p53 is recognized as a critical regulator of the cell cycle and apoptosis. Mounting evidence also suggests a role for p53 in the differentiation of several cell types including neuronal precursors. Using the PC12 cell model derived from adrenal chromaffin cells of ectodermal lineage, we studied the transcriptional role of p53 during neuronal differentiation induced by nerve growth factor (NGF) treatment. Differentiation in PC12 cells by NGF is characterized by growth arrest and neurite extension. The transcription factor p53 contributes to each of these processes but the mechanisms are incompletely understood. We hypothesized that p53 contributed to PC12 differentiation through the regulation of gene targets distinct from its known transcriptional targets during apoptosis. This study demonstrated that p53 protein was transcriptionally activated and contributed to NGF-mediated neurite outgrowth during differentiation of PC12 cells. Using a genome-wide chromatin immunoprecipitation cloning technique, we identified and validated 14 novel p53-regulated genes following NGF treatment. Furthermore, we describe stimulus-specific regulation of a subset of these target genes by p53. The most salient differentiation-relevant target genes included wnt7b involved in dendritic extension and the tfcp2l4/grhl3 grainyhead homolog implicated in ectodermal development. Additional targets included brk, sdk2, sesn3, txnl2, dusp5, pon3, lect1, pkcbpb15 and other genes. These studies demonstrate that receptor-mediated p53
transcriptional activity is involved in PC12 differentiation and may suggest a contributory role for p53 in neuronal development.

Since NGF signaling stabilizes p53 and enables the transcriptional regulation of various target genes, including \textit{wnt7b}, we tested the hypothesis that \textit{wnt7b} expression is involved in p53-mediated neurite outgrowth in NGF-differentiated PC12 cells. \textit{Wnt7b} transcript increased within 3 days, while protein levels of \textit{wnt7b} are rapidly and efficiently increased within 24 hours of NGF exposure. Stable silencing of p53 by shRNA reduced \textit{wnt7b} protein levels and halted neurite outgrowth in NGF-treated cells. Immunofluorescence showed \textit{wnt7b} was distributed throughout the cytosol but changed to peri-cytoplasmic, nodal localization during \textit{wnt7b} overexpression in transfections of mitotic and NGF-differentiated cells. Overexpressed \textit{wnt7b} produced marked neurite extensions in the presence of NGF and was sufficient to restore neurite outgrowth in p53-silenced cells. Therefore, \textit{wnt7b} is a p53-regulated neuritogenic factor that in conjunction with NGF signaling is capable of eliciting potent induction of neurite outgrowth in PC12 cells.

NGF is also recognized for its role in neuronal differentiation and maintenance. We investigated NGF influence over p53 activity during NO-induced apoptosis by sodium nitroprusside in differentiated and mitotic PC12 cells. NGF-differentiation produced increased p53 levels, nuclear localization and sequence-specific DNA binding. Apoptosis in mitotic cells also produced these events but the accompanying activation of caspases 1-10 and mitochondrial depolarization were inhibited during NGF differentiation and could be reversed in p53-silenced cells. Transcriptional regulation of \textit{PUMA} and \textit{survivin} expression were not inhibited by NGF, although NO-induced
mitochondrial depolarization was dependent upon \textit{de novo} gene transcription and only occurred in mitotic cells. Therefore, NGF mediates prosurvival signaling through factors such as Bcl-2 and p21^{Waf1/Cip1} without altering p53 transcriptional activity to inhibit apoptosis.

These studies demonstrate that NGF potently activates p53 transcriptional regulation of target genes involved in cell cycle arrest and neurite outgrowth. Although NGF potentiates p53 DNA-binding activity, it also inhibits p53-dependent apoptosis. In summary, receptor-mediated NGF signaling represents a comprehensive mechanism through which many functions of p53 activity may be regulated in responsive neuronal cells.
NERVE GROWTH FACTOR REGULATION OF TRANSCRIPTION FACTOR p53 ACTIVITY

by

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I dedicate this work to:

my loving wife Rachel,
for her constant and unending
support, patience and faith

and

my Mother and Father,
who instilled in me many values,
including faith and the virtues of hard work
BIOGRAPHY

I am Christopher Brynczka, son of Jozef and Maria Brynczka. My father and mother were respectively born and raised in Frydman, Poland and Sao Paulo, Brazil. While their arrival and union within the United States may be the subject of discourse reserved for a later time, I am the second of three children, preceded by brother Marc and succeeded by sister Lisa. Born in the city of Passaic, New Jersey and raised in the town of Oakland, New Jersey, my upbringing was steeped in traditional eastern European values and faith. Nowhere were these values more evident than in my parochial primary education and weekend participation in Polish language school.

I thank my parents who instilled the importance of education in me during my youthful years. Upon graduation from Indian Hills High School in 1996, I entered Rutgers University in New Brunswick, New Jersey with an interest in biology, and left in 2000 with a B.S. in Animal Science-Preveterinary Medicine and a broad understanding of the many scientific fields. I was subsequently employed for several years at IDEXX Diagnostics in Totowa, NJ as a Medical Technician, which provided my first real glimpse of a dynamic corporate environment. During this period of time, I took several continuing education courses within the Biochemistry department at Rutgers University to satiate my continued interest in the molecular basis of disease. It was here that I became acquainted with Dr. Keith Cooper and several other faculty, who I credit with sparking my interest in the field of Toxicology.

My acceptance into and ultimate enrollment in the Department of Environmental and Molecular Toxicology at NCSU represented a thrilling period of time within my life.
At the cusp of earning my PhD within this department, I can safely say that the years spent here will be viewed in hindsight with the same amount of enthusiasm.
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1. INTRODUCTION

1.1 Prologue

The transcription factor p53 is recognized as a central node in the negative regulation of cell cycle arrest and the positive induction of programmed cell death. As such, mutations in the tumor protein 53 (tp53) are a frequent occurrence in the development of the majority of cancer types [1]. Discovered in 1979, p53 protein was originally identified through its physical association with the transforming simian virus SV40 large T antigen and was subsequently believed to operate as an oncogene within the cell [2, 3]. Early studies supported this categorization, since overexpression of p53 cDNA led to cellular transformation [4] and its expression in a large number of tumor types was believed to lead to a poor clinical prognosis [5, 6]. This collection of early data suggested the p53 protein was involved in tumor pathogenesis and supported its classification as oncogene.

The initial misinterpretation of the role of the tp53 gene within physiologically normal and cancer cells continued until it was demonstrated that, in a large percentage of colorectal carcinomas, chromosomal aberrations existed within a region including the tp53 locus, 17p13.1 [7]. This discovery was consistent with the concept that p53 was involved in carcinogenic development, but led to reevaluation of the mechanisms through which p53 induced cellular transformation. Multiple research groups reported cDNA sequences encoding for p53 [8, 9] that led to cellular transformation by its overexpression. Insights into the true biological nature of p53 protein grew from a seminal report detailing the mutation requirement in the gene for p53 to elicit its transforming ability [10]. Subsequent studies described p53-mediated cell cycle arrest [11, 12]. As a result, it was recognized that p53 mutation or functional inactivation is an
important determinant for cellular transformation [13, 14], suggesting that most earlier published reports had been made using mutated p53 cDNA derived from tumor cells. Subsequently, the improper characterization of p53 function as oncogene was recognized and its subsequent characterization more accurately fit the emerging concept of a “tumor suppressor gene” [15]. The years that followed the discovery of p53 as a tumor suppressor gene revealed continued insights into its more fundamental roles in biology.

1.2 p53 gene and protein structure

p53 is typified as a 393 amino acid protein of 43.6 kDa theoretical molecular weight encoded by a 19.8 kb gene on human chromosome 17. The protein is named for its observed molecular weight following characteristically slow migration through polyacrylamide under reducing conditions at roughly 53 kDa. Cloning of p53 from varied sources has led to the identification of 368 unique cDNAs, including 10 substantially different RNAs which encode 8 or more putative isoforms of the p53 protein [16]. The vast majority of cDNA sequences derived from p53 transcripts vary from a single base pair through roughly 30 base pairs, often identified within tumor cells. Endogenous isoforms of the p53 protein have been identified [17, 18], but more research is needed to assign unique molecular functions to each isoform.

Three major functional domains comprise the 393 amino acids of the full-length p53 protein. The activation domain makes up residues 1 through 50, the DNA binding domain is from residues 102-292 and the tetramerization domain lies within residues 326-356 [19]. Within the tetramerization domain is a nuclear localization signal, providing the necessary cue for nuclear import. Furthermore, the amino- and carboxy- termini of p53 contain nuclear export
signals, functioning in opposition to nuclear localization signals to enable the rapid nucleocytoplasmic shuttling of p53 protein in response to intracellular signals as necessary [19].

The identification of mutations in the p53 gene leading to tumorigenesis have led to increased understanding of these functional domains and identification of critical amino acid residues responsible for their function. Missense mutations comprise the majority of p53 gene alterations in which amino acid substitutions result in a loss-of-function of its recognized tumor suppressor activity. So frequent is p53 mutation in various malignancies that it has been suggested by some researchers that the p53 primary sequence may be utilized as a “blueprint” to identify the carcinogenic agent responsible for tumorigenesis [1], provided sufficient predictive information is available for mutagenic toxicant exposures. p53 loss-of-function is generally associated with mutations within the DNA binding domain, the most commonly mutated region within this gene. The high mutation rate of the DNA-binding domain in spontaneous and chemically-induced tumorigenesis allude to important p53 functions within the normal cell as a transcription factor, whose transactivation and repression of target genes regulate cell cycle and cell death.

In addition to localization signals coded within its primary sequence, p53 serves as an integrator of diverse cellular signals related to stress, DNA damage and growth through posttranslational modifications by a variety of protein ‘sensors’. These modifications include phosphorylation, acetylation, ubiquitination, neddylation, sumoylation and methylation [20, 21]. Critically, phosphorylation of serine residue 15 within the amino terminus of the p53 protein is recognized as a key mediator of p53 activation [22-24], occurring prior to and prerequisite for additional modifications [25, 26]. Phosphorylation of serine/threonine residues within the p53 amino terminus by the ATM/ATR, Chk1/Chk2, DNA-PK, CK and p38 kinases [27] are believed
to disrupt the association of p53 protein with its major negative regulator, MDM2 [24] and prime p53 for DNA binding. Acetylation of the p53 carboxy-terminus occurs through the acetyltransferase activity of p300/CBP and PCAF [27]. These enzymes are recruited by DNA-bound p53 for the purpose of histone acetylation, resulting in an open chromatin conformation that enables gene transcription. Furthermore, some studies have demonstrated that acetylation of the p53 DNA-binding domain results in the selective activation of pro-apoptotic target genes but has little effect on the transcriptional regulation of cell cycle arrest genes [28, 29]. While the exact sequence of posttranslational events leading to p53 activation remains unclear, it is understood that modification of the p53 protein is necessary for full activity of p53 in response to cellular stress or injury.

1.3 Regulation of p53 RNA and protein

Homeostatic levels of p53 protein are generally maintained at low levels through the E3 ubiquitin ligase MDM2 (murine double minute 2) [30-32]. Ubiquitination of p53 by MDM2 is required for its recognition by the proteasomal protein degradation complex. Efficient ubiquitination of p53 ensures rapid turnover of cellular p53 protein in the unstressed cell. p53 associates with MDM2 at the activation domain of the carboxy terminus [31]. Disruption of the MDM2-p53 association by stress-induced posttranslational phosphorylation of critical p53 serine residues within the activation domain inhibits MDM2-mediated p53 ubiquitination. The result is a nuclear accumulation of the p53 protein [24]. The maintenance of low p53 levels within the unstressed cell is of critical importance, as demonstrated by the rescue of MDM2-null mouse lethality upon subsequent loss of p53 function [33]. The finely choreographed modulation of p53 by ubiquitination, phosphorylation and ultimately the p53-dependent transactivation of
MDM2 to reestablish baseline levels represents a remarkable molecular feedback pathway that so often occurs in transcription factor regulation.

Regulation of p53 activity is also achieved by the MDM2 family member MDM4 (alias MDMX), believed to also associate with p53 by binding to its transactivation domain in a manner similar to MDM2 [34, 35]. MDMX binding inhibits p53 transcriptional activity [36]. MDMX-null mutations are developmentally lethal, while loss of p53 is sufficient for rescue from the lethal phenotype [37]. p53 rescue suggests a non-overlapping role for p53 regulation by MDMX with MDM2. While MDMX is a negative regulator of p53 transcriptional activity [38], it also functions to regulate MDM2 levels [39] and vice versa [40]. Interestingly, the MDMX-p53 association appears to prevent MDM2-mediated ubiquitination of p53 [38] and may therefore serve to negatively regulate MDM2 activity while maintaining inactive p53. Therefore, in addition to the regulatory mechanisms controlling p53 activity, the regulation of MDM2 and MDMX in both p53-dependent and independent functions are similarly complex and continue to be actively investigated.

1.4 p53 activation

In order to alleviate E3 ubiquitin ligase-mediated targeting for proteasomal degradation, post-translational modifications of the p53 protein by various stress-activated kinases function to increase p53 levels and enable its transcriptional function as the regulator of target genes. Collectively, the pathways that function to enable p53 accumulation are considered p53-activating events. As a critical mediator of cell cycle arrest and apoptosis, p53 is activated by multiple signals including DNA damage and oncogenic stress to facilitate the cellular response between life and death [41].
DNA damage, in the form of single- or double-stranded breaks, efficiently activates p53 through ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3 related (ATR) kinase phosphorylation of the p53 activation domain [24, 42]. DNA damage-induced signaling also results in the phosphorylation of MDM2 by ATM [43], disrupting its ability to associate with p53 [44]. Ultimately, the extent of DNA damage and repair capacity dictates whether the cell undergoes cell cycle arrest or apoptosis in response to p53 activation [45].

Proto-oncogenes, such as p21Ras, function normally within the cell to regulate proliferation and growth [46]. Oncogenic stress, as would be derived from constitutive activation of the p21Ras oncogene through mutation [47] or through expression of the adenoviral E1A oncogene [48], lead to p53 activation through the induced expression of p19ARF [49, 50]. Expressed from an alternate reading frame of the Ink4a cyclin dependent kinase inhibitor gene [50, 51], p19ARF elevates p53 levels through the association with and inactivation of MDM2 [52].

In addition to DNA damage and oncogenic stress, p53 is reportedly activated by the ribosomal stress pathway [53, 54], hypoxia [55] and loss of cell-matrix contact [56]. Furthermore, p53 is also activated through receptor-mediated signaling pathways. Growth factor receptor activation typically results in proto-oncogene signaling and may be similar to the oncogenic stress pathway of p53 activation, while death receptor-mediated activation of p53 occurs through direct activation of programmed cell death pathways [57, 58]. Remarkably, the diverse array of known p53-activating stimuli are generally distilled into the functions of cell cycle arrest or apoptosis, however recent evidence suggests that the transcriptional program regulated by p53 leading to these outcomes may be dependent upon the activating stimulus [55, 59].
1.5 p53 DNA binding

Among the best characterized functions of p53 are its roles as transcription factor. Tetramerization of p53 is thought to be prerequisite for the efficient binding to gene regulatory regions, and occurs through the physical association of C-terminus tetramerization domains of individual p53 subunits [60]. The degenerate consensus half-site binding sequence for p53 is 5’-PuPuPuC(A/T)(T/A)GPyPyPy-3’, where a spacer of 0-13 base pairs between half-sites permits p53-DNA association with a large number of putative regulatory sequences [61]. Specific half-site sequences are believed to be important for the p53-dependent association with and bending of DNA [62]. Increased DNA bending up to 50° at each half-site favors p53-DNA stability and transactivation [63]. Although not completely understood, the spacer length between half sites appears to be important for p53 gene regulation, irrespective of half-site sequence [64]. Spacer length requirements may be related to the number of helical turns between half-sites. Interestingly, the p53 target gene siah-1b contains an atypically long spacer of 33 bp [65], while the binding site within the p53-repressed target gene survivin has a 3 bp spacer that when deleted generates a transactivating element [66]. These exceptional events suggest that the rules with respect to spacer sequence and length guiding p53 DNA binding continue to be refined with the discovery of new p53 regulatory sites. Therefore, both the specific sequence of the p53 response element and the number of bases separating half-sites are important factors in the recognition and effect of p53-DNA binding.

Binding of p53 to promoter regions of transcriptional target genes generally results in the transactivation or repression of gene expression, although binding may also occur in the absence of active gene regulation. Surprisingly, little distinction can be made between DNA binding sequences of activated and repressed gene targets, suggesting there may not be specific
consensus sequences for transactivation or transrepression. Alternative models suggest that either the specific recruitment of transcriptional cofactors or association with the transcriptional machinery may lend specificity to the transcriptional outcome of p53 DNA binding [67, 68]. Importantly, p53 does not recruit or maintain members of the transcriptional initiation machinery in the same manner across all target genes [69], although the mechanisms regulating these associations are not yet fully understood.

1.6 Nerve growth factor

Nerve growth factor (NGF) was identified for its involvement in the differentiation and maintenance of sympathetic and sensory neuronal populations [70-73]. Mature NGF is composed of 3 polypeptide subunits (α, β, γ) which associate to form the mature protein [74], although the β subunits are explicitly involved in the neurotrophic actions of NGF. NGF is a member of the neurotrophin family, along with brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). Each neurotrophin is associated with its cognate receptor tyrosine kinase (Trk) receptor, where NGF mediates its signaling specifically through TrkA, BDNF through TrkB, NT-3 through TrkC and NT-4/5 also through TrkB. All members of the neurotrophin family are capable of binding to and signaling through the low affinity p75<sub>NTR</sub> receptors. Neurotrophins are initially expressed and assembled as pro-neurotrophins from which mature neurotrophins are generated upon protease cleavage. As the Trk and p75 receptors induce different signaling paradigms upon ligand binding and activation, protease cleavage may therefore control the signaling specificity of neurotrophin signaling. The p75<sub>NTR</sub> receptor is preferentially bound by the pro-neurotrophi [75], while the Trk receptors are bound by their cognate mature neurotrophin ligand. NGF is considered a pro-survival neuronal
factor [76], however pro-NGF signaling through the \( p75^{\text{NTR}} \) receptor is known to induce apoptosis [75, 77].

NGF promotes \textit{in vivo} neonatal sympathetic nervous development and survival [70, 71, 73]. NGF also promotes catecholamine generation within both chromaffin cells of the adrenal medulla and sympathetic cervical ganglia by modifying levels of tyrosine hydroxylase [78-80]. Tyrosine hydroxylase activity represents the rate-limiting enzyme in dopamine synthesis from the amino acid tyrosine [81], and its expression is often regarded as a marker for dopaminergic neuronal cells. Isolation and propagation of the NGF-responsive \textit{rattus norvegicus} PC12 pheochromocytoma cell line [82] has enabled the biochemical study of NGF-regulated signaling within a neuronal cell type serving as an \textit{in vitro} experimental model of neurotrophin-dependent differentiation for dopaminergic neurons.

1.7 Nitric oxide

The ubiquitous nitric oxide (NO) radical is recognized as an atmospheric pollutant and is present as a biological product throughout taxonomic groups. NO is a bioactive radical originally described as endothelin-derived relaxing factor [83] generated through activity of the nitric oxide synthases, eNOS (endothelial NOS), iNOS (immune/inducible NOS) and nNOS (neuronal NOS). NOS enzymes each have different tissue distributions, however they all catalyze the production of NO from the conversion of L-arginine to L-citrulline [84, 85].

The NO radical is not persistent and once synthesized the biological effects of NO are mediated through its reactivity with cellular components. Its cellular half-life ranges from 3-30 seconds, typically below 5 seconds, depending upon availability and location relative to reactive sites [86]. As with other diatomic radical gasses, NO reacts with amino acid thiol groups and
enzyme heme or zinc centers [87, 88]. Furthermore, NO reacts efficiently with superoxide to produce peroxynitrite, a highly reactive cytotoxic compound utilized by the immune system in inflammatory defense [87]. Because NO reacts indiscriminately with cellular components, diffusion of NO is considered unlikely for intracellular signaling. NO production and activity is therefore believed to be regulated in part through the direct association of NOS enzymes with reactants [89].

NO produces a biological signal through direct reactivity with a wide variety of proteins in nearly all organ systems of the body. The cysteine sulfhydryl group is S-nitrosylated by NO in a variety of proteins, serving in some cases as a posttranslational modification enabling activity through allosteric protein modulation [86]. Cardiovascular NO functions in vessel dilation through the direct interaction with the heme group of soluble guanylate cyclase [86, 90], inducing production and biochemical signaling of the second messenger cGMP.

The toxicology associated with NO is complex, primarily due to the wide variety of its cellular reactants. NO reacts with the active sulfhydryl group of glutathione which results in protein inactivation [91], however a similar reaction within thioredoxin results in protein activation [92]. S-nitrosylation also inactivates aldehyde dehydrogenase [93], arylamine-N-acetyltransferase [94], cytochrome P450s [95] and other Phase I and Phase II detoxification proteins. NO is also reported to inhibit mitochondrial respiration by masquerading as molecular oxygen and binding to cytochrome c oxidase [96]. A similar reaction occurs between NO and the heme group in hemoglobin, however erythrocyte consumption of NO may be a mechanism through which vascular tone is regulated [97] and may therefore act as a reservoir of bioavailable NO [98]. In conclusion, the biological effect of NO reactivity cannot be generalized across
protein or reaction types, since even the $S$-nitrosylation of residues within proteins do not produce comparable outcomes.

1.8 Nitric oxide in neurodegeneration

Extrapyramidal nervous dysfunction due to dopaminergic neuronal loss characterizes the outward signs of Parkinson’s disease. While this disease afflicts nearly one million Americans and is closely correlated with age, little is known concerning its etiology or the risk factors contributing to disease formation. Three distinct factors have been considered responsible, in whole or in part, for disease progression: inflammation, proteasomal disorders and mitochondrial dysfunction.

The abnormal accumulation of proteins into intraneuronal Lewy bodies is a pathological hallmark of Parkinsonism. However, the mechanisms by which Lewy bodies accumulate are unknown and may be related to disease pathogenesis. Immunohistochemical studies have identified $\alpha$-synuclein as the predominant component of Lewy bodies in PD and other neurodegenerative diseases, termed synucleopathies [99]. In a family with autosomal dominant inheritance of PD, three copies of the $\alpha$-synuclein gene were present, suggesting an amplification process may underlie familial susceptibility to the disease and to Lewy body formation [100]. Amplification is rare, however, and is unlikely to contribute to idiopathic Lewy body formation found in majority of PD patients. In a drosophila model of PD, dopaminergic neurons were selectively killed in response to expression of human $\alpha$-synuclein, and intracellular inclusions were found [101]. Therefore, the selective accumulation of protein aggregates into Lewy bodies may not require synuclein amplification but instead may be related to an abnormal intracellular process or some type of neuropathogenic exposure. Ultimately, the consequence of Lewy body
aggregation is neuronal cell death leading to gradual loss of dopaminergic signal output within the substantia nigra and corpus striatal nerve tracts.

Several prospective epidemiological studies have determined a link between the regular administration of NSAIDs and dietary vitamin E with a reduced risk of developing Parkinson’s disease, suggesting an inflammatory or oxidative process is basic to disease progression [102, 103]. In addition, chronic overstimulation of microglia in vivo leads to neurodegeneration in rat models, a phenomenon that NSAID administration can partially prevent [104]. Evidence for inflammation in PD continues to be reported in the literature, and connections between inflammatory stress and PD-specific physical lesions are slowly being described.

Neurologic inflammation and glial activation creates reactive oxygen and nitrogen species, which may be harmful to surrounding neurons. An example is nitric oxide (NO), a cell-permeable inflammatory mediator released by microglia during inflammatory processes [105]. In an inflammatory response, microglia surrounding dopaminergic neurons in the substantia nigra may produce and release increased levels of NO into the surrounding intercellular milieu. While the source of such an inflammatory response has yet to be fully characterized [106, 107], there is increasing evidence that NO release potentiates the neurodegenerative response seen in rodent models of Parkinson’s disease [108, 109]. These findings support the belief that NO may play a similar role in human Parkinsonism. In humans, postmortem analysis of PD-afflicted brains by in situ hybridization shows a direct relationship between the number of microglia positive for activation-specific cell surface markers and relative degeneration of the substantia nigra [110]. Abnormal protein nitration has been demonstrated in the human Parkinsonian brain [111] as well as in other neurodegenerative diseases [112], implicating that NO release and reactivity may be a relevant factor in human disease. However, it remains unclear whether NO
production is a cause or an effect of disease processes. Exposure to excess NO may have significant and deleterious effects upon multiple cellular components and therefore may suggest a prominent role for NO toxicity in synucleopathies. In addition, the central nervous system is uniquely susceptible to oxidative and nitrosative stress because lower antioxidant enzyme abundance and high lipid content provide abundant targets for peroxidation reactions.

Mitochondrial dysfunction has also been suggested as a contributing factor to Lewy body aggregation [113]. The acquired form of PD can be characterized by interruption of mitochondrial Complex I involved in oxidative phosphorylation, resulting in decreased ATP levels and resultant creation of reactive oxygen species [114]. The inflammatory mediator NO can inhibit mitochondrial complex I, which in turn promotes production of reactive oxygen species [115]. In order to understand the pathophysiological effects of mitochondrial respiratory inhibition by NO, comparisons may be drawn to studies involving other compounds. Interestingly, interruption of Complex I by the pesticide rotenone and the 1-methyl-4-phenylpyridinium iodide ion (MPP+) \textit{in vivo} leads to the deposition and accumulation of Lewy bodies in neurons and development of alpha-synuclein cytoplasmic granules in glial cells [114]. This pathogenesis markedly resembles the inherited forms of Parkinson’s disease, suggesting a common denominator between the two [116]. Inhibition of rat mitochondrial complex I respiration by rotenone has been shown to produce marked amounts of superoxide [117], which may contribute or induce α-synuclein aggregation into fibrils \textit{in vitro} [118]. Furthermore, disruption of the mitochondrial electron transport chain by specific inhibitors causes formation of detergent resistant aggregates in \textit{in vitro} experimental models [119]. These aggregates are similar in protein content and physical characteristics to Lewy bodies, and they can be removed by restoring normal mitochondrial metabolism [119]. Also, mitochondria may be involved in
PD pathogenesis through their contribution to apoptosis by permeability transition pore opening. The anti-Parkinsonian drug rasagiline elicits a protective effect on neurons partly by decreasing Bax protein levels and increasing the anti-apoptotic proteins Bcl-2 and Bcl-XL [120]. These results suggest that effects attributed to mitochondrial dysfunction in PD may be due to oxidative stress within the neuron, due to Complex I inhibition. Also, inhibition of Complex I with the pesticide rotenone causes both oxidation and reduced activity of the 20S proteasome.

Interuption of the ubiquitin-proteasomal pathway contributes significantly to Lewy body formation. The E3 ubiquitin ligase protein, parkin, functions normally in proteasomal activity by marking proteins for destruction through attachment of ubiquitin residues [114]. Polymorphisms found in the parkin gene in hereditary PD implicate alterations in the ubiquitin-proteasome pathway in Lewy body formation and pathogenesis, although parkin was not found in Lewy bodies of these patients [116]. Onset of sporadic PD is also likely to involve reduction in parkin activity, possibly through sequestration in Lewy body deposits and concomitant loss of activity [121]. It has been demonstrated that proteasomal inhibition in cells overexpressing parkin leads to increased protein inclusions in vitro [116]. In addition, parkin has been shown to colocalize with α-synuclein in both neuroblastoma cell culture and human brain homogenates of PD patients [110]. These results suggest that abnormal ubiquitination processes may contribute to Lewy body formation. S-nitrosylation of proteins by NO may further prevent their recognition and degradation by the proteasome complex [122].

Disruption of the 20/26S proteasome may also be involved in Lewy body formation. Individuals with late-onset sporadic PD have been found to have alterations in the α-subunit of the 20/26S proteasome, resulting in altered proteasomal enzymatic properties [123]. Accumulated α-synuclein can inhibit the 20/26S proteasome, creating a potential source for
amplification of Lewy body formation [124]. Thus, the ubiquitin-proteasomal pathway may be disrupted both through aberrant ubiquitination and inhibition of protein degradation. Alone, however, disruptions in this pathway cannot entirely explain the pathogenesis of PD. A postmortem analysis of sporadic Parkinsonian brains (divided by area of the brain) revealed that proteasomal activity was not decreased relative to age-matched controls [125]. Although these data may be intriguing, the assays performed in determining proteolytic activity used entire brain regions. Such an experimental approach may not accurately determine proteasomal enzymatic differences in the catecholaminergic substantia nigra from other regions in the brain, as would be needed to accurately describe changes seen in the Parkinsonian brain.

Still, the relative susceptibility of dopaminergic neurons in the substantia nigra when compared to surrounding brain areas remains an anomaly. While Parkinsonian brains show that defects in areas beyond the substantia nigra do occur somewhat randomly, the loss of nigral neurons is most dramatic and occurs in every case of Parkinson’s disease. Neurons in the substantia nigra may possibly be exploited in PD pathogenesis because of their differences compared to other neurons. The neurotransmitter dopamine may become oxidized within storage vesicles by nitric oxide, leading to impairment of mitochondrial respiration [126]. Also, the nearest cellular neighbors of nigral dopaminergic cells may predispose them in PD. Activation of the microglia surrounding the substantia nigra and subsequent NO release may target those cells, particularly because NO has an extremely short half-life [105, 127]. Although both of these scenarios may have some bearing in neuronal outcome in PD, continued study is necessary to accurately describe this phenomenon.
1.9 Specific Aims

The studies described herein were carried out using the in vitro PC12 cellular model, a NGF-responsive cell line derived from a *rattus norvegicus* pheochromocytoma [82]. Upon treatment with NGF, PC12 cells differentiate into neuron-like cells positive for the dopaminergic marker tyrosine hydroxylase [79, 128], representing a tractable model through which both neuronal differentiation and dopaminergic cellular toxicity could be approximated.

The following Chapters broadly aimed to determine the role of the tumor suppressor p53 protein within normal and cytotoxic events in the differentiating neuronal cell. In particular, the specific aim for each Chapter was to determine i) the transcriptional role of p53 within neuronal differentiation, ii) factors through which p53 may regulate the physiological changes seen in neuronal differentiation, and iii) the role for p53 within NO-induced cytotoxicity of the neuronal cell. These specific aims were carried out through direct experimentation based on the following main hypotheses, i) NGF-stabilized p53 protein contributes to neuronal differentiation through the transcriptional regulation of target genes, ii) the p53 target gene *wnt7b* regulates NGF-induced, p53-dependent neurite outgrowth in differentiating neuronal cells, and iii) NGF regulates apoptotic p53 activity in differentiated neuronal cells upon NO cytotoxic exposures. Collectively, these studies have determined a previously uncharacterized biological role for p53 protein within the differentiating neuronal cell.


2. NGF-MEDIATED TRANSCRIPTIONAL TARGETS OF P53 IN PC12 NEURONAL DIFFERENTIATION. Brynczka, C, Labhart, P and Merrick BA.

2.1 Abstract

p53 is recognized as a critical regulator of the cell cycle and apoptosis. Mounting evidence also suggests a role for p53 in the differentiation of several cell types including neuronal precursors. Using the PC12 model derived from adrenal chromaffin cells of ectodermal lineage, we studied the transcriptional role of p53 during nerve growth factor-induced differentiation into neuron-like cells. We hypothesized that p53 contributed to PC12 differentiation through the regulation of gene targets distinct from its known transcriptional targets during apoptosis. This study demonstrated that p53 protein was activated by NGF and contributed to NGF-mediated neurite outgrowth during differentiation of PC12 cells. Using a genome-wide chromatin immunoprecipitation cloning technique, we identified and validated 14 novel p53-regulated genes following NGF treatment. Furthermore, we describe stimulus-specific regulation of a subset of these target genes by p53. The most salient differentiation-relevant target genes included wnt7b involved in dendritic extension and the tfcp2l4/grhl3 grainyhead homolog implicated in ectodermal development. Additional targets included brk, sdk2, sesn3, txnl2, dusp5, pon3, lect1, pkcbpb15 and other genes. We conclude that receptor-mediated p53 transcriptional activity is involved in PC12 differentiation and may suggest a contributory role for p53 in neuronal development.
2.2 Introduction

The tumor suppressor p53 is recognized as a critical regulator of cell cycle progression and apoptosis, incorporating signals from DNA damage and other cellular stressors to decide cell fate [1]. Disruption of p53 activity through direct mutation or regulatory dysfunction is a demonstrated causal factor in a large proportion of human malignancies [2]. As a transcription factor, many of the functional cellular roles of the p53 protein are elicited through direct DNA binding to sequence-specific cis-regulatory elements [3] leading to the transactivation or transrepression of target genes. Many of these target genes have been described through both computational and biochemical methods [4-6].

Evidence suggests a functional role for p53 in differentiation and development, particularly within the nervous system. While initial p53-null mouse models were described as phenotypically normal at birth with markedly increased tumor development later in life [7, 8], later studies demonstrated a proportion of p53−/− mice had defects in neural tube closure resulting in exencephaly [9, 10]. In vivo measurements in p53-responsive lacZ transgenic mice demonstrated elevated p53 transcriptional activity in the embryonic and early gestational nervous system [11], while high levels of p53 expression have been described throughout the chick, rat, mouse and Xenopus embryo [12-16] through midgestation. p53 functional activity has been further implicated in the differentiation of neuronal precursors [9, 10, 15] and is transcriptionally active within the nucleus of differentiating primary hippocampal neurons and oligodendrocytes [17], while p53 is inactive and localized to the cytoplasm of postmitotic differentiated sympathetic neurons [18]. p53 transcriptional activity may be further involved in the differentiation of other non-neuronal cell types. Transcriptional activity of p53 plays a direct role in embryonic stem cell differentiation by suppression of the Nanog gene [19], and can
directly induce *Xenopus* homeobox gene expression [20]. Involvement of p53 has also been shown in spermatogenesis [21], eye development [22], renal development [23], osteogenesis [24], immune development [25], lung development [26] and muscle differentiation [27]. Despite data supporting the transcriptional activity of p53 protein in differentiation, few genetic targets have been described and fewer still within a neuronal context.

Discovery of non-genotoxic mechanisms through which p53 protein may be activated within tumor cells harboring wild-type p53 remains an important therapeutic objective, both in effort to alleviate traditional off-target chemotherapeutic side-effects and to prevent the unpredictable mutagenicity characteristic of these therapies. Wild-type p53 can be activated in several malignancies by treatment with nerve growth factor [28] or pharmacologic agents [29-32], occurring through mechanisms sparing cells from the concomitant genotoxicity following chemotherapy. Recent evidence demonstrates that while p53 transcriptional regulation of target genes may be stimulus-dependent (e.g., DNA damage), promoter occupancy is generalized and may occur independently of the activating genotoxic stimulus [33]. Whether this paradigm is also applicable to nongenotoxic p53 stimuli compared to DNA damage-induced p53 activation is unclear. NGF-mediated p53 activation mainly results in decreased proliferative ability of tumor cells and not apoptosis [28], raising the question of whether NGF-regulated p53 activity is distinguishable from other means of p53 activation like DNA damage. While the mechanism of NGF-induced p53 activation is uncertain, inquiry into the transcriptional program following such activation is warranted to discern possible receptor-dependent p53 transactivational selectivity compared to genotoxic agents.

In the current study, the p53 transcriptional program was studied during nerve growth factor-induced differentiation of the ectoderm-derived rat PC12 pheochromocytoma cells. A
global chromatin immunoprecipitation-based screen was used to identify genetic elements bound to and regulated by p53. Treatment of PC12 cells with NGF induces differentiation into neuron-like cells marked by cell cycle arrest and neurite extension [34] along with marked changes in gene expression [35] and signaling pathway activity [36]. NGF-responsive p53 activity [37] has a functional contribution towards NGF-mediated PC12 differentiation [38-40], but a role in gene-specific transactivation has not yet been established. We hypothesized that marked accumulation of p53 protein within differentiating PC12 cells would be accompanied by concomitant transcriptional regulation of genes involved in differentiation and cell cycle arrest. Furthermore, we hypothesized that genomic elements regulated by p53 during neuronal differentiation may be unique from those regulated during genotoxic stress and apoptosis. We now report that p53 protein is transcriptionally activated in NGF-mediated neuronal differentiation and describe its binding to a number of novel and previously unreported genomic regions as well as the transcriptional outcome of this binding. We identify and validate genomic targets of p53 activity within the differentiating neuron-like cell, and demonstrate selectivity between NGF-activated versus genotoxicant-activated p53 transcriptional activity in a select number of p53 target genes.

2.3 Results

NGF induces activation of p53 protein within PC12 cells - PC12 morphologic changes and neurite extension consistent with differentiation were visible within 12 hours of NGF addition to the media and became marked over the course of 7 days (Fig 2.1A). NGF-dependent differentiation after 7 days treatment in PC12 cells was evidenced by expression of the dopamine transporter, a marker for dopamine generation [41] and transport, and decreased nestin
immunoreactivity, indicating loss of undifferentiated phenotype [42], (Fig 2.1B). In agreement with previous reports [43], cell cycle analysis by flow cytometry using FITC-BrdU labeling of total and newly synthesized DNA, respectively, revealed the population of cells within S phase was decreased to ~1% of the total cell population within 96 hours of NGF treatment (Supplementary Fig 2.1A). Reduction of S phase cells was accompanied by a concomitant increase in the number of cells within G1 phase after 7 days of NGF exposure (Supplementary Fig 2.1B).

p53 protein levels were significantly elevated in response to NGF treatment in PC12 cells as previously described by others [43, 44]. Accumulated of p53 protein became detectable at 8 hours following NGF exposure and remained highly elevated over the course of 7 days (Fig 2.1C). Phosphorylation of p53 protein at serine 15, which is typically needed for transactivation, began at 8 hours after NGF treatment and remained highly phosphorylated throughout 7 days (Fig 2.1C). Accumulation of p53 protein can be attributed to protein stabilization since levels of p53 mRNA remained unchanged over a 7-day course of NGF treatment (Fig 2.1D). Virtually all p53 protein was localized within the nucleus in 7 day NGF-differentiated cells, as determined by immunoblot of nuclear and cytoplasmic fractions (data not shown). Accumulated p53 protein was transcriptionally active, as demonstrated by luciferase expression using the p53TA-luc reporter vector in differentiated compared to naïve cells (Fig 2.1E).

The role of p53 activity in differentiation of NGF treated PC12 cells was investigated in anti-p53 shRNA-expressing PC12 cells (Fig 2.2A). Three stable PC12 lines expressing different anti-p53 shRNAs were created to observe p53-dependent function in PC12 differentiation. p53 RNA silencing resulted in reduced p53 protein levels, with p53#3low having the lowest levels, followed by p53#2mid and p53#4mid. While no apparent morphological differences were noted
in the shRNA expressing cell lines maintained in the naïve state, NGF differentiation of these cells demonstrated reduced early neurite extension compared to their WT counterparts. Within 3 days of NGF exposure, p53\#3low cells had markedly reduced neurite outgrowth compared to wild-type cells (Fig 2.2B), lasting up to 7 days (data not shown). These results agree with prior work showing disruption of morphological differentiation in both dominant-negative and temperature-sensitive p53 transfection into PC12 cells [17, 38, 45].

**Chromatin immunoprecipitation and cloning of ChIP-generated tags** - Seven-day differentiated PC12 cells were studied for p53 DNA binding via chromatin immunoprecipitation (ChIP) analysis. Preliminary assay validation demonstrated p53 binding to a known, well-defined binding site within the \( p21^{Waf1/Cip1} \) promoter at a 2-fold greater level in differentiated versus naïve cells (Supplementary Fig 2.2). Having validated p53 occupancy to a known gene promoter, ChIP-enriched DNA from differentiated PC12 cells was amplified, concatemerized and cloned. Cloned concatemers were sequenced and separated, resulting in 7,184 individual DNA tags representative of putative p53-occupied locations within the PC12 genome (Supplementary Table 2.1). Tags were aligned to the *Rattus norvegicus* genome and locations with >1 tag mapping within a 2 kb area (“clusters”) were identified. Sequence data was obtained for 500 bp upstream and downstream of each tag, single or clustered, and tags were recompiled to represent the original p53-bound ChIP-enriched fragments. Since the number of sequenced tags was non-saturating compared to the total population of p53-occupied fragments, both clusters and single tags were potentially informative. Tags representing putative p53-occupied genomic fragments were found to span the entire *Rattus norvegicus* genome in addition to known p53 binding...
regions within the *MDM2*, *cyclin G1* and other known target promoters, validating this experimental approach for the study of other novel putative targets.

Recompiled tag sequences were analyzed for predicted p53 binding sites using the p53MH algorithm [46] and sorted according to both binding site score and location relative to nearby genetic elements. Stringency for p53 binding site probability score was set at a cutoff of 90 (out of 100) as determined by the p53MH algorithm using likelihood ratio scoring and a 14 base gap size limit between half sites. Results using these criteria demonstrated 364 p53 binding sites within tag sequences located in upstream 5’, downstream 3’ and intergenic gene regions. Of those tags with a p53MH binding site score $\geq$ 90 (out of 100), 56% mapped to within a gene, 25% to the upstream 5’ region and 19% to the downstream 3’ region (Fig 2.3B). Observed p53-occupied sites in the PC12 genome were visualized by plotting normalized tag alignment locations relative to a hypothetical gene size of 100kb (Fig 2.3C). Scored p53 binding sites clustered to both the 5’ and 3’ gene regions, while intergenic binding sites were observed throughout the entire length of the gene.

*Validation of p53 occupancy and transcriptional regulation* - We selected a total of 100 clusters and single tags combined for ChIP-based validation of p53 occupancy based on either the presence of a putative p53 binding site as determined by the p53MH algorithm, or tag location relative to a genomic element of interest. We recognized that selection based on high p53 binding scores alone may pose a constraint on putative regulated genes because p53-mediated transrepression can occur in both the absence of a consensus binding site [47, 48] or by binding to sites sufficiently distinct from the consensus sequence as to fall below our accepted p53MH binding site prediction level [49]. A preponderance of genes whose known functions may be
relevant in neuronal differentiation also factored into our selection of tags and clusters for validation. ChIP analysis demonstrated significantly elevated (p≤0.05) p53 occupancy in an NGF-dependent manner for roughly 30% of the 100 studied validation targets. Those targets to which p53 binding was significantly increased in NGF-treated cells are shown in Figures 2.4, 2.5 and 2.6. As expected, reproducible binding was demonstrated to promoters of known p53-inducible target genes including p21^{Waf1/Cip1}, MDM2 and cyclin G1 (Fig 2.4, 2.5, 2.6). In addition to NGF-responsive genomic p53 binding sites, roughly 40% of targets showed significant p53-binding independent of NGF treatment (Supplemental Table 2.3). These targets were significantly enriched in ChIP validation when compared to negative control regions, although no NGF-responsive changes were noted in p53 binding. Absence of NGF-dependent 53 binding to these regions precluded further analysis as they were beyond the objectives of this study. While these locations were ChIP-enriched, cloned and demonstrated to be enriched once more in a validation ChIP experiment, further study will be necessary to demonstrate p53-dependent transcription.

In order to determine the transcriptional outcome of p53 binding to NGF-responsive target regions described above, RT-PCR was performed to compare mRNA levels for indicated targets among naïve and differentiated PC12 cells (Fig 2.4, 2.5, 2.6). p53-dependence on target gene expression was verified using three stable anti-p53 shRNA-expressing PC12 lines with varying p53 knockdown efficiencies. We discovered ten novel, p53-inducible targets regulated in an NGF-responsive manner (Fig 2.4), including the sesn3 and nme1 targets recently described to be p53 regulated either by association or indirect experimental evidence [50, 51]. In addition, the dusp5 gene was an NGF-inducible p53 target, recapitulating earlier reports of this gene being p53-regulated [52]. Including known targets, a total of 14 genes were p53-induced in
differentiating PC12 cells. Four novel gene targets demonstrated a p53-dependent repression (Fig 2.5), including the pkcbpb15/trib3 locus, where transcripts of both genes were elevated in p53-silenced cells. We further demonstrate eleven p53 occupied regions where transcriptional changes in nearby genes during NGF-mediated differentiation could not be accredited solely to p53 activity in the study (Fig 2.6), including the previously described p53 targets snk [53] and nck2 [5]. Additionally, new p53-occupied binding sites were identified for the MDM4 and dyrk3 genes, for which different p53 binding locations have previously been described [5].

The majority of p53MH-predicted p53 binding sites within regions of validated occupancy contained either no spacer or a short spacer, in agreement with earlier data [5]. However, there are several notable exceptions to this trend including wnt7b, grhl3, shmt1 and sesn3 binding sites, which contain atypical spacers from 5-13 bp separating the individual half-sites, similar to the described p53-binding site within the siah promoter [54]. Consensus binding sites derived from our data are shown in Figure 2.7 using the WebLogo [55] sequence analysis tool. Notably, we report little deviation from the p53 consensus sequence for transactivation compared to repression except for the substitution of purine for pyrimidine at site 11 (Fig 2.7b).

*Stimulus-dependent transcriptional regulation* - Experiments were conducted to determine if p53-regulated genes in differentiation were unique from those following genotoxicity. Expression levels of NGF-dependent p53 target genes measured after DNA-damage by bleomycin and 5-fluorouracil were compared to NGF treatment to discriminate stimulus-dependency of p53-mediated transcription. As expected, expression levels for p21Waf1/Cip1 and cyclin G1 were elevated following all treatments, confirming the transcriptional activity of p53 in all groups (Fig 2.8A). Increased expression levels in three of the NGF-responsive targets, wnt7b,
*pkebp15* and *lect1* (Fig 2.8B) were not recapitulated in 5-FU and bleomycin treated cells. Results demonstrate an opposite transcriptional regulation for these p53-regulated targets depending upon the treatment stimulus. Although the absolute levels varied, expression levels and direction of the remaining p53-regulated target genes were similar regardless of the treatment (Fig 2.8C). Expression levels of all gene targets were similar to those found earlier using RT-PCR, with absolute differences attributed mainly to differential assay sensitivities. These data demonstrate that the gene expression at chosen time points for some, but not all, p53 target genes is NGF-dependent. The remaining target genes studied, however, were induced or repressed both by NGF receptor-mediated or genotoxic p53-inducing stimuli.

2.4 Discussion

The p53 tumor suppressor is widely appreciated as a central node in the regulation of proliferation and apoptosis in response to various genotoxic insults. Its role within cellular differentiation, however, is uncertain. p53 has been considered non-essential to development since p53 null mice are viable and succumb to tumors rather than developmental malformations [7]. The discovery of low frequency neural tube defects in p53-deficient mice [10] is an apparent paradox, suggesting the role of p53 in development may be more complex than initially believed. Interpretation of developmental effects in p53-deficient models at the organismal level are complicated by the overlapping transcriptional functions of the p53 family members p63 and p73 [56] which include such vital targets as *p21^{Waf1/Cip1}* , Bax and MDM2 and likely other unknown target genes. In addition, regulation of growth control and apoptosis can also be carried out through p53-independent cellular mechanisms which could circumvent developmental defects in a majority of p53-deficient mice.
The marked accumulation of p53 protein and serine 15 phosphorylation levels suggesting its activation during NGF exposure led us to investigate a possible transactivational role for p53 in neuronal differentiation. Using the PC12 cell model of NGF-induced neuronal differentiation, we hypothesized that p53 is involved in differentiation through specific transcriptional regulation of target genes. The contribution of p53 to PC12 cell differentiation is suggested by the reduction of neurite outgrowth observed during p53 knockdown studies described here, which is consistent with previous work showing similar findings using a temperature-sensitive p53 mutant [38, 39]. We demonstrate p53-dependence for target gene transcription using three PC12 cell lines with varying levels of shRNA-mediated p53 silencing, further enabling inference into the transcriptional sensitivity of each target gene to reduced levels of p53 protein.

Here we describe a number of novel p53-occupied genomic sites within the NGF-differentiated *Rattus norvegicus* neuronal PC12 cell line, and further identify corresponding genes regulated by p53 binding to these regions. The transcriptional targets described here represent enticing mechanisms through which PC12 cellular differentiation may be controlled by activated p53. Particularly interesting is the regulation of *wnt7b* and the *tfcp2l4* grainyhead homolog gene. Transcriptional regulation of Wnt pathway genes by p53 has been described for several factors, including the negative regulator Dickkopf-1 [57], the transcription factor Tcf-4 [58] and beta-catenin [59]. Until now, direct DNA binding and transcriptional regulation of Wnt genes themselves has not been previously reported for p53. Wnt7b functional activity is necessary for dendritic development and neuronal connectivity in hippocampal neurons [60], consistent with the generalized lack of neurite extension observed in NGF-treated p53 knockdown PC12 cells in this study. The transcriptional regulation of *wnt7b* by p53 may be extended to other cell types. For example, macrophage-derived *wnt7b* has been reported as an
apoptotic initiator in vascular endothelial cells of the developing eye [61], raising the possibility of p53 apoptotic control in immune cells through localized secreted factors.

The transcription factor cp2-like 4 gene, a Rattus norvegicus grainyhead family homolog, is homologous to the grhl3 mouse and human family members. In Drosophila, the grainyhead gene product participates in wnt-frizzled signaling in the establishment of wing planar polarity [62] and in cuticle barrier formation and function [63]. The latter function is conserved in mammals, where grainyhead homologs are involved in epidermal stratification and wound repair [64]. The importance of the grhl3 transcription factor during development is underscored in the grhl3-null mouse, in which neural tube defects are prevalent [65, 66]. Absence of the p53-tfcp2l4 network in p53-null mice may represent a potential mechanism through which a proportion of these mice are born with neural tube defects. The role of grhl3 in ectodermal differentiation and specification into epidermal tissue and also within epidermal wound healing is strikingly similar to the demonstrated involvement of the p53 family member p63 in these events [67]. Since p63 has an overlapping transcriptional program with p53 [56], investigation into joint regulation of tfcp2l4 is warranted as a possible common juncture of in vivo redundant transcriptional control in development.

Other transcriptional targets of p53 found in our study may play roles within the differentiating PC12 cell or developing organism. The nme1/nm23 gene is a positive regulator of neuronal differentiation through regulation of Rb2/p130 expression [68]. The Kctd (potassium channel tetramerization domain containing) family, of which FLJ12242 is a member, is involved in the left-right symmetry of developing zebrafish brain [69]. We found FLJ12242 to be the most highly expressed p53-regulated gene in NGF-mediated differentiation. Txnl2 is involved in P19 cardiac cell differentiation [70], while BRK promotes enterocyte differentiation [71]. The
Sdk2 neuronal adhesion molecule is involved in synaptic connectivity [72]. Dusp5 is a negative regulator of ERK signaling [73], thus regulating MAPK signal propagation. The Lect1/Chm1 gene, an anti-angiogenic factor in differentiating cartilage [74] and a bone remodeling factor [75], may be involved in bone development. Furthermore, the additional unannotated p53-regulated targets described here such as FLJ32743, LOC366671 and LOC362557 may be important in yet unknown developmental, differentiation or signaling processes.

The large number of p53-occupied genomic regions identified by ChIP for which mRNA expression changes do not coincide with PC12 differentiation provide additional examples of putative stimulus-specific p53 transcriptional activity. Since several of these targets have been described as p53-regulated under different experimental conditions, including Snk [53], MDMX, Nck2 and Dyrk3 [5], expression of these genes may not be dependent upon p53 promoter binding alone but also on the availability of other transcriptional cofactors. As the above targets were previously identified as p53-regulated, by extension, the genes shmt1, rnf10, hmmr, trim34, znf609 and ovary-specific MOB-like protein are also likely to be regulated under other p53-activating conditions. Along with the NGF-dependent transcription of the wnt7b, pkcbpb15 and lect1 gene targets described here, the p53-occupied targets shmt1, rnf10, hmmr, trim34, znf609 and ovary-specific MOB-like protein may provide additional examples of stimulus-dependent p53 transcriptional targets.

NGF receptor-mediated induction of p53 activity through TrkA [38, 43] may represent a useful therapeutic avenue for development of nongenotoxic chemotherapeutics [28]. While activation of the p53 protein in response to NGF treatment within PC12 cells has recently been described [43], it is less clear what particular signaling proteins are responsible for the marked increase in p53 protein stability and transactivational selectivity. Changes in p53 activity are
related, directly or indirectly, to TrkA activated signaling pathways, such as Ras/MAPK [76]. It is believed that activation of the low affinity NGF receptor p75\textsuperscript{NTR} by NGF results in apoptosis in Schwann cells [77] through the activity of p53 [78]. TrkA high affinity and p75\textsuperscript{NTR} low affinity NGF receptors may represent a useful model through which the divergent ‘choices’ between cell cycle arrest/differentiation and apoptosis by p53 protein function can be studied.

The potential utility in NGF receptor signaling for understanding p53 activation is underscored by data presented here, where NGF-induced PC12 cell differentiation results in little or no detectable apoptosis, despite markedly increased p53 levels and transcriptional activity over the extended time course of several days. It is thus likely that both the inducing agent (i.e. stimulus) and cell type lend specificity to the p53-controlled transcriptional response observed for other p53-responsive conditions [79]. In addition, the continued transcription of MDM2 and cyclin G1 described here is in opposition with the observation that p53 protein levels are consistently elevated following NGF treatment for the duration of at least 7 days. MDM2 functions as the major negative regulator of p53 protein within the cell [80], while cyclin G1 exerts its negative regulatory role through MDM2 dephosphorylation and subsequent stabilization [81]. However, continued stabilization of p53 protein during NGF-induced differentiation occurs despite the increased MDM2 and cyclin G1 RNA levels shown here and maintained MDM2 protein levels (data not shown). This suggests the presence of an intermediate event either inhibiting MDM2 function, or protecting p53 protein from MDM2 function, in differentiating PC12 cells. The incongruent time frames of activation for NGF-induced p53 compared to the relatively rapid stabilization via genotoxic agents further suggests the presence of a mechanism distinct from DNA damage through which p53 stabilization and activation may occur during NGF-mediated signaling.
The approach used here in the identification of p53-regulated elements in neuronal differentiation represents a useful strategy amenable to the analyses of DNA binding by other transcription factors. The recognized roles of NGF-induced p53 transcriptional targets identified in this report raise new implications for a role of p53 in differentiation and development and also provide meaningful evidence for transactivational selectivity by activated p53 protein.

2.5 Materials and Methods

*Cell culture and treatments* - PC12 cells (ATCC, Manassas, VA) were propagated in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10% horse serum (Invitrogen), 5% fetal bovine serum (Invitrogen) and appropriate antibiotics in a humidified incubator maintained at 37° with 5% CO₂. PC12 cells (passage 10-25) were differentiated on rat tail collagen-coated culture vessels (Sigma, St. Louis, MO) in RPMI 1640 medium containing 1% horse serum, 2 mM L-glutamine and antibiotics with 50 ng/mL mouse nerve growth factor, (NGF 2.5S) (Chemicon, Temecula, CA) for indicated time intervals. Differentiation medium was replaced and fresh NGF 2.5S was added every third day to differentiating cells. PC12 cells were incubated with genotoxic agents at the following concentrations, at the indicated time points: bleomycin, 10 µg/mL (Sigma) and 5-fluorouracil, 375 µM (Sigma). Reporter assay was performed by transient transfection of p53TA-luc vector (BD Pharmingen, San Diego, CA) into naïve PC12 cells plated onto collagen-coated dishes using Lipofectamine 2000 (Invitrogen). Cells were treated with NGF the following day and luciferase activity was quantified using the Luciferase Assay System (Promega, Madison, WI) at the indicated time intervals on a Tropix (Bedford, MA) TR717 luminometer. Statistical analysis of
reporter assay data was performed using one-way ANOVA followed by Tukey’s HSD post-hoc test for significance.

*Generation of stable shRNA expressing cells* - Stable anti-p53 shRNA expressing PC12 cells were generated via lentiviral-mediated infection, integration and stable selection (BlockIt Lentiviral Expression System, Invitrogen). Hairpin sequences were designed and synthesized (IDT, Coralville, IA) with appropriate overhangs for cloning into the pENTR/U6 entry vector. The informative p53-targeting sense hairpin sequences are: p53-2mid - 5’-GCATACGGATTTCCTTCCACC-3’, p53-3low - 5’-ATATCCGACTGTGAATCCTCC-3’, p53-4mid - 5’-AAAATTAGGTGACCCTGTCGC-3’. shRNAs were designed using the 4-base loop sequence CGAA. Hairpin sequences were cloned into the pENTR/U6 vector and selected clones were sequence-verified. The U6 promoter-shRNA cassettes were transferred into the pLenti6-BLOCK-iT-DEST vector. The resulting destination clones were used to generate lentiviral particles by transfection of HEK293FT cells along with the pLP1, pLP2 and pLP/VSVG plasmids in equal amounts using FuGene (Roche Diagnostics, Indianapolis, IN) lipid reagent. Transfection efficiencies were monitored by concomitant tranfection of the pEYFP-C1 vector (BD Clontech, Mountain View, CA), demonstrating efficiency ≥ 90% in all cases. Lentiviral-laden media was collected 48 hours following transfection, clarified by centrifugation and used with 4 µg/mL polybrene (Sigma) in transduction of naïve PC12 cells plated on collagen. Selection using 6 µg/mL blasticidin was initiated and maintained for 7 days until stable lines were enriched. Cells were maintained for 14 days before differentiation and experimentation. Experiments using stable shRNA-expressing PC12 cells were compared to wild-type PC12 cells of the same original population and passage number in all cases.
Western blotting - Sample preparation for SDS-PAGE was performed as previously described [82]. Antibodies used were against nestin (Rat401, Pharmingen), dopamine transporter (D2442, Sigma), p53 (pAb122, Pharmingen), phospho-serine 15 p53 (No. 9284, Cell Signaling, Davers, MA) and actin (MAb1501, Chemicon). Proteins were visualized using either ECL reagent (GEH Amersham, Piscataway, NJ) or SuperSignal reagent (Pierce, Rockford, IL).

Chromatin immunoprecipitation assays - ChIP assays were performed similarly to previous reports [83, 84]. Briefly, NGF-differentiated and naïve (dividing) PC12 cells were crosslinked by 1% formaldehyde with rocking for 15 minutes before terminating the crosslinking reaction with 125 mM glycine. Cells were washed, lysed by Dounce homogenization and resuspended in a buffer containing sodium deoxycholate, SDS and Triton X-100 [83]. Chromatin was sheared to an average length of 500 bp by sonication on ice and samples were clarified by centrifugation at 10,000g. Sheared, crosslinked chromatin complexes were precleared with protein A agarose. p53-DNA complexes were immunoprecipitated using anti-full length p53 polyclonal antibody (FL-393, Santa Cruz Biotech, Santa Cruz, CA) or with control rabbit IgG (I-5006, Sigma). Immune complexes were captured with protein A agarose and beads were washed as described [83, 84]. Protein/DNA complexes were eluted and crosslinks reversed by incubating at 65 °C for at least 6 hours. To assay for p53 binding sites in purified ChIP DNA, target specific primers (Supplementary data, Table 2) were used to measure amounts of target sequence in immunoprecipitated samples by qPCR using SYBR Green-based detection (BioRad, Richmond, CA). Experimental qPCR values were normalized against values obtained for 25 ng of input DNA using the same primer set.
Cloning and analysis of enriched ChIP DNA - Cloning of enriched ChIP DNA was performed by using Genpathway’s (San Diego, CA) FactorPath Discovery approach, as previously described [84]. Briefly, enriched ChIP DNA was amplified via ligation-mediated PCR [85] and purified products were subjected to random priming/extension and gel-purification [86] to generate a 40-50 bp tag library. Tags were concatemerized, cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced. Individual tag sequences were aligned to NCBI Rat Genome Build v3.4 using MEGABLAST v2.2.11. Tags producing low-scoring or multiple alignments were eliminated from consideration. Clusters were generated by grouping of tags mapping to within 2 kb of each other. Alignments were associated with annotated or predicted genes mapping to within 10 kb of each tag. The presence of a consensus p53 binding site was determined in each tag by querying 500 bp of bilateral genomic sequence flanking each tag using the p53MH algorithm [46]. Functional assessment of genetic elements associated with individual tags was performed using GoMiner [87] and grouped into ontological subsets for further analysis and selection. To confirm candidate p53 binding sites, PCR primers targeting a region within 200 bp of each selected alignment or cluster (Supplementary data, Table 2) were used to measure the amount of sequence in immunoprecipitated sample by qPCR as described above.

Quantitative reverse-transcriptase and real-time polymerase chain reactions - PC12 cells were NGF-differentiated as above and harvested at indicated intervals. Total RNA was harvested (Qiagen, Valencia, CA) according to manufacturer’s protocol. RNA concentration was determined using a NanoDrop spectrophotometer (BioRad). Equal amounts of RNA sample were used for SuperScript II first-strand cDNA synthesis (Invitrogen). RT-PCR was carried out...
using 1 µL of cDNA per sample, HotStart master mix (SuperArray, Frederick, MD) and 0.25 µM each primer (IDT) in a GeneAmp 9700 PCR instrument (Applied Biosystems, Foster City, CA) for indicated number of cycles. Equivalent volume of each PCR reaction was run on 2% TBE agarose gels containing ethidium bromide and photographed under UV illumination. Each target was amplified using an appropriate, empirically determined cycle number allowing gel-based visualization of samples within the exponential amplification phase of each reaction. Band intensity was measured by integration using ImageJ software (http://rsb.info.nih.gov/ij/) and normalized relative to naïve expression value.

qRT-PCR expression analysis was performed with cDNA prepared as above in reactions using the Syber Green qRT-PCR master mix (ABI) and analyzed by an ABI Model 7900 Prism instrument. All reactions were carried out in triplicate and measurements analyzed using the relative quantitation $2^{-\Delta\Delta Ct}$ method. The housekeeping gene GAPDH was used as an endogenous control to which sample measurements were normalized. The ROX passive reference dye was used to normalize reaction conditions across sample wells. Naïve PC12 RNA levels for each gene were used as the reference calibrator to which the NGF-treated sample RNA levels were compared. Statistical analysis of qRT-PCR data was carried out using Student’s t-test for significance at $p\leq0.05$ comparing treatment to naïve gene expression levels.
2.6 References


Figure 2.1. PC12 neuronal differentiation involves p53 stabilization and activation.  A) Representative image of naïve PC12 cells compared to 7-day differentiated cells following 50 ng/mL NGF 2.5S treatment.  B) Immunoblot of the dopamine transporter and nestin in naïve compared to 7-day differentiated cells, with total actin protein levels serving as loading control. C) Immunoblot depicting total and phospho-serine 15 p53 protein levels over time following 50 ng/mL NGF 2.5S, with total actin protein levels serving as loading control. D) RT-PCR levels of p53 mRNA over time following NGF 2.5S treatment compared to β-actin loading control. E) Luciferase activity in PC12 cells transfected with p53TAluc reporter vector following NGF 2.5S treatment at indicated time intervals, where p≤0.05. Error bars represent standard deviation.
Figure 2.2. Analysis of p53 knockdown PC12 cell lines. A) RT-PCR and immunoblot depicting levels of p53 mRNA and protein in wild-type naïve PC12 cells compared to 7-day NGF (50 ng/mL) differentiated wild-type and anti-p53 shRNA expressing PC12 cells. GAPDH mRNA levels serve as loading control for RT-PCR, while actin serves as immunoblot loading control. p53 and GAPDH mRNA levels in each sample were quantitated using ImageJ and normalized to naïve RNA levels, depicted under each band. PCR cycle number used is shown on right of gel image. p53 RNA and protein levels (and knockdown efficiency) were lowest in p53#3low cells, followed by p53#2mid and p53#4mid cell lines. B) Representative image of wild-type and p53#3low PC12 cells following 3 days treatment with 50ng/mL NGF.
Figure 2.3. Identification of p53-occupied genomic sites. A) Schematic of chromatin immunoprecipitation (anti-p53, FL-393) cloning strategy. See methods for more detail. B) Location of tags containing putative p53 binding sites relative to known genes within the rat genome. Binding sites scoring 90 or above out of 100 using the p53MH algorithm were included in this analysis. C) Hypothetical 100 kb gene map depicting relative location of 364 p53MH-predicted binding site-containing tags scoring ≥ 90 located in upstream 5’, downstream 3’ and intergenic gene regions.
**Figure 2.4.** p53-dependent induction of target genes. Panels A-N show site-specific qPCR ChIP results on left with standard deviation, where significance at $p \leq 0.05$ is designated by *. Center tile depicts gene map of predicted binding site location and sequence relative to annotated genes, along with exon structure and direction of transcription as per NCBI MapViewer conventions. Right tile represents RT-PCR gel image of each mapped target gene. RT-PCR analyses was performed multiple times for each target gene and gel images are representative of consistent expression levels. mRNA levels of each gene in wild-type naïve PC12 cells was compared to 7-day NGF (50ng/mL) differentiated wild-type and three anti-p53 shRNA expressing PC12 cells (p53#3low, p53#2mid, p53#4mid). ImageJ quantitation, normalized to naïve RNA levels, is depicted under each band, while PCR cycle number is depicted on right of gel image. GAPDH is shown in A and represents a typical loading control.
FIG 4

A. Chr.20: 7370k-7446k

B. Chr.7: 56995k-57058k

C. Chr.10: 25776k-25783k

D. Chr.7: 123530k-123558k

E. Chr.5: 154313k-154388k

F. Chr.3: 170337k-170354k

G. Chr.18: 71066k-71154k

H. chr.10: 103420k-103700k
Figure 2.4 continued

**Chr.6: 101309k-101365k**

**LOC366671**

Similar to LSm8 (pseudogene)

**GGGCTTGTTT**

**Chr.8: 11060k-11125k**

**AAGCAAGCAC**

**ATAAAT**

**Chr.10: 82570k-82603k**

**GAACTAGCTC**

**TCCAGC**

**ACACATGATT**

**Chr.1: 197175k-197208k**

**Dusp5**

**Chr.1: 259745k-259775k**

**TGCCAAGTTC**

**AAGCAAGTCT**

**Tst**

**Mpst**

**LOC300317**

**Fig 4**
**Figure 2.5.** p53-repressed target genes. Panels A-D show site-specific qPCR ChIP results on left with standard deviation, where significance at p≤0.05 is designated by *. Display and analyses are as described in Figure 4 legend.
**Figure 2.6.** Targets with NGF-dependent increase in p53 occupancy, but where p53-dependent transactivation could not be determined using anti-p53 shRNA expressing cell lines. Panels A-L show site-specific qPCR ChIP results on left with standard deviation, where significance at p≤0.05 is designated by *. Display and analyses are as described in Figure 2.4 legend.
Figure 2.6 continued

**I.**
Chr. 13: 44075k-44124k
GGACTTGCCT, GAGCTTGTTT

LOC498225 (similar to ligatin)

**J.**
Chr. 1: 162081k-162113k
GGGCAAGCCC, AGGCAAGTCA

Trim34, predicted
RGD1304579, predicted

**K.**
Chr. 8: 69952k-70055k
TAACAAGTCC, AGACTTGCCA

LOC363412 (similar to Znf609)
Figure 2.7. WebLogo multiple sequence alignment of putative p53 binding sites. Known p53 consensus binding site is 5'-PuPuPuC(A/T)(T/A)GPypPyPy-3'. Individual base letter height indicates level of conservation within each binding site position. Color used is specific to each nucleotide. A) Consensus sequence of p53-induced targets shown in Figure 4. B) Consensus sequence of p53-repressed targets shown in Figure 5.
Figure 2.8. qRT-PCR expression analyses of p53 targets following genotoxic exposure. A) mRNA expression levels of known p53 targets comparing naïve, 7-day NGF (50 ng/mL), 6h 5-fluorouracil (375 µM) and 6h bleomycin (10 µg/mL) exposure. Expression was measured via real-time PCR and normalized to naïve expression levels using $2^{-\Delta\Delta ct}$, where significance at $p \leq 0.05$ is designated by *. Error bars represent standard deviation. B) Same as in Panel A, depicting genes whose expression levels were not regulated in same manner between NGF treatment and genotoxic treatment. C) Same as in Panel A, depicting genes with comparable regulation between NGF treatment and genotoxic exposure.
Supplemental Figure 2.1. FACS cell cycle analysis of NGF-treated PC12 cells. Cells were treated with NGF and harvested at indicated time intervals using FITC-BrdU kit (Pharmingen) according to manufacturer’s recommended protocol and analyzed on a Becton Dickinson FACSort flow cytometer.
Supplemental Figure 2.2. p53 increasingly binds DNA upon NGF treatment. qPCR of chromatin immunoprecipitation-enriched fragments occupied by p53 in naïve and 7 day NGF-treated PC12 cells. Primers were designed to the known p53 binding site within the proximal p21Waf1/Cip1 promoter and to a negative control region. Immunoprecipitation was performed using either the negative control pre-immune IgG or anti-p53 polyclonal FL-393 antibody. Relative occupancy was determined by normalization of PCR amplification to pre-IP input chromatin amplification using the same primer set. Statistical analyses compared occupancy levels of negative control IgG to levels observed with anti-p53 FL-393 antibody by at-test, where significance at p≤0.05 is designated by *. Error bars represent standard deviation.
Supplemental Figure 2.3. Cluster frequency across the *rattus norvegicus* genome. A) Number of cluster alignments to the *rattus norvegicus* genome. B) Relationship between cluster size and frequency.
**Supplemental Table 2.1. ChIP statistics**

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### Supplemental Table 2.2. ChIP primers

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| Apc2         | GTTCCCATCCAGCATTTATTG  
               | TGTGGTAAGAGGCAAGGATG |
| BRK          | TCTCCCCTCTGACACCTTTG  
               | CCATGTGCGCAATATAGCAAG |
| Ccn1         | CAAAAGAGGCAGCTAGTCAG  
               | AGGTCAGAGGCAGGTGAATC |
| Cdc42ep4     | CCTCGAAGGAAGTCCATAGAG  
               | CCAGGGCTGTGTCTCAGTC |
| Cnnm2        | GTTGCTTGGAAAGGAAGAAG  
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| Csnk1d       | GCCATCTACTGCTAGGCAAG  
               | GTGACCATCCCTAACCAGAAG |
| Drd4         | AAGCCCTGCTCTATGGACAC  
               | GCTGCATGTACCACATAAC |
| Ddx25        | TCGCCAACCTGTGGTCCTACG  
               | ATGGCTCTGCAAGAAGAAG |
| Dlx2         | AGGGGAGCAGGAGATCAGTATT  
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| Dusp5        | AATCTCTCTCCCATTCGTGTG  
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| Dvl3         | AGAGCAAGGAGATCAGGATG  
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| Dyrk3        | CCTCCCAGCTCAAAGATTAC  
               | CATTCAATCCCTTGATAG |
| Ednrb        | GACCCTGCAAGGAACAGTAC  
               | CGAGGGAGGAGAGAAGAG |
| FAK          | GAATCACACCTAATTTCCAGTG  
               | GTGCTGTAGGGTTGAGT |
| Fgf2         | TTACACGGCACACCTGACTG  
               | GGGAAAGCTGACTGAGAAG |
| FLJ32743     | TCAACTAACCTGTAAGCCGATTT  
               | ATTAATGCTGCTGCTATCAG |
| Gsk3b        | TTCTTTGAAAGCATGAGCAGAT  
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**Supplemental Table 2.4.** gene-specific RT-PCR primers

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3. THE p53 TRANSCRIPTIONAL TARGET \textit{wnt7b} REGULATES NGF-MEDIATED NEURITE OUTGROWTH IN NEURONAL PC12 CELLS. \textit{Brynczka, C and Merrick, BA.}

3.1 Abstract

Differentiation in PC12 cells by neural growth factor (NGF) is characterized by growth arrest and neurite extension. The transcription factor p53 contributes to each of these processes but the mechanisms are incompletely understood. NGF signaling stabilizes p53 which enables the transcriptional regulation of various target genes, including a newly identified target, \textit{wnt7b}. We tested the hypothesis that \textit{wnt7b} expression is involved in p53-mediated neurite outgrowth in NGF-differentiated PC12 cells. \textit{Wnt7b} transcript increased within 3 days, while protein levels of \textit{wnt7b} are rapidly and efficiently increased within 24 hours of NGF exposure. Stable silencing of p53 by shRNA reduced \textit{wnt7b} protein levels and halted neurite outgrowth in NGF-treated cells. Immunofluorescence showed \textit{wnt7b} was distributed throughout the cytosol but changed to peri-cytoplasmic, nodal localization during \textit{wnt7b} overexpression in transfections of mitotic and NGF-differentiated cells. Overexpressed \textit{wnt7b} produced marked neurite extensions in the presence of NGF that was sufficient to restore neurite outgrowth in p53-silenced cells. In conclusion, data show that \textit{wnt7b} is a p53-regulated neuritogenic factor that in conjunction with NGF signaling is capable of eliciting potent induction of neurite outgrowth in PC12 cells.
3.2 Introduction

PC12 cells have been extensively studied as an *in vitro* model of neuronal differentiation [6]. Nerve growth factor (NGF) induces neuronal differentiation of PC12 cells and concomitantly elevates transcription factor p53 protein levels [7]. Neurite outgrowth in differentiating PC12 cells is a p53-dependent process [1, 3] and the aberration of p53 transcriptional activity leads to generalized lack of neurite extensions [3]. In order to understand the specific function of p53 within NGF-induced differentiation, our lab has recently identified a number of p53 transcriptional targets in NGF-treated PC12 cells, including the *wnt7b* signaling molecule.

Wnt-regulated pathways have been shown to influence a varied spectrum of cellular processes including development, cell proliferation and migration, while deregulated Wnt signaling has been associated with cellular transformation and tumorigenesis [9]. *Wnt7b*-induced signaling has been described to act through both canonical [13, 15] and noncanonical signaling pathways [11], depending upon both cell type and receptor availability. Previous reports have suggested that *wnt7b* is transcriptionally regulated during hindbrain development by Pax6 in mice [12] and by the TTF-1, GATA6, and Foxa2 transcription factors in cultured lung epithelium from binding sites within the 5’ promoter region of these genes [14]. In addition to these factors, we identified a region within the first *wnt7b* intron containing a p53 consensus binding site that was occupied by p53 in an NGF-dependent manner during PC12 cell differentiation. Wnt7b stimulates dendritic development through non-canonical signaling in hippocampal neurons [11]. In the p53-silenced PC12 cell, we observed both a generalized lack of neurite extension upon treatment with NGF and a decrease in expression of the p53 target gene *wnt7b*. These results suggested that *wnt7b* signaling may be a mechanism in which p53-
regulated neurite outgrowth occurs. Furthermore, NGF-dependent expression of the wnt7b protein has not been described within the wild-type PC12 cell [4]. We therefore hypothesized that wnt7b function may be related to p53-dependent neurite outgrowth in PC12 differentiation and aimed to characterize expression of wnt7b within PC12 cells.

3.3 Materials and Methods

Rat PC12 cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen), 4 mM L-glutamine (Invitrogen) and penicillin/streptomycin antibiotics in a humidified 37°C incubator maintained at 5% CO₂. All experiments were carried out using cells of less than 25 passages. Cells were plated on rat tail type I collagen (Sigma, St. Louis, MO) prior to experimentation and were differentiated by the addition of 50 ng/mL NGF 2.5S (Chemicon, Temecula, CA) in RPMI 1640 with 1% horse serum and antibiotics. Overexpression studies were performed by transfection of the wnt7b open reading frame cloned into the pDREAM 2.1 vector (Genscript Corp., Piscataway, NJ) using Lipofectamine 2000 (Invitrogen) into specified cells.

RT-PCR was performed following total RNA isolation according to manufacturer’s protocol (Qiagen, Valencia, CA). RNA concentration was determined using a NanoDrop spectrophotometer (BioRad, Richmond, CA). Equal amounts of each RNA sample (1.0 µg) were used for first-strand cDNA synthesis (Invitrogen). RT-PCR was carried out using 1 µL of cDNA per sample, HotStart master mix (SuperArray, Frederick, MD) and 0.25 µM each primer (IDT) in a GeneAmp 9700 PCR instrument (Applied Biosystems, Foster City, CA). Equivalent volume of each PCR reaction was run on 2% TBE agarose gels containing ethidium bromide and
photographed under UV illumination. Western blotting was performed as indicated previously [8] using the following antibodies: wnt7b (Q-13, Santa Cruz Biotechnology, Santa Cruz, CA), wnt7a/b (H-40, Santa Cruz), wnt7a (K-15, Santa Cruz) and actin (MAb1501R, Chemicon, Temecula, CA).

Indirect immunofluorescence of wnt7b protein was performed after fixation of cells with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were permeabilized with 0.4% Triton X-100 and incubated with H-40 anti-wnt7a/b polyclonal antibody (Santa Cruz) and detected after incubation with goat anti-rabbit Alexa 594-conjugated secondary antibody (Invitrogen). Samples were mounted using ProLong Gold reagent (Invitrogen) containing the nuclear counterstain DAPI and photographed using an Olympus IX70 inverted microscope (Olympus, Center Valley, PA) and appropriate filter sets. Image exposure time for each fluorophore was maintained across all samples. Merging of images was performed using AxioVision software (Carl Zeiss, Oberkochen, Germany). Morphologic analysis of fixed PC12 cells treated as indicated was performed at 40x magnification using microscope and software as described above.

3.4 Results/Discussion

Wnt7b expression increased in a time-dependent manner after NGF treatment of PC12 cells. Wnt7b transcript levels were significantly elevated from basal (naïve) amounts within 3 days of NGF exposure and maintained for at least 7 days (Figure 3.1A). Intracellular wnt7b protein levels also increased within 24 hours of NGF treatment and were maintained over the course of 7 days (Figure 3.1B). Remarkably, intracellular wnt7b protein levels increased rapidly with NGF treatment relative to mRNA levels which suggested that either translation of wnt7b
RNA was highly efficient or protein levels are efficiently stabilized during PC12 neuronal differentiation.

In order to verify whether wnt7b protein levels were dependent upon p53 availability, we used two anti-p53 shRNA-expressing PC12 cell lines in which p53 RNA and protein levels were stably decreased relative to wild-type PC12 cells. We observed that wnt7b protein levels were highly elevated within 24 hours of NGF treatment in wild-type cells as expected but wnt7b levels in p53-silenced cells treated with NGF were significantly lower (Figure 3.1C), demonstrating that wnt7b protein expression was also dependent upon p53 availability. Silencing of p53 levels was more efficient in p53sh#3 than p53sh#2 cells which was reflected in the graded reduction of wnt7b levels within each cell line. Furthermore, these results demonstrated that p53-mediated activity was the major route through which wnt7b was expressed in the differentiating PC12 cell.

The wnt7b protein was identified as a single, 25 kDa band via immunoblotting (Figure 3.1B) within PC12 cells. However, the calculated molecular weight of wnt7b derived from accession NP_001009695 is 39.3kDa, as demonstrated by wnt7b immunoblot from COS cells [2]. We noted a wnt7b RNA variant containing an alternate first exon has been described within the developing chick eye [5], a region in which p53 is highly expressed [10]. Thus, we aimed to determine whether the observed wnt7b protein was encoded by a full-length transcript. The wnt7b locus is comprised of three distinct exons. We tested if p53 bound to and activated transcription from the first intronic region of the wnt7b gene. Using primers to amplify each exon specifically within wnt7b cDNA, we determined that exons 1-3 were represented in the expressed product (Figure 3.2A) and found that amplicon levels representing each exon were increased upon NGF treatment. Differences in detection levels between exons 1 through 3 were attributed to the ratio of PCR product size to ethidium bromide intercalation and differing
amplification efficiencies. Therefore, each of three exons were represented in the wnt7b transcript upon NGF-induced differentiation of PC12 cells despite SDS-PAGE migration at 25 kDa in reducing conditions, with transcript levels remaining low in the mitotic cell as previously described [4].

In order to determine the function of wnt7b during PC12 differentiation, we overexpressed wnt7b cDNA to remove p53-driven expression from the first intron. PC12 cells were transfected with pDREAM-wnt7b and treated with NGF as indicated. We observed increased wnt7b protein expression in mitotic cells transfected with pDREAM-wnt7b (Figure 3.2B). NGF treatment led to elevated wnt7b levels in control cells that were further increased in transfected cells (Figure 3.2B). A decrease in migration or multiple bands was not observed in transfected cells, suggesting that wnt7b may be post-translationally processed (i.e. proteolytic cleavage) in the differentiating PC12 cell.

Immunofluorescent measurement of wnt7b within mitotic and differentiating PC12 cells demonstrated low nearly undetectable levels within mitotic cells, while transfection with the pDREAM-wnt7b vector greatly increased detectable wnt7b fluorescence (Figure 3.3). In particular, overexpressed wnt7b was found to localize as nodal points within or at the plasma membrane surface of the mitotic PC12 cell. Following NGF treatment, wnt7b was readily detected and evenly distributed throughout the cytoplasmic compartment with localized nodal regions apparent upon transfection with pDREAM-wnt7b. Because the antibody used was capable of detecting both wnt7a and 7b, we analyzed wnt7a expression to verify the specificity of immunofluorescence measurements. Immunoblotting demonstrated that no wnt7a immunoreactive bands were detected in PC12 cells in either the mitotic or NGF-differentiated state (data not shown). These results demonstrated that wnt7b localized within the cytoplasmic
cellular compartment upon NGF stimulation in PC12 cells and increasingly localized to nodal peri-cytoplasmic regions when overexpressed. In addition, the wnt7a family member is not expressed in either the mitotic or NGF-differentiated PC12 cell.

In order to determine whether wnt7b has a physiological effect when expressed in PC12 cells, we studied neurite extension in both the wild type and p53-silenced p53sh#3 cell line (Figure 3.4). NGF treatment for 24 hours induced neurite extension in the wild-type cells while neurite formation was largely inhibited in p53sh#3 cells. Upon transfection with pDREAM-wnt7b, no observable changes in neurite growth was detected in mitotic cells but markedly increased numbers and length of neurites were observed in cells concurrently treated with NGF compared to control cells. In addition, transfection of pDREAM-wnt7b was sufficient to recover neurite outgrowth in p53sh#3 cells. The effects of wnt7b overexpression were marked where distance between cells were less than 4-5 cell diameters, suggesting wnt7b secretion or localization to the plasma membrane may be important in establishing and maintaining neurite growth.

In conclusion, we demonstrated that wnt7b was expressed in NGF-differentiating PC12 cells in a p53-dependent manner and was involved in neurite outgrowth and extension within these cells. The lack of neurite outgrowth in mitotic cells transfected with the pDREAM-wnt7b expression vector suggested that wnt7b alone was not sufficient for neurite development but cooperated with NGF-regulated signaling pathways to increase both the number and length of neurite extensions. In addition, recovery of neurite outgrowth in pDREAM-wnt7b transfected p53sh#3 cells suggested that wnt7b regulation was a central pathway through which p53 promoted neurite outgrowth in NGF-differentiating PC12 cells. In conjunction with other
recently described p53 target genes [3], wnt7b represents a unique transcriptional target as a secreteable morphogen through which p53 may regulate the neuronal morphology.
3.5 References


Figure 3.1. Wnt7b is expressed in a p53-dependent manner upon NGF treatment in PC12 cells. A) RT-PCR for PC12 wnt7b cDNA following NGF treatment for indicated intervals. Each reaction was performed on 1 µL cDNA derived from 100 µg DNase-treated RNA. PCR was performed for 36 cycles. Actin amplification is shown as loading control, and was amplified for 23 cycles. B) Immunoblot for wnt7b performed on 15 µg PC12 whole cell lysate following treatment with NGF for indicated interval. Actin is shown as loading control. C) Immunoblot for wnt7b using 15 µg whole cell lysates from either wild-type PC12 or p53sh#3 PC12 lines treated with NGF for 24 hours. Actin is shown as loading control.
Figure 3.2. Wnt7b is generated as a full-length transcript. A) Exon-specific RT-PCR in wild-type PC12 performed on 1 µL cDNA derived from 100 µg DNase-treated RNA. PCR was performed for 39 cycles. GAPDH is shown for loading control along with no RT negative controls using same GAPDH primer set, performed at 25 cycles. B) Immunoblot for wnt7b using 15 µg PC12 whole cell lysate in pDREAM-wnt7b transfected and untransfected cells treated with NGF for 24 hours as indicated.
Figure 3.3. Expressed wnt7b is localized to cytoplasm. Indirect immunofluorescence for wnt7b in either naïve 24 hour NGF treated pDREAM-wnt7b transfected or control PC12 cells. Secondary antibody is conjugated to Alexa594 (red). Nuclei are stained with DAPI (blue).
Figure 3.4. Wnt7b expression rescues NGF-induced neurite outgrowth in p53-silenced PC12 cells. 40x magnification of fixed naïve or 24 hour treated PC12 cells transfected with pDREAM-wnt7b as indicated.
4. NERVE GROWTH FACTOR POTENTIATES p53 DNA BINDING BUT INHIBITS NITRIC OXIDE-INDUCED APOPTOSIS IN NEURONAL PC12 CELLS. Brynczka, C and Merrick, BA.

4.1 Abstract

NGF is recognized for its role in neuronal differentiation and maintenance. Differentiation of PC12 cells by NFG involves p53, a transcription factor that controls growth arrest and apoptosis. We investigated NGF influence over p53 activity during NO-induced apoptosis by sodium nitroprusside in differentiated and mitotic PC12 cells. NGF-differentiation produced increased p53 levels, nuclear localization and sequence-specific DNA binding. Apoptosis in mitotic cells also produced these events but the accompanying activation of caspases 1-10 and mitochondrial depolarization were inhibited during NGF differentiation and could be reversed in p53-silenced cells. Transcriptional regulation of PUMA and survivin expression were not inhibited by NGF, although NO-induced mitochondrial depolarization was dependent upon de novo gene transcription and only occurred in mitotic cells. We conclude that NGF may mediate prosurvival signaling through factors such as Bcl-2 and p21Waf1/Cip1 without altering p53 transcriptional activity to inhibit apoptosis.
4.2 Introduction

Control over the cell death process is critical in tissues throughout the body, particularly within the nervous system. During development, apoptotic manicuring of nervous tissue is mandatory to ensure proper neuroanatomy and connectivity, as demonstrated in mice lacking key mediators of the apoptotic process [1]. Conversely, aberrant neuronal apoptosis within the mature nervous system may play an important role in neurodegenerative diseases such as Parkinson’s, Alzheimer’s, amyotrophic lateral sclerosis and Huntington’s as well as ischemic brain conditions [2].

The machinery executing the apoptotic program is largely conserved throughout diverse cell types and the transcription factor p53 is recognized as a central node in apoptosis. A variety of cellular stresses transcriptionally activate p53 and its effector genes such as the cdk inhibitor \( p21^{Waf1/Cip1} \) for growth arrest or apoptotic BH3 family members such as \( Bax \) and \( PUMA \). These two cell fates are triggered by signal transduction pathways that converge on p53 to increase its nuclear localization, alter DNA-binding specificity by post-translational modifications and recruit specific coactivators to tip the balance between expression of apoptotic and prosurvival genes [3]. In addition, direct cooperation of cytosolic p53 with apoptotic BH3-domain proteins can contribute to mitochondrial-mediated apoptosis [4, 5]. Both apoptotic mechanisms of p53 involve the mitochondria, where permeabilization by apoptotic BH3-domain family members induce the cytosolic release of cytochrome c and initiation of the cell death program [6].

Recent evidence indicates the presence of p53 transcriptional activity in neuronal differentiation and neurite outgrowth in the nervous system [7, 8]. In particular, nerve growth factor (NGF) mediated differentiation of rat PC12 pheochromocytoma cells is associated with elevated p53 protein levels, increased p53 transcriptional activity and a rise in \( p21^{Waf1/Cip1} \)
expression which is prevented by a temperature sensitive mutant p53 [9]. NGF is a neurotrophin whose signaling through the TrkA receptor [10] maintains neuronal differentiation in PC12 cells [11]. NGF is believed to act as a survival factor in neuronal cells [12] through mechanisms that include activation of protein kinase B/Akt [13, 14] and ERK kinase through Ras activation [15]. NGF signaling also upregulates heme oxygenase-1 [16, 17], increases glutathione synthase activity [18] and induces inhibitor of apoptosis (IAP) proteins [9, 19]. Consistent with its pro-survival role, NGF treatment confers some protection against a variety of cytotoxic insults including nitric oxide although the mechanism is unknown [20].

Nitric oxide (NO) is a diffusible diatomic nitrogen radical involved in signal transduction throughout the body [21] and is involved in NGF-induced signaling during PC12 differentiation [22]. Within the nervous system, NO normally functions as an endogenous neurotransmitter [23] but exposure to levels beyond those necessary for physiological activity may be highly toxic in neuronal cells [24]. Low doses of NO can also be cytoprotective [21], mainly through a cGMP-mediated signaling mechanism [21, 25-27] or through caspase inhibition [28]. Elevated levels of NO can activate the p53 pathway [29] and induce apoptosis in some cell types [30, 31]. Local immune hyperstimulation [32-34] is the major route through which neuronal cells are exposed to supraphysiological levels of reactive oxygen and nitrogen species including NO [35], and is thought to contribute to neurodegenerative conditions such as Parkinson’s disease [36]. As an agent of known neurotoxicological relevance, NO represents a biologically relevant mediator of apoptosis through which cellular NGF and p53 signaling can be studied.

We hypothesized that NO-induced apoptosis involves p53 signaling in propagating PC12 cells and that NGF-induced differentiation of these cells would modulate the role of p53 during apoptosis. These studies demonstrated that NO-induced apoptosis in propagating PC12 cells was
dependent on p53 activity and directly involved apoptotic gene transcription, caspase activation, depolarization of the mitochondrial membrane and nuclease cleavage of DNA. NGF-mediated differentiation was sufficient to substantially inhibit mitochondrial depolarization, caspase activation and DNA cleavage following nitroprusside treatment. However, transcriptional activity of representative p53-regulated apoptotic genes was comparable to propagating cells. We conclude that NGF mediates potent anti-apoptotic signaling through factors such as Bcl-2 and p21\(^{Waf1/Cip1}\) in neuronal PC12 cells without directly altering p53 transcriptional activity.

4.3 Materials and Methods

*Cell culture and treatments*

Rat PC12 cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum (Invitrogen), 5% fetal calf serum (Invitrogen), 4 mM L-glutamine (Invitrogen) and penicillin/streptomycin antibiotics in a humidified 37° C incubator maintained at 5% CO\(_2\). All experiments were carried out using cells of less than 25 passages. Cells were plated on rat tail type I collagen (Sigma, St. Louis, MO) prior to experimentation and were differentiated by the addition of 50 ng/mL NGF 2.5S (Chemicon, Temecula, CA) in RPMI 1640 with 1% horse serum and antibiotics. Medium and NGF were replenished every other day until differentiation was completed at 7 days.

Sodium nitroprusside (Sigma) was dissolved into sterile ddH\(_2\)O and added directly into culture medium at indicated concentrations for treatments. Medium was replaced prior to nitroprusside treatments in all experiments in order to maintain glucose levels and avoid widespread cellular death via necrosis [37]. Cell number was maintained at roughly 3x10\(^4\) cells per cm\(^2\) of vessel surface area to ensure equivalent nitroprusside dosage per cell across
experiments and culture vessel types. Cells were exposed to ultraviolet light as a positive apoptosis-inducing control at indicated doses using a Stratalinker (Stratagene, La Jolla, CA). Transcriptional inhibition was performed by incubation with 1 µM Actinomycin D (Sigma) for 1 hour prior to the indicated treatments.

**Generation of stable shRNA expressing cells**

Stable anti-p53 shRNA expressing PC12 cells (p53sh#3) were generated via lentiviral-mediated infection, integration and stable selection (BlockIt Lentiviral Expression System, Invitrogen). Hairpin sequences were designed and synthesized (IDT, Coralville, IA) with appropriate overhangs for cloning into the pENTR/U6 entry vector. The informative p53-targeting sense hairpin sequence is - 5'-ATATCCGACTGGAATCCTCC-3'. shRNA was designed using the 4-base loop sequence CGAA. Hairpin sequences were cloned into the pENTR/U6 vector and selected clones were sequence-verified. The U6 promoter-shRNA cassettes were transferred into the pLenti6-BLOCK-iT-DEST vector. The resulting destination clones were used to generate lentiviral particles by transfection of HEK293FT cells along with the pLP1, pLP2 and pLP/VSVG plasmids in equal amounts using FuGene (Roche Diagnostics, Indianapolis, IN) lipid reagent. Transfection efficiencies were monitored by concomitant transfection of the pEYFP-C1 vector (BD Clontech, Mountain View, CA), demonstrating efficiency ≥ 90% in all cases. Lentiviral-laden media was collected 48 hours following transfection, clarified by centrifugation and used with 4 µg/mL polybrene (Sigma) in transduction of naïve PC12 cells plated on collagen. Selection using 6 µg/mL blasticidin was initiated and maintained for 7 days until stable lines were enriched. Cells were maintained for 14 days before differentiation and experimentation.
Western blotting

Sample preparation for SDS-PAGE was performed as previously described [38]. Cell lysate protein determination was performed using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Antibodies used were against p53 (pAb122, BD Pharmingen, San Diego, CA), phospho-serine15 p53 (9284, Cell Signaling, Danvers, MA), p21Waf1/Cip1 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (DC21, Santa Cruz Biotechnology), Bcl-X(L) (2762, Cell Signaling), actin (mAb1201, Chemicon) and SULT 2A1 (Abcam, Cambridge, MA). Proteins were visualized using either ECL reagent (Amersham Biosciences, Piscataway, NJ) or SuperSignal (Pierce Biotechnology).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed similarly to previous reports [39, 40]. Briefly, NGF-differentiated and naïve (dividing) PC12 cells were crosslinked by 1% formaldehyde for 15 minutes before terminating the crosslinking reaction with 125 mM glycine. Cells were washed, lysed by Dounce homogenization and resuspended in a buffer containing sodium deoxycholate, SDS and Triton X-100 [40]. Chromatin was sheared to an average length of 500 bp by sonication on ice and samples were clarified by centrifugation at 10,000xg. Sheared, crosslinked chromatin complexes were precleared with protein A agarose. p53-DNA complexes were immunoprecipitated using anti-full length p53 polyclonal antibody (FL-393, Santa Cruz Biotechnology) or with control rabbit IgG (I-5006, Sigma). Immune complexes were captured with protein A agarose and beads were washed as described [39, 40]. Protein/DNA complexes were eluted and crosslinks reversed by incubating at 65 °C for at least 6 hours. To assay for p53
binding sites in purified ChIP DNA, target specific primers were used to measure amounts of target sequence in immunoprecipitated samples by qPCR using SYBR Green-based detection (BioRad, Richmond, CA). Experimental qPCR values were normalized against values obtained for 25 ng of input DNA using the same primer set.

Cell viability and caspase assays

Cell viability was determined by measuring mitochondrial reduction of the MTS dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent into a soluble formazan product (Promega, Madison, WI). PC12 cells were plated on collagen-coated 96-well plates and then treated as indicated for 24 hours prior to MTS addition directly to culture medium. Absorbance measurements at 490 nm were carried out within 1-2 hours using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA). Statistical analysis was performed using one-way ANOVA followed by Tukey’s Honest Significant Difference post-hoc test for group comparisons. Comparison of viability between naïve and differentiated cells was performed by two-tailed t-test. Data were statistically significant at p<0.05.

Caspase 3/7 activity was determined by measuring cleavage of the Z-DEVD-R110 substrate into fluorogenic product (Promega). PC12 cells were plated as above, nitroprusside-treated for 24 hours and lysed in-well along with the addition of caspase substrate. Cleavage of substrate to the fluorogenic product was measured within 2-4 hours at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a SpectraMax M2 spectrophotometer as above. Pan-caspase activity was measured following described treatment in 50 µg of cellular lysates essentially as above using AFC-conjugated caspase 1-10 substrates (BioVision).
Individual caspase activities were determined by measuring generation of the cleaved substrate fluorophore using excitation and emission wavelengths of 400 and 505 nm, respectively, as above. Statistical analyses were performed using the two-sided Student’s t-test comparing indicated treatments.

Fluorescence microscopy

Microscopy was performed using an Olympus IX70 inverted microscope (Olympus, Center Valley, PA). Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) was performed according to manufacturer’s instructions (Clontech, Mountain View, CA). Naïve or differentiated wild-type and anti-p53 shRNA expressing cells were plated on poly-L-lysine/collagen (Sigma) coated Lab-Tek chamber slides (Nunc, Rochester, NY) and treated as indicated. Samples were fixed in 4% formaldehyde and permeabilized in 0.2% Triton X-100 / PBS. Cleaved apoptotic DNA ends were labeled with fluorescein-dUTP by the TdT enzyme for 60 minutes at 37°. Samples were washed and mounted using ProLong Gold reagent (Invitrogen) containing the nuclear counterstain DAPI and photographed under fluorescence microscopy with appropriate filter sets. Image exposure time for each fluorophore was maintained across all samples. Merging of images was performed using AxioVision software (Carl Zeiss, Oberkochen, Germany).

Mitochondria were visualized using the fixable fluorescent dye MitoTracker Red CMXRos (Invitrogen Molecular Probes, Carlsbad, CA), a cell permeable dye that is selectively sequestered in mitochondria retaining their membrane potential ΔΨ. Naïve or differentiated wild-type and shRNA expressing cells were plated on coated Lab-Tek chamber slides as above and treated as indicated. Cell populations were then exposed to 400 nM CMXRos for 10
minutes. Samples were subsequently fixed using 3.7% formaldehyde and permeabilized with 0.2% Triton X-100 / PBS for 5 minutes. Permeabilization was performed to improve CMXRos signal retention as per manufacturer’s recommended protocol. Samples were mounted using ProLong Gold antifade reagent containing DAPI, and visualized as described above with equivalent image exposure times for all samples.

Indirect immunofluorescence for p53 protein was performed on both wild-type naïve and NGF-differentiated PC12 cells plated as above. Cells were treated as indicated and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). Cells were permeabilized with 0.4% Triton X-100 and incubated with FL-393 anti-p53 polyclonal antibody (Santa Cruz). p53 protein subcellular localization was labeled by incubation with goat anti-rabbit Alexa 594-conjugated secondary antibody (Invitrogen). Samples were mounted as described above and visualized using appropriate filter sets. Image exposure times were maintained across samples. Merged images were generated using AxioVision software as above.

Quantitative reverse-transcriptase polymerase chain reactions

PC12 cells and anti-p53 shRNA expressing cells were maintained in the propagating state or NGF-differentiated as above, treated and harvested at indicated intervals. Total RNA was isolated according to manufacturer’s protocol (Qiagen, Valencia, CA). RNA concentration was determined using a NanoDrop spectrophotometer (BioRad). Equal amounts of each RNA sample (1.5 µg) were used for first-strand cDNA synthesis (Invitrogen). RT-PCR was carried out using 1 µL of cDNA per sample, HotStart master mix (SuperArray, Frederick, MD) and 0.25 µM each primer (IDT) in a GeneAmp 9700 PCR instrument (Applied Biosystems, Foster City, CA). Equivalent volume of each PCR reaction was run on 2% TBE agarose gels containing
ethidium bromide and photographed under UV illumination. Each target was amplified using an appropriate, empirically determined cycle number allowing gel-based visualization of samples within the exponential amplification phase of each reaction. Primer sequences are available upon request.

4.4 Results

NGF reduces nitroprusside-induced loss of cell viability

Cell viability was measured in both naïve and NGF-differentiated PC12 cells following treatment with increasing concentrations of nitroprusside in both naïve and NGF-differentiated PC12 cells using the MTS reagent (Figure 4.1). We demonstrate that nitroprusside exposure reduces cell viability in both naïve and NGF-differentiated PC12 cells in a concentration-dependent manner, with differentiated cells significantly less susceptible to loss of cell viability at higher concentrations. At doses of 0.8 mM and 1 mM nitroprusside, 22% and 15.21% more mitotic (naïve) cells, respectively, were non-viable at these concentrations compared to differentiated cells.

Nuclear p53 accumulation in both naïve and NGF-differentiated cells

p53 protein was found to accumulate in both a concentration- and time-dependent manner following nitroprusside treatment in naïve PC12 cells (Figures 4.2A, 4.2B), with levels proportionately elevated following doses of 0.3 mM through 1 mM over the course of 24 hours. Accumulated p53 protein was posttranslationally phosphorylated at the serine 15 residue in a dose-dependent manner, consistent with p53 transcriptional activation [41] in these cells (Figure 4.2A). p53 protein levels were quickly elevated over time in naïve cells following 0.8 mM
nitroprusside, with levels of p53 protein visible within 30 minutes (Figure 4.2B). p53 phosphorylation at serine 15 occurred in a delayed manner compared to levels of total p53 protein (Figure 4.2B) which is typically observed during p53 activation.

As previously reported [7, 9], p53 protein levels were highly elevated in response to NGF treatment alone in PC12 cells over the course of 7 days (Figure 4.2C). Elevated p53 protein levels were accompanied by NGF-dependent increases in DNA binding to regulatory elements of the known target genes $p21^{Waf1/Cip1}$ and $MDM2$ (Figure 4.2D) as determined via chromatin immunoprecipitation (ChIP) assay. Binding to the proximal p53 binding site (relative to the +1 transcriptional start site) within the $p21^{Waf1/Cip1}$ promoter was increased 3.3-fold following NGF treatment, while binding to the distal p53 response element was elevated 3.9-fold. Occupancy of the intronic primary p53 binding site within the $MDM2$ gene was also elevated in response to NGF alone, increasing by 3.7-fold compared to naïve cells. Following nitroprusside treatment of differentiated cells, p53 protein levels were moderately decreased at 2 hours and quickly returned to the elevated levels observed in unstressed differentiated cells within 4 hours (Figure 4.2E). Therefore, NGF reduces nitroprusside-induced losses of cell viability in differentiated PC12 cells compared to naïve cells in the presence of highly activated p53 protein during differentiation.

Compared to untreated controls, we observed both an elevation in p53 levels and an increase in nuclear localization in nitroprusside-treated naïve PC12 cells within 4 hours (Figure 4.2F). Untreated NGF-differentiated cells contained prominent nuclear p53 protein as determined by both immunofluorescence and subcellular fractionation coupled with immunoblotting (data not shown), while nitroprusside exposure further increased p53 nuclear localization within 4 hours of treatment. These results demonstrate that nuclear localization of p53 protein is similar following nitroprusside in both naïve and NGF-differentiated cell states.
**NGF attenuates caspase activity**

Both naïve and differentiated PC12 cells were treated with 0.8 mM nitroprusside. Pan-caspase activity (caspases 1-10) was measured at 18 hours, the time point following treatment at which the greatest fluorescence was generated based on preliminary experiments. Pan-caspase activity in naïve PC12 cells was significantly activated within 18 hours following nitroprusside treatment (Figure 4.3A). Caspase 3 showed the most significant activation resulting in a 2.3-fold activity increase relative to untreated naïve cells. In contrast, NGF-differentiated cells demonstrated insignificant changes in the activities of caspases 1-10 following nitroprusside treatment over the course of 18 hours (Figure 4.3B).

We questioned whether attenuated caspase activity in differentiated PC12 cells was dose-dependent relative to naïve caspase activity. As expected, naïve PC12 cells underwent marked caspase 3 activation across a range of nitroprusside doses (Figure 4.3C), with a 2.2-fold increase in activity following 0.8 mM nitroprusside, consistent with previous caspase activity data. Caspase 3 activity was also elevated at lower concentrations (0.1 through 0.5 mM) of nitroprusside in naïve cells although significant levels of apoptosis were not yet visually observed. In contrast, no tested concentration of nitroprusside elicited an increase in caspase 3 activity within differentiated cells (Figure 4.3D).

**Nitroprusside-induced apoptosis is p53-dependent**

PC12 cell lines were generated which stably express anti-p53 shRNA from a constitutively active U6 promoter to maintain lower cellular levels of p53 RNA and protein compared to their wild-type counterparts. Levels of p53 RNA in shRNA-expressing p53sh#3
cells were significantly reduced compared to both the naïve and differentiated wild-type PC12 cells (Figure 4.4A). Stable silencing of p53 RNA efficiently maintained low p53 protein levels as determined by p53 immunoblot even in the presence of NGF (Figure 4.4B), which was observed earlier (Figure 4.2C) to effectively elevate p53 protein levels in PC12 cells.

We hypothesized that if nitroprusside-induced caspase activation produced apoptosis by a p53-dependent mechanism then reduction of p53 should limit apoptosis in PC12 cells. Apoptosis was measured in both wild-type and p53-silenced naïve and differentiated PC12 cells by TUNEL assay (TdT biotin-dUTP Nick End Labeling). Naïve wild-type PC12 cells undergo marked apoptosis after nitroprusside exposure as determined by the proportion of fluorescent TUNEL-positive cells compared to untreated control (Figure 4.4C). The number of apoptotic TUNEL-positive cells were substantially reduced in naïve p53-silenced p53sh#3 cells, demonstrating that apoptosis is p53-dependent following nitroprusside exposure in naïve cells.

NGF-differentiated cells were substantially more resistant to nitroprusside-induced apoptosis, in contrast to naïve cells (Figure 4.4D). However, a small number of differentiated cells were observed as TUNEL-positive after nitroprusside which was similar and not further reduced in differentiated p53sh#3 cells. The significant decrease in caspase activation and apoptosis in differentiated cells suggested that NGF may have affected the process of apoptosis or the transcriptional activity of p53 directly.

Nitric oxide is known to produce cell death through mixed necrosis and apoptosis in some cell types [37, 42]. Using the stain trypan blue, we scored cells for membrane permeability as an indicator of necrosis within naïve and NGF-differentiated cells following 20 hours 0.8 mM nitroprusside exposure. In naïve cells, nitroprusside treatment produced 17.2% ± 3.4% trypan blue-positive cells (n=3) and 11.9% ± 0.64% trypan blue-positive cells in NGF-differentiated
PC12 cells (n=3). These data suggest that NGF has insignificant effect on NO-induced necrosis despite its antiapoptotic effect in differentiated cells.

*Mitochondrial involvement in nitroprusside toxicity*

Mitochondrial depolarization has been shown to contribute to apoptosis in various cell types [43-46], including nitroprusside-treated PC12 cells [47]. However, the role of NGF and p53 in nitroprusside-induced mitochondrial depolarization is not known. Maintenance of the mitochondrial membrane potential $\Delta \Psi$ following nitroprusside treatment was studied in both naïve and differentiated PC12 cells using the cell permeant dye, MitoRed CMXRos, which accumulates selectively within mitochondria maintaining a potential difference across their membrane.

Naïve PC12 cells exposed to 0.8 mM nitroprusside experienced marked loss of mitochondrial membrane potential compared to untreated cells (Figure 4.5A), as evidenced by decreased mitochondrial MitoRed dye retention. Loss of mitochondrial membrane potential in naïve cells was concurrent with pyknotic nuclei in DAPI counterstained naïve cells suggesting a positive relationship between apoptosis and mitochondrial pathology. Rare large nuclei consistent with necrotic pathology were also observed, both with and without MitoRed staining, in nitroprusside-treated naïve cells although less frequently observed in NGF-differentiated cells. The importance of p53 to nitroprusside-induced pathology was demonstrated by inhibition of mitochondrial depolarization in p53-silenced naïve p53sh#3 cells reflected by their MitoRed fluorescence retention. Furthermore, nuclear changes consistent with apoptosis were visibly absent in nitroprusside-treated naïve p53sh#3 cells, suggesting that p53 was involved in both mitochondrial and nuclear pathology following nitroprusside treatment in the naïve cell.
Differentiated PC12 cells were substantially less susceptible to nitroprusside-mediated loss of mitochondrial membrane potential (Figure 4.5B). Both wild-type and p53sh#3 cells treated with NGF retained MitoRed fluorescence following nitroprusside exposure, in contrast to naïve PC12 cells. These results indicated that, similar to TUNEL detection of apoptosis, p53-dependent, nitroprusside-induced mitochondrial and nuclear pathologies were significantly reduced in NGF-differentiated cells.

*Intact p53 apoptotic transcriptional activity during NGF protection*

Since p53-dependent apoptosis can occur by both transcriptional and non-transcriptional mechanisms [4, 5, 48], we examined the pathways for nitroprusside-induced apoptosis in naïve PC12 cells for comparison to NGF-differentiated cells. Although rapid p53 nuclear partitioning was demonstrated in response to nitroprusside exposure (Figure 4.2F), we attempted to rule out alternative functions of cytoplasmic p53 as a mechanism leading to nitroprusside toxicity.

Cellular RNA synthesis was inhibited by pretreatment with 1 µM Actinomycin D one hour prior to nitroprusside treatment to determine if p53 transcriptional function was upstream of mitochondrial depolarization. No apparent nuclear or mitochondrial pathology was detected following treatment with Actinomycin D alone (Figure 4.6A). As expected in naïve PC12 cells, nuclear pyknosis and mitochondrial depolarization were apparent following nitroprusside treatment (Figure 4.6A). In combination with nitroprusside, Actinomycin D significantly alleviated, although did not completely prevent, chromatin pyknosis and mitochondrial depolarization in naïve cells. These results suggested the *de novo* transcription of apoptotic genes was largely responsible for development of mitochondrial pathology and apoptosis following nitroprusside exposure in naïve PC12 cells within the tested time frame.
Both p53 silencing and generalized transcriptional inhibition by Actinomycin D were protective against nitroprusside-mediated apoptosis in naïve PC12 cells. Therefore, it was likely that p53-dependent transcriptional regulation of apoptotic target genes controlled the response to nitroprusside treatment. We hypothesized that if NGF inhibited apoptosis by directly modulating p53 activity then p53-dependent transactivation or repression of target genes would be affected. Using both wild type and p53sh#3 cells, we found that gene expression of the known target genes $p21^{Waf1/Cip1}$, $MDM2$ and $cyclin G1$ were increased in both naïve and NGF-differentiated cells following nitroprusside treatment in a p53-dependent manner (Figure 4.6B). As expected by earlier ChIP analyses in Figure 2D, the transcript levels of $p21^{Waf1/Cip1}$ and $MDM2$ were highly elevated in NGF-differentiated compared to naïve cells during nitroprusside toxicity. We observed that expression of the apoptotic gene, $PUMA$, was highly increased in a p53-dependent manner with nitroprusside in NGF-differentiated cells, but only moderately increased in the naïve cell. Anti-apoptotic $survivin$ expression was also repressed following nitroprusside in all cell types. Although $survivin$ is a recognized target of p53 in apoptosis [49, 50], this gene may also be regulated by additional mechanisms following p53 suppression. mRNA levels of the apoptotic BH3-domain protein, $Bad$, were increased slightly at 4 hours following nitroprusside treatment in differentiated cells, while no changes were observed in expression levels of the apoptotic BH3-domain family members, $Bid$ or $Bax$, in either the naïve or NGF-differentiated state following nitroprusside treatment. These results demonstrated that transcription of p53 targets involved in both apoptosis and cell cycle arrest were highly similar in both the naïve and NGF-differentiated cells treated with nitroprusside.

As a potential mechanism of NGF-induced protection against apoptosis, levels of the anti-apoptotic proteins Bcl-2 and Bcl-X(L) were studied during PC12 differentiation. We
observed that Bcl-2, but not Bcl-X(L), protein levels were increased over the course of 7 days during NGF differentiation (Figure 4.7A). Upon nitroprusside treatment, anti-apoptotic Bcl-2 protein levels in naïve cells decreased within 7 hours while expression in NGF-differentiated cells remained constant (Figure 4.7B), in agreement with observed levels of apoptosis (Figure 4.4C, 4.4D). In contrast, p21Waf1/Cip1 protein levels increased over time in naïve cells upon nitroprusside treatment, with greatest amounts seen within 12-24 hours (Figure 4.7C). In accordance with p53 occupancy and mRNA expression data (Figure 4.2D and 4.6B, respectively), p21Waf1/Cip1 levels were highly elevated in the untreated differentiated cell and remained elevated with no change in p21Waf1/Cip1 expression observed after nitroprusside exposure (Figure 4.7C).

4.5 Discussion

NGF-mediated inhibition of apoptotic p53 signaling was particularly interesting because NGF itself strongly induces the nuclear accumulation and transcriptional activity of p53 during neuronal PC12 differentiation. As described here, NGF-mediated activation of p53 does not result in apoptosis within the differentiating PC12 cell, suggesting the presence of an NGF-dependent mechanism through which apoptotic p53 activity was suppressed. These studies demonstrated that NGF inhibited p53-dependent apoptosis induced by supraphysiological levels of NO after nitroprusside treatment. Furthermore, NGF-regulated pathways promoted neuronal cell survival during differentiation even though p53-dependent transcription of apoptotic effector proteins was increased. We therefore conclude that NGF anti-apoptotic activity was not mediated by the direct alteration of p53 transcriptional activity.
Expression of factors affecting mitochondrial integrity in the differentiation process may be important for cell survival. NGF treatment enhanced PC12 cell survival during nitroprusside exposure despite concurrent expression of apoptotic p53 target genes PUMA and Bad, and over the repression of anti-apoptotic survivin expression. NGF alone increased Bcl-2 expression as previously described [51], which might account for some of NGF’s pro-survival effects in PC12 cells although the exact role for Bcl-2 in preventing mitochondrial depolarization remains under study [52, 53]. An alternative mechanism through which apoptosis may be inhibited in the NGF-differentiated cell was by the anti-apoptotic activity of the p53 target gene p21^{Waf1/Cip1}. The cyclin-dependent kinase inhibitor p21^{Waf1/Cip1} has long been recognized as the major mechanism through which p53 regulates G1 cell cycle arrest [54]. Recent findings also suggest that p21^{Waf1/Cip1} acts in a dominant manner to inhibit apoptotic signaling [55], while loss of p21^{Waf1/Cip1} sensitizes cells to apoptosis [56]. As shown here, NGF-activated p53 binds to the p21^{Waf1/Cip1} promoter and activates its transcription. Furthermore, p21^{Waf1/Cip1} protein levels were highly increased with NGF alone as previously described [9, 57]. NGF may therefore prime PC12 cells for survival by increasing levels of the anti-apoptotic factors p21^{Waf1/Cip1} and Bcl-2 during differentiation.

NO signaling occurs through a series of reactions including nitrosylation of the guanylate cyclase heme moiety [58], cysteine thiol S-nitrosylation [59] and tyrosine nitrosation [60]. The outcome of NO-mediated signaling may therefore be dependent upon both target availability and local NO concentration. A multitude of effects accompany NO-mediated signaling, including the inhibition of caspases at low NO levels [61]. In this study, levels of NO from nitroprusside exposure acted as an apoptotic stimulus. We report that activities of multiple caspases were reduced following nitroprusside treatment in differentiated but not naïve PC12 cells. Reduced
caspase activity may therefore be the result of NGF anti-apoptotic activity rather than direct NO-mediated caspase inhibition. In addition, induction of heme oxygenase-1 has been shown to protect against nitrosative stress [62] through decreased cytotoxicity [63]. Our studies demonstrated that p53-regulated apoptotic gene expression upon nitroprusside exposure was enhanced in NGF-differentiated cells, suggesting that the cytotoxic insult generated by NO was similar in differentiated cells and mitotic cells. NGF is also known to activate Akt/PKB signaling in differentiating PC12 cells [13]. While Akt/PKB is necessary for the trophic effects of NGF [64, 65], the role of the Akt/PKB pathway in pro-survival signaling elicited by NGF continues to be studied [66, 67] and may be dependent upon the apoptotic stimulus. Akt/PKB survival signaling may be carried out by increasing Bcl-2 levels during NGF-mediated differentiation [68]. We therefore concluded that NGF-mediated inhibition of apoptosis occurs upstream of mitochondrial pathology upon nitroprusside treatment, and may include multiple protective mechanisms described above.

Changes in neurotrophin levels and their receptors within the nervous system has been suggested in neurodegenerative disorders such as Alzheimer’s [69] and Parkinson’s disease [70]. Because of their protective effect within the nervous system, neurotrophin replacement has been studied as a therapeutic for spinal cord injury and excitotoxicity [71, 72] and has been suggested as a therapeutic intervention for neurodegenerative conditions [73]. The studies described here suggest that the neurotrophin NGF has the ability to protect neuronal cells from an apoptotic stimulus in vitro, and supports the idea of NGF supplementation as a protective in vivo therapeutic agent. However, our studies demonstrated that while NGF may antagonize apoptotic stimuli in PC12 cells, it did not protect against necrotic cell death. Therefore, NGF
supplementation therapy may be of limited value as a broad-spectrum protective agent against cell death *in vivo*.

*In vitro* exposures examining cytotoxicity of endogenous compounds like NO are not always applicable to complex tissue responses *in vivo* such as the central and peripheral nervous system where neurotrophic factors exert powerful receptor-mediated signaling [74]. Cell cycle-dependent effects in immortalized neuronal cell models may significantly alter the toxic response compared to post-mitotic neuronal cells in culture [75, 76]. While NGF-differentiated neuronal cultures may imperfectly model the complexity of NO-mediated events *in vivo*, they point to the importance of using post-mitotic cells for study of neuronal cell death and neurodegeneration.
4.6 References


Figure 4.1. NGF-differentiation of PC12 cells protects from NO-induced toxicity. Cell viability 20 hours following indicated nitroprusside doses was measured using the MTS reagent (n=3). The percent viable cells were determined compared to the untreated control. NGF-treated cells were allowed to differentiate for 7 days prior to exposure. Significance was determined by comparing treated to untreated for either naïve or differentiated cells using one-way ANOVA (n=3, p ≤ .05) followed by Tukey’s HSD post-hoc test and designated by *. Comparison of naïve to differentiated viability for each treatment was performed using Student’s t-test (p ≤ .05) and designated by **. Error bars represent standard deviation.
Figure 4.2. Protein expression and nuclear localization of p53 in naïve and NGF-differentiated PC12 cells following sodium nitroprusside (SNP) exposure.  A.) Immunoblot for total p53 and serine-15 phosphorylated p53 protein after nitroprusside exposure for 20 hours in naïve PC12 cells.  Actin protein levels are presented as loading control.  B.) Immunoblot for p53 protein and serine-15 phosphorylated p53 over time after nitroprusside.  Actin protein is shown as loading control.  C.) Immunoblot for p53 protein in PC12 cells after NGF treatment (50 ng/ml).  Actin is the loading control.  D.) qPCR of chromatin immunoprecipitation-enriched DNA demonstrating p53 occupancy in promoter sequences of target genes in naïve and differentiated PC12 cells.  Statistical analysis was performed using Student’s t-test (n=3, p ≤ .05) and significance is designated by asterisk *.  Error bars represent standard deviation.  E.) Immunoblot for p53 protein in 7-day NGF-differentiated cells following 0.8 mM nitroprusside treatment over time.  Actin is the loading control.  F.) Subcellular p53 localization by indirect p53 immunofluorescence in naïve and 7-day differentiated PC12 cells after 4 hours of 0.8 mM nitroprusside. Image of p53 localization (red) was overlaid that of nucleus (blue) taken at 400X magnification under oil. Nuclear localization of p53 is indicated by pink color.
Figure 4.3. Nitroprusside exposure induces caspase activation in naïve but not differentiated PC12 cells. A.) Activity of caspase 1-10 was measured in naïve PC12 cells after 18 hours of 0.8 mM nitroprusside, compared to untreated cells. Student’s t-test was used to compare activity levels between untreated and nitroprusside-treated (n=6, p ≤ .05) for each measured caspase. B.) Caspase activity was measured as in A for 7-day NGF-differentiated PC12 cells treated with 0.8 mM nitroprusside. C.) Caspase-3 activity was measured at 18h after nitroprusside in naïve PC12 cells. Ultraviolet light (254 nm, 120 mJ/cm², 25 sec.) was used as positive control. Significance (n=6, p≤.05) was determined using one-way ANOVA followed by Tukey’s HSD post-hoc analysis comparing treated activity to untreated control. Error bars represent standard deviation. D.) Caspase-3 activity was measured in 7-day NGF-differentiated PC12 cells and statistical analyses performed as in C.
Figure 4.4. NGF reduces p53-dependent apoptosis from nitroprusside in PC12 cells. A.) RT-PCR showed silencing of p53 mRNA levels in p53sh#3 compared to wild-type PC12 cells. GAPDH is shown as loading control. B.) Immunoblot showing lack of p53 protein following 7-day NGF treatment in p53sh#3 cells compared to wild-type PC12 cells. C.) TUNEL assay for apoptosis detection performed 20 hours following 0.8 mM nitroprusside treatment in naïve wild-type and p53sh#3 PC12 cells. Nuclease-cleaved DNA was detected by FITC labeling (green). Nuclei are stained by DAPI (blue). Overlaid images are shown. D.) TUNEL assay in 7-day differentiated wild-type and p53sh#3 PC12 cells as in C.
Figure 4.5. NGF limits p53-dependent mitochondrial depolarization by nitroprusside exposure. A.) Mitochondrial membrane potential as measured by MitoRedCMXRos dye retention (red) in naïve wild-type and p53sh#3 PC12 cells following 20 hours 0.8 mM nitroprusside. Loss of peri-nuclear red fluorescence indicated mitochondrial membrane depolarization. Nuclei are stained by DAPI (blue). B.) Retention of mitochondrial membrane potential in 7-day NGF-differentiated wild-type and p53sh#3 PC12 cells as measured in A.
Figure 4.6. p53 transcriptional function was similar in naïve and NGF-differentiated PC12 cells after nitroprusside. A.) Mitochondrial membrane potential in naïve PC12 cells was measured in Figure 5. Cells were treated with 1 µM Actinomycin D, 0.8 mM nitroprusside or both for 20 hours. Actinomycin D pretreatment occurred for 1 hour prior to 0.8 mM nitroprusside. C.) RT-PCR measured p53 and transcriptional target mRNA levels. Wild-type and p53sh#3 cells in both naïve and 7-day NGF-differentiated state were treated with 0.8 mM nitroprusside and cellular RNA collected after 0.8 mM nitroprusside treatment. GAPDH is the loading control.
Figure 4.7. Bcl-2 and p21Waf1/Cip1 levels were increased in differentiated cells and unchanged by nitroprusside. A.) Immunoblot of Bcl-2 and Bcl-X(L) levels following 50 ng/mL NGF 2.5S treatment over time. Bcl-X(L) levels are shown as loading control. B.) Immunoblot of Bcl-2 levels in naïve and differentiated cells over time following 0.8 mM nitroprusside. Actin (naïve) and prominent non-specific band (differentiated) are shown as loading control. C.) Immunoblot of p21Waf1/Cip1 levels over time in naïve and differentiated cells following 0.8 mM nitroprusside. SULT 2A1 (naïve) and actin (differentiated) levels are shown as loading controls.
5. CONCLUSION

5.1 Preface

The research presented here demonstrates the regulation of tumor suppressor p53 transcriptional activity by NGF signaling within the PC12 cell line as an experimental model of dopaminergic neuronal differentiation. These studies have resulted in three significant discoveries: 1) NGF potently activates p53 transcriptional regulation of target genes during in vitro neuronal differentiation. My research has identified and extensively validated 14 novel p53-regulated targets of NGF-mediated transcriptional activity, including genes of unique relevance and function in neuronal differentiation. 2) While NGF potentiates p53 DNA binding activity, it is also highly effective in the inhibition of p53-dependent apoptosis induced by NO. NGF-mediated inhibition of apoptosis occurs immediately downstream of p53 transcriptional activity but upstream of mitochondrial pathology and may include the pro-survival role of NGF-inducible factors. 3) p53-dependent neurite outgrowth within the differentiating PC12 cell is mediated in part through direct transcriptional regulation of the secreted morphogen wnt7b. Furthermore, wnt7b expression is sufficient to recover neurite outgrowth upon NGF treatment in the p53-silenced cell.

This research provides evidence that neurotrophic signaling enables p53 to transcriptionally participate in neuronal differentiation and neurite outgrowth. Few examples exist of receptor-mediated, nongenotoxic regulation and sustained activation of p53 activity. Elevation of p53 protein levels, increased transcriptional activity and inhibition of apoptotic signaling by NGF provide an encompassing and comprehensive mechanism through which receptor-mediated neurotrophin signaling may coerce cell cycle exit of neuronal cell precursors.
while maintaining their survival. The putative NGF-p53 axis as described in these studies may be extensible to other cells expressing the TrkA or p75NTR NGF receptors. Finally, identification and characterization of receptor-mediated regulation of p53 transcriptional activity provides a proof-of-principle analysis of an avenue through which p53 by nongenotoxic pharmacological principles that should be amenable to receptor agonists and antagonists.

5.2 p53 stabilization by NGF treatment

NGF potently elevated and maintained p53 levels over a course of at least 7 days (Fig 2.1C). Elevated p53 levels were accompanied by nuclear localization (Fig 4.2F), DNA binding (Fig 4.2D) and transcriptional activation of a reporter gene (Fig 2.1E) and genomic targets (Fig 2.4, 2.5 and 2.6). Maintenance of the differentiated state was dependent upon continued NGF availability [1, 2] and accumulated p53 protein remained active with continued NGF stimulation. Despite markedly elevated p53 protein levels, minimal apoptosis of untreated differentiated cells could be detected by TUNEL analysis (Fig 4.4D). Remarkably, NGF treatment was also sufficient to inhibit p53-dependent apoptosis induced by the NO donor sodium nitroprusside (Fig 4.4C and 4.4D). Therefore, we queried whether the function of p53 within NGF-stimulated PC12 cells may have been related to the process of differentiation itself as opposed to being a mechanism through which cellular apoptosis might be regulated.

The mechanism through which p53 remained elevated over the course of NGF differentiation remains to be explored. In contrast to p53-activating DNA-damaging agents, the time over which p53 protein levels were elevated with NGF stimulation was considerably longer than the time course of p53 stabilization upon genotoxic damage. In particular, p53 protein levels did not return to baseline levels throughout the 7 day period of continuous NGF
stimulation (Fig 2.2). By comparison, p53 levels following 6-hydroxydopamine-induced genotoxic stress in PC12 cells have been reported to return to baseline levels within 6 hours of stimulus [3]. One possibility was that stabilization of p53 through NGF receptor TrkA signaling results in a positive feedback loop, where transactivation of trkA by p53 [4] resulted in maintained or increased receptor availability. While the initiating p53-activating stimulus may have been preserved in this context, it is not clear how p53 protein within the differentiating PC12 cell may be spared from the innate negative feedback loop that exists in the p53-MDM2 regulatory pathway.

Present research demonstrated that NGF-activated p53 protein regulated mdm2 and cyclin G1 gene transcription through increased occupancy of high-affinity binding sites residing within both the first mdm2 intron and in the upstream cyclin G1 promoter (Fig 2.4). As a result, NGF stimulation caused increased transcription of mdm2 and cyclin G1 mRNA in a p53-dependent manner. Increased mdm2 RNA was followed by elevated MDM2 protein levels over the course of at least 7 days. Since both the MDM2 and cyclin G1 proteins negatively regulate p53 intracellular levels, an intermediate event either inhibiting MDM2 function, or protecting p53 protein from MDM2 function, may exist in differentiating PC12 cells.

Because p53 mRNA levels remained in a steady-state over time in PC12 cells following NGF treatment, initial elevation in p53 protein levels may have occurred through a posttranslational mechanism enhancing its stabilization. This was supported by our observation of increased p53 phosphorylation on serine 15 upon NGF treatment (Fig 2.1C). The extended duration of p53 phosphorylation upon NGF exposure suggested either a de-regulation of phosphatase activity or continued kinase activity on p53 protein. The latter is supported by the necessity of continued NGF stimulation [1, 2] in PC12 neuronal differentiation. Experimental
evidence to support phosphatase regulation of p53 activity upon NGF treatment is not available, although evidence in other cell types supports a role for the regulation of p53 transcriptional activity by PP2A subunits [5].

p53 levels may have been stabilized by NGF treatment through protection from MDM2 ubiquitination, which may occur through the interference of p53-MDM2 association. Ras-regulated MAPK activation is reported to occur through NGF signaling in PC12 cells [6] and is known to induce the expression of the p19ARF tumor suppressor [7]. Although no precedent exists for p19ARF induction during NGF-mediated differentiation of PC12 cells, its expression is reportedly responsible for p53 accumulation. p19ARF directly binds to MDM2 and prevents MDM2-mediated ubiquitination of p53 [8]. Alternatively, MDM2 splice variants containing only the RING-finger domain elevate p53 levels by binding to and sequestering full-length MDM2 protein [9] in a manner similar to p19ARF. Expression of the MDM2 RING-finger in MEF cells reduces cell proliferation and is consistent with p53-mediated cell cycle arrest [10].

Furthermore, the MDMX-s variant of the MDM2 family member MDMX encodes a protein containing only the p53-binding domain and has a higher affinity for p53 than MDM2. However, MDMX-s reportedly represses p53 transcriptional activity [11]. MDM2-family splice variants have been described as oncogenic in several normal tissues [12] but little is known about the endogenous regulation and function of these variants. Indeed, preliminary evidence in the conduct of this research suggested the presence of one p53-regulatory MDM2 splice variant during NGF differentiation but continued study would be necessary to confirm this finding.

Ultimately, stabilization of p53 upon NGF treatment appears to be a late response attributed to NGF signaling. Results demonstrated that elevated p53 levels are detectable within 8 hours of NGF exposure but did not become significantly elevated until 48 hours of continued
NGF exposure (Fig 2.1C). Furthermore, p53 transcriptional activity as measured by reporter assay was increased significantly only after 24 hours of NGF treatment (Fig 2.1E). In contrast, the c-fos immediate-early responder to NGF treatment is transcribed within 5 minutes and reaches maximal mRNA levels within 30 minutes [13]. Therefore, the delayed activation of p53 within differentiating PC12 cells suggested that multiple levels of signaling activity must occur prior to p53 activation.

Uncovering the mechanism through which p53 was stabilized and transcriptionally activated upon NGF binding to the TrkA receptor may hold significant value in the treatment of malignancies harboring wild-type p53. Recently, several reports have demonstrated that restoration of p53 function within autochthonous lymphomas [14] and liver carcinomas [15] lead to near-complete tumor regression. The genotoxic nature of many chemotherapeutics agents currently in use elicit a cytotoxic cellular response involving p53 activation. Genotoxic and negative growth effects in normal tissue brought about by chemotherapeutic activation of the p53 pathway can produce serious clinical side effects. The utility of potentially non-genotoxic mechanisms through which p53 might be pharmacologically manipulated represents an attractive therapeutic avenue for further study.

5.3 p53 transcriptional function in NGF-mediated PC12 differentiation

In order to identify the NGF-stimulated role for p53 within PC12 differentiation, we aimed to identify the genomic targets of activated p53 protein. Initially, we selected an approach utilizing the recently developed chromatin immunoprecipitation (ChIP) assay [16] combined with PCR amplification of individual enriched genomic fragments bound to p53. The relative genomic occupancy of p53 was measured after NGF stimulation compared to naïve cells which
served as untreated controls. The ChIP procedure measures occupancy of known genes with sequence-specific PCR primers but is unable to identify novel p53 target genes. For example, increased p53 DNA-binding activity to the p21\textsuperscript{Waf1/Cip1} promoter was validated upon NGF treatment in preliminary ChIP experiments.

Development of a ChIP-cloning protocol [17] ultimately enabled the identification of novel transcription factor targets within the entire genome. The detailed procedure outlined by Weinmann et al. [17] was adapted for use in these experiments with NGF. However significant limitations were experienced which precluded the identification of novel p53-occupied genomic regions in the differentiating PC12 cell. First, either through limitations of assay/antibody specificity or through the non-specific association of p53 protein with genomic DNA, cloned fragments contained large numbers of simple repetitive elements and sequences devoid of p53 consensus binding sites. The enrichment of repetitive sequence DNA may have been attributable to p53 protein binding non-specifically to DNA [18] through its C-terminal domain [19]. Although the genomic locations of cloned fragments were identified, we were unable to identify reproducible, NGF-dependent regions of the PC12 genome occupied by p53 protein. Specific PCR amplification of repetitive DNA was difficult to achieve and therefore binding was not verifiably confirmed in these studies, although p53 occupancy of repetitive DNA may hold biological significance and has been previously described [20]. Second, the isolation and sequencing of individual clones containing single ChIP-enriched fragments proved insufficient for the identification of novel targets when compared to the high enrichment of nonspecific, repetitive clones. Continued progress in ChIP assay development had led to the coupling of traditional ChIP assay with the Serial Analysis of Gene Expression procedure [21]. The coupling of ChIP and SAGE enabled the high-throughput identification of specific ChIP-
enriched DNA fragments [22, 23]. The high-throughput identification of enriched ChIP clones using this approach ultimately provided a robust high-throughput method through which multiple parameters could be observed that are amenable to statistical analyses, namely the relative frequency of clone identification, occupancy position within the genome and generation of consensus binding site sequences, provided tag sequencing was performed to saturation [24].

Using a modified ChIP-SAGE procedure (Fig 2.3A), this study identified the genomic occupancy of p53 following 7 days of NGF stimulation. Up to 7,184 tags, each representative of a single p53-occupied DNA fragment, were sequenced and aligned to the PC12 genome. This number of sequenced tags represented a non-saturating, small fraction of the total number of ChIP enriched fragments, as each assay was performed with several million cells, with each cell containing a multitude of p53 proteins available for DNA binding. Furthermore, genomic extrapolation based on chromosomal sequence analysis for chromosomes 21 and 22 has suggested up to 1,600 p53 binding sites could be predicted within the entire human genome [25], without the inclusion of non-specifically occupied genomic DNA. Although some repetitive elements were again occupied by p53 a sufficiently large number of DNA sequences were identified for statistically sound and biologically relevant genomic targets of p53.

Upon identification of binding sites, ChIP-based validation was used to determine NGF induction of p53 DNA binding for identified genomic regions. 29 unique binding sites were identified where p53 occupancy increased with NGF treatment, and 38 binding sites were identified where p53 DNA occupancy occurred independently of NGF treatment. Expression analysis using three stable PC12 cell lines in which p53 was silenced ultimately identified 14 novel target genes for which NGF treatment resulted in p53-dependent expression increases or decreases. Further, 11 NGF-dependent p53 binding locations were identified for which no
transcript expression changes were seen upon NGF treatment. Ultimately, these experiments demonstrated that, for p53, 1) binding does not necessarily imply gene regulation, 2) p53 binding may occur in regions distant from regulated gene targets, and 3) regulation of gene targets may occur through the binding to enhancer elements outside of prototypical upstream promoter regions.

Of the novel p53 gene targets which were verified to be regulated by NGF treatment, 10 were found to be p53-induced (Fig 2.4) and 5 repressed (Fig 2.5). p53-inducible targets included morphogens (Wnt7b), transcription factors (Tfcp2l4), kinases (Brk, Sdk2, Nme1), redox regulators (Txnl2), phosphatases (Dusp5), genes with unknown functions (Sesn3) and unannotated genes (FLJ32743, LOC366671, LOC300317). Repressed genes included transcriptional cofactors (Pkcbpb15), membrane glycoproteins (Lect1), redox regulators (Pon3), kinases (Trib3) and unannotated genes (LOC362557). There were an additional 11 targets for which defined NGF-dependent p53 occupancy did not correlate with changes in gene expression (Fig 2.6). These targets are involved in multiple cellular pathways, although further study will be required to determine the action of p53 binding and gene regulation upon stimulation with other p53-activating agents.

Prospective p53-regulated genes from NGF treatment in the current study had minimal overlap with a similar ChIP-cloning approach by others with the apoptotic agent 5-fluorouracil to stimulate p53 activity in HCT116 human colorectal carcinoma cells [24]. I speculated that p53 transcriptional regulation of target genes may be dependent upon the stimulus-dependent availability of transcriptional cofactors. These experiments compared expression levels of newly discovered target genes following NGF treatment or DNA damage. Presumably, the stimuli for receptor-mediated induction of p53 with NGF compared that for DNA damage with 5-FU would
both activate p53 by putatively different pathways. We identified 3 targets upon which DNA damage was insufficient for expression or was NGF-regulated in a manner distinct from DNA damage, including wnt7b, lect1 and pkcbpb15 (Fig 2.8). p53 regulation of the secreted morphogen wnt7b was of particular interest, both because of the known developmental roles of wnt family genes and because direct regulation of a wnt gene by p53 had not been previously described.

5.4 p53 regulation of NGF-mediated neurite outgrowth

This research project and others [4, 26, 27] have demonstrated that transcriptionally active p53 is required for neurite outgrowth in differentiating neuronal cells. Multiple techniques have been utilized to demonstrate this role for p53, including overexpression of a non-functional temperature sensitive mutant [4], dominant negative p53 overexpression [26], pharmacological activation [27] and through the engineered expression of anti-p53 shRNA-expressing stable PC12 cell lines driven from a constitutively active U6 promoter (Fig 2.2B). Collectively, these data show that p53 activity contributed to differentiation after NGF treatment through the positive regulation of a signaling process resulting in the axon-like outgrowths characteristic of neuronal cells. As a transcription factor, it was considered likely that p53 controlled the process of neuronal differentiation through the transcriptional regulation of target genes. While both the actin binding protein coronin 1B and GTPase Rab13 were identified as p53-regulated target genes that functioned in the process of neurite outgrowth [26], no other gene targets had been described. Furthermore, we speculated that the significant levels of nuclear p53 protein induced upon NGF treatment regulated target genes in addition to the previously described p21^Waf1/Cip1,
cyclin D1 and cyclin G [28], which suggested that a large number of p53 targets in neuronal differentiation remained undiscovered.

Neurite outgrowth represents a prominent visual representation of neuronal differentiation. Alterations in neurite outgrowth can be a valuable observation in the identification of factors involved in or regulating neuronal differentiation. Through this method, multiple pathways including transcription factors, kinases and GTPases have been discovered whose disruption are ultimately manifested by a lack of neurite outgrowth in NGF-treated PC12 cells. The ERK/MAPK [29] and PI3K/Akt [30] pathways have been recognized as the major contributors to the neurotrophic action of NGF, acting as master regulators of the differentiation and survival program. Ultimately, our identification of a role for p53 within neurite outgrowth in the current research project suggests the presence of novel p53-regulated pathways distinct from effector genes mediating cell cycle arrest and apoptosis. We reasoned that the wnt7b p53 target gene may have contributed to this process because of known developmental functions of Wnt family genes.

The wnt7b protein is a member of the Wnt family of secreted glycoproteins, which are widely recognized for their role in developmental tissue specification, cellular polarity, and cellular renewal [31]. Although it had not previously been described as an expressed gene in PC12 cells [32], we determined that p53 induced wnt7b mRNA expression at low levels following NGF treatment (Fig 2.4, Fig 3.1A) and required up to 36 PCR cycles to be performed on 1 µL cDNA generated from 1 µg of isolated RNA in order to be visualized with ethidium bromide. However, translation of wnt7b transcripts occurred with high efficiency (Fig 3.1B). We further demonstrated that no other major mechanism for wnt7b expression existed in the NGF-differentiating PC12 cell, as p53 silencing was sufficient for the reduction of wnt7b protein
levels (Fig 3.1C). These results illustrate the limits of solely transcript-based approaches for the identification of all expressed proteins within a cell or disease state, as multiple transcriptomic studies have been performed on NGF-treated PC12 cells [33, 34] without detecting the \textit{wnt7b} transcript. Importantly, considerable signal to noise issues of many global transcriptomic approaches limit the detection of rare transcripts with confidence.

The efficient translation of \textit{wnt7b} mRNA was highlighted in efforts to generate stable anti-\textit{wnt7b} shRNA-expressing PC12 cells. Three stable, antibiotic-resistant cell lines were generated in which \textit{wnt7b} transcript levels were suppressed by at least 60\% with NGF treatment compared to the wild-type cell. However, no shRNA-expressing cell line showed reduced levels of \textit{wnt7b} protein upon treatment with NGF. The inability to appreciably reduce \textit{wnt7b} protein levels in shRNA-expressing cell lines did not make them useful for functional studies. Unexpectedly, the PC12 cell line with most efficient silencing reproducibly expressed levels of \textit{wnt7b} protein that were elevated over the wild-type control with NGF treatment. These experiments suggested that high levels of \textit{wnt7b} transcript were not necessary in order to generate sufficient \textit{wnt7b} protein to elicit a physiological effect. Because of the paradoxical and uncertain association between \textit{wnt7b} RNA versus protein expression in these cells, they were not used for further studies despite the remarkable phenotypic changes that were observed upon treatment with NGF.

Western blot of PC12 \textit{wnt7b} protein under reducing conditions in SDS-PAGE yields a single immunoreactive band at 25 kDa. However, the predicted MW of \textit{wnt7b} based on amino acid composition is 46kD suggesting unusual expression properties of \textit{wnt7b}. I investigated the possibility that p53-regulated \textit{wnt7b} was expressed from an alternative transcriptional start site, resulting in a transcript without the first exon. This hypothesis was supported by the presence of
a p53 binding site noted within the first intron. In addition, the calculated molecular weight of a wnt7b protein variant from only the second and third wnt7b exons could produce a gene product similar in size to the observed migration distance. However, experimental evidence (Fig 3.2A, 3.2B) did not support this hypothesis of an alternative p53-regulated start site. Therefore, either the physical properties of wnt7b permit more rapid migration in polyacrylamide gels or the nascent protein may be processed via post-translational cleavage to produce a mature lower molecular weight protein in PC12 cells.

Overexpression studies of wnt7b were conducted to gain insight into its potential effects in PC12 cells, specifically upon neurite growth. Importantly, wnt7b overexpression was sufficient to rescue neurite outgrowth within p53-silenced PC12 cells (Fig 3.4). Furthermore, wnt7b overexpression was also capable of increasing the rate of neurite extension in the wild-type PC12 cell, although it was insufficient to induce neurite extension within the mitotic cell in the absence of NGF. Therefore, wnt7b was likely to cooperate with other NGF-regulated proteins or receptors that were not under the transcriptional control of p53 to enable neurite outgrowth in PC12 differentiation. Since wnt7b has been described to regulate both the canonical [35, 36] and noncanonical [37, 38] signaling pathways, the mechanism leading to PC12 neurite extension warrants further study. Dendritic arborization in developing hippocampal neurons is regulated by wnt7b noncanonical signaling [38], suggesting that this mechanism may also regulate neurite extension within PC12 cells and other neuronal types. Since wnt7b has recently been implicated in branch formation of the developing mammary bud [39], potential involvement of p53 in morphological development of non-neuronal tissue types may be a promising area for further investigation.
Recently, p53-dependent expression of both an actin-binding protein (Coronin 1b) and GTPase (Rab13) were identified as necessary components for neurite outgrowth in neuronal cells [26]. Our discoveries relating p53 neurite extension to *wnt7b* transcriptional regulation were complementary to these findings since the signaling program induced by wnt ligand binding through the non-canonical pathway regulates multiple cytoskeletal and GTPase proteins [40, 41]. Interestingly, the non-canonical Wnt signaling pathway is reportedly involved in GTPase regulation with a demonstrated importance in developmental cellular organization [42] and metastatic tumor cell migration [43, 44]. Gross overexpression of *wnt7b* has been described within a small percentage of breast tumor tissue [45], suggesting its regulation may be through factors other than p53 in certain tissue types.

A significant research question to be answered is why the p53 gene has evolved to regulate not only differentiating neuronal cells but also the developmental progression of a considerable number of other tissues within the body [46]. It is not yet clear how early within development p53 might be involved in the formation of neural tissues, especially in germ layer specification and neural tube closure. The studies described here provide novel information about transcriptional activity of p53 within an *in vitro* differentiation model. *In vivo*, p53 is transcriptionally active in the embryonic murine brain from E11 through at least P1 [47]. While the p53-null mouse model is generally viable, some phenotypic defects in these mice have been noted such as incomplete neural tube closure and resultant exencephaly which occur at a frequency of 8-16% [48, 49]. Furthermore, the developmental defects in p53-null mice are dependent upon both sex and the background strain [48]. Neural defects in mouse models implicate a more fundamental role for p53 function in differentiation and development, yet no
progress has been made in this potentially unique area of p53 biology despite these initial phenotypic observations made nearly two decades ago.

The incomplete penetrance of neural tube defects within p53-null mouse models may suggest compensatory mechanisms may exist for the role of p53 in the ectodermal germ layer from which the neural crest and ultimately neural tube form. While many of the actions of p53 are ascribed to a tumor suppressor function, the other family members p63 and p73 are well recognized regulators of development [50]. Both p63 and p73 are fully capable of the transcriptional activation of some p53 target genes in the absence of p53 [51, 52]. Surprisingly, mutations within the p63 and p73 genes are rare in mutagenic development, suggesting they may not be significant tumor suppressors within many cell types [53]. However, loss of p63 and p73 increases spontaneous tumor development [54] and p73-dependent apoptosis may occur in the absence of p53 following chemotherapeutic treatment [55]. The multiple isoforms of each of the p53 family members further complicates our understanding of their developmental roles [56]. Evidence suggests that p53 family members may have overlapping functions with respect to apoptosis and some developmental processes, with the implication that the overlap and compensation of p53 family members is not complete and may therefore explain some of the neural defects within a relatively small proportion of p53-null mice.

The contribution of p53 to development may be related to its functions within cell cycle control. Neuronal differentiation is dependent upon cell cycle withdrawal [57], with cell cycle-related factors including p27^Kip1 [58], p57^Kip2 [59], Rb [60], p130 [61] and others having functions in differentiation that are complimentary to those in cell cycle regulation. As the genes regulating developmental cell differentiation and those targeted in mutagenesis are often found to be the same, theories regarding tumorigenesis have centered upon deregulated cellular
differentiation as one basis of cancer. Therefore, it is interesting to consider that tumor suppressor functions from genes such as p53 may have evolved to enable proper cellular differentiation and organismal development. While speculative, the distinct parallels between development and tumorigenesis warrant continued inquiry.

5.5 NGF and p53-mediated apoptosis

NGF treatment and differentiation of PC12 cells did not result in increased levels of apoptosis, despite highly elevated p53 levels (Fig 4.4D). However, trophic factor removal from differentiated PC12 cells results in asynchronous apoptosis [62, 63] accompanied by concomitant mitotic reentry [63] of remaining cells. Surprisingly, NGF deprivation-induced apoptosis does not appear to significantly involve p53 signaling [64, 65], but is instead largely dependent upon the activity of E2F transcription factors [66, 67] and cyclin dependent kinase expression [68]. One hypothesis is that cell death may be related to aberrant cell cycle reentry [69]. I questioned whether p53 was capable of inducing apoptosis in PC12 cells in the presence of NGF, particularly because my experiments described a pro-differentiation role for p53 upon NGF treatment in the PC12 cell.

Nitric oxide (NO) is an endogenous inflammatory mediator that may contribute to the pathological effects characteristic of several neurodegenerative conditions [70]. NO is a biologically relevant signaling molecule that was used to study neuronal cell death in PC12 cells. Since NO generation may contribute to neurodegenerative diseases, it was hoped that study of NO cytotoxicity within PC12 cells might simulate biologically relevant pathways involving p53-mediated apoptotic signaling. The NO donor, sodium nitroprusside, was used in order to generate the unstable nitric oxide radical to which PC12 cells were exposed. This compound
was chosen because of its experimental history in in vitro research and extensive use as an antihypertensive in humans. We found that high nitroprusside levels within the range of 500-800 µM were needed in order to generate sufficient NO to induce loss of cell viability (Fig 4.1). This dose-response was a perfectly characteristic example of the truth underlying the words of Paracelsus, where "All things are poison and nothing is without poison; only the dose makes a thing a poison". Since nitric oxide is an endogenous signaling radical, exceeding cellular detoxification capabilities required doses of NO that were beyond the physiological range of normal exposures.

Cytotoxicity within naïve PC12 cells increased rapidly at nitroprusside levels of > 800 µM (Fig 4.1), while loss of viability was elevated in NGF-differentiated cells only in a manner proportional to concentration. This observation suggested potential differences in mechanisms of NO-mediated cell death in mitotically active (naïve) and NGF-differentiated PC12 cells. The cytotoxic mechanism of NO in naïve cells was hypothesized as a pathway-dependent response rather than a proportional increase of cell death that might be expected with increased NO-mediated electrophilic binding of cellular nucleophiles in differentiated cells.

Nitroprusside increased p53 levels in both a dose- and time-dependent manner in naïve cells. However, no detectable changes in p53 levels (Fig 4.2E) or subcellular localization (Fig 4.2F) could be observed in differentiated cells with nitroprusside treatment. The elevation in p53 protein levels and transcriptional activity along with nuclear subcellular localization in NGF-differentiated cells were expected based on previous results (Chapter 2). Interestingly, despite high levels of p53, differentiated cells did not demonstrate caspase activation (Fig 4.3) or mitochondrial membrane depolarization (Fig 4.5) upon nitroprusside exposure as was observed in naïve cells. NO-induced mitochondrial depolarization and apoptosis were p53-dependent in
 naïve cells (Fig 4.4C, 4.5A). However, NGF differentiation potently inhibited each of these processes in differentiated cells (Fig 4.4D, 4.5B). These results demonstrate that the usually high levels of p53 expression during PC12 differentiation do not necessarily translate into apoptosis, contrary to some previously held concepts in the field [71].

Dependence upon p53 transcriptional activity for NO-induced apoptosis in naïve cells (Fig 4.6A) led us to investigate expression of several known targets of active p53 (Fig 4.6B). Surprisingly, we demonstrated that no difference of p53-dependent expression in the spectrum of cell cycle arrest or apoptotic genes existed between naïve and differentiated cells upon NO exposure. High expression of PUMA within differentiated cells did not result in apoptosis, although previous reports have suggested that PUMA expression can be both sufficient and necessary for apoptosis within neuronal cells [72, 73]. These findings provided novel evidence for NGF pro-survival signaling in PC12 cells that potently superseded p53-dependent apoptosis pathways. Furthermore, these results also determined that NGF-mediated activation of p53 was not sufficient to preclude p53 involvement in apoptotic transcriptional activity.

Studies described in Chapter 4 focused specifically on apoptotic cells. However, the NO generated from 800 µM nitroprusside was likely sufficient to induce concomitant necrosis. MTS cell viability assay and mitochondrial membrane potential, which both measure mitochondrial functions, can reflect both necrosis and apoptosis. Inclusion of the nuclear fluorophore DAPI in the MitoRed experiments could discriminate between apoptosis and necrosis by permitting the visualization of shrunken pyknotic nuclei characteristic of apoptosis along with MitoRed determination of mitochondrial membrane potential within the same cell. Mixed cell-death mechanisms have been described following nitroprusside treatment [74] and could be expected for studies utilizing other NO donors [75].
There are inherent difficulties in extrapolating the meaning of *in vitro* experiments to the complexities of clinical diseases such as Parkinsonism. Despite limitations of the current study, toxicological analysis of NO-induced apoptosis in differentiated PC12 cells was used to effectively explore an interesting biochemical function of NGF in its regulation and inhibition of apoptotic cell death within the differentiated neuronal cell.

The impact of supraphysiological levels of NO has not been well established in an etiology of Parkinsonism that might occur during long-term inflammation of the central nervous system during gliosis. While the contribution of glial-derived immunologic stress in the Parkinsonian brain remains a well-studied potential risk factor [76], the pathophysiological effect of neuroinflammation as it relates to Parkinson’s disease is not easily categorized as overt necrosis or mixed necrosis/apoptosis of neurons within the substantia nigra [77]. However, NO exposure does accurately model the mitochondrial pathology characteristic of several neurodegenerative conditions [78]. Ultimately, I have attempted to study cellular components and responses of neuronal cells under the influence of NGF that may share features with complex neurodegenerative diseases such as Parkinsonism.

5.6 Concluding Remarks

In summary, these results have demonstrated that p53 is transcriptionally activated by NGF signaling and is involved in the differentiation of neuronal PC12 cells. p53-regulated factors such as \( p21^{\text{Waf1/Cip1}} \) were effectors of cell cycle arrest while \( \text{wnt7b} \) directly influenced neurite outgrowth upon NGF stimulation. Furthermore, while NGF potently activates p53 transcriptional activity, NGF produced potent pro-survival signaling upon exposure to NO. Pro-survival signaling upon NGF-induced neuronal differentiation was sufficient to directly supplant
p53-dependent transcriptional activation of apoptotic target genes. In conclusion, NGF receptor-mediated signaling is both capable of inducing p53 transcriptional signaling while concomitantly inhibiting p53-dependent apoptosis.
5.7 References


