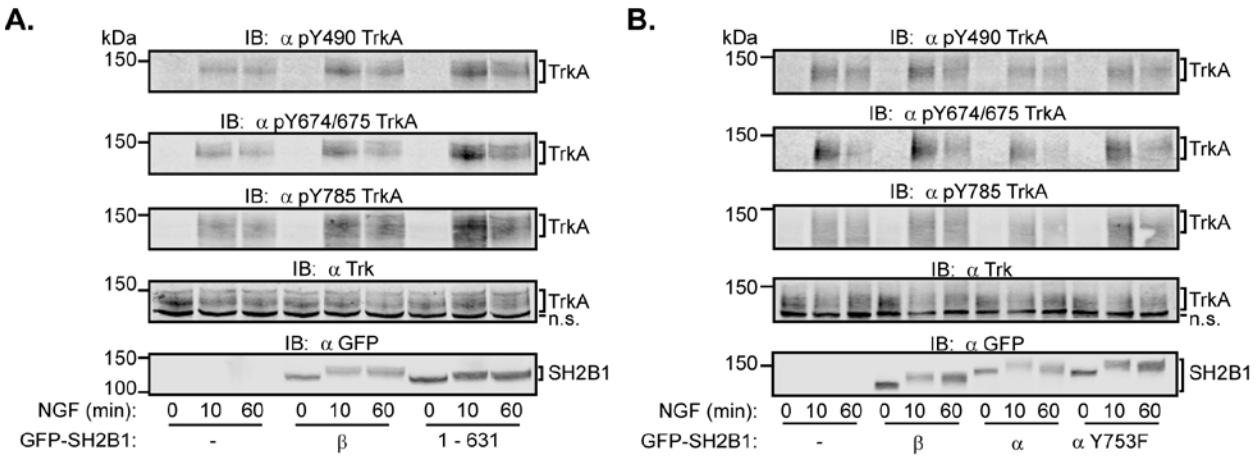

Figure 3.6. SH2B1's C-terminal tails modulate NGF-mediated TrkA phosphorylation. A, B) Serum-starved (15 h) PC12 cells stably overexpressing GFP(-) or the indicated GFP-tagged SH2B1 were then treated with 100 ng/mL NGF for 10 and 60 min. Proteins in cell lysates were separated using SDS-PAGE (9% acrylamide) and immunoblotted with the indicated antibodies. GFP and GFP SH2B1β lanes for panels **A** and **B** are aliquots of the same cell lysates. The blot is representative of 3 independent experiments.

from the endoplasmic reticulum and activation of protein kinase C (123,147). GFP-SH2B1β appeared to enhance the phosphorylation of all of these Tyr residues in TrkA, although to variable extents (compare Figures. 3.6A and 3.6B). In contrast, overexpression of SH2B1α had no impact on the phosphorylation of any of these Tyr residues in TrkA unless Tyr 753 in SH2B1α was mutated to Phe. Phosphorylation of these Tyr in TrkA in cells expressing SH2B1 1-631 resembled that observed with in cells expressing SH2B1β. These data are consistent with the α tail preventing SH2B1 from enhancing TrkA activity and subsequent autophosphorylation of Tyr490 and Tyr785, unless Tyr753 in the α tail is mutated to Phe.

TrkA phosphorylates multiple Tyr in SH2B1α and β, including Tyr753 in SH2B1α.

Since mutating Tyr753 to Phe restores the ability of SH2B1α to promote NGF-induced neurite outgrowth and gene expression, phosphorylation of Tyr753 in the α tail likely inhibits the actions of SH2B1. We have shown previously that SH2B1β is phosphorylated on Tyr in response to NGF (26), presumably by the NGF receptor TrkA. We therefore wondered whether Tyr753 is a substrate of TrkA. To gain insight into whether TrkA can phosphorylate Tyr753 in SH2B1α and/or other Tyr within SH2B1α and β, we used site-directed mutagenesis to mutate each individual Tyr in Myc-tagged SH2B1α or β to Phe. The Myc tag was used because it does not contain any tyrosine residues. We then transiently co-expressed each mutated SH2B1 with TrkA in 293T cells, immunoprecipitated each myc-SH2B1 using αMyc and immunoblotted with αpTyr. Figure. 3.7C reveals that both SH2B1α and β are highly phosphorylated on Tyr when

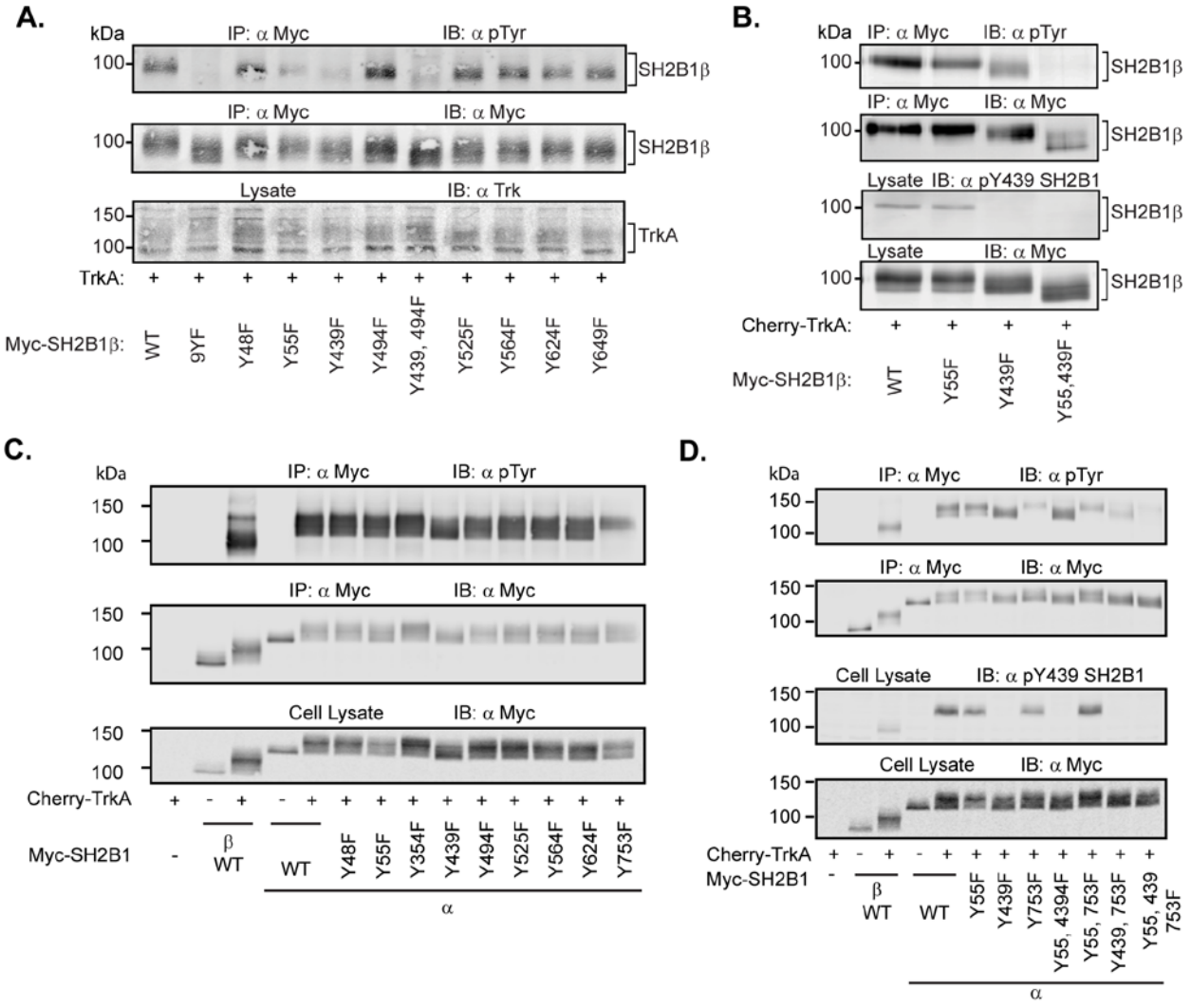

Figure 3.7. **TrkA phosphorylates Tyr753 in SH2B1α as well as Tyr439 and potentially Tyr55 in SH2B1β and SH2B1α.** 293T cells transiently co-expressing indicated myc-tagged SH2B1 with either TrkA (Panel A) or mCherry TrkA (Panel B-D) were lysed and proteins immunoprecipitated with αmyc. Cell lysates and α myc immunoprecipitates (IP) subjected to SDS-PAGE (9% acrylamide) and immunoblotted with the indicated antibodies. The blots shown are representative of 3 (Panels A, B) or 2 (Panels C, D) independent experiments.

co-expressed with TrkA, but not in its absence. Mutating Tyr55 or Tyr439 causes a substantial reduction in levels of Tyr phosphorylation of SH2B1β (Figure. 3.7A). Mutating both Tyr55 and 439 abrogates Tyr phosphorylation of SH2B1β (Figure. 3.7B, top panel), suggesting that Tyr55 and 439 are the two main substrates of TrkA in SH2B1β. Immunoblotting cell lysates with Ab highly specific to pTyr439 in SH2B1 (92) confirmed that co-expression of SH2B1β with TrkA results in phosphorylation of Tyr439 in SH2B1β (Figure. 3.7B, 3rd panel). Phosphorylation of Tyr439 was not appreciably altered by mutating Tyr55. In the case of SH2B1α, mutating Tyr753 caused a major reduction in its tyrosyl phosphorylation (Figure. 3.7C). Mutation of other individual Tyr did not cause a reproducible reduction in Tyr phosphorylation of SH2B1α suggesting Tyr753 is the primary substrate of TrkA. However, SH2B1 Y753F retained some Tyr phosphorylation, indicating that TrkA phosphorylates additional Tyr in SH2B1α. Since Tyr55 and 439 in SH2B1β appear to be phosphorylated by TrkA (Figures. 3.7A, 3.7B), we tested whether mutating Tyr 753, 55 and 439 in different combinations would prevent TrkA phosphorylation of SH2B1α (Figure. 3.7D, top panel). Only when all three were mutated did we see almost total absence of Tyr phosphorylation, suggesting that SH2B1α is phosphorylated on Tyr55 and/or 439 in addition to Tyr753. Immunoblotting cell lysates with pTyr439 Ab confirmed that co-expression of SH2B1α with TrkA results in phosphorylation of Tyr439 (Figure. 3.7D, 3rd panel). Levels of phosphorylation of Tyr439 were not significantly affected by mutating Tyr753 or Tyr55 alone or together. Collectively, these data are consistent with Tyr753 in the unique C-terminal tail of SH2B1α being a major substrate for TrkA. Additionally, Tyr 439 and most likely Tyr55, both in the N-terminal region shared by all 4 known isoforms of SH2B1, appear to be

substrates of TrkA in both SH2B1α and β.

SH2B1α inhibits SH2B1-induced neurite outgrowth of PC12 cells.

Dimerization of both TrkA and SH2B1 has been implicated in their function (30,63). This observation, combined with our findings that the unique C-terminal tail of SH2B1α inhibits the ability of SH2B1 to promote NGF-induced tyrosyl phosphorylation of TrkA and NGF signaling pathways, led us to ask whether SH2B1α inhibits the ability of SH2B1β to promote neurite outgrowth. PC12 cells stably expressing GFP, or GFP-SH2B1β, α or α Y753F were transiently transfected with Td-tomato (Tdt) or Tdt-tagged SH2B1β, α or α Y753F. NGF (25 ng/ml) was added for 2 days and neurite outgrowth assessed in cells stably expressing both the GFP and Tdt tags (compare Figure. 3.2A and 3.8). As expected, transient overexpression of Tdt itself resulted in a profile of neurite outgrowth similar to that observed in the absence of Tdt (see Figure. 3.2A). Transient overexpression of Tdt-SH2B1β increased neurite outgrowth in cells expressing GFP or GFP-SH2B1α but had minimal effect on cells stably expressing GFP-SH2B1β or SH2B1α Y753F, suggesting that maximal SH2B1 stimulation of neurite outgrowth had been obtained in the latter cell types. While overexpression of Tdt-SH2B1α had no effect on neurite outgrowth in the PC12-GFP or PC12-GFP-SH2B1α cells, it inhibited neurite outgrowth in the PC12-GFP-SH2B1β and PC12-GFP-SH2B1α Y753F cells by about 50%. This inhibitory effect was lost when Tyr753 in Tdt-SH2B1α was mutated to Phe. These results are consistent with SH2B1α competing with SH2B1β to bind TrkA via their shared SH2 domains. Phosphorylation of Tyr753 in SH2B1α,

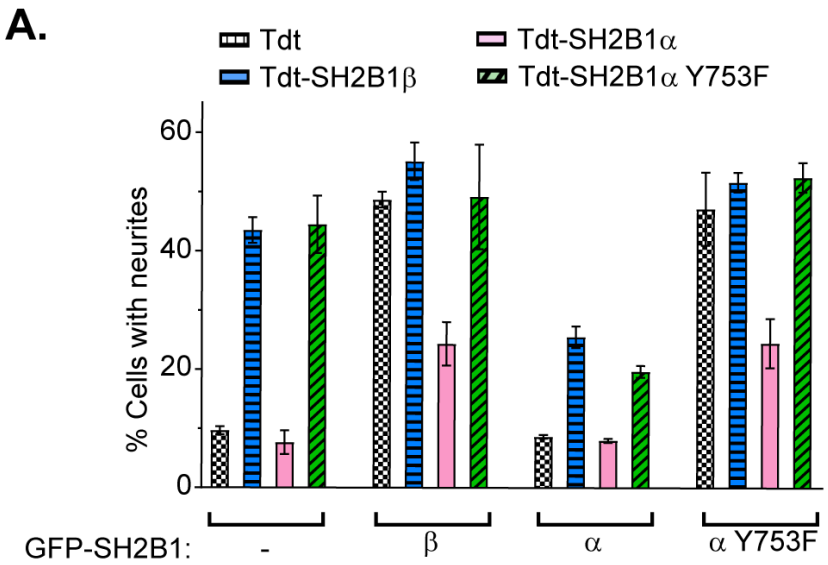

Figure 3.8. Tyr753 in SH2B1α regulates the ability for SH2B1β to enhance NGF-mediated neurite outgrowth. Live PC12 cells stably expressing GFP (-) or the indicated GFP-SH2B1 were transiently transfected with the indicated Td-Tomato (Tdt)-tagged SH2B1 and incubated with 25 ng/ml NGF for 2 days. The percentage of cells expressing both Td-Tomato and GFP neurite outgrowths at least twice the length of the cell body length was determined. Means and ranges from 2 independent experiments are shown.

presumably by TrkA, would be expected to inactivate SH2B1α, resulting in the inability of a portion of the total bound SH2B1 to enhance NGF signaling gene expression and neurite outgrowth. Dephosphorylation of Tyr 753 or prevention of its phosphorylation by TrkA would relieve or prevent, respectively, this inhibition, allowing SH2B1α to act like SH2B1β to enhance NGF signaling and responses.

Discussion

It is becoming increasingly evident that alternative splicing plays an important role in increasing the diversity of cellular responses both between and within different tissues. In this study, we show that two different isoforms of the scaffold protein SH2B1, SH2B1α and SH2B1β, which differ only in their C-terminal amino acids representing only 15 and 6% of the total protein sequence, respectively, have profoundly different effects on NGF signaling. SH2B1β enhances NGF-dependent phosphorylation of TrkA, Akt and PLCγ, gene expression, and neurite outgrowth of PC12 cells whereas SH2B1α does not. We further provide strong evidence that the α tail inhibits the ability of the 631 N-terminal amino acids shared by all isoforms of SH2B1 to enhance these NGF functions, rather than the β tail enabling them. This is supported by the findings that SH2B1 1-631 is as effective, if not more so, than SH2B1β in all of these activities. Finally, we identify potential TrkA substrate sites within SH2B1α and β and provide strong evidence that phosphorylation of a single amino acid (Tyr753) in the C-terminal tail of SH2B1α is responsible for the inhibition of SH2B1 functions by the α tail.

Our favored hypothesis that phosphorylation of Tyr753 inhibits the ability of SH2B1α to serve as a signaling protein for NGF is largely based on our finding that mutating Tyr753 to Ala restores the ability of SH2B1α to enhance functions of NGF. Further support for a regulatory role for phosphorylation of Tyr753 comes from our

finding that mutating Tyr753 to the phosphomimetic Glu did not restore the ability of SH2B1α to enhance neurite outgrowth. Further, TrkA is capable of phosphorylating Tyr753 in SH2B1α. Thus, when TrkA and SH2B1α are co-expressed in 293T cells, SH2B1α is highly phosphorylated on Tyr in a TrkA-dependent fashion. The level of Tyr phosphorylation is greatly reduced when Tyr753 is mutated to Ala. Our finding suggesting that TrkA can phosphorylate Tyr753 in SH2B1α is supported by unpublished results mentioned, but not shown or discussed, by Qian et al. (25). The finding that TrkA can highly phosphorylate Tyr753 suggests that once SH2B1α is recruited to TrkA, Tyr753 becomes phosphorylated, which essentially turns SH2B1α off until it can be dephosphorylated. It is also possible that Tyr753 is phosphorylated by other Tyr kinases. Such crosstalk would enable other ligands that bind to receptor tyrosine kinases to negatively modulate neurotrophic factor functions by reversibly "turning off" a positive regulator of those functions (ie SH2B1α). While we think it less likely, we also cannot rule out the possibility that mutating Tyr753 additionally or alternatively causes a conformational change independent of phosphorylation that relieves the inhibitory action of the α tail on SH2B1 function.

Consistent with phosphorylation of Tyr753 playing an important role in regulating the function of SH2B1α, the C-terminal tail of SH2B1α, including the Tyr753- containing motif RAINNQY$_{753}$SFV is highly conserved within mammals. In addition, comparable tyrosines are found in the C-termini of SH2B1 family members SH2B2/APS (Tyr618) and SH2B3/Lnk (Tyr536). Tyr618 in SH2B2/APS has been shown to be phosphorylated by the insulin receptor. PhosphoTyr618 is believed to recruit the E3-ligase c-Cbl and facilitate the tyrosine phosphorylation of c-Cbl by the insulin receptor, association of c-

Cbl with Crk, and ultimately the translocation of GLUT4 glucose transporters to the plasma membrane (109). In contrast to SH2B1 and SH2B2/APS, which tend to enhance the functions of the receptors to which they are recruited, Lnk appears to inhibit the functions of the receptors to which it is recruited. In fact, in contrast to SH2B1β, Lnk has been shown to inhibit TrkA signaling (146). It also inhibits functions of the JAK2-associated erythropoietin receptor in erythroblasts (148). Mutation of Tyr536 to Phe modestly impaired the ability of Lnk to inhibit erythropoietin-dependent 32D cell proliferation and erythroblast survival. Interestingly, though, the same mutation substantially enhanced the ability of Lnk to inhibit interleukin 3-dependent proliferation of bone marrow mast cells (111). Synthetic peptides corresponding to the sequences surrounding Tyr536 (or phosphoTyr536) were conjugated to agarose beads and incubated with cell lysates from lymph node lymphocytes. Immunoblotting the proteins bound to the beads with phosphoTyr536 but not unphosphorylated Tyr536 revealed PLCγ1, Grb-2 and phosphatidyl inositol 3'-kinase (149), suggesting that ligand stimulates the association of PLCγ1, Grb-2 and PI3K to an LNK containing complex.

So how might phosphorylation of Tyr753 inhibit the actions of SH2B1α? The presence of a potential PDZ binding motif (150) containing Tyr753 raises the possibility that the C-terminal tail of SH2B1α binds to a PDZ domain-containing protein that inhibits the actions of SH2B1 and phosphorylation of Tyr753 promotes that binding. Arguing against this hypothesis is our finding that mutating other critical residues expected to disrupt the putative PDZ binding domain does not restore the ability of SH2B1α to enhance NGF signaling. Alternatively, phosphorylation of Tyr753 might promote the binding of a PDZ domain-containing protein, which inhibits the actions of SH2B1. A

second hypothesis stems from SH2B2/APS data. The Tyr in SH2B2/APS homologous to Tyr753 in SH2B1α (Tyr618) has been shown to bind to c-Cbl (109,151), raising the possibility that phosphoTyr753 recruits c-Cbl or another member of the Cbl family of E3 ligases which could promote degradation of Sh2B1α or associated TrkA. Arguing against this, we have not seen decreased degradation of SH2B1α or TrkA in PC12 cells stably expressing SH2B1α Y753F compared to SH2B1α. Although phosphoTyr536 in Lnk has been proposed to recruit PLCγ1, Grb-2 and PI3K (149), it is hard to imagine how recruiting signaling proteins that are thought to be important for NGF-induced neurite outgrowth would prevent, rather than enhance, the ability of SH2B1 to augment NGF-dependent neurite outgrowth.

In addition, or alternatively to facilitating or blocking interaction of SH2B1α with another protein, phosphorylation of Tyr573 may, through a conformational change, cause the α tail to block the NLS. Such an interference could help explain why SH2B1α appears to bind to the plasma membrane to a reduced extent compared to SH2B1β and also show a greatly reduced ability to cycle through the nucleus, both of which are thought to be important for the actions of SH2B1 to enhance NGF functions. This reduced binding was no longer observed when Tyr753 was mutated to Phe. Further experiments are clearly required to identify whether phosphorylation or dephosphorylation of Tyr753 regulates the binding of the C-terminal tail of SH2B1α to specific proteins that interfere with SH2B1 function and/or causes a conformational change that interferes with SH2B1 function.

Previously, we showed that SH2B1β is phosphorylated on Tyr 439 and 494 by JAK2, both in the context of a 293T overexpression system (91) and in response to GH

stimulation of 3T3-F442A cells (92). Here we provide evidence that TrkA, like JAK2, is able to phosphorylate SH2B1β on Tyr439. We also provide evidence that the second major TrkA substrate is Tyr55 rather than Tyr494. Tyr55 and 439 in SH2B1α also appear to be TrkA substrates, although they appear to be phosphorylated to a lesser extent than Tyr753. Both Tyr55 and 439 were implicated as substrates in SH2B1α and β because of a reduced anti-phosphotyrosine signal when they were individually mutated and a further reduction when both were mutated together. Tyr439 was confirmed as a substrate using a Tyr439 phosphospecific antibody and Tyr55 was mentioned as a substrate of TrkA without documentation by Qian et al. (25). Thus, TrkA and JAK2 appear to share at least one substrate site in SH2B1α and β (Tyr439) but differ in at least one other (Tyr55 for TrkA vs Tyr494 for JAK2), allowing for some shared and divergent functions. Tyr439, along with Tyr494, has been implicated in SH2B1β enhancement of GH-stimulated membrane ruffling in 3T3-F442A fibroblasts (91) and GH-stimulated motility of RAW264.7 macrophages (92). Thus, it is tempting to speculate that in response to NGF, phosphoTyr439 in SH2B1β and SH2B1α Y753F recruit some protein important for neuronal migration or the cytoskeletal rearrangements that occur during neuronal differentiation. The function of phosphoTyr55 remains to be determined.

In addition to being phosphorylated on Tyr, we have shown previously that SH2B1 is phosphorylated on multiple Ser/Thr (64). Phosphosite Plus (http://www.phosphosite.org/homeAction.action) lists 22 different phosphoserines in SH2B1 isoforms that have been detected by mass spectrometry, including the 8 phosphoserines (pSer96, 124, 126, 127, 154, 161, 453 and 613) detected by our

laboratory (78). Mutational analysis has implicated Ser 154, 157, 161 and/or 165, which are proximal to the NLS, as being important in regulating the plasma membrane and nuclear localization of SH2B1β (78). Preventing phosphorylation by mutating all four of these Ser, or even just Ser 161 and 165, to Ala redistributes SH2B1β almost exclusively to the plasma membrane and prevents their cycling through the nucleus whereas mimicking phosphorylation by mutating the Ser to Glu causes SH2B1β to leave the plasma membrane and enter the nucleus. Phosphorylation of Ser 161 and 165 has also been implicated in GH-induced macrophage motility, based on the finding that mutating Ser161 and 165 individually or together inhibits GH-dependent motility whereas mutating the Ser to Glu enhances basal motility to the level seen in the presence of GH (92). Because of this critical role of phosphoserines, we were interested to see that NGF stimulates an upward shift in migration of SH2B1α and a broadening of the SH2B1βα band in immunoblots, similar to that seen previously with SH2B1β (64). Digestion with phosphatases indicated that this shift was due to Tyr and Ser/Thr phosphorylation. However, the shift with SH2B1α was more transient than that seen with SH2B1β. Additionally, mutation of Tyr753 restored the migration pattern to resemble that seen with SH2B1β. This raises the possibility that sustained Ser/Thr phosphorylation of SH2B1 contributes to or is necessary for SH2B1's stimulatory effects on NGF-dependent neurite outgrowth and gene expression. Presumably some of the Ser are constitutively phosphorylated, but clearly some are phosphorylated in response to NGF, as has also been reported for SH2B1β that is recruited to the PDGF receptor (24) or the GH-receptor-JAK2 complex (4). The Ser/Thr kinases that are responsible for the extensive phosphorylation are not known. But since phosphorylation occurs very

soon after NGF binding, it seems possible that some of the sites are phosphorylated by kinases that are activated by NGF. Indeed, Rui et al. (64) provide evidence that SH2B1β Is phosphorylated on multiple Ser/Thr in response to NGF by kinases within the MEK/ERK cascade. It will be intriguing to find out what kinases actually phosphorylate the various Ser/Thr with the various isoforms of SH2B1, and how that phosphorylation regulates their function.

Despite significant effort, it is still not totally clear how SH2B1β enhances NGF-induced neurite outgrowth. Previous work and work presented here (25,26) indicate that SH2B1β is recruited via its SH2 domain to phosphorylated Tyr in TrkA and is phosphorylated by TrkA on multiple Tyr. Our work using phosphospecific antibodies to various Tyr in TrkA indicate that this binding of SH2B1β to TrkA increases the activity of TrkA (indicated by increased phosphorylation of the activating Tyr674/675 in TrkA), which appears to increase phosphorylation of other tyrosines in TrkA. In our hands, SH2B1β (and SH2B1 1-631) reliably increased phosphorylation of Tyr785, which would be expected to recruit and activate more PLCγ (123,147). Consistent with this, we found that stable expression of SH2B1β and 1-631 in PC12 cells consistently enhanced and prolonged phosphorylation of PLCγ. Thus, we think it likely that one of the functions of SH2B1 important for its enhancement of NGF functions is to increase and prolong the activity of PLCγ. We found SH2B1β enhancement of phosphorylation of Tyr490 to be more variable, being increased in some of our studies by SH2B1β and 1-631. pTyr490 has been shown to recruit Shc and FRS-2 proteins, both implicated in activating Erks 1/2 by initiating the Shc/grb2/SOS/Ras/Raf/MEK pathway, and the latter by prolonging the activation of Rap1 (120,123,147,152). However, consistent with the more variable

SH2B1β-induced increase in Tyr490 phosphorylation, we do not consistently show SH2B1β enhancement of phosphorylation of the activating Tyr/Thr in ERKs 1/2, leading us to conclude that the major of effect of SH2B1β on neurite outgrowth is unlikely to be mediated primarily via changes in NGF activation of this pathway. In contrast, SH2B1α neither stimulated the Tyr phosphorylation of any of the tested sites in TrkA, nor did it stimulate the phosphorylation of Erks 1/2 or PLCγ. However, all of these were restored upon mutation of Tyr753. Similar results were seen with SH2B1 stimulation of phosphorylation of Ser473 in Akt, another signaling protein implicated as being critical for NGF-induced neurite outgrowth as well as with NGF-induced gene expression. Thus, SH2B1β, SH2B1 1-631, and SH2B1α Y753F robustly stimulated NGF-dependent expression of *Plaur, Mmp10, FosL1* and *Mmp3* whereas SH2B1α had no effect. The discrepancy between the robust SH2B1 induced enhancement of NGF-dependent neurite outgrowth and gene expression and the more modest increases in phosphorylation of TrkA and downstream signaling pathways suggest either that there are some as yet to be determined signaling pathways or nuclear functions of SH2B1 that promote these functions, or that the robust end point responses are the consequence of multiple small changes that result in a substantial effect on neurite outgrowth and gene expression.

These data together suggest a working model in which the ability of SH2B1β, and inability of SH2B1α, to enhance neurite outgrowth and gene transcription starts at the level of the TrkA receptor. Since SH2B1β and α share a functional SH2 domain, we would expect them to compete for binding to a phosphorylated Tyr in TrkA. When SH2B1α binds, TrkA phosphorylates Tyr753, which by some unknown mechanism

inactivates SH2B1α, thereby preventing enhancement of TrkA activity by either the bound SH2B1α or by SH2B1β that prevents it from binding to TrkA. Because all SH2B1 isoforms share the same SH2 domain and SH2B1 isoforms are reported to bind to multiple neurotrophic factor receptors, we predict that SH2B1α would similarly compete with other isoforms of SH2B1 for binding to TrkA and other neurotrophic factor receptors, including the receptor for BDNF, TrkB. One can envision that by so doing, SH2B1α helps to fine tune the response to neurotrophic factors which not only help to form but also help to prune neuronal projections, including projections in the brain that regulate energy expenditure.

This Chapter will be submitted for publication under the title “The C-terminal Tail of the α Isoform of SH2B1 Inhibits the Ability of SH2B1 to Enhance NGF Functions and Alters SH2B1 Subcellular Localization, Both by a Phosphorylation-Dependent Mechanism” by Ray Joe, Anabel Flores, Joel M. Cline, Erik Clutter, Paul Vander, Lawrence S. Argetsinger, and Christin Carter-Su. I performed neurite outgrowth assays, confocal microscopy, quantification of microscopy and qPCR, NGF signaling immunoblots, TrkA phosphorylation of SH2B1α immunoblots, and SH2B1α, and SH2B1β neurite outgrowth competition assay. I generated all the figures and first draft text of the manuscript.

Chapter 4

Effect on the Ability of SH2B1 Human Obesity-Associated Variants of SH2B1 to Enhance Nerve Growth Factor Functions

Abstract

The scaffold protein SH2B1 has been implicated in energy homeostasis through its promotion of cell signaling pathways for various cytokines and growth factors, including leptin, insulin, and nerve growth factor. Alternative splicing of *Sh2b1* generates two universally expressed (β and γ) isoforms and two brain-specific (α and δ) isoforms that share a common N-terminus but have unique C-terminal tails that vary in amino acid content and length. We and others have previously reported unique variants in the common N-termini (P90H, T175N, R270W, P322S, F344LfsX20, T546A) and unique C-terminal tails (α-A662V, α-V695M, α-L723V, β-T655I, and γ-P673S) of SH2B1 isoforms in individuals with obesity and insulin resistance. Here, we characterize three more novel variants (R227C, G238C, and E299G) within or near the PH domain of SH2B1 that appear associated with human obesity and insulin resistance. These new variants and the recently reported variants R270W and β-T655I impaired the ability of SH2B1β to enhance NGF-mediated neurite outgrowth in PC12 cells. However, the γ-P673S variant, which is the same variant as β-T655I, had no effect on the ability of SH2B1γ to enhance neurite outgrowth. In addition, we have determined that the P90H and P322S mutations impair the ability of SH2B1β to enhance expression of the NGF-responsive

genes *Plaur, Mmp10,* and *FosL1* yet do not detectably influence NGF-mediated phosphorylation of signaling proteins PLCγ, Akt and ERKs 1 and 2. Lastly, variants specific to the SH2B1α isoform (A662V, V695M, L723V) did not positively or negatively influence the ability of SH2B1α Y753F to enhance NGF-mediated neurite outgrowth. Collectively, these data suggest that: 1) the PH domain of SH2B1 is a potential regulatory region of SH2B1 that is important for SH2B1 enhancement of NGF-mediated neurite outgrowth; 2) Obesity variants impair a subset of the transcriptome enhanced by SH2B1β and NGF without detectably affecting known NGF-signaling pathways; and 3) there exists a variant within the C-terminal tail of SH2B1β that has isoform specific effects on neurite outgrowth. Variants within the C-terminal tail that have isoform specific effects on SH2B1 enhancement of neurite outgrowth and/or human physiology provide further evidence of isoform-specific responses to that single ligand. Further analysis of its PH domain and C-terminal tails will be critical to identifying the physiological role of SH2B1.

Introduction

The scaffold protein SH2B1 has been shown to promote metabolic homeostasis; disruption of SH2B1 promotes an imbalance in energy homeostasis resulting in severe obesity and insulin resistance. Individuals with chromosomal deletions spanning regions that include *SH2B1* were found to exhibit severe obesity, hyperphagia, and insulin resistance disproportionate to their level of obesity. (12,13,15). Recently, individuals with obesity, hyperphagia, insulin resistance, maladaptive behavior were found to have non-synonymous variants encoding for P90H, T175N, P322S, and T546A, which are all located in the region of SH2B1 common to all four known isoforms of SH2B1 (6,135). Additional variants encoding for A662V, V695M, and L723V, which are specific to SH2B1α, were found in individuals who exhibited obesity and insulin resistance. However, maladaptive behaviors were not observed in these latter individuals (135). Similar to humans with the human obesity-associated mutations in SH2B1, mice with targeted homozygous deletion of *Sh2b1* are obese, insulin resistant, and hyperphagic. However, it is still not clear exactly how SH2B1 regulates energy balance (18,19).

SH2B1 is composed of multiple domains that help mediate its effects on cell signaling in response to key receptor tyrosine kinases and cytokine family receptors that bind to Janus kinases (JAKs). These include receptors for leptin, insulin, growth hormone, insulin-like growth factor-1 (IGF-I), nerve growth factor (NGF), brain-derived neurotrophic factor, and glial-derived neurotrophic factor (125). All SH2B1 isoforms

share several proline-rich domains, a dimerization domain, nuclear localization sequence (NLS), nuclear export sequence (NES), pleckstrin homology domain (PH), and Src-homology 2 domain (SH2). Alternative splicing of *Sh2b1* produces four unique isoforms(α, β, γ, and δ) that share 631 amino acids and vary at the C-terminus (2). SH2B1β has the shortest tail (39 amino acids) and SH2B1α has the longest tail (125 amino acids). These tails have been shown to partially regulate the shared N-terminus (103) (see Chapter 3). SH2B1β and γ are universally expressed throughout all human tissues and SH2B1α and δ have been detected primarily in the brain (6). Raising the question of the role of SH2B1 in the brain. Targeted expression of SH2B1β in the brain of SH2B1-deficient mice largely restores the lean phenotype (54). SH2B1β enhances NGF-mediated neurite outgrowth in PC12 cells (26), and enhance BDNF-induced neurite length and branching in primary hippocampal and cortical neurons (27). However, SH2B1α is unable to enhance NGF-mediated neurite outgrowth, cell signaling pathways, and gene expression in PC12 cells unless a conserved Tyr753 in the C-terminal tail is mutated (135) (see Chapter 3), suggesting that phosphorylation of Tyr753 inhibits the function of SH2B1α. The human obesity mutations (P90H, T175N, P322S, F344LfsX20, T546A) within the N-terminus of SH2B1 shared by all four isoforms impair the ability of SH2B1β to enhance NGF-mediated neurite outgrowth (6,135). However, it is unclear exactly how these mutations impair the ability of SH2B1β to enhance NGF-dependent neurite outgrowth.

In this study, we examine the ability of three novel SH2B1 mutations associated with human obesity, R227C, G238C, E299G, the previously identified mutation R270W (153), and the SH2B1 variant (g.9483C/T) that encodes for SH2B1β T655I and

SH2B1γ P673S (17) to enhance NGF-dependent neurite outgrowth in PC12 cells. We also investigated the effect of the P90H and P322S mutations on the ability of SH2B1β to affect NGF-dependent phosphorylation of signaling proteins (PLCγ, Akt, ERKs 1 and 2) and expression levels of *Plaur, Mmp10, Mmp3*, and *FosL1* genes. Lastly, because the Tyr753Phe mutation restores the ability of SH2B1a to enhance neurite outgrowth, we tested the ability of the human obesity-associated SH2B1α specific mutations to impair neurite outgrowth in the presence of the Tyr753Phe (Y753F) mutation. These results collectively suggest that the PH domain is a key component of SH2B1 isoforms required for enhancement of NGF-mediated neurite outgrowth. Further, based on the gene expression studies, the human obesity associated mutations negatively impact only a subset of the SH2B1β and NGF regulated genes by signaling pathways not yet identified.

Materials and methods

Antibodies

Mouse monoclonal antibodies to Akt (αAkt; 2920S; 1:2000) and Erks 1 and 2 (αErk 1/2; 4696S; 1:2000); rabbit monoclonal antibodies to phospho-Akt (Ser473) (αpAkt-Ser473; 4058L; 1:1000); and rabbit polyclonal antibodies to doubly phosphorylated Erks 1 and 2 (Thr202/Tyr204) (αpErk 1/2; 9101S; 1:1000), PLCγ (αPLCγ; 2822S; 1:1000) and phospho-PLCγ (Tyr 783) (αpPLCγ-Tyr 783; 2821S; 1:500) were from Cell Signaling. Rabbit polyclonal antibody to GFP (αGFP; #632592; 1:2000) came from Clontech. IRDye800-conjugated, affinity purified anti-goat GFP (αGFP (600-132-215); IB: 1:20,000) came from Rockland Immunochemicals and IRDye 800- (827-11081) and IRDye 700- (926-32211) conjugated affinity-purified anti-mouse IgG and anti-rabbit IgG were from Li-cor Odyssey.

Plasmids

cDNA encoding mouse SH2B1α and SH2B1γ was a kind gift of H. Riedel (2). cDNAs encoding GFP- rat SH2B1β have been described previously (64). The sequence encoding mouse SH2B1α (AF421138) and SH2B1γ (AF421139) was subcloned into pEGFPC1 (Clontech) containing an N-terminal GFP. Additional mutations were introduced into SH2B1β, SH2B1α, SH2B1γ using Quickchange II Site-Directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

Multiple mutation sites were introduced into SH2B1α in successive steps using the same primer pairs.

Cell culture and transfection

PC12 cells (CRL-1721; ATCC) were plated on rat tail type I collagen (BD Biosciences) coated dishes and maintained at 37°C with humidified air at 5% CO_2 in normal growth medium (RPMI-1640 medium [Life Technologies], 10% horse serum [HS; Atlanta], and 5% fetal bovine serum [FBS; Life Technologies]). PC12 cells were transiently transfected using Lipofectamine LTX (Life Technologies) per manufacturer's instructions for 24-72 h. PC12 cell lines stably overexpressing GFP or GFP tagged SH2B1β WT, P90H and P322S were generated by transfecting cells with the indicated plasmid using Lipofectamine LTX for 72 h and then selecting in G418 medium (normal growth medium containing 0.5 mg/ml G418 [Cellco; Corning]) for 5 days. Cells were expanded and maintained for 30 days. The top 60% of GFP-positive cells were gated and sorted from non-GFP expressing cells by fluorescence-activated cell sorting (FACs [Beckman Coulter MoFlo Astrios, University of Michigan Flow Cytometry Core]). Sorted cells were maintained in G418 medium. Prior to experimental use, PC12 cells were incubated overnight in deprivation medium (RPMI-1640 containing 1% bovine serum albumin (BSA; Proliant Biologicals)).

Neurite outgrowth

Transiently or stably transfected PC12 cells were plated in 6-well dishes. Cells were treated with 25 ng/ml 2.5S mouse NGF (BD BioSciences, 356004; or Harlan, BT.5025) in RPMI-1640 medium containing 2% HS and 1% FBS. GFP- positive cells were

visualized by fluorescence microscopy (20X or 40X objective, Nikon Eclipse TE200) after 1, 2, or 3 days. 100 GFP- positive cells were scored in three different areas of each plate for the presence of neurites 2 times the length of the cell body. Three independent experiments were performed for Figure 4.1 (300 cells per experiment for a total of 900 cells per condition) and Figures 4.2- 3 (200 cells per experiment for a total of 600 cells per condition) were counted. Two independent experiments were performed for Figures 4.8-4.10 (300 cells per experiment for a total of 600 cells per condition). The percentage of cells with neurites was determined by dividing the number of cells with neurites by the total number of GFP- positive cells counted.

Immunoblotting and immunoprecipitation

Stably transfected PC12 cells were seeded at $1.0x10^7$ cells per 10-cm collagen-coated dish in normal growth medium. After 24 h, cells were incubated overnight in deprivation medium. Cells were incubated at 37°C with NGF (25 or 100 ng/ml) as indicated, placed on ice and washed two times with phosphate buffered saline (10 mM $NaPO_4$, 140 mM NaCl, pH 7.4; PBS) containing 1 mM Na_3VO_4 pH 7.3 (PBSV). Cells were then lysed on ice for 10 min with ice-cold 20mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin), pH 7.5. Cell lysates were centrifuged at ~15,000 x g for 10 min at 4°C and protein concentrations of the supernatant were determined using DC protein assay (Bio-Rad). Equal amounts of proteins were resolved by SDS-PAGE using 5-12% gradient acrylamide gels.

Quantitative polymerase chain reaction (qPCR)

PC12 cells stably overexpressing GFP or the indicated GFP-SH2B1 were incubated at 37°C in deprivation medium overnight. Cells were treated with or without 100 ng/ml NGF (Harlan) for 6 h. Total RNA was extracted using Trizol (Ambion by Life Technologies) according to the manufacturer's instructions and RNA quality confirmed by OD via Nanodrop spectrophotometer. cDNA was generated from the RNA using Taqman Reverse Transcription Reagent Kit (Applied Bio-Systems; N808-0234) per manufacturer's instructions. Transcript levels of *Plaur, Mmp10, Mmp3 (79), FosL1, Hprt (141),* and *Cyclophilin* A (142) were determined in triplicate per gene by qPCR using the TaqMan kit (Invitrogen), SYBR green I (Life Technologies) and an Eppendorf Realplex2 (using Mastercycler ® software). Cycle threshold values were normalized to the geometric mean of the cycle thresholds for *Hprt* and *Cyclophilin A* whose expression did not differ between the different cell lines or with NGF treatment (143). Results were then normalized to those obtained with GFP-SH2B1β cells treated with NGF.

Statistics

Statistics were performed using GraphPad Prism 7.02. For neurite outgrowth and western blot quantification, time course curves were converted to area under the curve (AUC). For qPCR experiments, relative transcript expression levels were normalized to GFP-SH2B1β NGF-treated values. One-way ANOVA with repeated measures and multiple comparison analysis with Dunnett's post-test (compared to GFP or GFP-SH2B1β) were used to test for statistical significance. Data shows the mean ± S.E.M for three independent experiments (N=3) (Figures 4.1-6). The mean ± range is depicted

(N=2) (Figures 4.1(P322S), 4.7-10). * indicates p-values ≤ 0.05, which was considered significant.

Results

Newly Identified SH2B1 variants associated with human obesity impair the ability of SH2B1 to enhance neurite extension.

Several new SH2B1 variants associated with severe-early onset obesity within SH2B1 have been identified by our collaborator, Dr. I. Sadaf Farooqi. These include Gly283Cys (G238C), Arg227Cys (R227C), and Glu299Gly (E299G). Volckmar et al. identified a variant present in both SH2B1β (T665I) and SH2B1γ (P673S) (17). Another study identified SH2B1 Arg270Trp (R270W) within the maturity onset diabetes of the young (MODY) database (153). The T655I/P673S mutation has been tested for its ability to inhibit SH2B1 enhancement of leptin-mediated STAT3 signaling in 293 cells transiently overexpressing SH2B1, LepRb and STAT3 (17). No effect of the mutations was observed. The impact of the other mutations on cellular effects of SH2B1 have not been tested. To start to gain insight into whether any of these human obesity-associated mutations affects the cellular actions of SH2B1, I tested the ability of these mutations to impair the SH2B1β enhancement of NGF-dependent neurite outgrowth in PC12 cells. Three of the four mutations within or near the PH domain of SH2B1(amino acids 267-376): G238C (Figure. 4.1), R270W (Figures 4.1), and E299G (Figure. 4.2) were shown to significantly impair SH2B1β enhancement of NGF-mediated neurite outgrowth in PC12 cells. The degree of inhibition was similar to that observed previously for the human obesity mutation P322S (Figures. 4.1 and 4.2) (6). The R227C mutation trended

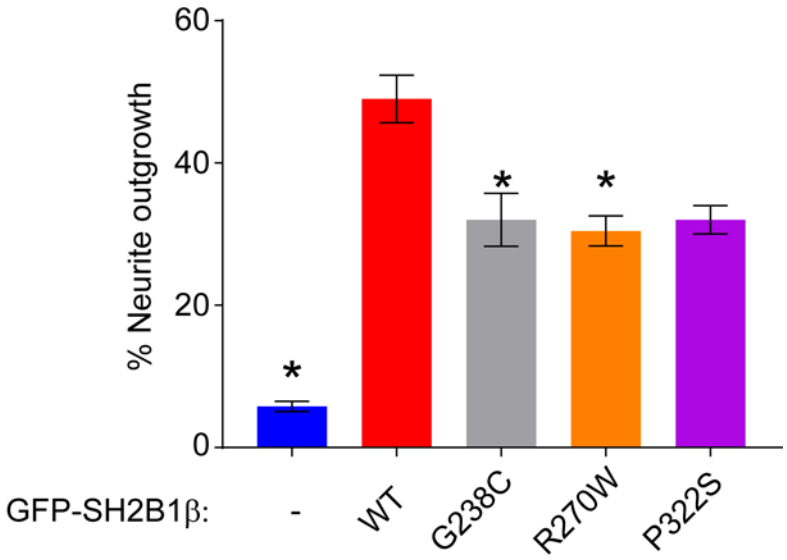

Figure 4.1. Human mutations G238C and R270W impair the ability of SH2B1β to enhance NGF-mediated neurite outgrowth. Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. Means ± s.e.m. are shown for 3 independent experiments except for P322S, which is an N=2 and mean ± range is depicted. * p≤0.05 compared to SH2B1β**.**

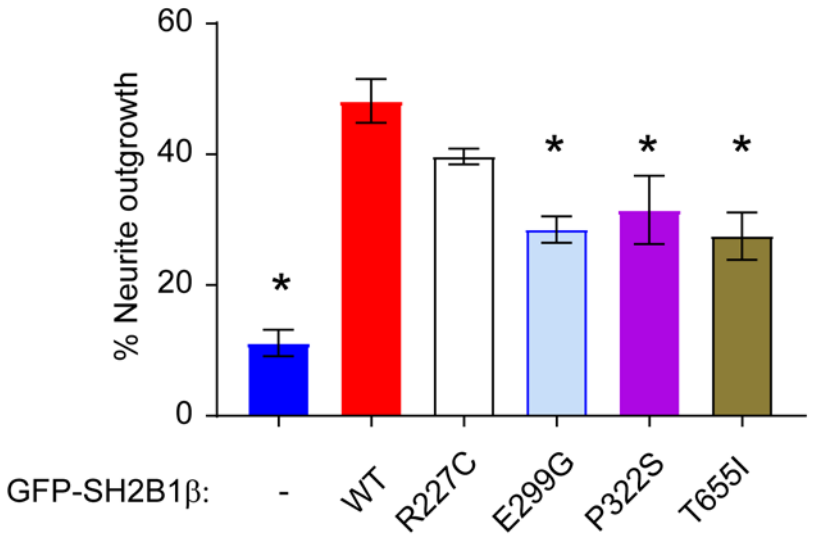

Figure 4.2. Human mutations E299G and T655I impair the ability of SH2B1β to enhance NGF-mediated neurite outgrowth. Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. Means ± s.e.m. are shown for 3 independent experiments. * p≤0.05 compared to SH2B1β.

toward modest impairment of SH2B1-stimulated neurite outgrowth (Figure. 4.2) but the effect did not reach statistical significance. The SH2B1β C-terminal tail mutant T655I also impaired NGF-mediated neurite outgrowth, to a similar extent as P322S (Figure. 4.2). Unlike T655I in SH2B1β, the shared variant in the SH2B1γ isoform, P673S, did not impair the ability of SH2B1γ to enhance neurite outgrowth (Figure. 4.3). Together these data show that multiple human obesity mutations within the PH domain of SH2B1 functionally impair SH2B1β's ability to enhance NGF-mediated neurite outgrowth. Furthermore, a single mutation shared by two isoforms can regulate the function of SH2B1 to different degrees depending upon the isoform.

SH2B1 human obesity mutations P90H and P322S minimally affect the ability of SH2B1 to enhance phosphorylation of NGF signaling proteins.

We previously reported that introducing the SH2B1 human obesity variants P90H and P322S into SH2B1β impaired the ability of SH2B1β to enhance NGF-mediated neurite outgrowth in PC12 cells (6). However, it was unclear how these mutations impaired SH2B1β's ability to enhance NGF-mediated neurite outgrowth. We therefore investigated the effect of the P90H and P322S mutations on the ability of SH2B1 to affect NGF signaling pathways. NGF-dependent recruit signaling proteins via their SH2 or PTB domains to activate multiple signaling pathways including those leading to rapid and prolonged activation of Erk1/2, Akt, and PLCγ (58,154). Our lab and others have previously shown that overexpression of SH2B1β in PC12 cells enhances and prolongs NGF-induced phosphorylation of activating amino acids in Akt (Ser473) and PLCγ

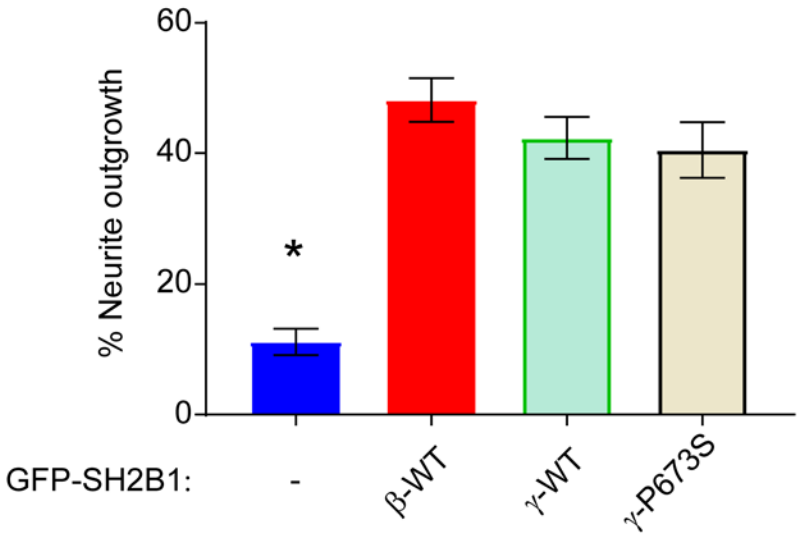

Figure 4.3. The human mutation P673S does not impair the ability of SH2B1γ to enhance NGF-mediated neurite outgrowth. Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. Means ± s.e.m. are shown for 3 independent experiments. * p≤0.05 compared to SH2B1γ.

(Tyr783) (70,146). Enhancement of phosphorylation of activating amino acids (Thr202, Tyr204) of ERK1/2 has not been observed in our studies (69,146), although others have reported an enhancement (63,146). We found that overexpression of SH2B1β WT enhanced and prolonged phosphorylation of Tyr783 in PLCγ compared to GFP control cells (Figure. 4.4). The mutations P90H and P322S did not impair that enhancement. Overexpression of SH2B1β WT, P90H or P322S did not enhance or prolong phosphorylation of Ser473 in Akt (Figure. 4.5A) or of Thr202/Tyr204 in Erks 1 and 2 (Figure. 4.6), together, these data and previously reported results (146) (see Chapter 3) suggest that SH2B1β reproducibly enhances and prolongs NGF-mediated phosphorylation of PLCγ. The introduction of the human obesity mutations to SH2B1β has no statistically significant impact on Erk1 and ERK2, Akt, and PLCγ phosphorylation, despite an effect on neurite outgrowths, suggesting SH2B1β may impact other or additional cellular events important for neurite outgrowth.

Post-translational modification of SH2B1 was observed after NGF treatment at 10 and 60 minutes as reported previously (64). The upward shift in SH2B1β has been shown previously to be due to primarily phosphorylation of Tyr and Ser/Thr (64). Phosphorylation on at least some of the Ser residues (Ser 161, 165) appears to be important for SH2B1 to leave the plasma membrane, enter the nucleus and enhance both NGF-induced neurite outgrowth and gene expression (78). Immunoblots of wild type SH2B1β and human obesity variants P90H and P322S stably expressed in PC12 cells reveal that NGF (Figure 4.4A, lower panel) causes a broadening and an upward shift in their migration in SDS-PAGE. For GFP-SH2B1β WT, the primary band after 60 minutes is the top of the broad band (Figure. 4.4A). The migration shift seen with

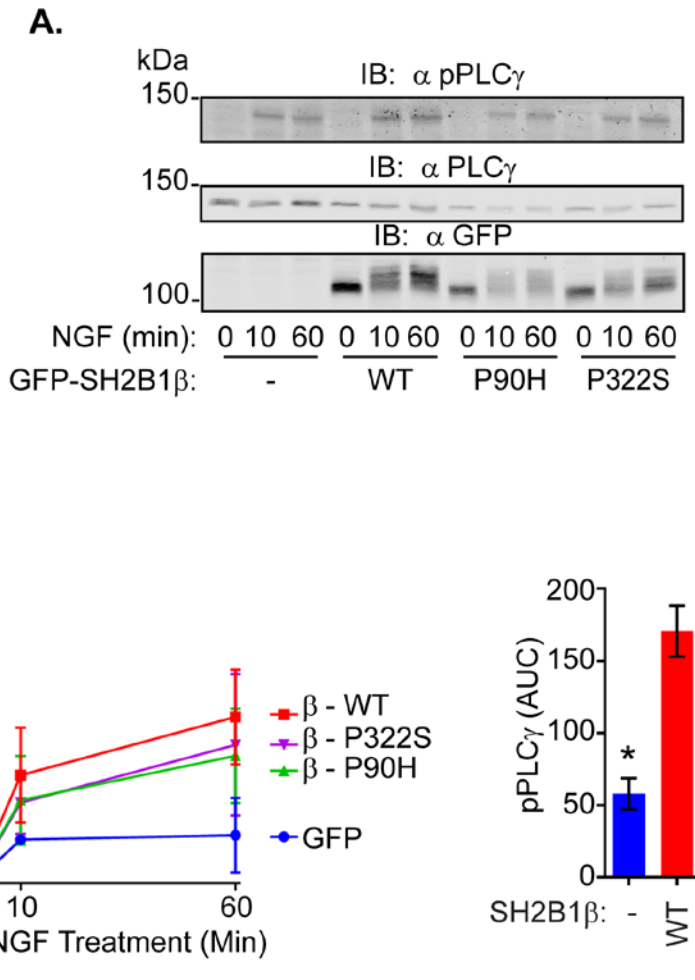

Figure 4.4. Human obesity mutations P90H and P322S do not impair the ability of SH2B1β to enhance and prolong NGF-mediated PLCγ phosphorylation. A) Serum-starved (15h) PC12 cells stably overexpressing GFP (-) or the indicated GFP-tagged SH2B1 were treated with 25 ng/mL NGF for 10 or 60 min. Proteins in cell lysates were separated by 5-12% acrylamide gradient SDS-PAGE gels and immunoblotted with the indicated antibodies. **B)** The relative signal intensities of the bands corresponding to pPLCγ and AUC determined from the relative signal intensities were determined and normalized to the GFP signal at 10 minutes. Means ± s.e.m. are shown from 3 independent experiments. * $p \leq 0.05$ compared to SH2B1β WT**.**

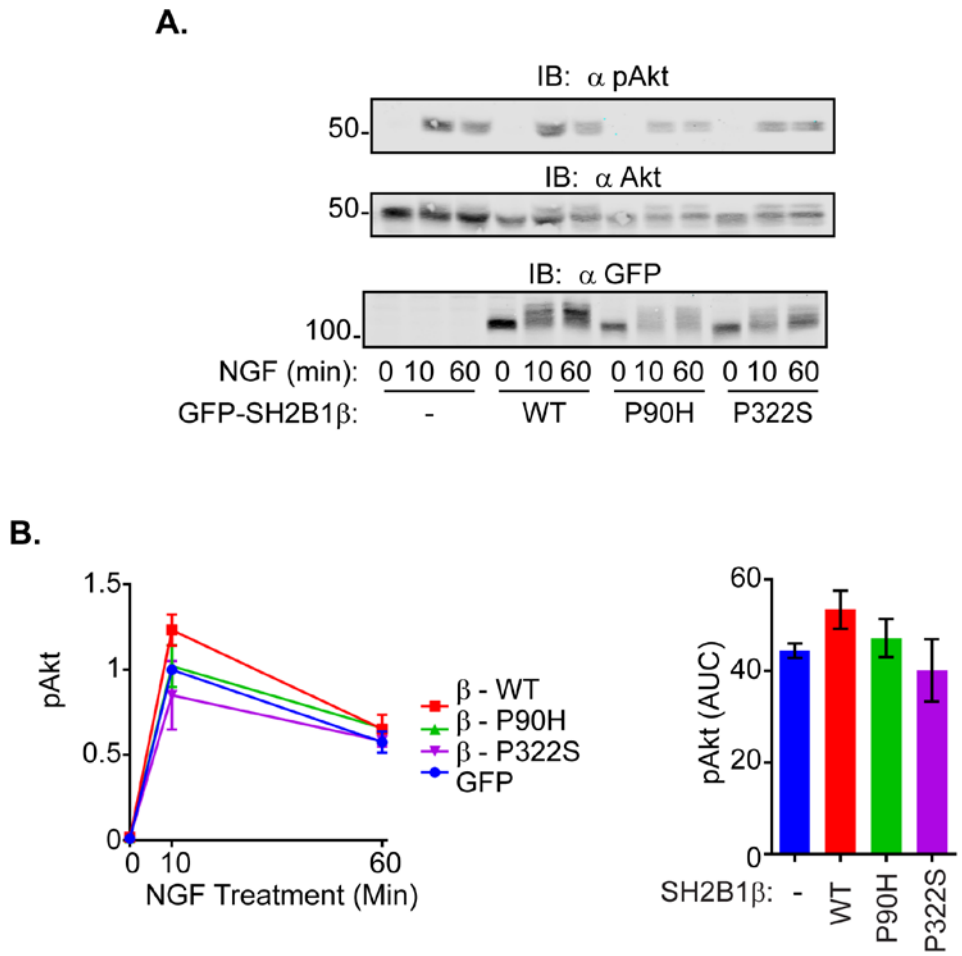

Figure 4.5. Like SH2B1β, human obesity mutations, P90H and P322S, do not significantly enhance NGF-mediated Akt phosphorylation. A) Proteins in the same cell lysates as in Figure 4.4 were immunoblotted with the indicated antibodies. **B)** The relative signal intensities of the bands corresponding to pAkt and AUC determined from the relative signal intensities were determined and normalized to the GFP signal at 10 minutes. Means ± s.e.m. are shown from 3 independent experiments.

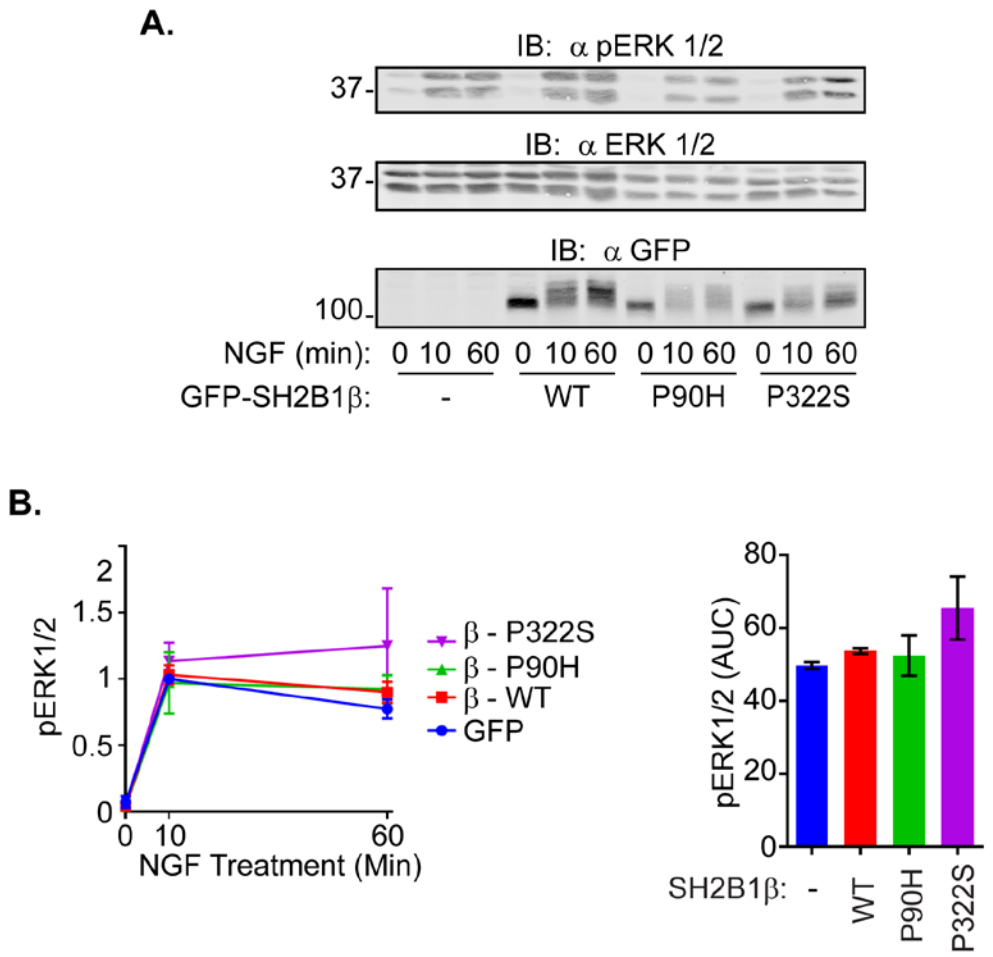

Figure 4.6. Like SH2B1β, human obesity mutations, P90H and P322S, do not significantly enhance NGF-mediated Erk 1 and 2 phosphorylation. A). Proteins in the same cell lysates as in Figure 4.4 were immunoblotted with the indicated antibodies. **B)** The relative signal intensities of the bands corresponding to pERK1/2 &and AUC determined from the relative signal intensities were determined and normalized to the GFP signal at 10 minutes. Means ± s.e.m. are shown from 3 independent experiments.

SH2B1β P90H and P322S is comparable to that seen with SH2B1β WT, however, no primary band is evident for P90H and the primary band for P322S at 60 min migration is slightly higher than that seen in the absence of NGF, but lower than that seen with WT SH2B1β (Figure. 4.4). These data suggest that the human mutations in SH2B1β may dysregulate the phosphorylation of SH2B1 in response to NGF.

SH2B1 human obesity mutations P90H and P322S impair the ability of SH2B1 to enhance the expression of NGF-responsive genes.

Previous studies showed that SH2B1β enhances the expression of a subset of NGF-responsive genes, including *Plaur, Mmp3, Mmp10,* and *FosL1* (79). *Plaur* is an early-response gene that encodes urokinase plasminogen activator receptor (UPAR). UPAR is located on the plasma membrane where it binds to and activates plasminogen activator (uPA). UPAR has been shown to be necessary for NGF-mediated neurite-outgrowth in PC12 cells (80,86). The matrix metalloproteases MMP3 and MMP10, along with uPA, are critical for degradation of the extracellular matrix to promote neuronal axon and dendrite extension (84,85). Fos-like antigen 1 (FosL1, Fra1*)* is a member of the Fos family of early-response genes (145). As previously reported, stable expression of GFP-SH2B1β significantly enhanced expression of *Plaur, Mmp10, Mmp3, and FosL1* compared to GFP control cells after 6 hours of 100 ng/ml NGF treatment (Figure. 4.7). Introduction of the P322S mutation impaired SH2B1β enhancement of NGF-dependent expression of *Plaur* (Figure. 4.7A), *Mmp10* (Figure. 4.7B), and *FosL1* (Figure. 4.7C) but had no effect on the expression of *Mmp3* (Figure. 4.7D). The P90H mutation impaired the ability of SH2B1β to stimulate NGF-induced expression of *Mmp10* (Figure. 4.7B),

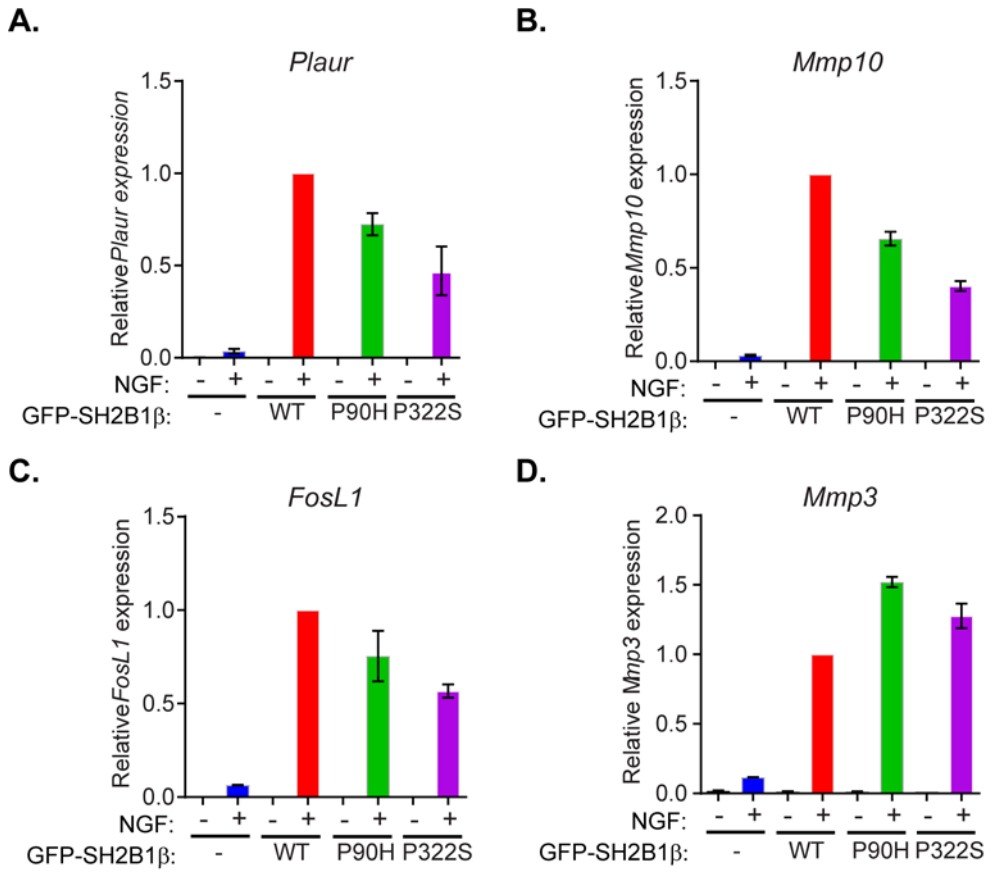

Figure 4.7. Human obesity mutations P90H and P322S impair the ability of SH2B1β to enhance NGF-mediated gene transcription. PC12 cells stably overexpressing GFP (-) or the indicated GFP-tagged SH2B1 were treated with 100 ng/mL NGF for 6 h. mRNA was collected and levels of *Plaur* (**A**.), *Mmp10* (**B**), *FosL1* (**C**), *Mmp3* (**D**) mRNA were determined by qPCR. Relative mRNA abundance was normalized to the geometric mean of the levels of *Hprt* and *Cyclophilin A* mRNA. Data were normalized to values obtained by GFP-SH2B1β cells treated with NGF. Means ± range are shown for 2 independent experiments. mRNA levels for untreated samples are plotted but were usually too low to be visible on the graph.

but had a much more modest effect on SH2B1β enhancement of expression of *Plaur* (Figure. 4.7A) and *FosL1* (Figure. 4.7C). Like P322S, P90H had no effect on SH2B1 enhancement of expression of *Mmp3* (Figure. 4.7D). Collectively, these data provide evidence that human obesity mutations impair the ability of SH2B1β to enhance the expression of at least a subset of genes (*Plaur, Mmp10, Mmp3, FosL1)* necessary for NGF-induced neurite outgrowth in PC12 cells.

SH2B1α-specific mutations A662V, V695M, and L723V do not alter NGF-mediated neurite outgrowth.

Previously, our laboratory showed that, unlike SH2B1β, SH2B1α does not enhance NGF-mediated neurite outgrowth (135). In Chapter 3, I showed that mutating Tyr753 in the C-terminal tail of SH2B1α enables SH2B1α to enhance NGF-mediated neurite outgrowth in PC12 cells to a similar degree as SH2B1β (see Chapter 3). However, in contrast to what we observed when mutations were introduced in SH2B1β, the SH2B1α A662V, V694M, and L723C mutations did not impair the ability of SH2B1α Y753F to enhance NGF-mediated neurite outgrowth (Figures. 4.8, 4.9, 4.10). These results suggest that the α-specific obesity mutations may not impair neurite outgrowth.

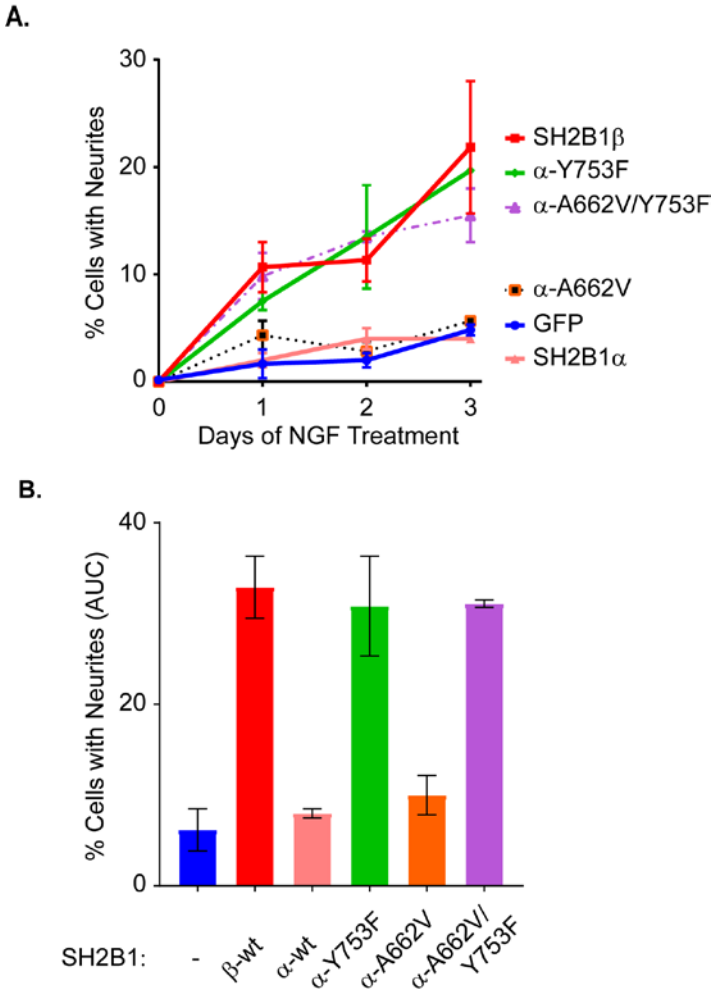

Figure 4.8. Human mutation A662V does not impair the ability of SH2B1α Y753F to enhance NGF-mediated neurite outgrowth. A) Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. **B)** AUC was determined from the data in Panel **A**. Means ± range are shown from 2 independent experiments.

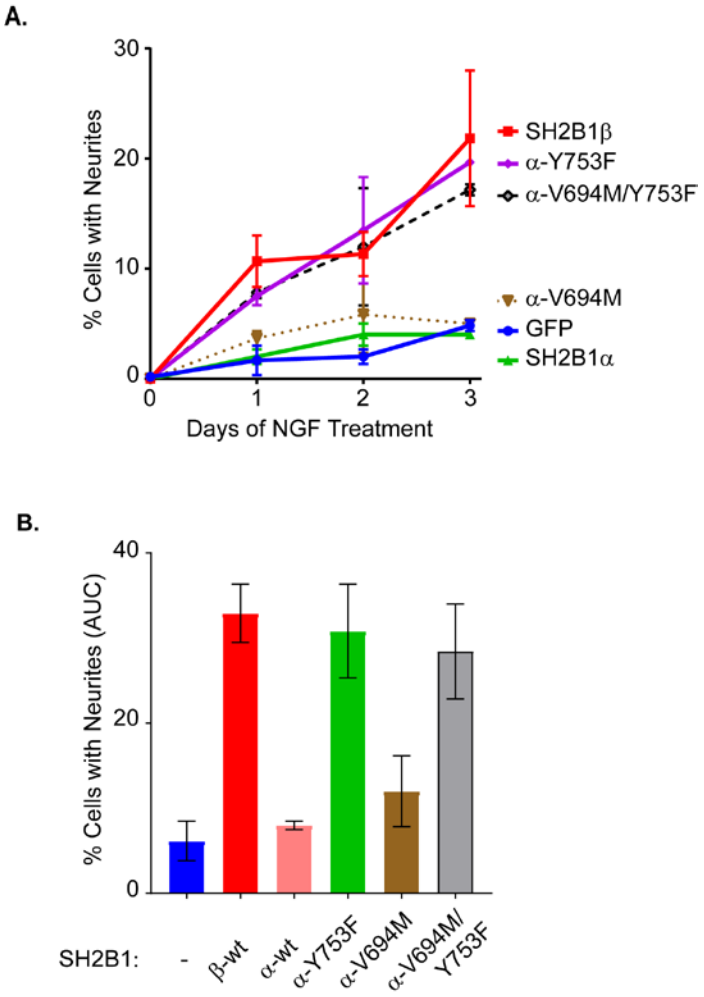

Figure 4.9. Human mutation V694M does not impair the ability of SH2B1α Y753F to enhance NGF-mediated neurite outgrowth. A) Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. **B)** AUC was determined from the data in Panel **A**. Means ± range are shown from 2 independent experiments. Mouse V694a amino acid corresponds to Human V694 amino acid.

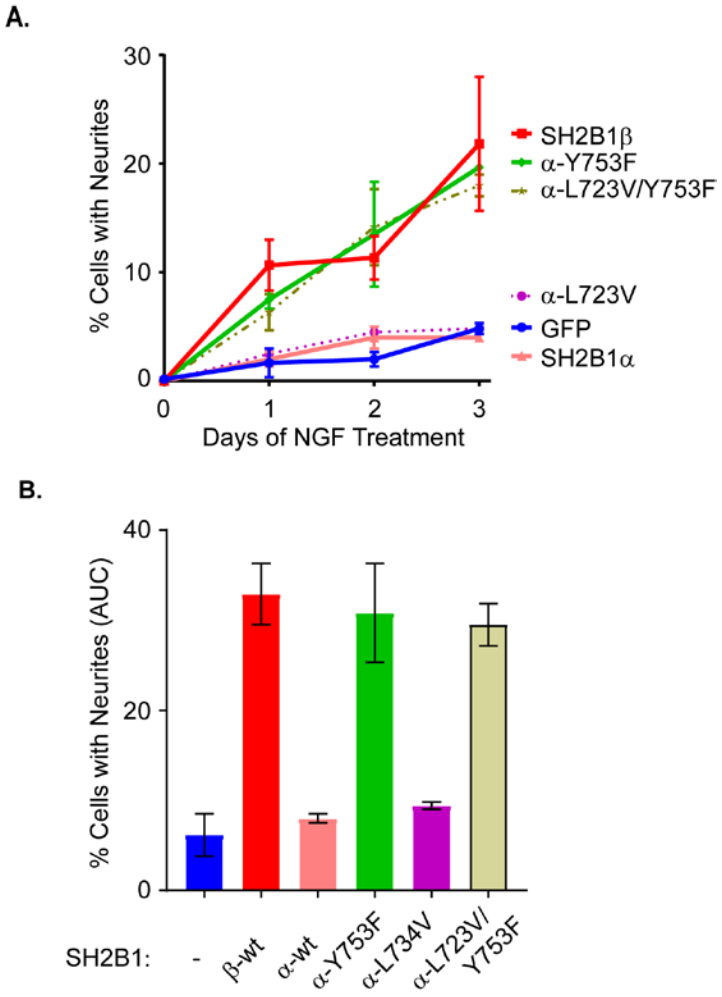

Figure 4.10. Human mutation L723V does not impair the ability of SH2B1α Y753F to enhance NGF-mediated neurite outgrowth. A) Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. **B)** AUC was determined from the data in Panel **A**. Means ± range are shown from 2 independent experiments. Mouse L723 amino acid corresponds to Human A723 amino acid.

Discussion

In this study, we have functionally characterized several new SH2B1 human obesity mutations common to all SH2B1 isoforms (R227C, G238C, R270W, E299G) as well as some unique to SH2B1β (T655I), SH2B1γ (P673S), or SH2B1α (A662V, V695M, L723V) for their ability to enhance NGF-mediated neurite outgrowth. In addition, we subjected the mutants SH2B1β P90H and P322S to cell signaling and gene expression assays to further reveal how these mutations disrupt the ability of SH2B1β to enhance NGF-induced neurite outgrowth.

We show that the newly identified human obesity mutations G238C and E299G impair the ability of SH2B1β to enhance NGF-mediated neurite outgrowth in PC12 cells to the same extent as the P322S mutation. The identified β-T655I mutation similarly impaired SH2B1β enhancement of neurite outgrowth to the same extent as P322S while R270W had no effect. However, the P673S mutation, which in the context of SH2B1β (T655I) impaired SH2B1 enhancement of neurite outgrowth, had no effect in the context of SH2B1γ. Collectively, most of the human mutations located in the shared N-terminal 631 amino acids of SH2B1 have been found to impair the ability of SH2B1 to enhance neurite outgrowth by approximately 50%. Most of these mutations are located within domains of SH2B1 that are likely to be involved in protein-protein interactions. P90H lies in a proline-rich region; G238C lies in the NES; R270W, E299G, P322S, F344LfsX20 are within the PH domain; and T546A is in the SH2 domain. Weakening the interaction

of a scaffold protein like SH2B1 to receptor tyrosine kinases or to cytokine family receptor-associated JAKs or to downstream signaling proteins could introduce relatively minor changes to cell signal fidelity that lead to an escalation of dysfunctional transcriptional responses, and a pathogenic state over time. The weakening of a protein-protein interaction due to the introduction of a disease-associated variant has been observed in euchromatic histone methyltransferase-1 (EHMT1) (155). Identification of a novel mutation encoding P809L in the conserved TPLX motif of EHMT-1 is associated with Kleefstra Syndrome (KS), which more commonly arises due to haploinsuffiency of EHMT-1. Individuals with KS exhibit severe intellectual disability with absent speech, hypotonia, congenital heart defects and abnormal facial features (156). The P809L mutation in EHMT-1 causes modest misfolding leading to irregular hydrogen bonding and improper hydrogen binding with its target protein demethylated H3K9 (155). These data provide insight into the structural instability induced by point mutations in proteins and their ability to dysregulate protein-protein interactions resulting in a pathogenic state. Examination of how the human obesity-associated mutations in SH2B1 disrupt the structural integrity of SH2B1 will greatly enhance our understanding of SH2B1's role in scaffolding signaling complexes.

Human obesity-associated mutations in SH2B1 lie in or adjacent to key domains and motifs in SH2B1. Mutations in the SH2 domain might be expected to decrease SH2B1 recruitment to receptor tyrosine kinases. Indeed, the F344LfsX20 mutation which results in a form of SH2B1 completely lacking an SH2 domain is unable to stimulate NGF-induced neurite outgrowth presumably due to an inability to bind via its SH2 domain to the NGF receptor TrkA (6). Whether SH2B1β T546A with a point

mutation in the SH2 domain has reduced ability to bind to TrkA has not yet been tested. The human obesity mutations R270W, E299G, and P322S in SH2B1 lie in the PH domain of SH2B1. The function of the PH domain in SH2B1 is unknown. However, many PH domains in signaling proteins have been reported to recognize phosphoinositides in the plasma membrane (77). Further examination of how the SH2B1 PH domain regulates SH2B1 function and determining whether the human obesity-associated PH domain mutations in SH2B1 alter interactions between SH2B1 and phosphoinositides may provide insight into the function of the PH domain in SH2B1. The G238C mutation in SH2B1 lies in the NES, potentially impairing the ability of SH2B1 to exit the nucleus. Proline-rich regions have been reported to bind to SH3 domains in signaling proteins (157). The P90H mutation in SH2B1 is located within a proline-rich motif potentially implicating the P90H mutation in disrupting interactions with SH3 domains containing signaling proteins. These human obesity-associated mutations that are positioned within key motifs and domains have provided regions of interests in SH2B1 whose function needs to be further examined.

The P90H and P322S mutations impair the ability of SH2B1 to alter the NGF-mediated transcriptome and neurite outgrowth (6) despite no statistically significant impairments to NGF-mediated phosphorylation of signaling proteins. SH2B1β P90H and P322S are similar to SH2B1β WT in their ability to alter NGF-mediated phosphorylation of cell signaling proteins PLCγ. However, in the experiments shown in Figure. 4.5, we did not detect a statistically significant change in NGF-mediated Akt phosphorylation between GFP control and SH2B1β, even though we and others have detected an increase in the past (shown in Figure 3.5C). Figure 4.5, we attribute the loss of a

statistical significant SH2B1-induced enhancement in Akt phosphorylation to use of a different set of stable cell lines. However, the trend of a modest SH2B1β induced increase in NGF-dependent Akt phosphorylation was still observed. Despite at best causing small changes in cell signaling, the P90H and P322S mutations exerted substantial effects on the expression of NGF-responsive genes *Plaur*, *Mmp10,* and *FosL1.* SH2B1β P90H and P322S were expressed to the same extent as SH2B1β WT in PC12 cells, thus removing the possibility of dosage differences in our overexpression model (6,135) (refer to Chapter 3). Further research using the stably overexpressed PC12 cells to identify how the SH2B1 P90H and P322S mutations alter the transcriptome will shed light on how these mutations alter the transcriptome.

The SH2B1α specific mutations A662V and A723V, but not V695M, in the C-terminal tail have been shown previously to inhibit GH-dependent motility of RAW264.7 macrophages (135). However, we have not yet identified any SH2B1 function in PC12 cells that is impaired by these mutations. Unlike SH2B1β and γ, SH2B1α does not enhance neurite outgrowth unless Tyr753 is mutated to Phe. However, even when A662, V694, and L723 are mutated in the context of SH2B1α Y753F, we saw no effect of the mutations on SH2B1α stimulation of NGF-induced neurite outgrowth. Thus, the role the SH2B1α specific mutations and the effect of SH2B1α mutations on neurons are not yet understood. Understanding the role of SH2B1α in neurons is important due to the tissue-specific expression of SH2B1α in neurons in the brain and the finding that individuals with α-specific mutations are obese but do not exhibit the maladaptive behaviors seen in individuals with mutations present in all isoforms (6,135).

Mutation of the TrkA substrate Tyr753 in the C-terminal tail of SH2B1α impairs the ability of the N-termini 1-631 amino acids to enhance phosphorylation of TrkA and its downstream signaling pathways (refer to Chapter 3). Co-expression of SH2B1α with SH2B1β reduces the capacity of SH2B1β to enhance neurite outgrowth, indicating that SH2B1α competes with SH2B1b and presumably SH2B1γ and δ isoforms for binding to neurotrophic factor receptors. This would allow for a role for SH2B1α in fine tuning signals for spatial and temporal control of neuronal development, since SH2B1α appears to prevent enhancement of neurite outgrowth by other SH2B1 isoforms when Tyr753 is phosphorylated, or to contribute to the enhancement when Try753 is unphosphorylated. This fine-tuning of a signal may also involve TrkA complexes recruiting the E3-ligase Cbl-b, which has been shown to inhibit NGF-induced neuroblastoma cell differentiation (158). The interactome data gathered by Emdal et al. (158), using their tet-inducible TrkA neuroblastoma cell line, SH-SY5Y-Tr-TrkA, suggest the possibility that SH2B adapter protein family member SH2B2 (APS) may facilitate the association of TrkA with Cbl-b. SH2B2 has been shown to recruit E3-ligases Cbl-b and C-Cbl to the phosphorylated C-terminal Tyr618 in response to insulin (109,159). Tyr753 and Tyr618 in SH2B1α and SH2B2, respectively, share homology with one another. The contribution of Cbl proteins to actions of SH2B1α in response to NGF-activated TrkA in PC12 cells has yet to be examined. Further, whether and how the human obesity-associated mutations in the C-terminal tail of SH2B1α affect the function of SH2B1α in neurons remains to be determined. However, since at least a subset of the mutations appear to affect GH-stimulated cell motility of macrophages (6,135), it is tempting to speculate that SH2B1α and the SH2B1α specific human obesity-associated

mutations may affect the cytoskeleton, thereby impacting neuronal migration and architecture.

Collectively, we have shown that an array of human mutations associated with obesity impair the ability of SH2B1 to enhance NGF-induced neurite outgrowth and gene transcription. Transcriptional data using cells expressing SH2B1β P90H and SH2B1β P322S provide the most striking example of human mutations that impair a function of SH2B1 thought critical for neuronal function. Further analysis of this transcriptional effect should provide insight into the molecular basis for how these mutations obstruct functions of the different isoforms of SH2B1.

Chapter 5

Summary and Future Directions

The goal of my thesis research was to identify cellular mechanisms by which SH2B1 isoforms affect neurotrophic functions in neurons. Individuals with variants in SH2B1 located within the first 631 amino acids shared by all isoforms of SH2B1 have been shown to have severe-early onset childhood obesity, insulin resistance, and maladaptive behaviors (6,135). Individuals with mutations specific to SH2B1α and δ were found to have the same phenotype except they did not exhibit maladaptive behaviors (135). The SH2B1α and δ isoforms are expressed almost exclusively in the brain (6). In addition, neuronal tissue-specific expression of SH2B1β largely restores the lean phenotype of SH2B1$^{-/-}$ mice (54). These data indicating that SH2B1 in the brain regulates energy balance, that certain isoforms are brain specific, and that isoform specific mutations are associated with human obesity suggest that it is critical to understand the function and role of the different SH2B1 isoforms in neurons. In further support of a critical role for SH2B1 in neurons, SH2B1 has been implicated in neurotrophin-induced neurite outgrowth of several different types of neurons (25-27). The Carter-Su lab had shown that the obesity mutations impair SH2B1β enhancement of NGF-mediated neurite outgrowth (6), yet the mechanisms by which the obesity mutations impair neurite outgrowth remained unclear. My studies examined how these

mutations impair the ability of SH2B1 to enhance NGF-mediated neurite outgrowth and further examined how the unique SH2B1α C-terminal tail regulates the ability of SH2B1 to enhance NGF-mediated neurite outgrowth. Future directions should focus on determining how SH2B1 isoforms mediate transcriptome level changes in response to a neurotrophin (i.e. NGF, BDNF) and how the brain-specific isoforms modulate neuronal circuits that regulate feeding behavior.

Our collaborator, I. Sadaf Farooqi (University of Cambridge), identified several rare mutations within *SH2B1* that encode for A662V, V695M, and A723V in the C-terminal tail of SH2B1α in individuals exhibiting severe-early onset childhood obesity, insulin resistance, and hyperphagia. To determine what known cellular functions of SH2B1α are impaired by the human mutations, we performed a series of functional studies that we had used previously to characterize the P90H, T175N, P322S, and F344LfsX20 mutations within the context of SH2B1β (6). None of the shared or α-specific SH2B1 mutations affected the ability of SH2B1α to enhance tyrosyl phosphorylation of IRS2 in response to leptin or insulin in a 293T overexpression system, suggesting that SH2B1α mutations do not contribute to obesity by dysregulating SH2B1α stimulation of leptin or insulin signaling. However, the high levels expression of SH2B1 and IRS2 or the use of a non-neuronal cell line could have masked mutation-induced changes in IRS2 phosphorylation. Lowering cDNA expression levels and performing leptin or insulin dose response curves may have improved our ability to detect subtle changes in IRS2 phosphorylation, if they existed. However, others have performed leptin dose response experiments in 293 cells, transiently co-expressing the long-form of leptin receptor and SH2B1β or γ wild-type or

mutations (βT655I/γP674S) and observed no effects of the mutations on SH2B1β enhanced changes in STAT3 response in a luciferase assay (17). This suggests that the SH2B1 mutations in general may affect only a subset of cellular responses that does not include the entire repertoire of insulin or leptin responses. We also found it difficult to reproducibly detect SH2B1β-mediated changes in NGF-mediated phosphorylation of signaling proteins (PLCγ, Akt, and ERKs 1 and 2) using stable expression of SH2B1β, so it is not surprising that we did not reliably detect changes in their phosphorylation due to the human obesity mutations (refer to Chapter 4). However, introduction of the mutations led to robust impairments in SH2B1β enhancement of the expression of the early-response genes, *Plaur* and *FosL1*. This suggests that we should design RNA-seq or microarray experiments to investigate how the SH2B1 human obesity-associated mutations alter the transcriptional landscape in response to leptin or insulin.

I have initiated experiments to determine the expression of SH2B1-dependent genes in leptin receptor-containing neurons using translating ribosome affinity purification (TRAP)-sequencing (TRAP-seq). The use of TRAP-seq provides the opportunity to identify the abundance of actively translating transcripts in a subset of cells within a heterogeneous population and reduce the transcriptional noise. Neurons containing the leptin receptor make up a small population of cells that are located within the hypothalamus. We received $LepR^{cre}$ mice with the cre-inducible ROSA26-L10-eGFP from Dr. Martin Myers (University of Michigan) and have been breeding these animals with $SH2B1^{flox/flox}$ mice from Dr. Liangyou Rui (University of Michigan) to obtain mice in which LepRb-expressing cells lack SH2B1 and express a GFP-tagged ribosomal L10

subunit (L10-eGFP). GFP immunoprecipitation will be used to purify GFP-tagged L10 along with any ribosome-associated mRNA species from LepRb neurons, which will then be analyzed by RNA-seq. This experiment should provide us with insight into cellular mechanisms controlling gene expression in leptin receptor neurons and specific transcriptome events facilitated by leptin and SH2B1.

SH2B1β has been shown to be a novel focal adhesion protein (93) that is required for GH-dependent migration of macrophages (92). All SH2B1 isoforms share an actin binding region located between amino acids 150-200 (89,103), providing an opportunity for all isoforms of SH2B1 to participate in actin cytoskeleton reassembly necessary for motility. Here, we showed that SH2B1α enhances GH-mediated macrophage motility to a similar extent as SH2B1β. We assessed the ability of the human obesity mutations in SH2B1α to enhance GH-mediated migration and identified a subset of the human mutations (P90H, P322S, T546A, A663V, A723V) that inhibit SH2B1α enhancement of GH-mediated macrophage migration. The P90H and P322S mutations impaired SH2B1α enhancement of motility as previously reported for SH2B1β (6). The A663V and A723V variants in the α-tail region also impaired SH2B1α enhancement of GH-dependent macrophage motility, these data suggest that cellular motility may be a key phenotype affected by the human obesity mutations within SH2B1α and β. SH2B1β has been shown to interact with the actin regulator insulin receptor tyrosine kinase substrate p53 (IRSp53) to promote neurite outgrowth in hippocampal neurons (139). Because axons, dendrites, and growth cones are highly dependent on the actin cytoskeleton for shape and motility, these data suggest that neuron-specific SH2B1α may have an important role in actin reorganization in neurons.

SH2B1 is a unique scaffold in its ability to localize to numerous regions of the cell. I found that SH2B1β localizes at a 2:1 ratio on the plasma membrane vs the cytoplasm (78) which would localize it appropriately to bind to receptor tyrosine kinases and to regulate the actin cytoskeleton in early signaling events (78). In addition, SH2B1β is capable of cycling into and out of the nucleus, and impairment of SH2B1β import into or export out of the nucleus disrupts its ability to enhance NGF-mediated gene expression and neurite outgrowth (69). Unlike SH2B1β, we found SH2B1α to localize equally between the plasma membrane and the cytoplasm and to be unable to accumulate in the nucleus in the presence of LMB indicating it is unable to enter the nucleus. Previously, our lab reported a direct correlation between the ability to cycle in and out of the nucleus and the ability to enhance NGF-mediated neurite outgrowth and gene expression (69,76). The human obesity mutations within the context of SH2B1β impaired the ability of SH2B1β to localize to the nucleus in the presence of LMB (6). Here, I report that none of the human obesity-associated mutations within the context of SH2B1α alter the localization of SH2B1α on the plasma membrane. We were unable to determine the ability of the mutations to impede nuclear localization of SH2B1α due to SH2B1α's inability to cycle in and out of the nucleus. However, the finding that mutating Tyr753 on SH2B1α enables SH2B1α to cycle in and out of the nucleus (see Chapter 3) provides an avenue to determine the ability of the mutations to dysregulate nuclear import and export. Preliminary data performed by the Carter-Su lab expressed GFP-tagged SH2B1β in cultured hippocampal neurons and observed GFP positive puncta undergoing retrograde and anterograde trafficking through axonal outgrowth (data not shown). This raises the possibility that the isoforms of SH2B1 may have a role in the

trafficking of plasma membrane proteins to the soma or nuclear proteins to the growth cones. Trafficking is critical for axonal growth and guidance and dendritic branching (160). Future experiments determining whether SH2B1 isoforms affect the subcellular localization of other proteins would provide functional insights into the role of SH2B1 motility in neurons.

Neuronal SH2B1α does not enhance NGF-mediated neurite outgrowth but inhibits the ability of other SH2B1 isoforms to enhance neurite extension. Mutating Tyr753 to Phe in the C-terminal tail of SH2B1α enhances NGF-mediated neurite outgrowth in PC12 cells as shown in Chapter 3, Figure 3.2 and prevents the SH2B1α-induced inhibition of SH2B1b enhancement of neurite outgrowth. I have found that introduction of the α-isoform specific subset of mutations (A662V, A695M, L723V) into SH2B1 Y753F do not impair the ability of SH2B1a Y753F to enhance NGF-mediated neurite outgrowth. Therefore, these mutations may not affect NGF-mediated neurite outgrowth. However, the ability of the α-isoform specific mutations to impair neurite outgrowth maybe be dependent on the NGF dosage. In a dose response experiment, I transiently expressed GFP-tagged SH2B1β and SH2B1α in PC12 cells, serum starved the cells overnight, and treated the cells with 10, 25, 100, 200 ng/ml of NGF. I then determined the percent neurite outgrowth after 2 days of NGF treatment (Figure 5.1). The 25 ng/ml dose of NGF used for my other neurite outgrowth experiments turns out to be a saturating dose based on the inability of PC12 cells exposed to higher doses of NGF to produce longer neurites within 2 days. Therefore, to tease out the potentially small changes brought about by the human obesity mutations, I propose future experiments to determine whether different doses of NGF shift the dose response

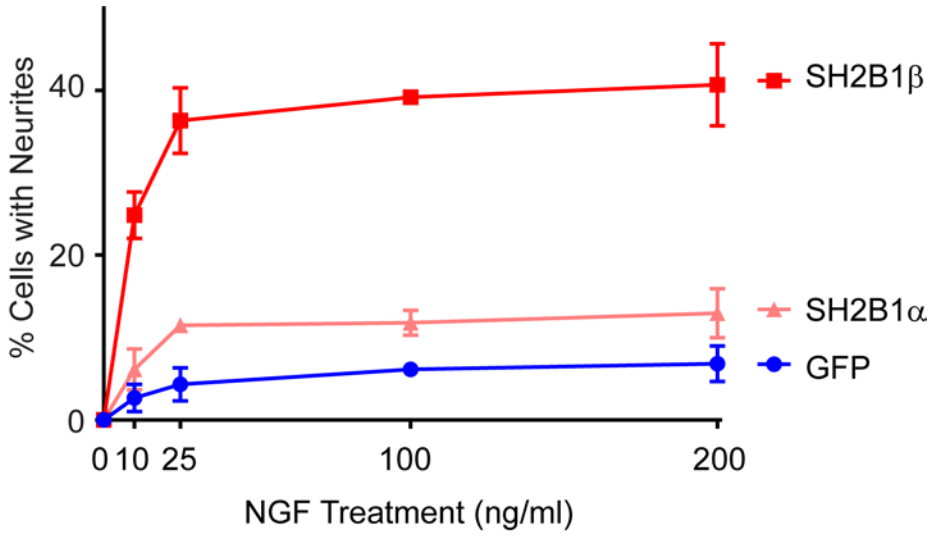

Figure 5.1. SH2B1β enhanced neurite outgrowth response by NGF is saturated. Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with indicated NGF dose. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. Means ± range are shown from 2 independent experiments.

curve.

We have primarily focused our neurite outgrowth studies using the neurotrophin NGF because PC12 cells both contain endogenous TrkA which is highly responsive to NGF and are amenable to assessing cellular effects of SH2B1. However, it would be informative to determine the role of the human obesity mutations in the context of brain derived neurotrophic factor (BDNF). Individuals with mutations in BDNF or TrkB have been shown to exhibit hyperphagia and obesity (72-74,161-163). SH2B1 has been shown to interact with the BDNF receptor, TrkB, and to enhance neurite outgrowths length (25,164). More recently, overexpression of SH2B1β was shown to promote neurite outgrowth and branching in cultured hippocampal neurons supplemented with BDNF (27). The roles of SH2B1 α, γ, and δ isoforms in BDNF signaling have not been studied nor has the effect of any of the human obesity-associated mutations on BDNF function been assessed. The human obesity mutations may be expected to have a bigger impact on SH2B1 regulation of BDNF than NGF signaling. Studying the effects of the different isoforms and the human mutations on BDNF signaling and functions may provide critical insight into SH2B1 regulation of neuronal function critical for energy balance.

Our current knowledge of how SH2B1 contributes to cellular signaling is based primarily on overexpression of the SH2B1β isoform. We have provided evidence that a post-translational modification on a unique C-terminal tail potentially regulates the shared N-terminal region (see Chapter 3). However, we are unable to determine definitively whether Tyr753 on SH2B1α is phosphorylated without the use of a phospho-specific antibody to Tyr753. An antibody to phosphoTyr753 would provide valuable

insight into whether Tyr753 is a substrate of TrkA or TrkB or other tyrosine kinases in the brain. Considering that Tyr753 is located 3 amino acids away from the C-terminal end of SH2B1α, development of a phospho-specific antibody to Tyr753 may be difficult to acquire. To overcome the lack of a phospho-specific antibody to Tyr753 on SH2B1α, experiments using stable isotope labeling with amino acids in cell culture (SILAC) or isobaric tag(s) for relative and absolute quantification (iTRAQ) mass spectroscopy to identify SH2B1α phosphorylation sites may shed light on the phosphorylation status of Tyr753 in SH2B1α. These experiments should identify other proteins whose phosphorylation is altered by SH2B1α, SH2B1α Y753F or SH2B1α with the human obesity-associated mutations. These experiments should elucidate traditional and novel signaling pathways that are regulated by SH2B1α.

Most of my experiments used in vitro model systems to elucidate the specific cellular functions of different SH2B1 isoforms and to understand how the cellular functions of specific isoforms are affected by human mutations identified in SH2B1. However, it has not been determined whether SH2B1 affects the ability to promote axonal projections and synapses in neurons (i.e. POMC neurons) implicated in energy balance in the hypothalamus. Based on our previous findings implicating SH2B1 in neurite outgrowth and cell motility, I predict that SH2B1 affects the neuronal projections from, or the number of neurons in, regions of the brain that are involved in energy balance. To test this, I would determine if SH2B1 regulates the number of POMC neurons or neuronal projections to the periventricular nucleus (PVN) of the hypothalamus. POMC neurons are located in the arcuate nucleus and serve a primary role in maintaining feeding behavior and energy homeostasis (165). Brains of *Sh2b1*$^{-/-}$

animals at various ages would be perfused and processed for anti-POMC immunohistochemistry and the number of POMC neurons and their locations determined (166). I predict that the absence of SH2B1 in POMC neurons will lead to a decrease in the number of neurons (SH2B1$^{-/-}$ have smaller brains, L. Rui, University of Michigan, personal communication) or neuronal projections to the PVN, resulting in abnormal synaptogenesis and impairment of POMC neurons in regulating energy homeostasis. However, if there is no change in POMC neurite projections to the PVN, there may be an imbalance of POMC neural projections to other brain regions (i.e. lateral hypothalamus) which could be examined.

The results in Chapter 2 have shed light on the impact of human obesity mutations within the context of SH2B1α. They provided key insights into how SH2B1 C-terminal tails have a functional role in the regulation of SH2B1. Further insight into regulation by SH2B1α of neuronal motility and circuit development could lay a foundation for identifying how the mutations contribute toward an obese phenotype. In Chapter 3, I investigated why SH2B1α, unlike SH2B1β and γ, does not promote NGF-dependent neurite outgrowth. I found that removal of the unique C-terminal tail of SH2B1α allows SH2B1α to enhance neurite outgrowth and to accumulate in the nucleus in the presence of LMB to a similar or greater extent than SH2B1β. These data provided evidence that the C-terminal tail regulates the N-terminus of SH2B1. The unique C-terminus of SH2B1β has been identified as an actin binding site (103) and the SH2B1δ C-terminus has the ability to localize SH2B1 to the nucleolus (167), providing further evidence that the alternatively spliced C-terminal tails regulate the N-terminal 631 amino acids shared by all isoforms of SH2B1. Chapter 3 also examines how the

SH2B1α tail attenuates the robust ability of the SH2B1 1-631 truncation mutant to stimulate NGF-induced neurite outgrowth.

In Chapter 3, I present evidence that tyrosine phosphorylation of Tyr753 in the C-terminal tail of SH2B1α inhibits the ability of the N-terminal 631 amino acids of SH2B1 to enhance NGF-mediated phosphorylation of TrkA and cell signaling proteins (PLCγ and Akt), as well as expression of *Plaur, Mmp10, Mmp3,* and *FosL1*, and neurite outgrowth in PC12 cells. In addition, phosphorylation of Tyr753 appears to interfere with the ability of SH2B1α to cycle in and out of the nucleus. Tyr753 in SH2B1α is located within a motif, R_{747}AXXNQY_{753}, that is conserved between other SH2B family members, SH2B2/APS and SH2B3/LNK, which suggests that this Tyr may be functionally important. X-ray crystallography of APS has shown Tyr618 to be a key residue that interacts with the E3-ligase C-Cbl. In addition, amino acids R612, A613, and N616 were found to interact with C-Cbl (168). I mutated conserved Arg747 to an Ala (R747A) in SH2B1α and truncated SH2B1α prior to Arg747 (1-746) and determined the effect of these mutations on the ability of SH2B1α to enhance NGF-mediated neurite outgrowth in PC12 cells. The 1-746 truncation enhanced neurite outgrowth to a similar extent as SH2B1α Y753F (Figure 5.2). SH2B1α R747A enhanced NGF-mediated neurite outgrowth to a lesser extent than SH2B1α Y753F, suggesting Tyr753 is the primary residue responsible for SH2B1α-mediated attenuation of NGF-mediated neurite outgrowth (Figure 5.2). TrkA has been shown to interact with C-Cbl, resulting in ubiquitination of TrkA and degradation of the complex (169). In a proteomic screen, Cbl-b was shown to interact with TrkA to promote TrkA ubiquitination and degradation in

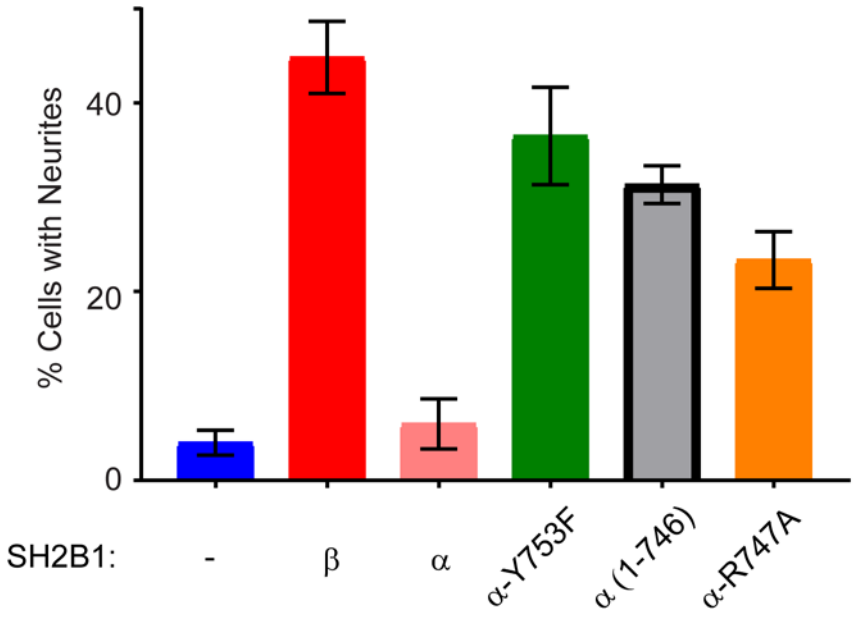

Figure 5.2. SH2B1α 1-746 enhances NGF-mediated neurite outgrowth to a higher extent than R747A. Live PC12 cells transiently expressing GFP or the indicated GFP-SH2B1 were treated with 25 ng/ml NGF. The percentage of GFP-expressing cells with neurite outgrowths at least twice the length of the cell body length was determined on the second day of treatment. Means ± range are shown from 2 independent experiments.

the neuroblastoma cell line SH-SY5Y (158). It is unclear whether C-Cbl or Cbl-b directly or indirectly associates with TrkA. These data raise the possibility that the Tyr753-containing motif may recruit C-Cbl or Cbl-b to SH2B1α, thereby bringing Cbl into close proximity to TrkA. Both Cbl family members have a conserved phosphotyrosine binding domain. Further experiments to determine whether Cbl binds to phosphorylated Y753 on SH2B1α could provide an explanation of how SH2B1α attenuates enhanced neurite outgrowth. An additional functional proteomics screen may provide insight into the role of SH2B1 isoforms as scaffold proteins and the impact of the C-terminal tails and human mutations on the proteins recruited to SH2B1.

In addition, we tested the ability of TrkA to phosphorylate the tyrosine residues in SH2B1β and SH2B1α. We determined that Tyr753 is likely to be the primary phosphorylation site for TrkA, and Tyr55 and Tyr439 appears to be secondary substrates for TrkA in 293T cells. Tyr55 and Tyr439 in SH2B1β also appear to be phosphorylated by TrkA. Tyr439 and Tyr494 lie in Y-X-X-L motifs and are phosphorylated by Jak1 and Jak2 (91). These tyrosine residues have been reported to be phosphorylated in response to GH in 3T3-F442A fibroblasts (92) presumably by GH-activated Jak2. Mutating Tyr439 and 494 to Phe decreases GH-mediated motility, membrane ruffling and macrophage motility (91,92). However, we were unable to observe any decrease in phosphotyrosine levels compared to wild-type SH2B1α or β when SH2B1α Y494 or SH2B1β Y494F were co-expressed with TrkA. Co-expression of SH2B1α Y55F, Y439F, or Y753F with TrkA produced migration shifts of SH2B1 in the immunoblots that suggested that phosphorylation of these tyrosines may regulate a subset of Ser/Thr residues on SH2B1α. Mutation of Ser161 and/or Ser165 to alanine in

SH2B1β has been shown to decrease macrophage motility (92). Together these results suggest that tyrosine phosphorylation of Tyr439 in SH2B1α and SH2B1β regulates cell motility, which leads us to speculate that phosphorylation of SH2B1α Tyr439 may aid in neuronal migration in the developing brain. Determining the migration of neural crest cells or neurons of specific regions of the brain such as the hypothalamus in SH2B1$^{-/-}$ mice and transgenic animals expressing SH2B1 isoforms would be critical to determine if and how SH2B1 isoforms participate in spatial placement of neurons in the developing brain. Further, experiments to verify the ability of TrkA to phosphorylate Tyr753 in SH2B1α and Tyr55 in various SH2B1 isoforms using phosphospecific antibodies would verify these tyrosines as sites of phosphorylation, and help in identification of kinases capable of phosphorylating them.

This thesis research has provided critical insights at the molecular and cellular level into the role of SH2B1α in the function of neurotrophic factors that utilize SH2B1α as a signaling protein. Future studies could be directed at the level of the whole animal to determine whether SH2B1α alters the ability of neurons involved in energy expenditure to make the appropriate synaptic connections. Because the human phenotype of patients with genetic mutations in SH2B1 includes obesity and insulin resistance, such insights should increase our understanding of the cellular pathways and functions that lead to obesity and insulin resistance. By providing valuable information about how seemingly relatively minor differences between isoforms can have a profound impact on the function of that protein, my studies also provide important insight into the impact of differential splicing on neuronal function.

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