# NRIF IS AN ESSENTIAL COMPONENT OF APOPTOTIC SIGNALING BY THE P75 NEUROTROPHIN RECEPTOR

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# CHAPTER I

# INTRODUCTION TO NEUROTROPHIN SIGNALING

## The neurotrophins

The neurotrophins are a family of proteins that are essential for the development of the vertebrate nervous system. These diffusible growth factors are synthesized and released by neurons and target tissues. The first identified neurotrophin was Nerve Growth Factor (NGF) (Levi-Montalcini, 1966), which was thought to be the only neurotrophic factor. However, it was subsequently discovered that few neurons in the central nervous system (CNS) and in the nodose ganglia were NGF-responsive. This led to the isolation of Brain-derived neurotrophic factor (BDNF) (Hofer and Barde, 1988) and subsequently Neurotrophin-3 (NT-3) and Neurotrophin 4/5 (NT-4/5) (Hohn et al., 1990; Berkemeier et al., 1991). The finding of multiple neurotrophins indicated that the fate of specific neuronal populations can be regulated by specific growth factors.

The neurotrophins are initially synthesized as precursors, or proneurotrophins, which are cleaved to produce the mature proteins. This intracellular cleavage is executed by the endopeptidase furin or proconvertases at a highly conserved dibasic amino acid cleavage site to release the carboxy-terminal mature proteins. The processed proteins are approximately 12 kDa in size and form stable, non-covalent dimers that are expressed at low levels during development.

The overall levels of neurotrophins regulate the balance between neuronal survival and apoptosis during development. Mice that have the neurotrophin genes

deleted die during the first few weeks after birth. Heterozygotes are viable but show other deficits. For example, *ngf* +/- mice have loss of neurons in the peripheral nervous system (PNS) (Crowley et al., 1994) and memory acquisition and retention deficits (Chen et al., 1997). *bdnf* +/- mice also have fewer PNS neurons and display enhanced aggressiveness, hyperactivity and hyperphagia (Lyons et al., 1999; Kernie et al., 2000; Rios et al., 2001). Finally, *nt-3* +/- mice have cardiovascular defects (Donovan et al., 1996) as well as neuronal loss in the PNS (Ernfors et al., 1994). Thus, these growth factors have critical roles in maintaining appropriate neuron numbers as well as modulating other aspects of development.

**The neurotrophin receptor system**

Neurotrophins bind two classes of transmembrane receptors, the Trks and the p75 neurotrophin receptor. The use of this dual receptor system allows diverse signals upon ligand binding to occur, such as cell survival through the Trk receptors or cell death through p75. The Trks and p75 can also directly interact upon neurotrophin binding to allow further fine-tuning of the downstream signal.

The Trks (tropomyosin-related kinase) are members of a family of receptor tyrosine kinases, including TrkA, TrkB, and TrkC. TrkA was first identified as a protooncogene from gene transfer assays of a carcinoma. When cloned, it consisted of the first seven of eight exons of nonmuscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase (Coulier et al., 1990). Due in part to the overlapping expression pattern of TrkA and NGF, it was hypothesized and subsequently proven that TrkA was the receptor for NGF. The *trkB* and *trkC* genes were identified due

to their high homology to *trkA*. The tyrosine kinase domains are the most highly conserved among these receptors (~80% amino acid identity) with the extracellular domains only having ~30% identity.

TrkA was originally termed the NGF receptor, due to its preference in binding this neurotrophin. However, it is now known that NT-3 can also be a less preferred ligand for TrkA. TrkB prefers BDNF and NT-4/5 while TrkC binds NT-3 preferentially, with less affinity for BDNF and NT-4/5. Therefore, while the Trk receptors bind the neurotrophins with specificity, these specificities are not absolute.

All three Trk receptors have splice variants that result in deletions in the extracellular domains or truncations of the intracellular domains, including the kinase domain (Shelton et al., 1995). Splice variants of TrkB and TrkC lacking the tyrosine kinase domain are expressed at high levels in the adult brain (Klein et al., 1990). The TrkB isoforms lacking the kinase domain, T1 and T2, are upregulated post-natally and predominate over full-length TrkB in mature brain (Fryer et al., 1996). They are also expressed in nonneuronal cells that do not express TrkB. While the biological roles of these variants are unclear, they are often suggested to be dominant negative modulators of Trk signaling. In support of this idea, the TrkB isoforms bind BDNF and may reduce its availability for full-length receptors (Biffo et al., 1995).

In contrast to the specificity of the Trk receptors, each of the neurotrophins binds p75 with similar affinity (Figure 1). P75 was the first member to be cloned of the TNF receptor superfamily, which includes both TNF receptors, Fas, CD40, and ~25 other members (Smith et al., 1994). This family is characterized by cysteine rich repeats in the

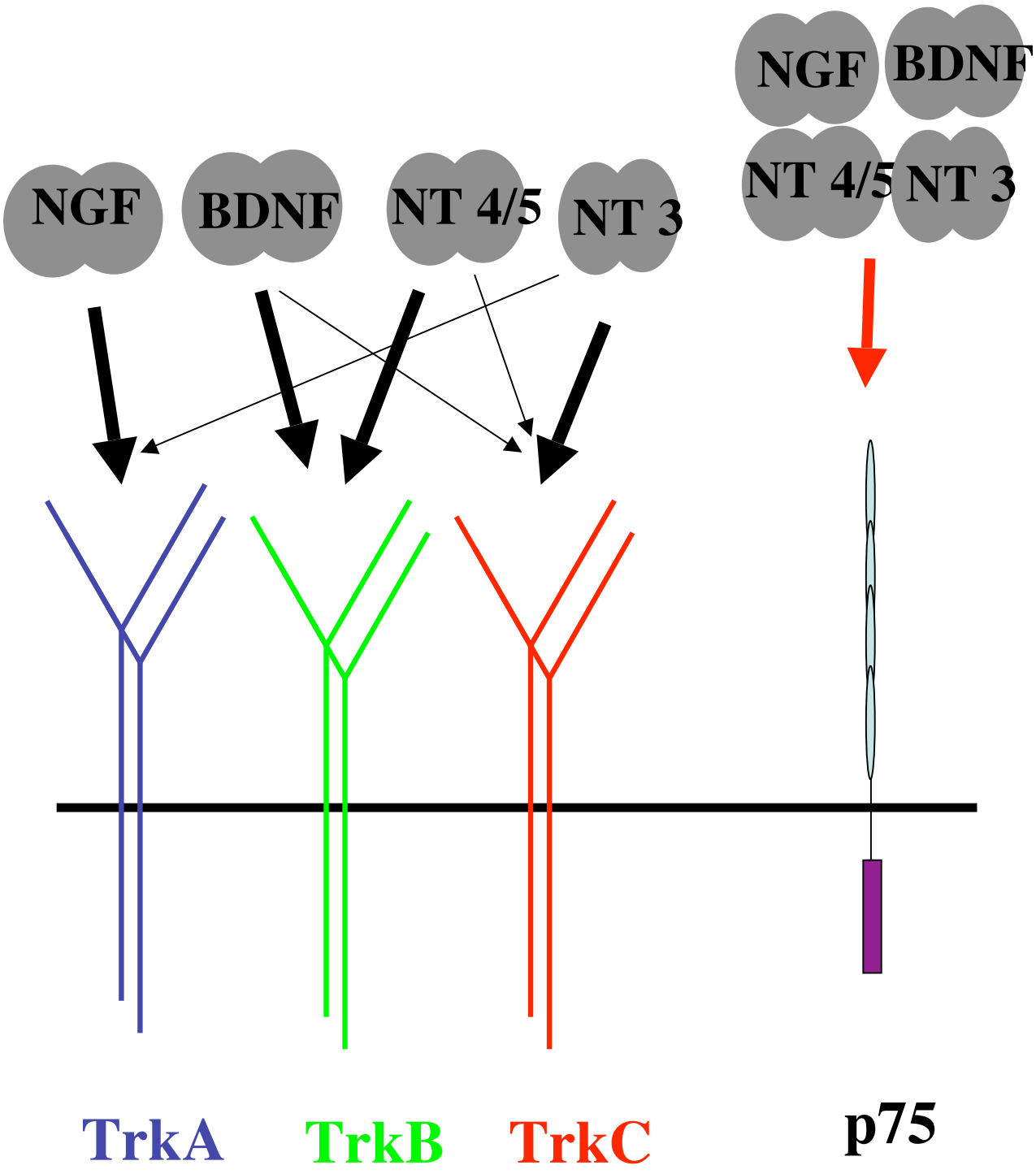



**Figure 1. The neurotrophins and their receptor binding specificity**. While each of the Trks bind a preferred neurotrophin, the specificity is not absolute. In contrast, each neurotrophin binds with similar affinity to the p75 neurotrophin receptor.

extracellular domain and a number of receptors in this family can induce cell death through an intracellular domain, termed the Death Domain (Feinstein et al., 1995). P75 also has a Death Domain (Liepinsh et al., 1997) and can initiate apoptosis through caspase activation (Gu et al., 1999; Wang et al., 2001; Troy et al., 2002). Unlike other TNF receptors, p75-mediated apoptosis is caspase-8 independent (Gu et al., 1999). None of these receptors possess any intrinsic catalytic activity and signaling likely occurs through association with, or dissociation from, cytoplasmic interacting proteins.

Most receptors of the family bind to ligands of the TNF-α family, which are trimeric and signaling occurs through a trimeric receptor complex. However, the crystal structure of mature NGF bound to p75 has been recently solved, demonstrating that the dimeric NGF binds a single chain of the receptor in a 2:1 stoichiometry (He and Garcia, 2004). The allosteric change upon dimeric ligand binding actually prevents the formation of p75 dimers. It should be noted that this structure was generated using only the extracellular domain of p75 and it remains possible that use of the full-length receptor would provide a different ligand binding stoichiometry.

P75 has been reported to bind ligands other than the neurotrophins, including cysteine-rich neurotrophic factor (CRNF), a protein isolated from the snail *Lymnaea stagnalis* with no sequence similarity to the neurotrophins (Fainzilber et al., 1996). Also, the rabies virus glycoprotein has been reported to bind p75 (Tuffereau et al., 1998). It is unclear what advantage these ligands binding to p75 provides and if there is any *in vivo* relevance to these interactions.

p75 homologs have also been described. *Xenopus* Neurotrophin Receptor Homolog 1 (NRH1, also called fullback) and rat NRH2 (also called PLAIDD) are two

novel genes that encode proteins with substantial sequence homology to p75 in the cytoplasmic and transmembrane domains. While there are no species where NRH1 and NRH2 are present together, NRH1 coexists with p75 in fish, amphibians and birds but is absent in mammals, where only NRH2 exists. However, NRH2 does not have the cysteine rich repeats that form the p75 ligand-binding domain. The simplest scenario for the evolution of this gene family is that a gene duplication event early in vertebrate development gave rise to NRH1 and p75 and a deletion mutation of NRH1 generated NRH2 at the point of avian and mammalian divergence. Studies are ongoing to determine the relevance of these homologs in p75 signaling but one study found that NRH2 interacts with TrkA and this leads to high affinity binding of NGF (Murray et al., 2004). In addition, it can be envisioned that NRH2 might also interact with p75 intracellular interacting proteins and either amplify or antagonize p75 downstream signaling.

**Outcomes of Trk signaling**

Neurotrophin binding to the Trk receptors leads to receptor tyrosine phosphorylation, which activates pathways leading to neuronal differentiation and the inhibition of apoptosis. There are three main signaling cascades that are regulated by ligand binding to the Trks: Ras/extracellular regulated kinase (ERK) pathway, phosphatidyl inositol-3 kinase (PI3 kinase) pathway, and phospholipase C-□(PLC- □) pathway (Figure 2).

The Ras/ERK pathway is activated by formation of adaptor molecule complexes with the Trks. Phosphorylated Src-homology protein (Shc) binds to the Grb2-SOS

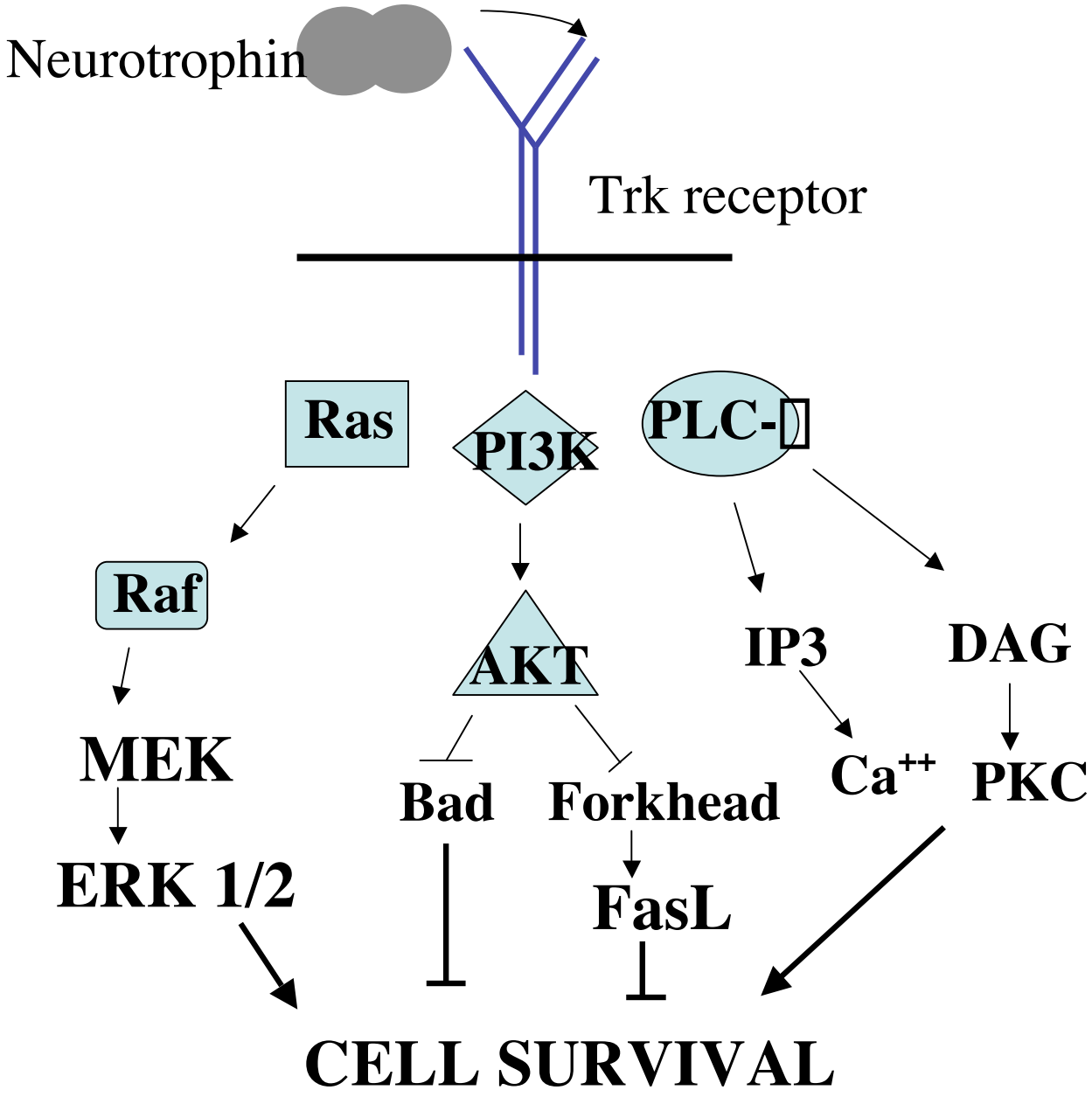



**Figure 2. Trk signaling pathways regulating neuronal survival**. Neurotrophin binding to Trks stimulates receptor tyrosine phosphorylation, activating the ERK, PI3K, and PLC-pathways. These pathways ultimately regulate cell survival.

complex which activates Ras and the downstream kinases MEK and ERK 1/2. This pathway is involved in synaptic plasticity, long-term potentiation, and the survival of neurons (Grewal et al., 1999). While NGF induces a sustained activation of MAPK in sympathetic neurons and PC12 cells, inhibition of MEK has minimal effects in NGF-dependent survival (Mazzoni et al., 1999). Thus, MEK/ERK activation can promote survival, but in these same cells, MEK activity is sufficient, but not necessary (Xia et al., 1995; Klesse et al., 1999; Korhonen et al., 1999; Mazzoni et al., 1999). Therefore, it has been suggested that the MEK/ERK survival pathway may protect neurons from death due to injury or toxicity, rather than trophic factor withdrawal. To this end, activation of the MEK/ERK pathway protects sympathetic neurons from cytosine arabinoside induced death (Anderson and Tolkovsky, 1999), cerebellar neurons from apoptosis due to oxidative stress (Skaper et al., 1998), and retinal ganglion cells from death after axotomy (Shen et al., 1999). However, the need for this pathway may be cell type specific; activation of the Erk pathway is required for the survival of cerebellar granule neurons because pharmacologically inhibiting this pathway reduced the TrkB-mediated survival of these neurons (Bonni et al., 1999). Finally, the MEK/ERK pathway is thought to be key for neurite outgrowth (Stephens et al., 1994).

The PI3 kinase pathway is activated by Trks interacting with Gab-1, an adaptor protein which binds and stimulates PI3K, as well as Ras. Ras directly interacts with PI3K in PC12 cells and Ras inhibition suppressed NGF-mediated PI3K activity (Rodriguez-Viciana et al., 1994). PI3K is implicated in neuronal survival via activation of the kinase Akt (also known as protein kinase B, PKB). Akt phosphorylates Bad (Datta et al., 1997), pro-caspase-9 (Cardone et al., 1998), and the transcription factor Forkhead

(Brunet et al., 1999). Phosphorylation of Bad, the proapoptotic member of the Bcl-2 family, induces association with 14-3-3 and prevents the association of Bad with the antiapoptotic family members, Bcl-2 and Bcl-Xl (Datta et al., 1997). The relevance of Bad phosphorylation and its role in neurotrophic factor mediated survival is debated, due in part to the finding that neurons from *bad* knockout mice do not have changes in apoptosis levels (Shindler et al., 1998). Akt inhibited proteolytic cleavage and activation of the pro-caspase-9 *in vitro* by overexpression experiments, suggesting a role for this phosphorylation event in growth factor mediated neuronal survival. However, there is no conserved Akt phosphorylation site in nonhuman pro-caspase-9 and the lack of reports describing Akt phosphorylation of endogenous pro-caspase-9 do not support a role for this phosphorylation in neuronal survival (Fujita et al., 1999). Forkhead is perhaps the best candidate for Akt in neurons. Data indicate that Forkhead stimulates apoptosis, in part, by increasing the levels of pro-death ligands such as Fas (Brunet et al., 1999).

The PI3K/Akt pathway may also suppress apoptosis indirectly, perhaps by increasing inhibitor of apoptosis proteins (IAPs), Bcl-2, or Bcl-Xl. Finally, this pathway also blocks the neuronal cell death pathway, JNK-p53 (described below).

The final signaling pathway induced by neurotrophin binding to the Trks is PLC-γ. This pathway plays an important role in neurotrophin release (Canossa et al., 1997) and synaptic plasticity (Minichiello et al., 2002). PLC-γ associates with Trks and regulates intracellular $Ca^{++}$ levels and protein kinase C activity via PIP2 cleavage to diacylglycerol and IP3. Activation of protein kinase C is involved in neurite outgrowth and activation of the ERK cascade (Corbit et al., 1999). However, mutation of the PLC-γ docking site (phosphorylated Y785) does not block neurite outgrowth, suggesting that

other Trk-mediated signals compensate to promote neuritogenesis. The PLC-γ pathway also plays a role in neuronal survival as pharmacologic activation of protein kinase C in sympathetic neurons prevents cell death after NGF withdrawal (Pierchala et al., 2004).

Neurotrophins are often localized in tissues at a substantial distance from the cell soma of the innervating neuron. While neurotrophins do have critical roles in signaling at the axon terminals, it is known that cell soma and nuclear signaling are essential to promote the survival and differentiation effects of these growth factors. Therefore, many studies have aimed at identifying the mechanisms of Trk retrograde signal transduction. Proposed mechanisms for retrograde signaling include propagation of calcium/phosphorylation waves along the axon (Senger and Campenot, 1997; MacInnis and Campenot, 2002), transport of activated signaling molecules along the axon (Kuruvilla et al., 2000; Watson et al., 2001), or transport of activated neurotrophin-Trk complexes (Bhattacharyya et al., 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999). The third possibility is referred to as the signaling endosome hypothesis and is the most favored. In this case, NGF-bound TrkA is internalized via clathrin coated pits (Grimes et al., 1997; Howe et al., 2001). Trk receptors and dynein directly interact and this provides a potential mechanism for trafficking of these signaling endosomes.

Trk receptor neurotrophin signaling uses multiple downstream pathways to amplify and diversify its signals. These signals are largely positive, such as mediating cell growth and differentiation. An open question remains as to how these different pathways are integrated in a given neuronal population.

**Neurotrophin signaling via p75**

P75 was originally considered to be a non-signaling receptor. Rather, it was believed to be a co-receptor for the Trks. In fact, upon neurotrophin addition, p75 and the Trks directly interact and form high affinity neurotrophin binding sites (Hempstead et al., 1991). The result is enhanced Trk signaling with the outcome of enhanced survival. The ratio of receptors is critical in dictating numbers of surviving cells and Trk-p75 interactions provide greater discrimination between different neurotrophins. For example, in sympathetic neurons, p75 regulates the ability of NT-3 to provide trophic support *in vivo* and dictates how these neurons respond to NT-3 activation of TrkA (Brennan et al., 1999). Also, during sympathetic neuron development, p75 expression enables these neurons to switch sensitivity from NT-3 to NGF (Kuruvilla et al., 2004). Finally, p75 binds to the proneurotrophins as well as the neurotrophins and signals in variety of receptor complexes, as well as on its own (Figure 3).

The neurotrophins are synthesized as pro-forms which are proteolytically cleaved to produce the mature neurotrophin. However, in some tissues, it is hypothesized that a portion of proNGF remains uncleaved, allowing for speculation that these proneurotrophins have biological functions (Beattie et al., 2002). Indeed, proNGF binds p75 with a higher affinity than mature NGF and is a potent inducer of p75-dependent cell death in sympathetic neurons (Lee et al., 2001), oligodendrocytes (Beattie et al., 2002), and a vascular smooth muscle cell line (Lee et al., 2001). ProNGF does not bind TrkA and is therefore thought to be an apoptotic ligand that is specific for p75.

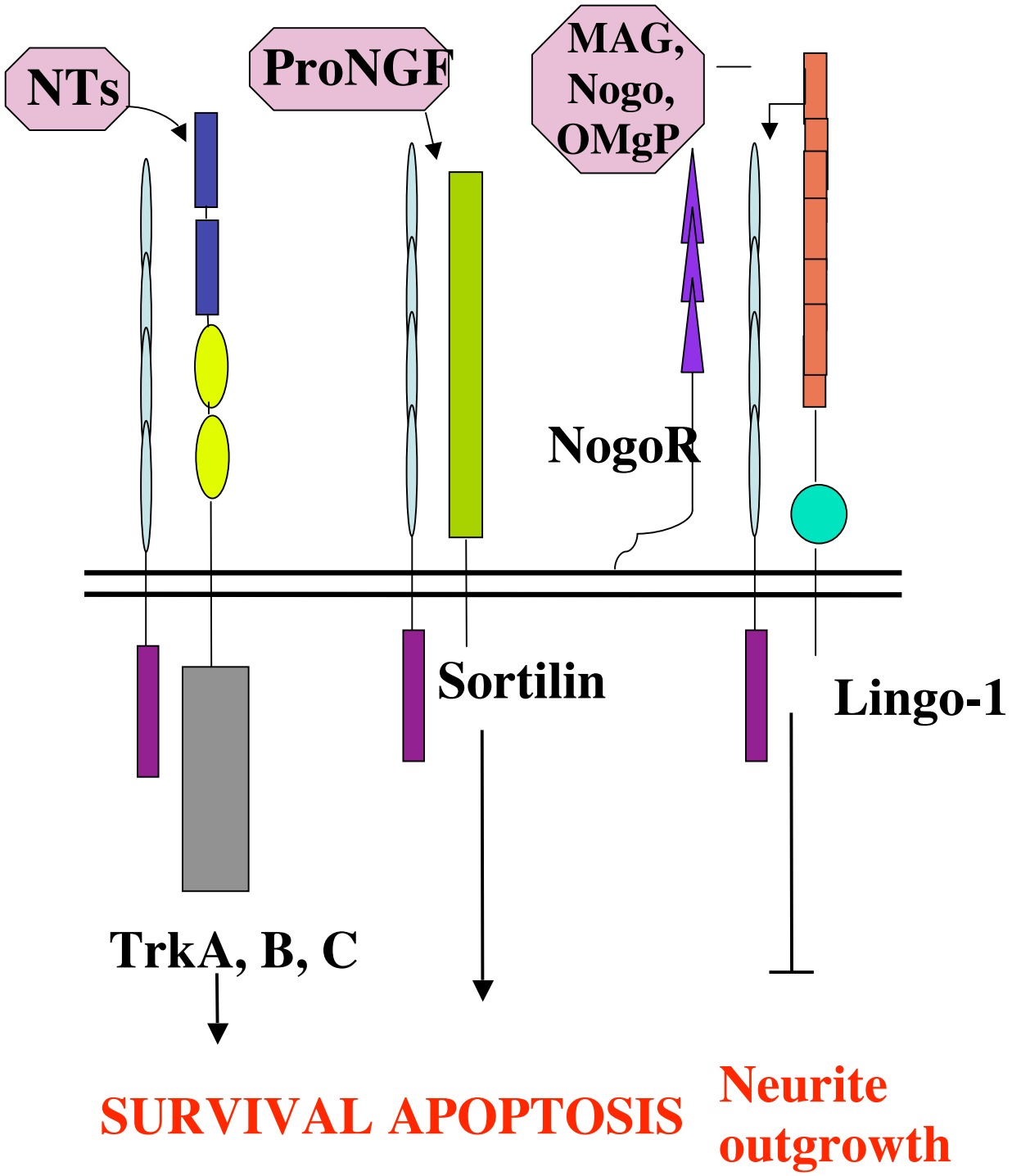



**Figure 3. p75 signaling complexes**. P75 interacts with the Trk receptors to enhance ligand-receptor specificity and Trk signaling. In conjunction with Sortilin, p75 binds proNGF and elicits cell death. When complexed with NogoR and Lingo-1, p75 mediates RhoA activation and thus inhibition of neurite outgrowth.

In binding proNGF, p75 forms yet another receptor complex with Sortilin, the neurotensin 3 receptor (Nykjaer et al., 2004). In addition, the NGF prodomain binds directly to the extracellular domain of Sortilin and forms a complex with p75 at the cell surface (Nykjaer et al., 2004). Both receptors appear to be required for proNGF to induce cell death because proNGF does not induce death in Schwann cells, which do not express endogenous Sortilin. However, after ectopic expression of Sortilin, these cells are sensitive to proNGF-induced apoptosis (Nykjaer et al., 2004).

Sortilin is type I transmembrane protein that is widely expressed but is abundant in the CNS during development and adulthood. It is a member of theVPS10 family, so named for the yeast gene that traffics cargo from the trans-Golgi network (TGN) to the vacuole. In mammalian cells, Sortilin is important for TGN to endosome and TGN to lysosome trafficking events and it is unknown whether p75 and Sortilin affect each other's subcellular distribution. As Sortilin binds proNGF in the absence of p75, it is possible that Sortilin functions not only as a cell surface co-receptor but also as a regulator of intracellular movement of newly synthesized proneurotrophins.

In addition to interacting with the Trks and Sortilin, p75 has also been identified as a member of a tripartite complex consisting of the Nogo receptor and Lingo-1 (Wang et al., 2002; Mi et al., 2004). In the injured CNS, myelin-based growth inhibitors, including myelin-associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMgP) and Nogo-66 bind the Nogo receptor and prevent neurite outgrowth by activation of RhoA. RhoA is a member of a family of small GTPases involved in neuronal migration, polarity, axon growth and guidance, dendrite plasticity, and synapse formation (Luo, 2000). The active, GTP bound form of RhoA binds specifically to the

intracellular domain of p75 (Yamashita et al., 1999). Unliganded p75 activates RhoA, leading to neurite outgrowth inhibition, and neurotrophin binding reversed this activation (Yamashita et al., 1999). When NGF binds the receptor, GTP is hydrolyzed and RhoA is inactivated. In the ciliary ganglia, which express p75 but not TrkA, this results in increased neurite length and cytoskeletal rearrangement. In contrast, the myelin inhibitory proteins bind the glycosylphosphatidylinositol (GPI)-linked Nogo receptor, which has no intracellular signaling capability, and interact with p75 to activate RhoA. The Nogo receptor and p75 interact through the extracellular domains of the two proteins and the association produces a repulsive effect on axonal growth (Wang et al., 2002). However, when expressed in fibroblasts, the Nogo receptor and p75 were not sufficient to reconstitute RhoA activation in response to MAG or Nogo-66. Most recently, Lingo-1 was identified as an essential component of the Nogo receptor-p75 complex (Mi et al., 2004). Lingo-1 interacts with the extracellular domains of both p75 and the Nogo receptor and can be immunoprecipitated with either receptor (Mi et al., 2004). Coexpression of all three proteins was sufficient for RhoA activation in COS cells, indicating that Lingo-1 is crucial for myelin-based growth inhibitor signaling.

p75 can also elicit downstream signals in the absence of other receptors. In some of the earliest data, it was discovered that p75 mediates sphingomyelin hydrolysis and production of ceramide following ligand binding (Dobrowsky et al., 1994). p75 also interacts with caveolin, and caveolae are presumably the site of neurotrophin-induced sphingomyelin hydrolysis (Bilderback et al., 1997). Because ceramide causes the activation of the transcription factor NF-$\kappa$B in some systems (Schutze et al., 1995), it suggested p75 may activate this pathway as well. NF-$\kappa$B is perhaps best known for its

role in the immune system and it is induced by members of the TNF receptor family. In Schwann cells, which express p75 but no catalytic Trk receptors, the addition of NGF resulted in NF-κB activation (Carter et al., 1996). Activation of NF-κB promotes cell survival in several contexts (Karin, 1998) and p75 signaling through this transcription factor is also antiapoptotic. NGF binding to p75 promotes the survival of a Schwannoma cell line (Gentry et al., 2000), sensory neurons (Hamanoue et al., 1999), and cultured Schwann cells (Khursigara et al., 1999), which is mediated by activation of NF-κB. In addition, NGF protects cortical neurons, which express p75 but not TrkA, against glutamate-induced cytotoxicity (Shimohama et al., 1993; Kume et al., 2000). p75 signaling promotes the survival of neocortical subplate neurons during development (DeFreitas et al., 2001); however, it is unclear whether NF-κB is involved in these effects. In sum, these data indicate that p75 is capable of mediating cell survival in the absence of the Trk receptors.

In the PNS, NF-κB activity is required for peripheral myelin formation (Nickols et al., 2003). As p75 is also required for proper peripheral myelination (Cosgaya et al., 2002), it is currently being investigated whether p75 activation leads to the induction of NF-κB, which in turn allows for myelination to occur.

In addition to its role in enhancing Trk survival signaling and activating NF-κB dependent survival pathways, p75 has a clearly established role in cell death. For example, sympathetic neurons of superior cervical ganglia (SCG) undergo a period of programmed cell death during ontogenesis and NGF, supplied by the tissues innervated, prevents the loss of these neurons through binding to a p75-TrkA complex (Chao and Hempstead, 1995). In contrast, specific activation of p75 (Bamji et al., 1998) or a p75-

Sortilin complex (Nykjaer et al., 2004) induces apoptosis in the neurons. In addition, genetic deletion of *p75* prevents the normal period of cell death in the developing SCG (Bamji et al., 1998; Brennan et al., 1999). Analysis of neurons of the SCG of *p75*-/-, *TrkA*-/- double knockout mice demonstrated that although *TrkA*-/- mice exhibit a dramatic loss in these neurons, codeletion of *p75* substantially rescued the number of neurons in the SCGs (Majdan et al., 2001). Moreover, in *bndf* -/- mice, there is hyperinnervation of the pineal gland by sympathetic neurons (Kohn et al., 1999). These neuronal projections reach this target at a time when only BDNF is being expressed. Since p75 is the only receptor expressed on these neurons that binds BDNF, it was suggested that normal pruning of these sympathetic neurons occurs through BDNF-dependent activation of p75. Together, these data demonstrate the key role of this receptor in regulating the survival of this neuronal population.

P75 also induces apoptosis in Schwann cells (Soilu-Hanninen et al., 1999; Syroid et al., 2000), oligodendrocytes (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999), cholinergic neurons in the basal forebrain (Naumann et al., 2002), and neuronal precursors in the developing retina and spinal cord (Frade and Barde, 1999). This receptor is also induced by a wide variety of insults in both the peripheral and central nervous system (Dobrowsky and Carter, 2000) and in several models it is responsible for the resulting cell death. For example, p75 mediates loss of hippocampal neurons following seizure (Troy et al., 2002), cortical neurons after severance of the cortico-spinal tract (Harrington et al., 2004) and oligodendrocytes in response to spinal cord lesions (Beattie et al., 2002). These findings suggest a role for p75 in injury responses in the nervous system. There is also speculation that this receptor may be

involved in neurodegenerative diseases because patients with multiple sclerosis (Dowling et al., 1999), ALS (Lowry et al., 2001), and Alzheimer's disease (Salehi et al., 2000a; Mufson et al., 2002) have altered p75 expression in the areas of neurodegeneration.

The stress kinase c-Jun N-terminal kinase, JNK, may also play a role in mediating this apoptotic signal. Neurotrophin activation of JNK through p75 correlates with the induction of cell death (Casaccia-Bonnefil et al., 1996) and inhibition of the kinase prevents receptor-mediated apoptosis (Yoon et al., 1998; Harrington et al., 2002; Yeiser et al., 2004). Interestingly, c-Jun, the downstream target of the kinase, is not required for the receptor to activate apoptosis (Palmada et al., 2002); however, other JNK substrates have been implicated in the p75 death pathway, including the pro-apoptotic Bcl-2 family member Bad (Bhakar et al., 2003) and the tumor suppressor p53 (Aloyz et al., 1998). P75-mediated cell death also involves caspase activation (Gu et al., 1999); however, the details linking these pathways are still unclear.

It should also be noted that p75 can induce cell death in the absence of ligand. These studies were performed in various cell culture paradigms where overexpression of p75 facilitated apoptosis. In one study, addition of NGF blocked this effect (Rabizadeh et al., 1993). However, these studies relied on supraphysiological levels of receptor and thus it is unclear whether unliganded p75 signals death *in vivo*.

A logical question is why neurotrophin signaling would elicit cell death. It is estimated that during development, approximately 50% of the neurons undergo programmed cell death. It is not fully understood what advantage this method of development provides, but it is clear that a functional nervous system cannot be created without the controlled pruning of massive numbers of immature neurons. This is

demonstrated in mice lacking *caspase-3*, as these mice die embryonically due to excessive exencephaly (Leonard et al., 2002). Without this critical mediator of neuronal apoptosis, there is an accumulation of unnecessary neurons, preventing the establishment of a functional nervous system.

The actions of the neurotrophins help maintain the homeostasis in the developing nervous system. A developing neuron will send projections out to various target tissues, each of which will be secreting a particular neurotrophin. Some of these projections will reach appropriate targets while those that fail to reach their targets will be eliminated through an apoptotic program that is initiated by a lack of Trk signaling. However, recent studies indicate some neurons that reach an inappropriate target or an appropriate target at the wrong time undergo an apoptotic cascade that is dependent on the specific activation of p75 (Kohn et al., 1999; Majdan et al., 2001).

The regulation of opposing pathways of survival and apoptosis from p75 is not well understood and is currently being investigated. It is hypothesized that a better understanding of the interaction of cytoplasmic proteins with the receptor's intracellular domain may lend insight to the dichotomy of p75 signaling.

**p75 interacting proteins**

In order to understand p75 function and regulation of downstream signaling, many cytosolic proteins have been identified that interact with the receptor's intracellular domain (Figure 4). These proteins have been largely isolated using yeast two-hybrid screens and include NRAGE, NADE, TRAF proteins, SC-1, and NRIF 1 and 2.

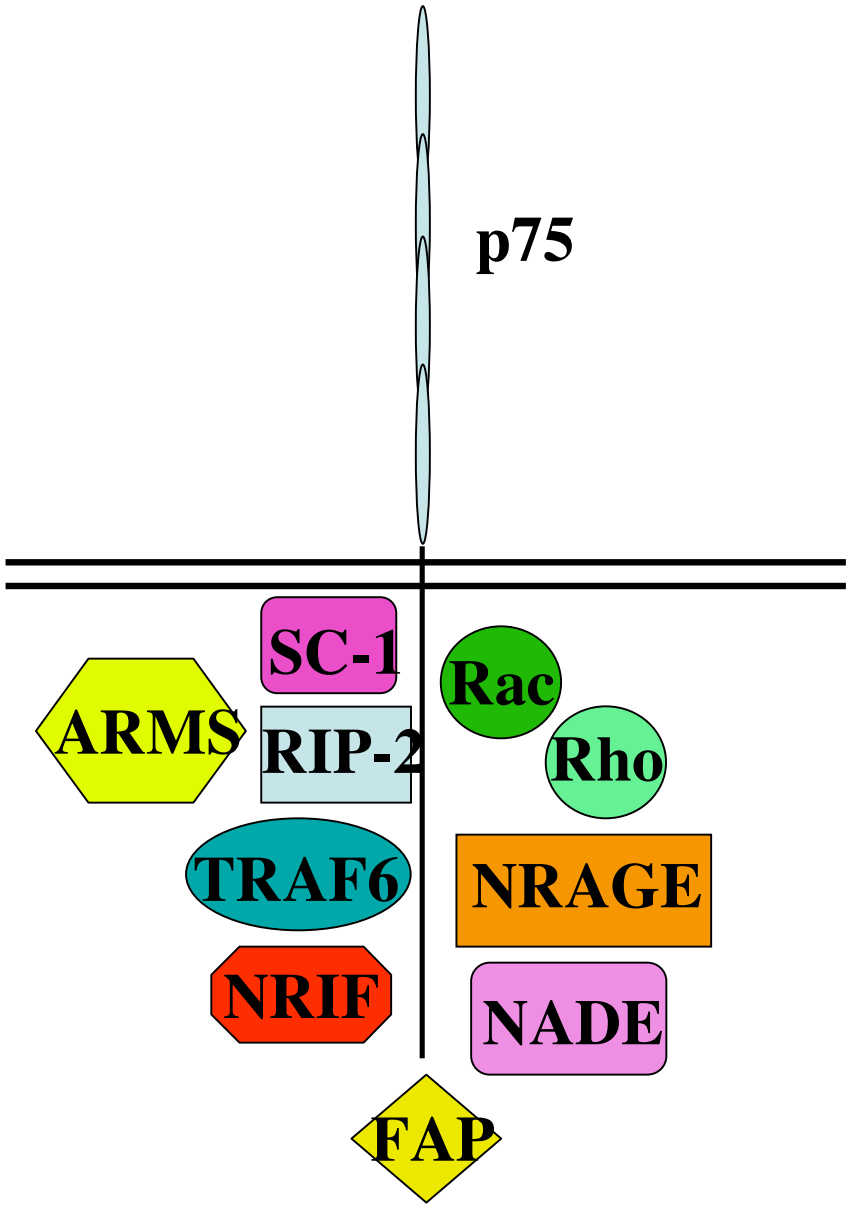



**Figure 4. p75 and its interacting proteins.** NRIF, NADE, NRAGE, SC-1, and Rac are associated with cell death and cell cycle arrest. Rip-2, TRAF6, and Fap-1 are involved in the activation of NF-□B. RhoA inhibition facilitates axon outgrowth while ARMS is involved in mediating cross-talk between p75 and the Trk receptors.

NRAGE (Neurotrophin Receptor interacting MAGE) is an 86 kDa protein that interacts in vivo and in vitro with the juxtamembrane region of p75 (Salehi et al., 2000b). It is a member of the MAGE family of proteins, which contain a MAGE homology domain (MHD). MAGE proteins are overexpressed in cancer where they can be processed to peptides and presented as MHC-associated tumor-specific antigens, but the cellular function of this family is essentially unknown. It is the MHD that is responsible for the binding of NRAGE to p75. During development, NRAGE and p75 are coexpressed in the trigeminal and dorsal root ganglia, the facial motor nucleus and in the marginal zone of the medulla (Frade and Barde, 1999). NRAGE expression alone is sufficient to decrease BrdU incorporation in cells while NRAGE expression was necessary for p75-dependent apoptosis of a neural crest cell line (Salehi et al., 2000b). Expression of TrkA abrogated this apoptosis and addition of NRAGE disrupted the p75 interaction with TrkA (Salehi et al., 2000b). This may represent a novel mechanism to modulate the p75-Trk interaction.

In addition to NRAGE, two other MAGE family members interact with p75 (Tcherpakov et al., 2002). Necdin and MAGE-H1 both possess the MHD and when ectopically overexpressed, coimmunoprecipitate with p75. In PC12 cells, a pheochromocytoma cell line commonly used to study neurotrophin signaling, Necdin and MAGE-H1 expression caused an acceleration of NGF-mediated differentiation (Tcherpakov et al., 2002). This acceleration was dependent on the presence of both p75 and TrkA because the effects were not observed in PC12 cells

lacking TrkA. These *in vitro* studies suggest that MAGE proteins may play an important role in p75 signaling; however, data regarding the physiological relevance of these proteins is lacking.

Another p75 interacting protein is the novel protein NADE (p75 neurotrophin receptor associated death executor). This protein was isolated in a yeast two-hybrid screen using the p75 death domain as bait (Mukai et al., 2000). The amino acid sequence of NADE reveals a functional nuclear export sequence, suggesting it acts in the nucleus. NADE binds to the death domain of p75 in pull down assays and its association with p75 is dependent on NGF in transfected cells. Cotransfection of NADE and p75 lead to NGF-dependent caspase activation and apoptosis in serum deprived 293T cells (Mukai et al., 2000). In PC12 cells that express both p75 and TrkA, a dramatic increase in cell death was observed when NADE was overexpressed, indicating that NADE may be able to override survival signals from Trks in some situations (Mukai et al., 2000). Endogenous NADE may have a role in p75-activated apoptosis in oligodendrocytes based on the increase of NADE mRNA following NGF treatment and the ability of a NADE fragment to block induction of cell death (Mukai et al., 2000).

TRAF (TNF receptor associated factor) proteins are a family of six adaptor proteins first identified based on their ability to mediate signaling through TNF receptors. These proteins are characterized by a carboxy terminal TRAF domain, which is subdivided into the TRAF-N and TRAF-C domains. TRAF-N domains mediate oligomerization while the TRAF-C domain determines receptor binding specificity as well as contributes to oligomerization. Several TRAFs interact with p75 (Ye et al., 1999), but the interaction of TRAF6 with p75 is the best-characterized interaction.

TRAF6 is expressed in the nervous system and associates with p75 (Khursigara et al., 1999). In addition, in cells cultured from *traf6* -/- mice, p75-mediated NF-κB and JNK activation is lost and there is a decrease in p75-mediated apoptosis in sympathetic neurons (Yeiser et al., 2004). These data suggest that interaction of TRAF6 with p75 is required for downstream signaling to occur.

Another p75 interacting protein thought to be important for NF-κB activation is Rip2 (also known as RICK or CARDIAK), a protein kinase that contains a caspase recruitment domain (CARD). This protein interacts with the TNF receptor complex (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998) and binds to p75 in Schwann cells in a ligand dependent manner (Khursigara et al., 2001). The association of Rip2 with p75 results in enhanced NF-κB activity in 293 cells and this activity was suggested to block NGF-mediated apoptosis in Schwann cells (Khursigara et al., 2001). Thus, TRAF6 may be involved in mediating p75 JNK activity while Rip2 is responsible for NF-κB activation, based on Rip2 and TRAF6 competing for binding to p75 in overexpression studies and thus determining the outcome of p75 activation. Alternatively, given that TRAF6 mediates both the NF-κB and the JNK signal (Yeiser et al., 2004), it is also possible that endogenous Rip2 and TRAF6 form a complex that determines the outcome of p75 signaling. For example, Rip2 binding may suppress the JNK pathway.

One putative transcription factor that interacts with p75 at its juxtamembrane region is the zinc finger protein SC-1 (Chittka and Chao, 1999). This protein has carboxy terminal Kruppel-type zinc fingers, a potential PEST domain in the amino terminus, and a positive regulatory/suppressor of variegation (PR/SET) domain. PR/SET domains are

modified SET domains which possess protein methyltransferase activity that can methylate histones, thus regulating chromatin structure and transcription (Rea et al., 2000; Strahl et al., 2002). SC-1 is present in the cytoplasm until NGF binds to p75, which results in its translocation to the nucleus. It is not known whether SC-1 possesses methyltransferase activitity but expression of SC-1 dramatically reduces BrdU incorporation in cells and the protein inhibits cell cycle progression by transcriptionally repressing cyclin E (Chittka et al., 2004). Both TrkA and p75 enhanced the repressive activity of SC-1 in cotransfection experiments, implying that SC-1 may be involved in NGF's differentiation signal elicited by the p75-TrkA complex (Chittka et al., 2004).

Another zinc finger protein, NRIF (neurotrophin receptor interacting factor, also known as ZFP 110), was originally identified in a yeast two-hybrid screen and also interacts with p75. This protein was suggested to function in p75 signaling based on its ability to bind the receptor (Casademunt et al., 1999; Benzel et al., 2001) and the fact that both *nrif*-/- and *p75*-/- mice have reduced levels of apoptosis in the embryonic retina (Casademunt et al., 1999). Despite this correlative evidence, a direct role for NRIF in p75 signaling has not been demonstrated. For example, the reduced cell death in the retina may be independent of p75 if NRIF is required for the generation of the p75-sensitive cells. Thus, prior to the studies described here, it was not conclusively demonstrated that NRIF is required for p75 signaling.

The expression pattern of p75 and NRIF during murine development is overlapping, although NRIF is more widely expressed suggesting it has a role beyond p75 signaling (Casademunt et al., 1999; Kendall et al., 2003). This is also reflected in the fact that the deletion of *nrif* in the BL6 strain of mice causes embryonic lethality around

day 11, a phenotype not observed in the p75 null mice (Casademunt et al., 1999). The cause of the embryonic death has yet to be determined.

Although only one NRIF gene has been identified in humans, a second NRIF homologous gene has now been cloned in mice. Termed NRIF2, the encoded protein has 85% amino acid homology to NRIF1 and can also bind p75 (Benzel et al., 2001). NRIF and NRIF2 can form heterodimers and possess the same structural motifs.

The amino acid sequence of NRIF reveals shared homology to C2H2, or Krüppel type zinc fingers, as well as a domain often associated with such zinc fingers referred to as a Krüppel-associated box (KRAB), which serve as transcription repressor domains (Margolin et al., 1994). There is also a SCAN domain, which likely mediates oligomerization (Williams et al., 1999). Thus, the structure of NRIF suggests that it regulates gene transcription, which was further supported by evidence that the protein can bind DNA through its zinc finger domain (Gentry, 2001). The ectopic expression of NRIF in 293 HEK cells also results in a significant portion of NRIF being localized to the nucleus (Casademunt et al., 1999; Gentry et al., 2004). In addition, the expression of NRIF1 or NRIF2 (Benzel et al., 2001) in 293T cells can block BrdU incorporation, suggesting that these proteins regulate the cell cycle.

Recently, NRIF was shown to bind to another p75 interacting protein, TRAF6 (Gentry et al., 2004). This interaction leads to the nuclear accumulation of NRIF as well as enhances TRAF6 activation of both NF-□B and JNK. This suggests that p75 signaling may involve TRAF6 mediating NRIF translocation to the nucleus where it could regulate genes involved in cellular viability.

**p75 receptor cleavage**

An important post-translational mechanism for regulating the function of some membrane-tethered proteins is regulated intramembrane proteolysis (RIP). This process involves the proteolytic processing of the protein, resulting in the release of the extracellular or ectodomain and generation of a bound fragment containing the transmembrane and cytoplasmic domains. A number of transmembrane receptors undergo ectodomain cleavage, including amyloid precursor protein (APP) (Hooper et al., 1997), Notch (Schweisguth, 2004), and ErbB4 (Ni et al., 2001). In addition, p75 is processed by ectodomain shedding (Weskamp et al., 2004). In the above cases, the protease responsible for ectodomain shedding is □ -secretase, TNF□ convertase (TACE), also known as ADAM 17. P75 was shed constitutively with the requirement for p38 MAPK activity and shedding can be stimulated by treatment with the phorbol ester PMA or treatment with the tyrosine phosphatase inhibitor pervanadate (Weskamp et al., 2004). In *adam 17*-/- cells, PMA and pervanadate stimulated shedding was abrogated but there was still a small amount of constitutive shedding (Weskamp et al., 2004). The functional consequence of p75 ectodomain cleavage is unclear but it may be involved in receptor signaling or the processing of the receptor.

Ectodomain cleavage also generates a membrane-tethered fragment containing the transmembrane and cytoplasmic domains. For p75, this membrane-spanning region is cleaved by a presenilin-dependent □-secretase. Such is also the case for APP, Notch and ErbB4. It is unknown if the proteolytic enzymes involved in RIP are themselves regulated and how this affects proteolysis of the substrate receptors. However, it has been suggested that the p75 intracellular domain (ICD) localizes to the nucleus. Thus, it

is tempting to speculate that, like the Notch ICD, nuclear translocation allows the p75 ICD to act as a transcriptional modifier or to interact with transcription factors like SC-1 and/or NRIF. It is also possible that the cleavage facilitates the nuclear translocation of these transcription factors.

**Summary and dissertation goals**

Although research into p75 and its signaling capabilities has intensified, there is still much to learn about this receptor. The identification of additional p75 co-receptor complexes (NogoR/Lingo-1 and Sortilin) and ligands (the proneurotrophins, Nogo, OMgP, and MAG) highlight the complexity of this signaling system. A central question that remains is what dictates whether p75 signaling induces survival or apoptosis.

The identification of p75 interacting proteins has given hints about the downstream signals but when my work was initiated, none of these interacting proteins had conclusively been placed within established signaling pathways. Indeed, much of the characterization of these interacting proteins has relied on ectopic expression systems rather than endogenous proteins in p75 signaling paradigms.

In an effort to understand the dichotomy of p75 signaling, I focused on the interacting protein NRIF. NRIF was previously implicated in p75-mediated apoptosis based on the phenotypic similarity between *nrif*-/- and *p75*-/- mice in the developing retina (Casademunt et al., 1999), yet a direct role for this interacting protein in signaling from p75 was not established. In Chapter II, I demonstrate that NRIF is required for p75 to induce cell death in sympathetic neurons. My results indicate that the activation of

JNK, but not NF-κB, by the receptor requires NRIF. In addition, I found that p75 induces apoptosis through a p53-dependent mechanism.

In Chapter III, I used ectopic expression of NRIF to understand the mechanism by which this protein functions. We demonstrate that NRIF is a proapoptotic protein that induces cell death in a variety of primary cell types. This apoptotic program results in caspase-3 activation as well as cytochrome c release from the mitochondria. Like p75-mediated apoptosis, NRIF induced cell death also required p53. The ability of NRIF to induce maximal apoptosis required the zinc finger domain, suggesting that DNA binding is required for this effect. Upon activation of p75 with proNGF, I found that NRIF accumulated in the nucleus, further suggesting that NRIF might be a transcription factor.

Given the proapoptotic role of NRIF as well as its ability to regulate the cell cycle, I assessed whether NRIF is a tumor suppressor by evaluating *nrif* -/- mice. I found that NRIF is certainly not a potent tumor suppressor, but future studies are warranted to determine the potential role of NRIF in tumor suppression.

In summary, I have established that NRIF is a critical mediator of p75 apoptotic signaling. In addition, I report that NRIF is a proapoptotic protein that induces cell death in primary cells and my localization studies support a nuclear role for this molecule. This dissertation research is the first to conclusively demonstrate that a p75 interacting protein is required for receptor signaling and sheds new light on the physiological function of NRIF.

CHAPTER II

# NRIF IS REQUIRED FOR P75-MEDIATED APOPTOSIS


## Abstract

Activation of the p75 neurotrophin receptor leads to a variety of effects within the nervous system, including neuronal apoptosis. Both c-Jun N-terminal kinase (JNK) and the tumor suppressor p53 have been reported to be critical for this receptor to induce cell death; however, the mechanisms by which p75 activates these pathways is undetermined. In this Chapter, we report that the Neurotrophin Receptor Interacting Factor (NRIF) is necessary for p75-dependent JNK activation and apoptosis. Upon NGF withdrawal, *nrif* -/- sympathetic neurons underwent apoptosis, while p75 mediated death was completely abrogated. The lack of cell death correlated with a lack of JNK activation in the *nrif* -/- neurons, suggesting that NRIF is a selective mediator for p75-dependent JNK activation and apoptosis. Taken together, these results establish NRIF as an essential component of the p75 apoptotic pathway.


## Introduction

The p75 neurotrophin receptor is involved in diverse signaling pathways, including cell survival, apoptosis, neurite outgrowth, and myelination. P75 survival pathways are largely initiated in response to p75 forming a high-affinity binding site with the Trk family of receptors (Hempstead et al., 1991) and enhancing Trk activation of these pathways. Neurite outgrowth and regulation of myelination are due to the

formation of p75 and Nogo receptor/Lingo-1 complexes, which interact with myelin proteins (Wang et al., 2001; Mi et al., 2004). The apoptotic signal is generated through mature neurotrophins binding p75 alone (Kohn et al., 1999; Majdan et al., 2001) or the proneurotrophins binding a complex of p75 and Sortilin (Nykjaer et al., 2004).

P75 plays a key role in regulating the survival of sympathetic neurons, as genetic deletion of *p75* prevents the normal period of cell death in this neuronal population (Bamji et al., 1998). It is not entirely clear how p75 elicits this death signal; however, the stress kinase JNK has been suggested to be downstream of receptor mediated apoptosis. Activation of JNK through neurotrophin binding p75 correlates with induction of cell death (Casaccia-Bonnefil et al., 1996) and inhibition of the kinase prevents this apoptosis (Yoon et al., 1998; Harrington et al., 2002). While c-Jun, the downstream target of JNK, is not required for p75-mediated cell death (Palmada et al., 2002), other JNK substrates have been implicated, including the proapoptotic Bcl-2 family member, Bad (Bhakar et al., 2003), and the tumor suppressor p53 (Aloyz et al., 1998). Mice lacking *p53* show a significant reduction in the normal attrition of sympathetic neurons during development and the viral p53 inhibitor E1B prevented p75-mediated apoptosis in these neurons (Aloyz et al., 1998).

In the present study, I examined the role of NRIF in p75-mediated cell death. NRIF was isolated in a yeast two-hybrid screen and encodes a 94kDa zinc finger protein of the Krüppel family (Casademunt et al., 1999). It was suggested to have a role in p75-mediated apoptosis based on the fact that mice lacking *nrif* display a significant reduction in cell death in the developing retina (Casademunt et al., 1999), a phenotype also

observed in p75 null mice (Frade et al., 1996). Despite this in vivo correlative evidence, it remains to be shown that NRIF is directly involved in signaling through the p75 receptor.

Here, I demonstrate that NRIF is required for p75-mediated apoptosis of sympathetic neurons, but is dispensable for cell death after NGF withdrawal. In addition, I establish a link between NRIF and both of the known components of p75 apoptotic signaling, JNK and p53.

## Methods

### Cell Culture

Mice lacking *nrif* were maintained on a 129Sv background and genotyped as previously described (Casademunt et al., 1999) and *p53*-/- mice on a mixed background were genotyped as previously described (Jacks et al., 1994). Sympathetic neurons were isolated from the superior cervical ganglia as described by Palmada et. al. (2002). Briefly, SCG from *nrif* or *p53* wild type, heterozygous or null animals were isolated at postnatal day 2-4, and the sympathetic neurons were dissociated with 0.25% trypsin and 0.3% collagenase for 30 min at 37°C. The non-neuronal cells were removed with a 2 hr preplating on uncoated, Falcon 60 mm plates (Becton Dickinson). The neurons were cultured on poly-L-ornithine and laminin coated 4 well slides (Nalge Nunc International) in Ultraculture medium (BioWhittaker) supplemented with 3% fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), and 20 ng/ml NGF (Harlan). The neurons were maintained for 4-5 d in the presence of NGF before being used for survival assays in NGF withdrawal and p75 activation experiments.

**ProNGF production**

ProNGF was generated by transfection of HEK 293 cells with a furin resistant, His-tagged construct and the protein purified using Ni-bead chromatography (Xpress System Protein purification, Invitrogen) as per the manufacturer's instructions using imidazole for elution, as previously described (Lee et al., 2001). Mature NGF was similarly produced and used for comparison in all experiments with ProNGF.

**Survival Assays**

For NGF withdrawal experiments, NGF was removed by washing the cultures twice in Ultraculture medium lacking NGF, and once with Ultraculture containing an antibody to NGF at 0.1 g/ml (Chemicon International). The procedure was similar for the p75 activation experiments except that after the anti-NGF wash, the neurons were switched to media containing anti-NGF together with 12.5 mM KCl, to promote survival, with or without 200 ng/ml BDNF (a gift from Regeneron Pharmaceuticals, Inc.). Forty-eight hours after the switch to NGF-free or BDNF-containing media, the cells were fixed in 4% paraformaldehyde and the number of surviving neurons, identified by DAPI staining the nuclei (Vector Labs), were counted. The induction of cell death was confirmed by evaluating condensed or fragmented nuclei, which was also verified by TUNEL staining in some experiments. For proNGF treatment, the neurons were plated directly in media containing 4.6 mM imidazole (used to elute pro- or mature NGF off the Ni beads), 20 ng/ml of NGF or proNGF and fixed 20 hr later. Schwann cells and MEFs infected with adeno-NRIF or GFP were similarly scored for apoptosis. In the assays done

with infected cells, only GFP-expressing cells were quantified. In all cases, at least 100 cells per condition were counted.

**NF-κB activation assays**

Activation of NF-κB was assessed in primary cultures of Schwann cells, one or two days after isolation, using a luciferase reporter 6XκB-Luc (a gift from Larry Kerr). The cells were transfected with 0.2 μg of 6κB-Luc reporter and 0.02 μg of RSV-*Renilla* (used as internal control for transfection efficiency) per well of a 24-well plate using Effectene (Qiagen) according to the manufacturer's protocol. After 24 hrs, the cells were washed twice in serum-free DMEM and treated with 100 ng/ml NGF for 4-6 hrs and lysed in 40 μl of reporter lysis buffer (Promega). Luciferase activity was measured according to the manufacturer's instructions (Promega) using a luminometer (Monolight 2010, Analytical Luminescence Laboratory). The results were normalized to the basal activity for each treatment and genotype. There was no consistent difference in the basal activity between genotypes, although there was considerable variability.

**Immunostaining**

Sympathetic neurons were isolated and treated with BDNF as described. Eighteen hours after treatment, neurons were fixed in 4% paraformaldehyde, permeabilized with 0.1% sodium citrate and 0.1% Triton X-100, blocked with 10% goat serum in PT, and immunostained with antiserum to c-Jun (Cell Signaling), diluted 1:500 in PT, followed by biotinylated secondary antibody (Vector Labs) and Cy-3 streptavidin. Nuclei were visualized by DAPI and viewed by fluorescence microscopy as described.

**Western Analysis**

Sympathetic neurons were isolated and treated with BDNF as described. Neurons from both CD-1 and 129Sv mice were used as control for comparison to those from *nrif*-/- animals; however, there was no difference between the control strains in the ability of BDNF to activate the kinase. Twenty four hours after treatment, neurons were lysed in lysis buffer (2% SDS, 20% glycerol, 0.1 M Tris HCl [pH 6.8] with protease inhibitors 0.5 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) and analyzed by Western blotting using antibodies at the following dilutions: 1:500 JNK (Santa Cruz Biotechnology) and 1:500 phospho-JNK (Cell Signaling). MEFs were infected as described or treated with 60 J/mm$^2$ ultraviolet light and 24 hours later, cells were lysed in RIPA buffer and analyzed by Western blotting using JNK and phospho-JNK antibodies as above.

**Real-time PCR analysis**

Total RNA was extracted from sympathetic neurons treated with BDNF using Trizol reagent (Gibco) per manufacturer's instructions. RNA was isolated from 3T3 cells using Trizol and used in a dilution series to control for input amount. cDNA was synthesized from 3 μg RNA using 4 μM random hexamers (Applied Biosystems) and 300 U MMLV-RT enzyme (Promega). This was incubated for 1 hour at 42°C, followed by 10 min at 95°C. For each PCR reaction, 2 μl of this reaction was used. Each sample was analyzed for *p53*, *p21*, *Puma*, *Noxa* or *GAPDH* mRNA using an iCycler (BioRad) and 12.5 μM of each specific primer (see below). Each PCR reaction was a volume of 25 μl, diluted using the SYBR green PCR master mix (Amersham). The following cycling parameters were used: 94°C for 60 sec, 60°C for 60 sec, 72°C for 60 sec for 40 cycles

with a final extension time of 5 min at 72°C. For each primer set, data is represented as percentage of wild-type neurons without BDNF addition ± SEM (n=3). The following are the primer sets that were used: p53 forward: 5'-TACTCTCCT CCCCTCAATAA-3'; p53 reverse: 5'-CTTGTAGTGGATGGTGGTAT-3'; p21 forward: 5'-AAGTGTGCCGTTGTCTCTTC-3'; p21 reverse: 5'-ACTTCAGGGTTTTCTCTTGC-3'; Puma forward: 5'-ATGGCGGACGACCTCAAC-3'; Puma reverse: 5'-AGTCCCATGAAGAGATTGTACATGAC-3'; Noxa forward: 5'-CTGGAAGCGAAAGCTAACACG-3'; Noxa reverse: 5'-GTACTTTCCCAGGAGTGGGAC-3'; GAPDH forward: 5'-TGCACCACCAACTGCTTAG-3'; GAPDH reverse: 5'-GATGCAGGGATGATGTTC-3'

**Results**

**NRIF is required for apoptosis mediated by the p75 receptor**

NRIF was first suggested to have a role in p75-mediated apoptosis based on the fact that mice lacking the *nrif* gene exhibit a reduction in the amount of naturally occurring cell death in the embryonic retina (Casademunt et al., 1999), a process known to depend on the p75 neurotrophin receptor (Frade et al., 1996). Therefore, to directly investigate whether *nrif* is essential for p75-mediated apoptosis we activated the receptor in sympathetic neurons isolated from the superior cervical ganglia from *nrif* +/+, +/-, and -/- mice. Sympathetic neurons require NGF binding the tyrosine kinase receptor TrkA for survival; however, they can be maintained in the absence of neurotrophin using 12.5 mM

KCl. These conditions allow selective activation of the p75 receptor by BDNF, resulting in neuronal apoptosis (Bamji et al., 1998; Palmada et al., 2002). Using this experimental paradigm, we found that neurons from the *nrif* null mice do not die in response to BNDF, in contrast to neurons from +/+ and +/- littermates (Fig. 5A). In addition, the proform of NGF (proNGF) can also induce apoptosis in these neurons through interaction with the p75 receptor (Lee et al., 2001). Similar to the results with BDNF, the *nrif*-/- neurons were resistant to proNGF (Fig. 5B). These results indicate that NRIF is essential for p75 to signal neuronal apoptosis.

The difference in the response to p75 activation between *nrif* -/- and +/+ was not due to loss of p75 expression (data not shown) nor was there any difference in general neuronal viability between the two genotypes. The NGF-mediated survival of the *nrif* -/- neurons was not different from the *nrif* +/+ cells (relative to wild type, 110 $\pm$ 29% of the *nrif* -/- neurons were viable after 48 hr in the presence of 20 ng/ml NGF) nor was the survival in KCl (relative to wild type, 99 $\pm$ 16% of the *nrif* -/- neurons were viable). In addition, there was no difference in the degree of apoptosis in response to NGF withdrawal between the genotypes (Fig. 6). Thus, although NRIF is required for p75-mediated cell death, it is not required for apoptosis that occurs when neurons are deprived of NGF.

**The activation of JNK by p75 is attenuated in the absence of NRIF**

Activation of the stress kinase JNK by p75 is necessary for the receptor to induce cell death (Yoon et al., 1998; Friedman, 2000; Harrington et al., 2002). The mechanism

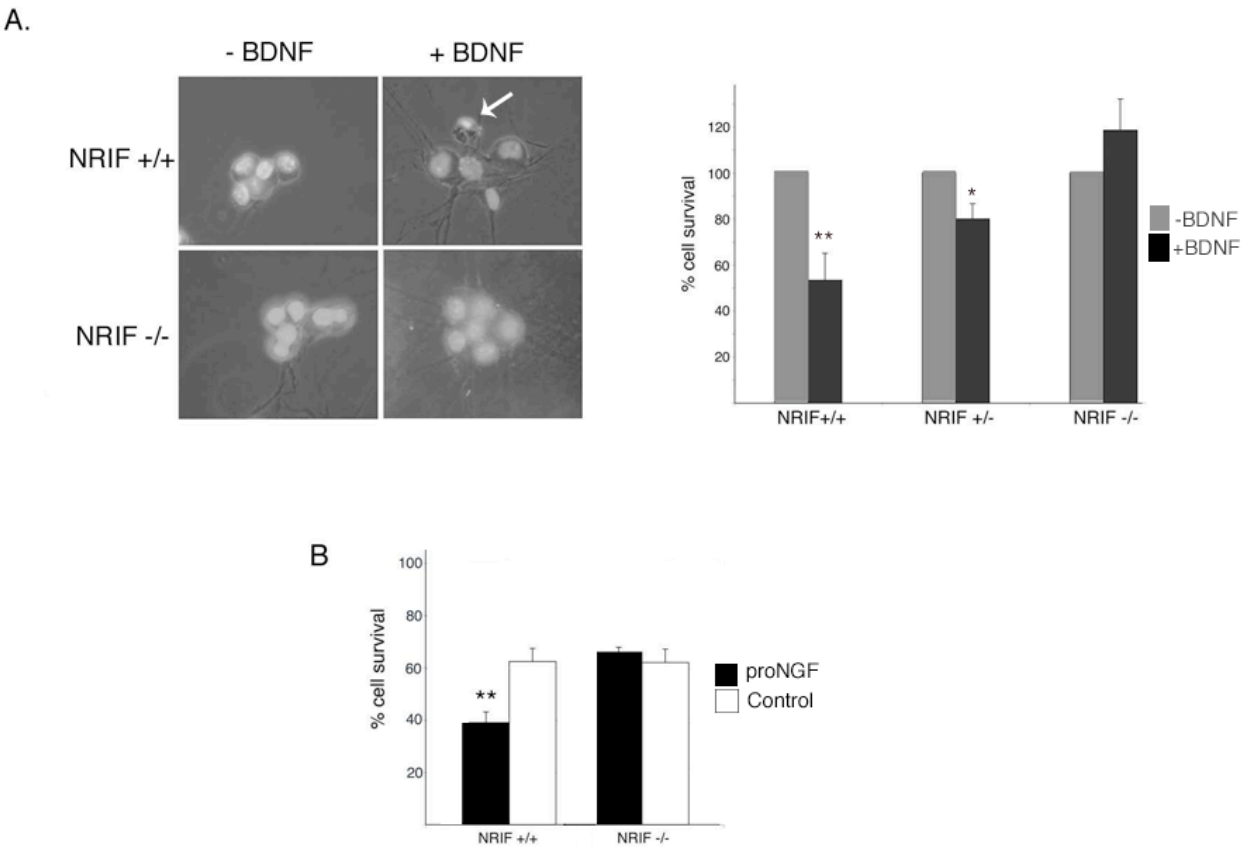



**Figure 5. *nrif* -/- sympathetic neurons are deficient in p75-mediated apoptosis.**
A. Sympathetic neurons from *nrif* +/+, +/-, and -/- mice were cultured in 20 ng/ml NGF for 4 days, rinsed and refed with media containing a neutralizing antibody to NGF and 12.5 mM KCl with or without the addition of 200 ng/ml BDNF. Forty-eight hours later, the cells were fixed and stained with DAPI to identify the non-apoptotic nuclei. Representative neurons are depicted in the left panels, with an arrow indicating an apoptotic neuron. The effect of BDNF expressed relative to survival of control cultures for each genotype is shown in the right panel. Gray bars indicate the untreated neurons and black bars indicate the addition of BDNF. The mean $\pm$ SEM is depicted (n=6). ** indicates statistical significance compared to neurons maintained in KCl based on student's t-test, $p<0.01$, * $p<0.05$. B. Sympathetic neurons from wild type and *nrif* -/- mice were cultured directly in media with diluent (control) or media containing 20 ng/ml mature or proNGF. Twenty hours later, the neurons were fixed and stained with DAPI to visualize the healthy nuclei. The effect of proNGF is expressed relative to survival of neurons cultured with mature NGF for each genotype. The mean $\pm$ SEM is depicted (n=3). ** indicates statistical significance compared to neurons maintained in the absence of NGF based on student's t-test, $p< 0.05$.

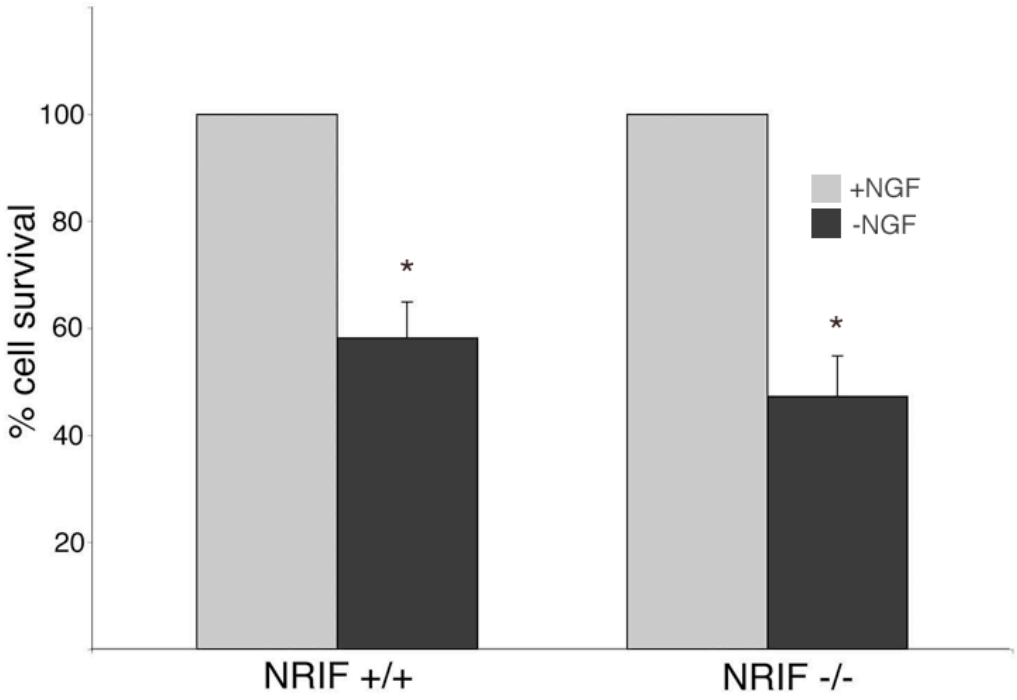



**Figure 6. *nrif* -/- sympathetic neurons undergo apoptosis after NGF withdrawal.** Sympathetic neurons were isolated from *nrif* +/+ and -/- mice and cultured for 4 days with NGF (20 ng/ml). The neurons were then rinsed and refed with media containing 20ng/ml NGF (gray bars) or with media containing a neutralizing antibody to NGF (black bars). After 48 hours, the cells were fixed and stained with DAPI to evaluate the non-apoptotic nuclei. The effect of NGF withdrawal is expressed relative to cultures maintained in NGF for each genotype. The mean ± SEM is depicted (n=4). * indicates statistical significance compared to neurons maintained in NGF based on student's t-test, $p<0.05$.

of JNK activation by p75 has not been delineated; however, a member of the TNF Receptor Associated Factor family (Khursigara et al., 2001) and the GTP binding protein Rac (Harrington et al., 2002) have been implicated as upstream activators. Furthermore, co-expression of NRIF, TRAF6 and p75 in 293 HEK cells reconstituted JNK activation by neurotrophin (Gentry et al., 2004). Therefore, I evaluated the stimulation of this kinase in neurons from mice lacking *nrif*. Sympathetic neurons isolated from *nrif* -/- or wild type mice were cultured in KCl, as for the cell death assays, BDNF was added to the culture media for 24 hrs and the activation of JNK was assessed by Western blotting for phospho-JNK. Unlike wild type neurons, there was no JNK activation detectable in those neurons from *nrif*-/- mice (Fig. 7A). We also investigated p75 regulation of this pathway on a cellular level by immunostaining for the transcription factor c-Jun. C-Jun is a well-characterized downstream target of JNK, which up regulates itself and accumulates in the nucleus following phosphorylation by JNK. Thus it can be used as a readout of the activation of the kinase, although c-Jun itself is not required for p75-mediated cell death (Palmada et al., 2002). In the absence of NRIF, the overall level of c-Jun was significantly reduced; however, BDNF treatment still elicited an accumulation of the transcription factor in the nucleus (Fig. 7B). This result suggests that in the absence of NRIF, the ability of p75 to activate the JNK-c-Jun pathway is severely attenuated, below detectable levels by Western blot, but it is not totally abrogated.

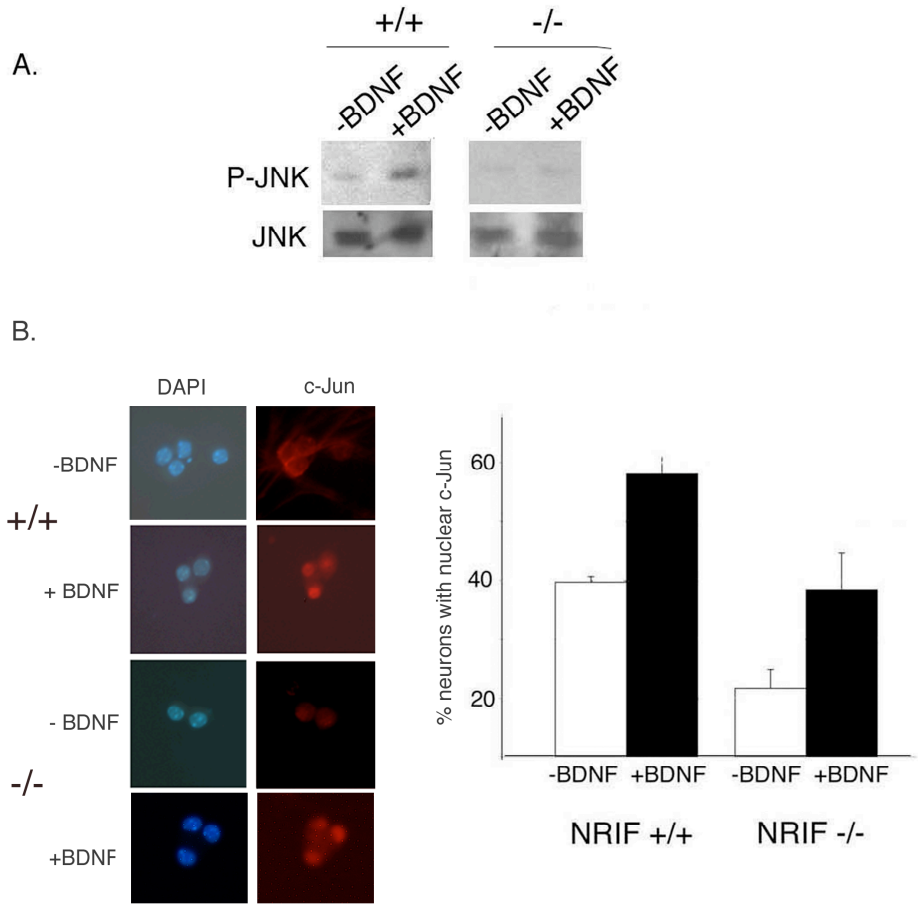



**Figure 7. p75 stimulation of the JNK pathway is attenuated in *nrif* -/- sympathetic neurons**. A. Sympathetic neurons from *nrif* +/+ and -/- mice were cultured in 20 ng/ml NGF for 4 days, rinsed and refed with media containing a neutralizing antibody to NGF and 12.5 mM KCl with or without 200 ng/ml BDNF. After 24 hours, the cells were lysed and immunoblotted using JNK and phospho-JNK antibodies. A representative blot from four experiments is shown. B. Sympathetic neurons from *nrif* +/+ and -/- mice were cultured as above, then treated with or without 200 ng/ml BDNF. After 18 hours, cells were fixed and immunostained using an antibody to c-Jun and DAPI to identify healthy nuclei. In the left panel representative photomicrographs of wild type (+/+) and *nrif* null (-/-) neurons immunostained for c-Jun are depicted. The right panel shows quantitation of the percent of neurons with nuclear c-Jun. White bars indicate KCl treatment and black bars indicate the addition of BDNF. The mean ±SEM is depicted (n=5).

**Deletion of *nrif* does not alter the ability of p75 to activate NF-κB**

Another signaling system that regulates cellular viability and is under the influence of p75 is the NF-κB pathway. This transcription factor is activated in Schwann cells by NGF binding to p75 and promotes cell survival (Khursigara et al., 2001). Therefore, I evaluated the stimulation of NF-κB by neurotrophins in Schwann cells from *nrif*+/+ and -/- mice using a luciferase reporter assay (Fig. 8). There was no significant difference between the genotypes in the ability of p75 to activate this transcription factor.

**p75-mediated apoptosis is p53 dependent**

The tumor suppressor p53 has also been implicated in p75-mediated apoptosis, based on the ability of the inhibitor E1B to prevent receptor induced death (Aloyz et al., 1998). To directly determine whether the neuronal apoptosis induced by p75 activation requires p53, sympathetic neurons were isolated from *p53* +/+ and -/- mice and subjected to NGF withdrawal or p75 activation by BDNF, as above. While the *p53* -/- neurons were totally resistant to cell death induced by p75 activation (Fig. 9A), they underwent apoptosis as well as the wild type following NGF removal (Fig. 9B). Thus, like NRIF, p53 is required for p75-mediated apoptosis but is dispensable for cell death occurring after NGF deprivation.

**p75-mediated apoptosis and induction of p53 target genes**

Because p75-mediated cell death requires the presence of both NRIF and p53, we hypothesized that in wild-type sympathetic neurons, p75 activation might activate p53 dependent transcription while in *nrif* -/- neurons, this would not occur. This data would

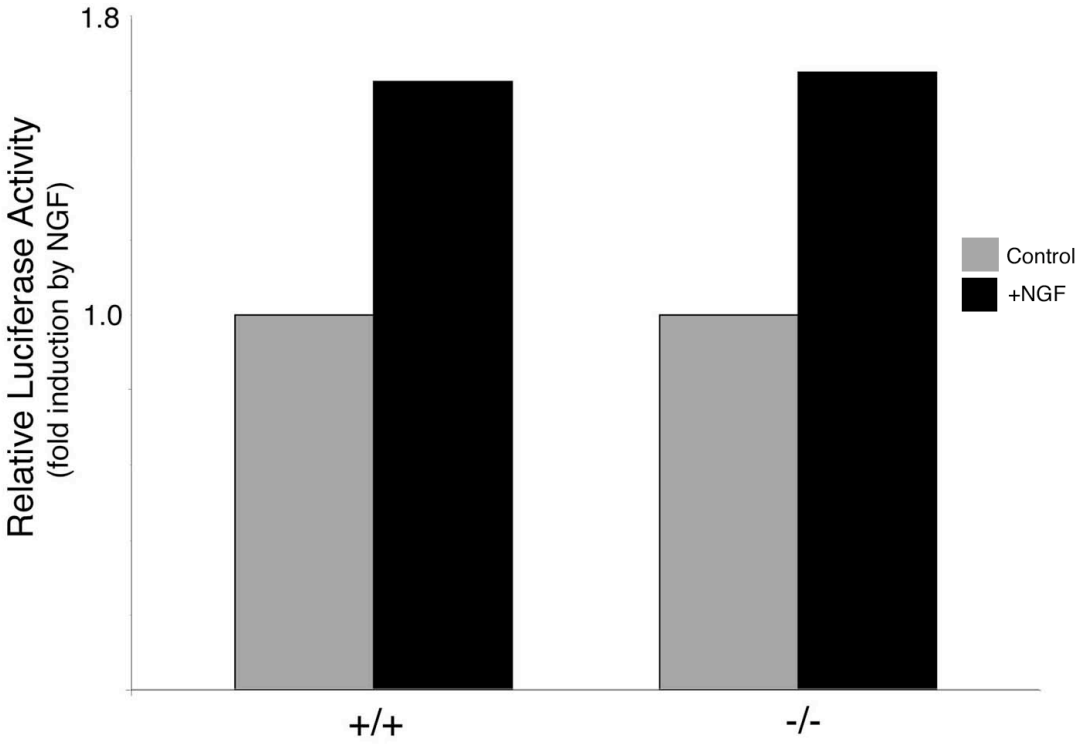



**Figure 8. The activation of NF-κB by p75 is unaffected in *nrif*-/- Schwann cells**.
Schwann cells were isolated from wild type and nrif null mice at postnatal day 2-4 and transfected with an NF-κB luciferase reporter 24 hours later. One day after transfection, cells were treated in serum free media with 100 ng/ml NGF or left untreated for 4-6 hrs. Lysates were collected and luciferase activity measured. All values were normalized to control cells (in serum free media alone). Gray bars indicate control and black bars indicate NGF treatment. The mean is depicted (n=4 for wild-type, n=2 for null).

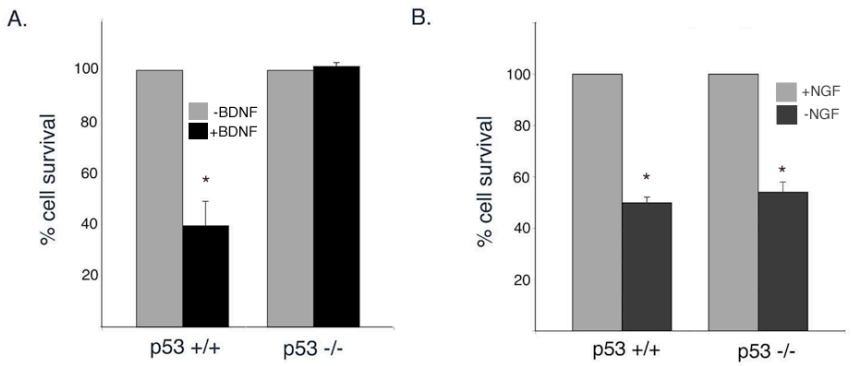



**Figure 9. p75-mediated cell death in sympathetic neurons require p53.** A. Sympathetic neurons from *p53* +/+ and -/- mice were cultured in 20 ng/ml NGF for 4 days, rinsed and refed with media containing a neutralizing antibody to NGF and 12.5 mM KCl with or without 200 ng/ml BDNF. Forty-eight hours later, the cells were fixed and stained with DAPI to identify the non-apoptotic nuclei. The effect of BDNF is expressed relative to survival of control cultures for each genotype. Gray bars indicate untreated neurons and black bars indicate the addition of BDNF. The mean ±SEM is depicted (n=4). * indicates statistical significance compared to neurons maintained in KCl based on student's t-test, $p<0.05$. B. Sympathetic neurons were isolated from *p53* +/+ and -/- mice and cultured for 4 days with NGF (20 ng/ml). The neurons were then rinsed and refed with media containing 20ng/ml NGF (light gray bars) or with media containing a neutralizing antibody to NGF (dark gray bars). After 48 hours, the cells were fixed and stained with DAPI to evaluate the non-apoptotic nuclei. The effect of NGF withdrawal is expressed relative to cultures maintained in NGF for each genotype. The mean ±SEM is depicted (n=5). * indicates statistical significance compared to neurons maintained in NGF based on student's t-test, $p<0.05$.

support the idea that NRIF is upstream of p53 in p75 death signaling. To this end, I treated neuronal cultures with BDNF and isolated total RNA. Using the sensitive method of real-time PCR, we examined the mRNA levels of the p53 target genes, p21, Puma, Noxa as well as the p53 message itself. As shown in Figure 10 for Noxa, we were unable to observe any significant or reproducible differences in the levels of these mRNAs. Similar results were obtained with Puma and p53 (data not shown). Thus, the pathway linking p75-mediated apoptosis to NRIF and p53 remains to be determined.

**Discussion**

In an effort to determine the molecular mechanism by which p75 signals cell death, a number of proteins have been identified that bind to the intracellular domain. Several of these have been implicated in the apoptotic pathway, including NRAGE (Salehi et al., 2000b), NADE (Mukai et al., 2000), TRAF2 (Ye et al., 1999), TRAF6 (Khursigara et al., 1999) and NRIF (Casademunt et al., 1999); however, these studies have relied primarily on ectopic, over-expression of the given p75 interacting protein. NRIF was previously implicated in p75-mediated apoptosis based on the phenotypic similarity between *nrif*-/- and *p75*-/- mice in the developing retina (Casademunt et al., 1999), yet a direct role for this interacting protein in signaling from p75 was not established. Here we demonstrate that NRIF is required for p75 to induce cell death in sympathetic neurons. My results indicate that the activation of JNK, but not NF-□B, by the receptor requires NRIF. In addition, I found that both NRIF and p75 induce apoptosis through a p53-dependent mechanism.

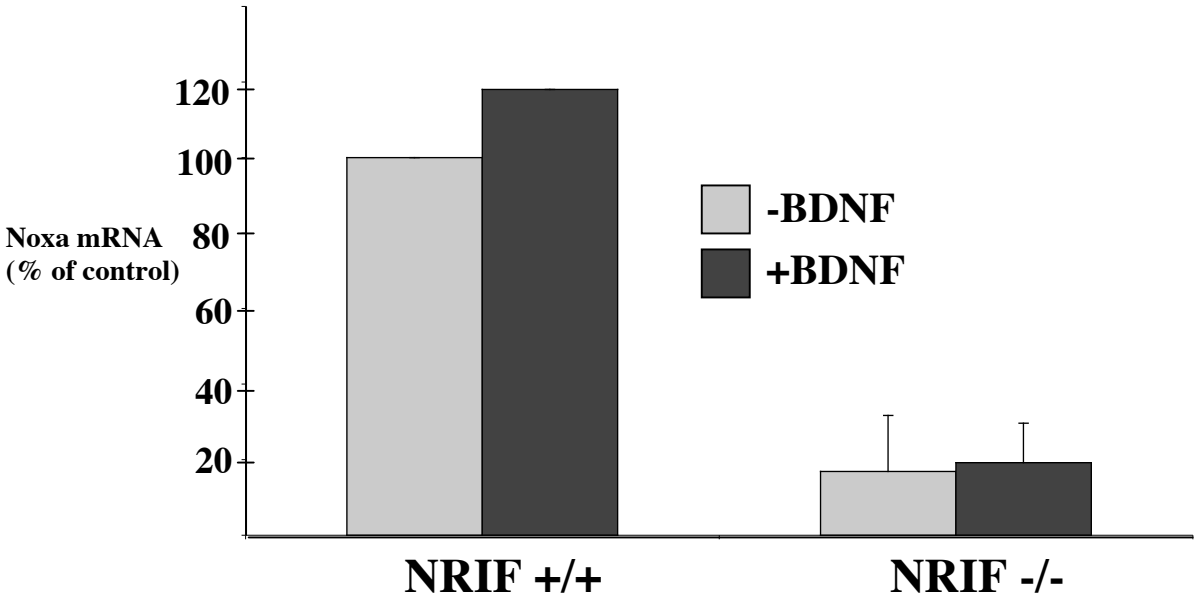



**Figure 10. Quantitation of Noxa mRNA levels in sympathetic neurons after p75 activation.** Sympathetic neurons were isolated from wild-type and *nrif* null mice and p75 was activated with BDNF as before. Twenty four hours after receptor activation, RNA was isolated, cDNA synthesized and used in real-time PCR using primers specific for Noxa. Data is represented as fold activation compared to untreated wild-type cells ± SEM (n=4). Similar results were observed with Puma and p53 primer sets.

NRIF, originally identified in a yeast two-hybrid screen, was suggested to function in p75 signaling based on its ability to bind the receptor (Casademunt et al., 1999; Benzel et al., 2001) and the fact that both *nrif*-/- and *p75*-/- mice have reduced levels of apoptosis in the embryonic retina (Casademunt et al., 1999). Moreover, the expression pattern of p75 and NRIF during murine development is overlapping, although NRIF is more widely expressed suggesting it has a role beyond p75 signaling (Casademunt et al., 1999; Kendall et al., 2003). This is also reflected in the fact that the deletion of *nrif* in the BL6 strain of mice is embryonic lethal around day 11, a phenotype not observed in the p75 null mice (Casademunt et al., 1999). The mechanism by which NRIF functioned, however, remained unclear. The data presented here demonstrates that NRIF is required for p75-mediated apoptosis.

Recently, NRIF was shown to bind to another p75 interacting protein, TRAF6, and their co-expression in 293 HEK cells resulted in a dramatic increase in nuclear accumulation of NRIF, suggesting that TRAF6 may facilitate the nuclear localization of NRIF and/or the p75 ICD (Gentry et al., 2004). The interaction of these two proteins also altered TRAF6 signaling. TRAF6 has been best characterized as a mediator of NF-□B and JNK activation by several receptors (Dempsey et al., 2003), including p75 (Khursigara et al., 2001; Yeiser et al., 2004). The interaction of TRAF6 with NRIF was reported to enhance TRAF6 mediated JNK activity by more than 3-fold while only marginally affecting NF-□B activation (Gentry et al., 2004). Moreover, co-expression of NRIF, TRAF6 and p75 in 293 HEK cells reconstituted the ability of NGF to stimulate JNK, while neither intracellular protein alone was sufficient (Gentry et al., 2004). In agreement with these results, we found that in the absence of NRIF, the ability of p75 to

activate the kinase was substantially reduced, but not ablated (Fig. 3). Thus, NRIF appears to serve as a facilitator for p75 regulation of this pathway through its interaction with TRAF6.

It should be noted that in addition to NRIF and TRAF6 there have been other signaling proteins implicated as having a role in p75 signaling to JNK, including the GTP binding protein Rac (Harrington et al., 2002) and the MAGE homolog, NRAGE (Salehi et al., 2000b) Whether these proteins form a multicomponent signaling complex is not clear; however, it has been reported that interleukin-1 (IL-1) signals through a pathway involving IRAK-1, TRAF6 and Rac1 (Jefferies et al., 2001). These authors focused on the activation of NF-□B and demonstrated that dominant negative Rac could block TRAF6 signaling, yet dominant negative TRAF6 could also block a Rac driven response; thus, supporting the notion of a signaling complex involving both of these proteins and possibly others.

Several reports have indicated that JNK activation is required for p75 to induce apoptosis. Inhibiting the kinase pharmacologically (Yoon et al., 1998) or by expressing a dominant negative JNK construct in oligodendrocytes (Harrington et al., 2002) or in sympathetic neurons (Yeiser et al., 2004) prevented cell death through p75. The most established target of this kinase is c-Jun, a member of the AP-1 family; however, Palmada et al. (2002) reported that deletion of c-Jun did not prevent sympathetic neurons from dying following p75 activation. Several alternative substrates of JNK have been proposed as mediating cell death in response to p75, including Bcl-2 family member Bad (Bhakar et al., 2003) and the transcription factor p53 (Aloyz et al., 1998). My findings further support a role for p53, because I show that *p53*-/- neurons are resistant to BDNF.

In contrast, p53 was not essential for cell death induced by withdrawal of NGF, in agreement with a previous report (Sadoul et al., 1996). Since the naturally occurring apoptosis in the superior cervical ganglia (SCG) is significantly reduced in mice lacking p53 (Aloyz et al., 1998), my results suggest that p75-mediated cell death plays a significant role in the normal loss of neurons during development. Miller and colleagues have also proposed such a role for p75 signaling based on the attenuated cell death in the SCG of p75-/- mice (Bamji et al., 1998) and the ability of deleting p75 to rescue the massive loss in neurons that occurs in the absence of TrkA, the NGF tyrosine kinase receptor responsible for survival signaling (Majdan et al., 2001).

We have been unable to detect any reproducible or significant differences in mRNA levels of p53 target genes after p75 activation. Therefore, we are unable to make any conclusions regarding p75 cell death signaling and the pathway leading to p53. There is likely some variability in the amount of activated p75 in different neuronal cultures and this may be why we were unable to reproduce our data. These experiments can be repeated using the more potent p75-activating ligand, proNGF. With more robust p75 activation, it may be possible to establish the link between p75 induced cell death and p53.

The data presented here demonstrate the requirement of NRIF in p75-mediated apoptosis. Moreover, we find that NRIF is required for JNK activation downstream of p75. This provides the first study to link a p75 interacting protein, NRIF, to well-established p75 signaling pathways.

# CHAPTER III

# NRIF INDUCES CELL DEATH IN PRIMARY CELLS


## Abstract

NRIF has been shown to be an essential component of p75-induced cell death in sympathetic neurons. However, there is still relatively little known about this protein. Here, we demonstrate that NRIF is capable of inducing apoptosis in primary cells in the absence of p75 activation. This apoptotic program involves the activation of caspase 3 and the release of cytochrome c from the mitochondria. NRIF expression in primary cells is not sufficient to activate JNK but NRIF-activated cell death requires both p53 and its upstream activator $p19^{Arf}$. NRIF is found in the nucleus after receptor activation, suggesting that this molecule may act as a transcription regulator. Consistent with this hypothesis, NRIF requires its putative DNA binding domain to maximally induce apoptosis. Finally, we examine the role of NRIF as a tumor suppressor and find that while this protein causes cell cycle arrest (Benzel et al., 2001) and apoptosis, the absence of *nrif in vivo* does not predispose mice to spontaneous tumor development.


## Introduction

NRIF was isolated from a yeast two-hybrid screen of an embryonic mouse cDNA library using the p75 intracellular domain as bait (Casademunt et al., 1999). This ubiquitously expressed 94 kDa protein is a zinc finger protein of the Krüppel family that interacts with the p75 neurotrophin receptor. I have demonstrated that NRIF is required

for p75-mediated cell death and that in the absence of *nrif,* p75 does not efficiently activate the JNK pathway (Chapter II). However, deletion of *nrif* is embryonic lethal at E12 in the BL6 mice, a phenotype not observed in p75 -/- mice (Casademunt et al., 1999). Moreover, NRIF has a broader expression pattern than p75 (Casademunt et al., 1999; Kendall et al., 2003). These findings suggest a broader role for this protein beyond p75 signaling. In an effort to better understand the function of NRIF, we investigated the effect of overexpressing NRIF in the absence of p75.

NRIF was originally hypothesized to be necessary for p75 signaling because disruption of *nrif* leads to a reduction in apoptosis of early retinal cells that is quantitatively indistinguishable from what is observed in *ngf* -/- and *p75* -/- mice. However, it remained possible that the reduced death in the retina was independent of p75. In Chapter II, I demonstrate that NRIF is required for p75 apoptotic signaling. However, much remains to be elucidated about how NRIF functions in apoptotic signaling.

NRIF also binds another p75 interacting protein, TRAF6 (Gentry et al., 2004). The interaction of these two proteins alters TRAF6 signaling as NRIF enhanced TRAF6-mediated JNK activity more than 3-fold. Additionally, co-expression of NRIF, TRAF6 and p75 in 293 HEK cells reconstitutes the ability of NGF to stimulate JNK, while neither interactor alone was sufficient (Gentry et al., 2004). These studies suggest the existence of a multi-component receptor signaling complex and highlight the importance of further studies that will examine NRIF function.

NRIF also induces cell cycle arrest in immortalized cells (Benzel et al., 2001). Transfection of NRIF into 293 HEK cells resulted in a decrease in the amount of BrdU

incorporation (Benzel et al., 2001). This effect was independent of expression of p75, suggesting a role for NRIF in cell cycle control in the absence of the receptor. Therefore, these authors suggest that an inappropriate level of NRIF *in vivo* will lead to deregulated expression of crucial cell cycle checkpoints, perhaps by transcriptional regulation of cell cycle genes. However, it is unclear how NRIF is modulating the cell cycle in these cells.

Considering its role in apoptosis and cell cycle control as well as its potential as a transcription factor, it is possible that NRIF functions as a tumor suppressor gene. Indeed, I have observed an increase in apparent tumors in *nrif* -/- mice and hypothesized that NRIF might function as a tumor suppressor. Such is the case with Zac-1, a zinc finger transcription factor that induces growth arrest and apoptosis in cells (Spengler et al., 1997). Zac-1-dependent growth arrest was independent of regulation of the cell cycle regulating factors Rb, p21, p27, and p16-INK4A. Instead, the anti-proliferative activity of Zac-1 was due to excessive induction of apoptosis (Spengler et al., 1997). Consistent with tumor suppressor activity, Zac1 abrogated tumor formation in xenografted nude mice (Spengler et al., 1997).

In an effort to better understand NRIF, I used an overexpression paradigm to evaluate its function in the absence of p75. I report that NRIF is proapoptotic in primary cells in the absence of p75 activation. NRIF-induced cell death is accompanied by caspase-3 activation and cytochrome c release from the mitochondria. Consistent with earlier reports (Gentry et al., 2004), the addition of NRIF alone is not sufficient for JNK activation. In addition, I found NRIF localized in the nucleus after p75 activation, suggesting that NRIF may regulate apoptosis by transcriptionally regulating genes necessary for cellular viability. Like p75-mediated death, NRIF-induced apoptosis

requires the presence of p53 as well as its upstream activator, $p19^{Arf}$. Despite its role in apoptosis and cell cycle regulation, the data presented here indicate that in the absence of *nrif*, there is no increase in spontaneous tumor formation in mice. This study of NRIF provides details as to how this protein functions, both in the presence and absence of p75.

## Methods

### Cell Culture

Rat Schwann cells were isolated from postnatal day 4 rats as described by Carter et al. (Carter et al., 1996) and maintained in DMEM supplemented with 10% fetal calf serum and 2 μM forskolin (Sigma). Cells were transfected with Effectene (Qiagen) per manufacturer's instructions. When adenovirally infected, cells were split the day before infection and infected with $4.5 \times 10^6$ pfu/cell of virus expressing NRIF and GFP bicistronically or GFP alone for mouse embryo fibroblasts or $7.5 \times 10^6$ pfu/cell for sympathetic neurons or Schwann cells. At the indicated times, cells were harvested for staining or immunoblotting, as indicated.

Mouse embryo fibroblasts (MEFs) were maintained in DMEM supplemented with 10% fetal calf serum. Cultures were split every 3 days and all experiments were done with cells that had been passaged fewer than 12 times.

### Immunostaining

Rat Schwann cells were maintained as described above. After transfection with adeno-GFP or GFP-NRIF, cells were fixed in 4% paraformaldehyde, blocked with 10%

goat serum in PT (PBS, 0.1% Triton X-100) and immunostained with antiserum to caspase 3 (a gift from Idun Pharmaceuticals, Inc.) diluted 1:1000 in PT, followed by a biotinylated secondary antibody (Vector Labs) and Cy-3 streptavidin. Nuclei were visualized by DAPI and slides were viewed by fluorescence microscopy with a Zeiss Axiophot microscope. For NRIF immunostaining, a previously described affinity purified antiserum to NRIF (Gentry et al., 2004) was used at a 1:100 dilution, followed by anti-rabbit Alexa546 at 1:500 and analyzed by confocal microscopy (Zeiss LSM 410).

**Western Analysis**

Schwann cells were cultured and transfected as described and 18 hr later, mitochondrially enriched fractions were prepared as described (Lipscomb et al., 2001). Briefly, each plate was rinsed twice in cold PBS before adding homogenization buffer (17 mM MOPS, 2.5 mM EDTA, 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 5 μg/ml aprotinin). Cells were scraped from the dishes, Dounce homogenized, and centrifuged at 750 x *g* for 10 min to pellet nuclei. The supernatants were centrifuged at 10,000 x *g* for 15 min and the resultant pellets were resuspended in homogenization buffer and centrifuged again at 10,000 x *g* for 15 min to enrich for mitochondria. The mitochondrial fraction was suspended in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGEPAL, 0.5% deoxycholate, 0.1% SDS, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin) and analyzed by Western blotting using antibodies at the following dilutions: 1:1000 cytochrome c (BD Pharmingen) and 1:1000 manganese superoxide dismutase (BD Biosciences).

**Generation of NRIF mouse colony**

NRIF knockout mice were created as described (Casademunt et al., 1999). However, for these studies, we generated littermate controls from the *nrif*-/- mice by back-breeding to the parental strain, 129Sv. All offspring were genotyped as previously described (Casademunt et al., 1999). Mice were separated into cages of males and females and were not allowed to breed during the course of the experiments. Thirty mice of each genotype (+/+, +/-, and -/-) were maintained for 18 months.

**Evaluation of Mice and Immunohistochemical analysis**

Mice were visually evaluated and palpitated bimonthly to evaluate the onset of potential tumors. Mice with observable tumors were sacrificed and abnormal tissues were fixed and paraffin embedded by the Vanderbilt Mouse Pathology Core lab. Sections of these tissues were then stained with hematoxylin and eosin and the tissue pathology examined by Dr. Mary Ann Thompson and Dr. Lillian Nanney.

## Results

**NRIF expression induces apoptosis in primary cells**

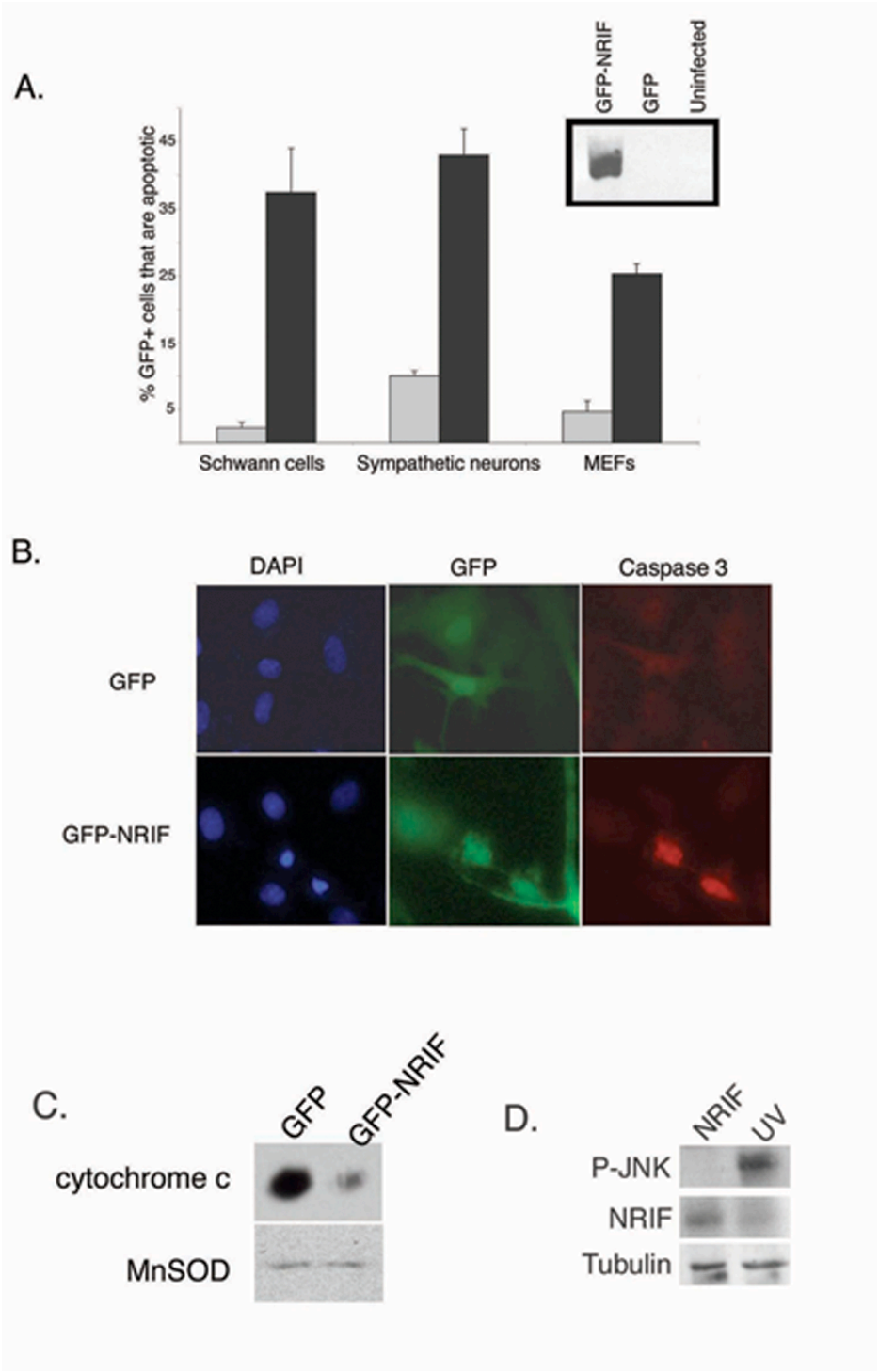
The observation that neurons do not undergo p75-mediated cell death or JNK activation in the absence of *nrif* suggested that this interacting protein mediates an apoptotic signal. Expression of NRIF in cell lines, such as 293 HEK cells, primarily caused cell cycle arrest (Benzel et al., 2001); however we considered the possibility that NRIF may activate cell death in primary, non-immortalized cells. To test this hypothesis,

NRIF was expressed in primary Schwann cells, sympathetic neurons or mouse embryo fibroblasts (MEFs). When mouse sympathetic neurons were infected with an adenovirus expressing GFP and NRIF bicistronically, approximately 40% of the GFP positive neurons were apoptotic, determined by DAPI staining of condensed or fragmented nuclei (Fig. 11A). In contrast, less than 10% of the GFP positive neurons were apoptotic when infected with the GFP virus alone. Similarly, when primary rat Schwann cells or mouse embryo fibroblasts (MEFs) were infected with the NRIF-expressing virus, a significant increase in apoptosis was observed as compared to the GFP control. Similar results were seen in the absence of the adenovirus when the Schwann cells were transiently transfected with NRIF or GFP expression vectors (data not shown). These results demonstrate that expression of NRIF is sufficient to induce cell death in multiple primary cell types.

**NRIF expression causes caspase-3 activation and cytochrome c release from mitochondria**

The cell death induced by NRIF expression involved activation of caspase 3 and cytochrome c release from the mitochondria. As depicted in Figure 11B, 24 hr after transfecting rat Schwann cells with GFP-NRIF, the GFP positive cells typically displayed a condensed or fragmented nucleus and were reactive to an antibody that recognizes the activated form of caspase 3. In many situations, the release of cytochrome c from the mitochondria precedes the activation of caspase 3. To determine if NRIF-mediated apoptosis involved cytochrome c release, Schwann cells were infected with adenovirus expressing NRIF or GFP, subjected to subcellular fractionation and the

**Figure 11. NRIF induces cell death in multiple primary cells and is accompanied by caspase activation and cytochrome c release.** A. Rat Schwann cells, mouse sympathetic neurons and mouse embryo fibroblasts were adenovirally infected with GFP control vector or with a bicistronic GFP-NRIF vector. After 48 hours, cells were fixed and stained with DAPI to identify apoptotic nuclei. Only GFP positive cells were counted. The mean ±SEM is depicted (n=4-6). Inset: a representative immunoblot in Schwann cells showing increased NRIF expression after adenoviral infection. B. Rat Schwann cells were infected with GFP or GFP-NRIF adenovirus as described and fixed 24 hour after infection. Cells were immunostained with an antibody to activated caspase 3 as described. A representative image from 4 experiments is shown. C. Rat Schwann cells were infected with GFP or GFP-NRIF adenovirus as described and 18 hours later, subcellularly fractionated into the mitochondrially enriched heavy membrane fraction, which was subjected to Western blotting using antibodies to cytochrome c. The fractions were also immunoblotted using an antibody to manganese superoxide dismutase as a mitochondrial marker. A representative blot from four experiments is shown. D. Mouse embryo fibroblasts were adenovirally infected with a GFP vector, a bicistronic GFP-NRIF vector or treated with 60 J/mm$^2$ ultraviolet light. Lysates were collected at 24 hours and immunoblotted using NRIF and phospho-JNK antibodies. Tubulin is used as a loading control.


A.
GFP-NRIF
GFP
Uninfected
% GFP+ cells that are apoptotic
45
35
25
15
5
Schwann cells
Sympathetic neurons
MEFs
B.
DAPI
GFP
Caspase 3
GFP
GFP-NRIF
C.
GFP
GFP-NRIF
cytochrome c
MnSOD
D.
NRIF
UV
P-JNK
NRIF
Tubulin

heavy membrane fraction, which is enriched with mitochondria, was evaluated for cytochrome c content by immunoblotting. At both 18hr and 24 hr (data not shown) after infection we observed a decrease in the amount of cytochrome c in the mitochondria from NRIF expressing cells relative to those infected with adeno-GFP (Fig. 11C). Taken together, we conclude that NRIF is a pro-apoptotic molecule that induces the release of cytochrome c from the mitochondria and activates caspase 3.

**NRIF is not sufficient for JNK activation**

In Chapter II, I reported that p75 was not able to maximally activate JNK in the absence of NRIF. Therefore, we considered the possibility that NRIF induced cell death by activating JNK; however, over expression of NRIF in Schwann cells (data not shown) or MEFs did not significantly increase the levels of phospho-JNK (Fig. 11D). Thus, NRIF is necessary, but not sufficient for stimulating the kinase, as was previously suggested based on ectopic expression of NRIF in 293 HEK cells (Gentry et al., 2004).

**NRIF localizes to the nucleus upon p75 activation**

Although NRIF is necessary for regulating JNK (see Chapter II), its amino acid sequence revealed that it has features of a transcription factor, namely three KRAB repression domains and C2H2 type zinc fingers (Casademunt et al., 1999). Moreover, a substantial portion of NRIF localizes to the nucleus when ectopically expressed in HEK 293 cells (Gentry et al., 2004). Therefore, we investigated the localization of NRIF in the sympathetic neurons in the presence of NGF or ProNGF. By immunostaining, we observed predominantly a nuclear localization either in the presence of proNGF or

following trophic factor withdrawal, compared to a more diffuse cytoplasmic staining observed when mature NGF was added to the culture media (Fig. 12A). This suggests that NRIF may serve a dual function, modulation of JNK activity as well as gene transcription. Indeed, it has been reported that NRIF is able to bind DNA (Gentry, 2001). Therefore, we postulated that the putative DNA binding domain of NRIF is required to induce apoptosis. To test this hypothesis, we used a mutant of NRIF that deletes its 5 zinc fingers and expressed this construct in rat Schwann cells. Relative to the full length NRIF construct, the mutant lacking the zinc fingers exhibited a significant reduction in the ability to promote cell death (Fig. 12B). It should be noted that the mutant NRIF was still able to induce cell death to a greater extent than GFP alone, perhaps due to oligomerization with endogenous NRIF. In order to further examine the hypothesis that DNA binding is required for NRIF to induce cell death, we expressed this mutant construct in Schwann cells derived from the *nrif* -/- mice. I found that in the absence of NRIF, the mutant lacking the zinc fingers was still able to induce apoptosis, albeit to a lesser extent than the full-length protein (Figure 13). While further experiments are needed to address the significance of this finding, this suggests that NRIF is capable of activating cell death in the absence of DNA binding.

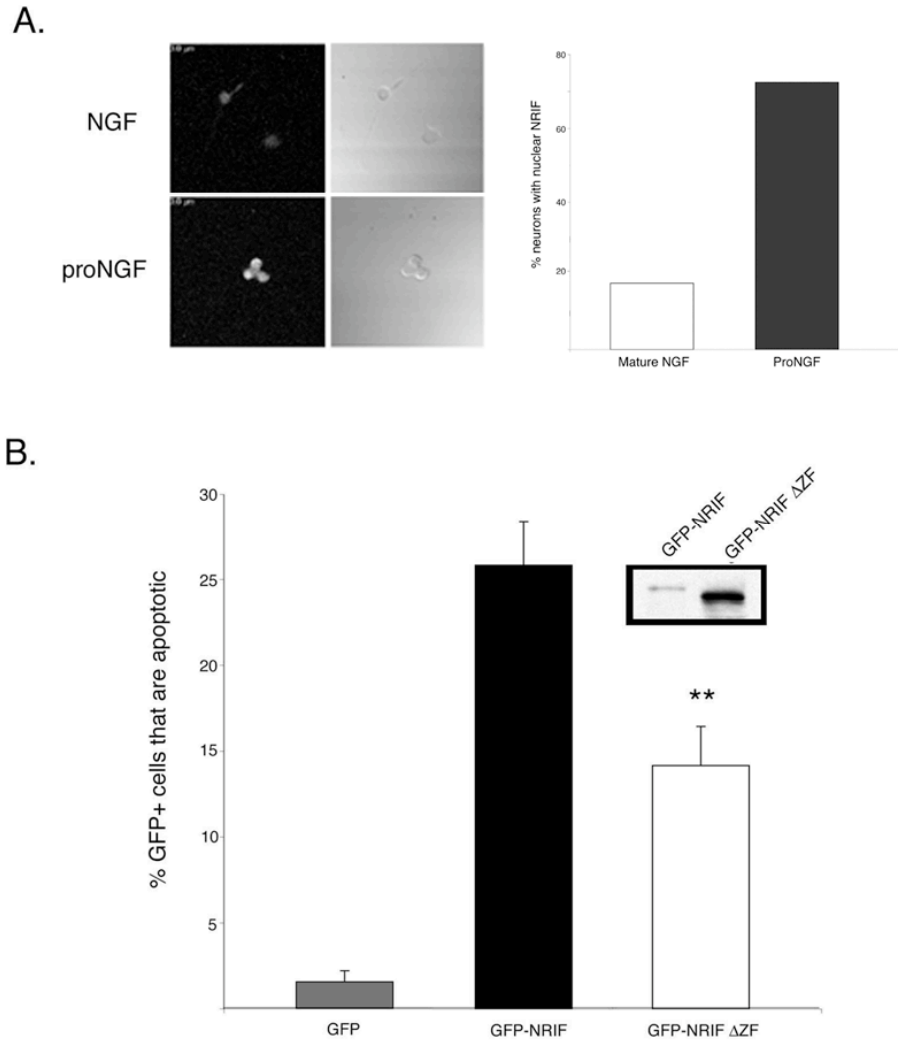



**Figure 12. Nuclear localization of NRIF following treatment with proNGF.**
A. Sympathetic neurons were treated with 20 ng/ml pro or mature NGF for 20 hours, then fixed and immunostained using an antibody to NRIF and analyzed by confocal microscopy. The same z-planes of representative neurons are depicted (representative of 2 experiments). Quantitation of these experiments is shown in the bar graph on the right. B. Rat Schwann cells were transiently transfected with a GFP, GFP-NRIF or GFP-NRIF□ZF, a construct that has the 5 zinc fingers deleted. After 48 hours, the cells were fixed and stained with DAPI to evaluate apoptotic nuclei. Only GFP positive cells were considered. The mean ± SEM are shown (n=5). Inset: A representative immunoblot showing the expression of GFP-NRIF and GFP-NRIF□ZF in 293 HEK cells after transfection. ** indicates statistical significance compared to full-length NRIF based on student's t-test, $p < 0.05$

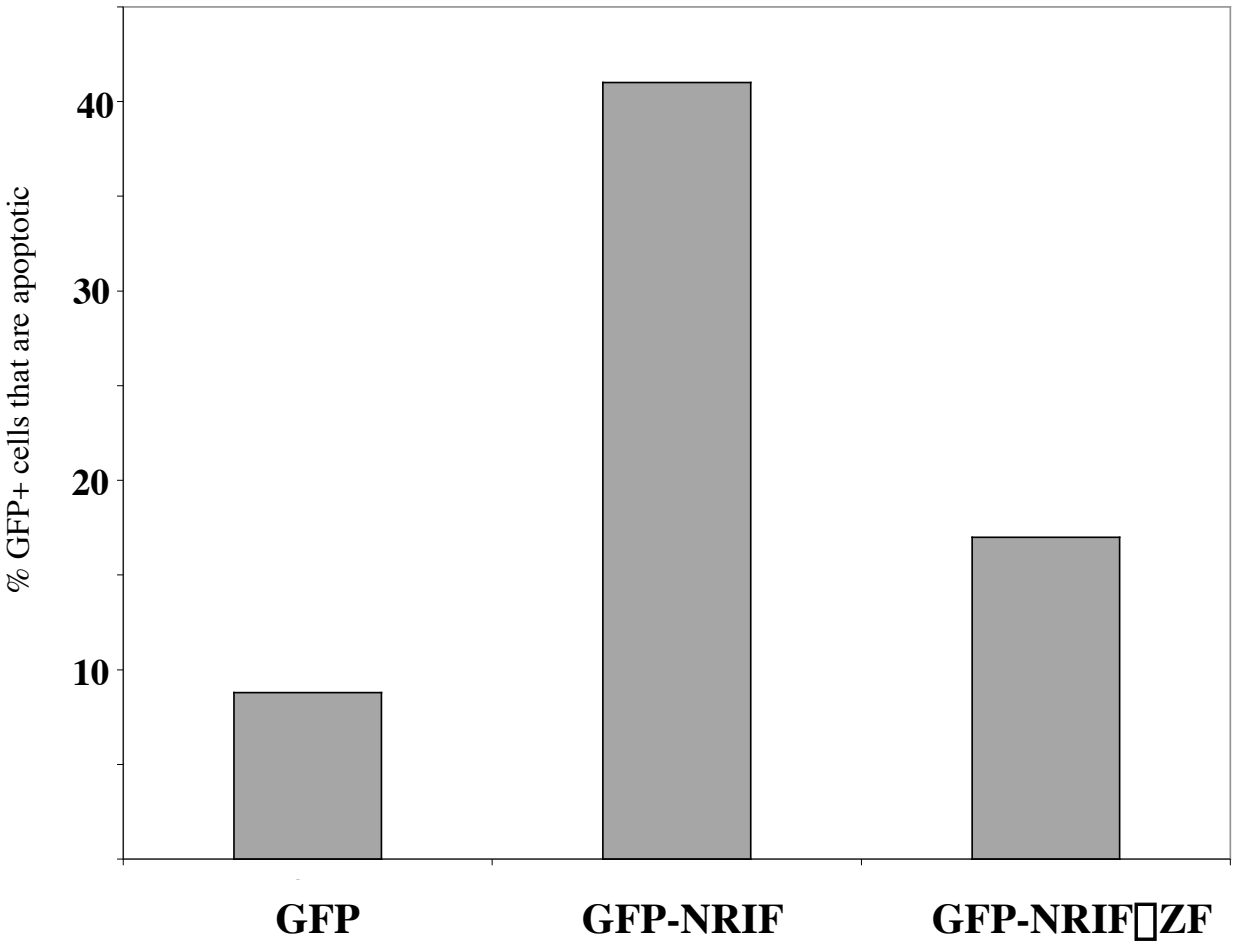



**Figure 13. Expression of GFP-NRIF and GFP-NRIFΔZF induces apoptosis in *nrif* -/- Schwann cells.** Schwann cells were cultured from *nrif* -/- mice as described and transiently transfected with GFP, GFP-NRIF, or GFP-NRIFΔZF**.** After 48 hr, cells were fixed and stained with DAPI to visualize apoptotic nuclei. Only GFP+ cells were considered. Results are an average of two experiments.

**p53 and $p19^{Arf}$ are required for NRIF-mediated apoptosis**

My previous results indicated that in sympathetic neurons, p75-mediated apoptosis occurs through a p53-dependent process. Therefore, I tested whether NRIF-induced death would also require p53. I cultured sympathetic neurons from p53 littermates and infected them with the GFP or NRIF expressing adenovirus and scored the number of apoptotic nuclei 48 hrs later. While the *p53* +/+ neurons underwent apoptosis when infected with NRIF, neurons derived from the *p53* -/- mice did not die when NRIF was ectopically expressed (Fig. 14A). Thus, like p75-mediated cell death, NRIF-induced apoptosis also required p53.

$p19^{Arf}$ is an upstream activator of p53 that acts by interacting with mdm2, thereby allowing p53 levels to increase. Therefore, to determine if this upstream activator of p53 was required for cell death induced by NRIF, we analyzed NRIF-mediated apoptosis in *$p19^{Arf}$* -/- MEFs. When NRIF was ectopically expressed in *$p19^{Arf}$* -/- MEFs, it was unable to induce cell death as compared to wild-type cells (Fig. 14B), indicating that like p53, NRIF-mediated apoptosis requires $p19^{Arf}$.

**The role of NRIF as a tumor suppressor**

NRIF induces apoptosis and inhibits cell cycle progression in immortalized cells. Given the essential roles of these processes in tumor suppression, we hypothesized that NRIF might be a tumor suppressor. In addition, preliminary evaluation of *nrif* -/- 129 Sv mice indicated that these mice develop tumors.

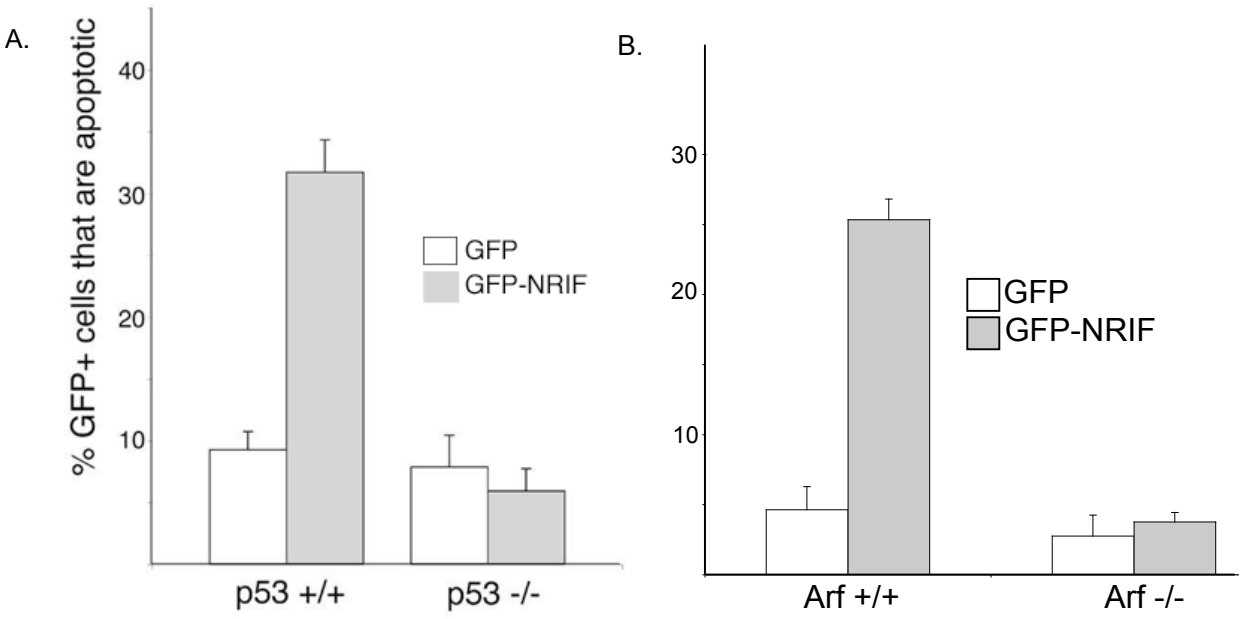



**Figure 14. NRIF does not induce apoptosis in p53 -/- or Arf -/- cells.**
A. Sympathetic neurons were isolated from *p53* +/+ and -/- littermate controls and cultured for 4 days with NGF (20 ng/ml). The cultures were then adenovirally infected with either GFP or GFP-NRIF as described. After 48 hours, the neurons were fixed and stained with DAPI to visualize the nuclei. Only GFP expressing cells were counted. White bars indicate cultures expressing the GFP vector and gray bars indicate cultures expressing the GFP-NRIF vector. The mean ±SEM is depicted (n=4). B. MEFs derived from *Arf* +/+ and -/- littermate controls were adenovirally infected with GFP or GFP-NRIF as described and treated as in A.

In our original colony, 6 of 30 (~20%) adult male mice developed tumors in a variety of tissues. Based on a histological examination, two of these mice appeared to have T cell lymphomas, one had a potential teratoma, one had a squamous cell carcinoma, while two had what appeared to be Wilms' tumors (Table 1). However, the age of onset of these tumors was unknown but the mice were >1 year of age. The spontaneous tumor incidence for the 129 mouse strain, based on the data available on the Jackson lab website (http://www.informatics.jax.org/external/festing/mouse/docs/129.shtml) is 7% for tumors of all types in male mice, 1% for teratomas, 2% for lymphomas and no Wilms' tumors were reported.

In order to further examine the incidence of tumors in *nrif* -/- mice, we bred our null mice with the 129Sv parental strain mice to obtain littermate controls. We used the offspring of this breeding scheme to get approximately 30 mice of each genotype, +/+, +/- and -/-. Each mouse was palpitated bimonthly for 20 months to evaluate the onset of any potential tumors.

**Table 1: Tumor Incidence in *nrif* -/- Mice**

| Mouse | Sex | Age | Histology |
|---|---|---|---|
| 1 | Male | > 1 year | Teratoma |
| 2 | Male | > 1 year | T cell lymphoma |
| 3 | Male | > 1 year | Squamous carcinoma |
| 4 | Male | > 1year | Wilms' tumor |
| 5 | Male | > 1 year | T cell lymphoma |
| 6 | Female | > 1 year | Wilms' tumor |

Table 1: Potential tumors observed in our original *nrif* null mouse colony. Mice with abnormal growths were sacrificed and tissues were paraffin embedded and sectioned. Sections were stained with hematoxylin and eosin, and the pathology of the tissues was examined by Dr. Mary Ann Thompson.

While we did observe 3 *nrif* -/- male mice that had abnormal growths, none of the pathology performed on these mice indicated tumors. Rather, these growths appeared to be epidermal inclusion cysts.

Epidermal inclusion cysts result from the implantation and proliferation of epidermal elements into the dermis. They are usually slow-growing and asymptomatic but may become inflamed or secondarily infected. Males are twice as likely to develop epidermal inclusion cysts as are females (Carson and Candel, 1998). Rarely, squamous cell carcinomas (Davidson et al., 1976; Miller, 1981) or basal cell carcinomas (Ikeda and Ono, 1990; Dini et al., 2001) can arise from epidermal inclusion cysts. In our mice with these cysts, however, we found no evidence of carcinomas. It remains possible that if mice with the epidermal inclusion cysts lived longer, they may have developed into carcinoma.

In addition, of the 86 mice we had in our colony at the end of our studies, 11 died without any observed tumor development. These mice were discovered dead by the Division of Animal Care and no autopsies or pathological exams could be performed. Of the mice that died, there were 6 females (1 +/+, 4 +/-, 1 -/-) and 5 males (3 +/- and 2 -/-) (Table 2). The average age of death for these mice was 10.3 months, with a range from 3 to 18 months. It is interesting to note that the mice who died later (at 12-18 months) were wild-types or heterozygotes while those mice who died at 3-11 months were homozygous null for *nrif*. Therefore, although we did not observe any obvious abnormal growths in these null animals, it is possible that they did in fact die of some type of tumor. These results suggest that NRIF is not a potent tumor suppressor.

**Table 2: Dead Mice Without Observed Tumors**

| | Sex | *nrif* genotype | Age of Death |
|---|---|---|---|
| 1 | Female | +/+ | 11 months |
| 2 | Male | +/- | 11 months |
| 3 | Male | +/- | 8 months |
| 4 | Female | +/- | 9 months |
| 5 | Male | +/- | 13 months |
| 6 | Female | +/- | 12 months |
| 7 | Female | +/- | 18 months |
| 8 | Female | +/- | 9 months |
| 9 | Male | -/- | 9 months |
| 10 | Female | -/- | 11 months |
| 11 | Male | -/- | 3 months |

Table 2: Eleven of the 86 mice died without any observation of tumor development. These mice were discovered dead by the Division of Animal Care and thus, no autopsies or pathological examination could be performed.

**Discussion**

NRIF was identified by virtue of its interaction with p75 intracellular domain (Casademunt et al., 1999). Although NRIF and p75 expression patterns overlap during murine development, NRIF is more widely expressed (Casademunt et al., 1999; Kendall et al., 2003). Moreover, deletion of nrif in the BL6 strain of mice is embryonic lethal around day 11, a phenotype not observed in the *p75* null mice (Casademunt et al., 1999). These data suggest a broader role for NRIF beyond p75 signaling; however, little is known about this putative transcription factor. Here, we report that NRIF is a proapoptotic protein that induces cell death through a mechanism dependent on $p19^{Arf}$ and p53. Moreover, NRIF can be localized to the nucleus and its ability to induce apoptosis depends on its DNA binding domain. Finally, I tested whether NRIF is a tumor suppressor and found that this protein is not a potent suppressor of tumor formation.

The amino acid sequence of NRIF revealed that it shared homology to C2H2, or Krüppel type zinc fingers, as well as a domain often associated with such zinc fingers

referred to as a Krüppel-associated box (KRAB), which serve as transcription repressor domains (Margolin et al., 1994). Thus, the structure of NRIF suggests that it regulates gene transcription, which was further supported by evidence that the protein can bind DNA through its zinc finger domain (Gentry, 2001). The ectopic expression of NRIF in 293 HEK cells results in a significant portion of NRIF being localized to the nucleus (Casademunt et al., 1999; Gentry et al., 2004) and we observed its nuclear accumulation in response to proNGF (Fig. 12A). Importantly, we found that the ability of NRIF to induce maximal cell death required the zinc finger domain (Fig. 12B), suggesting that DNA binding is required for this effect and, perhaps, for p75 to induce apoptosis. Therefore, I suggest that NRIF is a transcription factor that regulates genes controlling cellular viability.

The nuclear localization of NRIF is interesting to consider in light of recent data demonstrating that p75 is cleaved by -secretase resulting in release of the intracellular domain (ICD), a fraction of which localizes to the nucleus (Jung et al., 2003; Kanning et al., 2003). It is possible that the interaction of NRIF with the ICD of p75 occurs primarily in the nucleus or that p75 cleavage facilitates NRIF translocation to the nucleus. A growing number of cell surface receptors undergo -secretase mediated cleavage, resulting in the nuclear translocation of their ICDs (Fortini, 2002). Notch was among the first receptors shown to undergo such processing and nuclear shuttling whereupon the ICD associates with a DNA binding complex that represses transcription, CBF1/Su(H)/Lag1 (CSL). The binding of the complex to Notch ICD releases the repression and recruits the co-activator Mam, facilitating transcription of Notch target genes (Schweisguth, 2004). The nuclear accumulation of NRIF following p75 activation

suggests that NRIF is recruited to DNA, as opposed to being released, as is the case for CSL in Notch signaling; however, it remains to be determined whether NRIF accumulates in the nucleus due to translocation, stabilization or up regulation.

In addition to its role in p75 signaling, we found that p53 and $p19^{Arf}$ were required for apoptosis induced by ectopic expression of NRIF (Fig. 14). This result provided an explanation for the ability of NRIF to activate a cell death program without stimulating JNK. Since JNK can phosphorylate and activate p53 (Hu et al., 1997; Fuchs et al., 1998), the requirement for p53 in NRIF-mediated apoptosis in the absence of JNK activation indicates that there may be two pathways activated that converge on this transcription factor, one dependent on the stress kinase and the other on NRIF. How NRIF regulates p53 remains to be elucidated.

The dependence of NRIF-mediated apoptosis on p53 and $p19^{Arf}$ implies that these tumor suppressors are downstream of NRIF; however, experiments performed to detect an increase in p53 or $p19^{Arf}$ protein expression or p53 stabilization upon NRIF expression have been inconclusive. In addition, the p53 target genes p21, PUMA and NOXA were evaluated by quantitative real-time PCR after NRIF expression but again, I was unable to observe any significant and reproducible differences in the levels of these mRNAs. The inconsistency in these experiments may be due to the low level of NRIF transfection efficiency in primary cells.

It is interesting to note that p53 was recently reported to act at the mitochondria in some cell death paradigms, independent of its more established role in the nucleus as a regulator of transcription. P53 was shown to activate Bax through a post-translational mechanism, resulting in permeabilization of the mitochondria and activation of a

cytochrome c death program (Chipuk et al., 2004). In addition, p53 triggers the release of pro-apoptotic proteins normally sequestered by Bcl-xL. Therefore, the role of p53 in cell death is complex and it remains possible that NRIF alters the ability of p53 to regulate mitochondrial function.

Since NRIF induces apoptosis and induces cell cycle arrest (Benzel et al., 2001), I hypothesized that in the absence of NRIF, animals would have defective apoptotic pathways and unregulated cell cycles that might allow for tumor development. Concomitantly, we observed apparent tumors in the *nrif*-/- mice from our original colony (Table 1). However, upon scaling up our mouse colony by breeding *nrif* heterozygotes, we did not see evidence for neoplasms. In fact, the abnormal growths we did observe in these mice appeared to be epidermal inclusion cysts, a non-malignant condition. While in rare cases epidermal inclusion cysts can lead to basal cell (Ikeda and Ono, 1990; Dini et al., 2001) and squamous cell carcinoma (Davidson et al., 1976; Miller, 1981), I did not observe either of these in our mice. It is, however, possible that had I allowed the mice to live longer with the cysts, we may have found evidence of carcinoma.

Although we did not record any malignant tumors, we did observe mice that died unexpectedly. These were mice that had no evidence of abnormal growths on prior examinations. Interestingly, the mice who died at earlier ages (3-11 months) were null for *nrif* while those who died at later ages (12-18 months) were *nrif* wild-types or heterozygotes (Table 2). The significance of this is unclear but it may indicate that some of the *nrif* null mice did have tumors that we did not detect.

Although our *nrif* null mice did not develop tumors, there are other experiments that can be performed that would potentially provide insight into the role of NRIF in

tumor suppression. Given that many tumor suppressors act on the cell cycle, it is not surprising that when mouse embryo fibroblasts (MEFs) are isolated from mid-gestation embryos, those cells obtained from mice deficient in tumor suppressor genes (e.g. p53) proliferate more rapidly in culture than their wild-type counterparts. Thus, if NRIF functions as a tumor suppressor, we would propose that *nrif* null MEFs would behave more like an immortalized cell line while the wild-type cells would proliferate more slowly.

*nrif* null MEFs could also be used in other assays that would help determine if it has properties similar to other known tumor suppressors. Specifically, these cells could be used to analyze cell cycle profiles and cell size as well as to determine if they arrest properly in response to stresses, such as serum deprivation or ionizing radiation. In addition, the null cells could be used in senescence assays to evaluate if they have any increased ability to form colonies when plated at low density. Finally, null cells could be used in oncogene transformation assays to determine if they can be transformed directly by a single oncogene (like Ras) or require the cooperation of another oncogene, such as c-Myc or adenoviral E1A, for co-transformation. Cells that lack oncogenic checkpoints will not arrest in response to an oncogene and therefore, hyperproliferate and transform. If deletion of *nrif* in our MEFs caused hyperproliferation and transformation after an oncogenic stimuli, it would indicate that NRIF has some properties of a tumor suppressor.

However, it is possible that in the above assays, *nrif* null cells would proliferate normally, exhibit unchanged cell cycle profiles and cell size, and respond normally to stress. These cells might not form colonies when plated at low density and might only

weakly be transformed with oncogenes and yet NRIF might still function as a tumor suppressor. Such is the case for the tumor suppressor p16-INK4A. This protein is a human tumor suppressor because reverse genetics studies indicated that mutant p16-INK4A is a hallmark of familial human melanoma (Kamb et al., 1994; Nobori et al., 1994; Okamoto et al., 1994). However, MEFs derived from p16-INK4A null mice proliferate normally (Krimpenfort et al., 2001; Sharpless et al., 2001), undergo senescence (Krimpenfort et al., 2001; Sharpless et al., 2001) and exhibit no other properties that indicate the gene is required for tumor suppression. These mutant mice show a subtle predisposition to spontaneous tumor formation within the first year of life (Sharpless et al., 2001). When treated with carcinogens, *p16-INK4A* null mice develop tumors more rapidly than wild-type littermates (Sharpless et al., 2001). Thus, *p16-INK4A* plays an important role in murine tumor suppression after carcinogen exposure.

My current data indicates that if NRIF is a tumor suppressor, it certainly is not a potent one. Unlike p16-INK4A, NRIF has not been studied in human disease and is not known to be a human tumor suppressor. However, if we treated our null mice with carcinogens or crossed them with *p53* +/- mice, we may observe an increased rate of tumorigenesis, similar to what was observed for the p16-INK4A mutant mice. Results from these experiments would provide insight into NRIF function and its role as a potential tumor suppressor. Finally, given the availabilty of many tumor banks, it would also be interesting to screen for restriction fragment linked polymorphisms (RFLPs) in human NRIF in such tissue samples.

In summary, I have found that NRIF is a proapoptotic protein that likely mediates death, in part, through regulation of transcription. NRIF is not a potent tumor suppressor

but may have certain qualities of these types of proteins. Finally, NRIF-mediated and p75-mediated apoptosis are similar in their requirements for p53 but seem to in the mechanisms by which they converge on p53. Activation of p75 activates JNK, while NRIF-mediated death is independent of the kinase. How NRIF modulates the $p19^{Arf}$–p53 pathway will be the subject of future studies.

# CHAPTER IV

## CONCLUSIONS

During development, cell death is critical for the establishment of a mammalian nervous system. Approximately 50% of neurons that are generated undergo apoptosis to ensure that a functional nervous system is created. In addition, apoptosis plays an important role in the loss of neural cells in response to injury and in neurodegenerative disorders. There is accumulating evidence to indicate that p75 plays an important role in regulating cell survival during developmental processes such as proper development of the SCG. The goal of the work outlined in this dissertation was to determine the role of the p75 interacting protein, NRIF, in cell death mediated by the receptor.

In Chapter II, the work focused on the role of NRIF in p75 signaling. I found that NRIF is required for p75-mediated apoptosis in sympathetic neurons but not for the cell death that occurs after the neurons are withdrawn from NGF (Figures 5 and 6). This work illustrates a clear difference in the requirements for unique proteins that are necessary for p75 induced cell death versus death that occurs after withdrawal of growth factor. Activation of p75 was known to activate the downstream signaling molecules, JNK and NF-κB. Activation of the stress kinase, JNK, in the nervous system is an established pro-apoptotic event in p75-mediated cell death. Previous data indicates that NF-κB counteracts the pro-death signal emanating from the receptor. The data in Chapter II shows that NRIF is required for activation of the JNK pathway but in the absence of *nrif*, the NF-κB mediated survival pathway is unaffected (Figures 7 and 8).

These data indicate that NRIF is uniquely involved in p75 death pathways, rather than any pathway activated by the receptor. This represents the only interactor known so far to be selectively involved in only one aspect of the dichotomous signaling initiated by p75.

Perhaps the best-characterized downstream target of JNK is the transcription factor, c-Jun. However, c-Jun is not required for p75-induced cell death (Palmada et al., 2002). Therefore, we evaluated the requirements of other proteins in p75-mediated apoptosis that are downstream of JNK in this pathway. The tumor suppressor p53 was reported to be a substrate of JNK in p75 signaling (Aloyz et al., 1998), so I hypothesized that in the absence of p53, p75 stimulation would not induce cell death in sympathetic neurons. Similar to the requirements for NRIF, *p53*-/- neurons do not undergo p75-mediated apoptosis but these same neurons do die when withdrawn from NGF (Figure 9). Taken together, this data indicates that p75-induced cell death requires both NRIF and p53.

These data challenge the long-standing neurotrophin hypothesis, which states that neurons send out axons to target tissues that are secreting growth factors in limited supply. If the axon reaches the target at the appropriate time, a survival signal is transduced through ligand binding to the Trk receptors. However, this hypothesis states that if a neuron reaches the target at an inappropriate time and the supply of neurotrophin is no longer available, that neuron undergoes programmed cell death that is due to lack of a Trk-mediated survival signal. Our data suggest that the apoptosis that occurs in sympathetic neurons *in vivo* may not be due to a lack of Trk survival signaling, but rather to a specific activation of p75 death signaling. We demonstrate that although NRIF and

p53 are not required for cell death after NGF withdrawal, both proteins are required for p75-mediated apoptosis. In addition, similar results were obtained for both c-Jun (Palmada, et al., 2002) and TRAF6 (Yeiser, et al., 2004). Moreover, if *p75* is genetically deleted, this hypothesis predicts that there would be no change in the number of neurons because the apoptosis is due only to a lack of survival cues from the Trk receptors. In fact, when *p75* -/- mice are examined, there are in fact more neurons, suggesting that in the absence of p75, there is an accumulation of neurons, due to lack of p75 initiated apoptosis (Bamji, et al. 1998). In light of these data, we argue that *in vivo*, it is not as simple as the classical neurotrophin hypothesis predicts and p75 is likely playing an active role in stimulating cell death.

While I have established that NRIF is essential for p75-mediated apoptosis, I have yet to identify how NRIF functions in this signaling paradigm. Given the requirement for p53 in p75-induced cell death, we hypothesized that p75 activation in these neurons would lead to an accumulation of p53 protein and that in the absence of NRIF, this would no longer occur. Experiments to date have been unable to resolve this hypothesis and it remains the subject of ongoing experiments. It is possible that NRIF is upstream of p53, but does not regulate its levels. In support of this, I found that NRIF-induced cell death depends on p53 but we have been unable to detect an increase in p53 levels after NRIF expression. Alternatively, NRIF may interact with p53 directly or as part of a protein complex and thus antagonize p53 function in mediating p75-induced cell death. Finally, if NRIF is in fact a transcription factor, it could interact with p53 at the promoters of specific target genes and modulate p53 function in this manner.

In Chapter III, I evaluated the function of NRIF in the absence of receptor signaling. I found that NRIF is a proapoptotic protein that is able to induce cell death in a variety of primary cell types. This apoptosis is accompanied by caspase-3 activation and cytochrome c release from the mitochondria (Figure 11). Since I found that in the absence of NRIF, p75 activation was unable to stimulate the JNK pathway, we wanted to determine if NRIF was capable of activating this stress kinase. Ectopic expression of NRIF in Schwann cells or MEFs was not able to activate JNK, indicating that NRIF is necessary, but not sufficient, for JNK activation.

NRIF has features of a transcription factor, including KRAB repression domains and C2H2-type zinc fingers. Therefore, I evaluated the subcellular localization of NRIF after activating p75 using proNGF. I observed a predominantly nuclear localization of NRIF when p75 was stimulated compared to a more diffuse staining in the presence of mature NGF (Figure 12). I hypothesized that the putative DNA binding domain of NRIF would be required for NRIF to induce apoptosis and found that a mutant of NRIF that deletes the zinc fingers could not induce apoptosis as efficiently as the wild-type protein. Together, these data suggest that NRIF may regulate cell death by translocating to the nucleus and regulating gene transcription. The notion that NRIF functions in apoptosis by regulating transcription is currently being investigated.

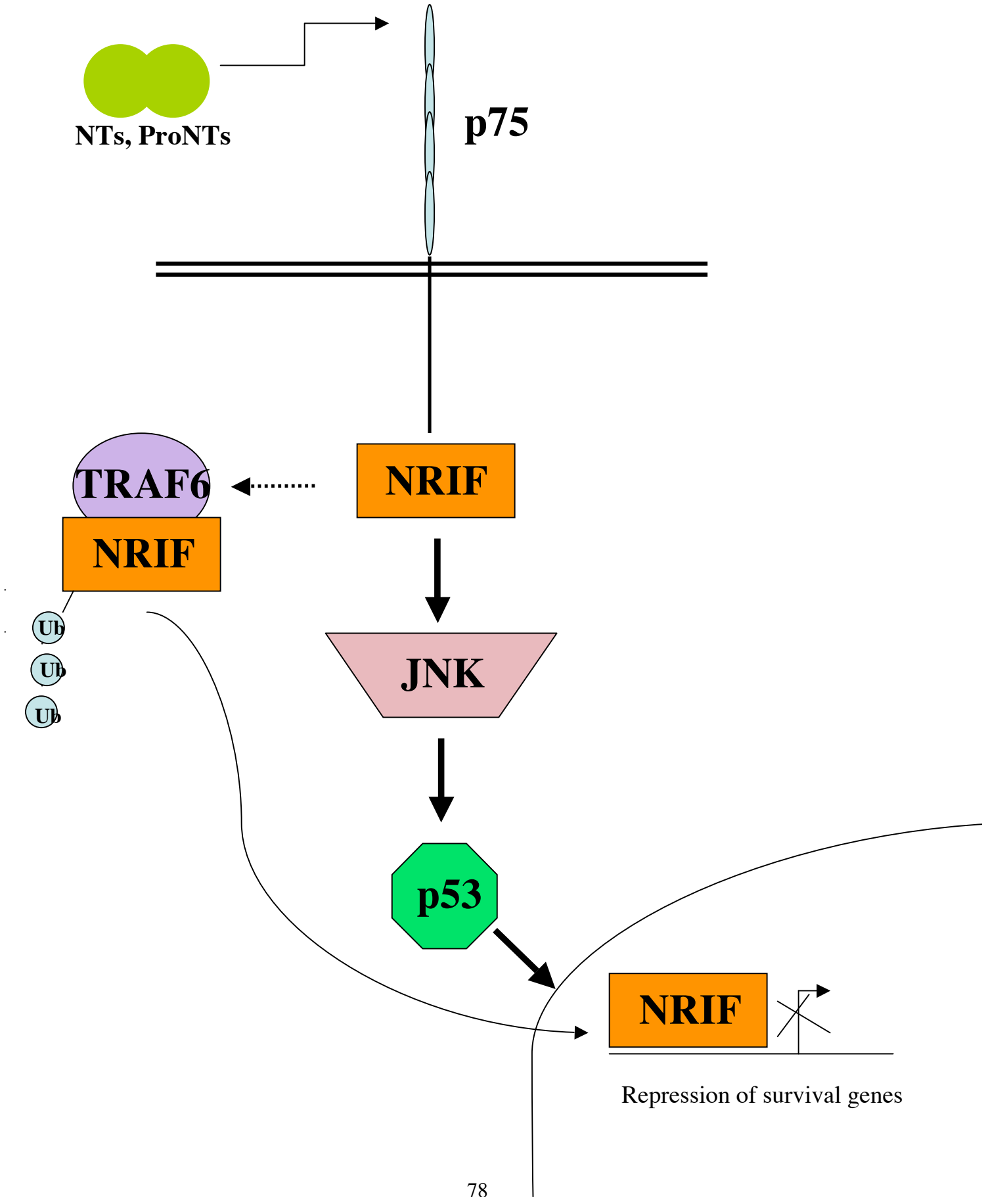
The data obtained so far indicate that NRIF is part of signaling pathway that is downstream of p75 and upstream of JNK. However, NRIF likely has a nuclear function based on its nuclear localization after p75 activation and the requirement for its zinc finger DNA binding domain to induce maximal levels of apoptosis. From these data, a model emerges as to how NRIF might function as both a cytoplasmic and nuclear protein

(Figure 15). Upon neurotrophin binding p75, NRIF activates a death pathway that leads to JNK activation, with p53 a potential downstream target of this stress kinase. In the absence of NRIF, there is no activation of JNK in sympathetic neurons and thus, p75-mediated cell death is attenuated. Likewise, p75-induced apoptosis cannot occur in the absence of p53. Since we also observed NRIF in the nucleus after proNGF activation of p75, one mechanism by which NRIF may translocate to the nucleus is by its interaction with TRAF6. TRAF6 has been reported to stabilize NRIF's expression in 293 HEK cells by an unknown mechanism (Gentry, et al., 2004). However, TRAF6 is an E3 ubiquitin ligase and recent data indicates that this may occur through TRAF6 modifiying NRIF by ubiquitination (M. Wooten, preliminary data) and thus stabilizing NRIF. Perhaps the interaction with TRAF6 allows NRIF levels to accumulate such that there is a population of NRIF in both the cytoplasm modulating JNK activity as well as in the nucleus regulating transcription. Moreover, both NRIF and TRAF6 are required to reconstitute JNK activity in 293 HEK cells so this protein complex is likely necessary for maximal activation of JNK signaling. In addition to stabilization and JNK activation, the TRAF6-mediated ubiquitination of NRIF may target it to the nucleus.

Alternatively, it has been suggested that upon cleavage with presinilin-dependent -□-secretase, the p75 ICD translocates to the nucleus. To this end, an interaction of NRIF with the p75 ICD was identified (R. Kenchappa, preliminary data), which would allow NRIF to translocate to the nucleus where it can regulate transcription of genes controlling cellular viability.

I also evaluated NRIF's role as a tumor suppressor in Chapter III. In the original *nrif* -/- mouse colony, I observed a tumor incidence of ~20% in males in a variety of

**Figure 15. A Proposed Model of NRIF function in p75 signaling.** In this model, ligand binding to p75 results in NRIF activating the JNK pathway. One substrate of JNK is p53, which can then translocate to the nucleus and regulate its target genes. P75 activation also induces the interaction of NRIF and TRAF6, which results in TRAF6 ubiquitinating NRIF. The ubiquitination of NRIF stabilizes the protein and allows for nuclear translocation. Once in the nucleus, NRIF likely functions as a transcriptional modifier to repress genes involved in cell survival.


NTs, ProNTs
p75
TRAF6
NRIF
NRIF
Ub
Ub
Ub
JNK
p53
NRIF
Repression of survival genes

tissues, compared to 7% for the parental strain. Upon scaling up the mouse colony, however, I did not see any increased incidence of tumors in *nrif* -/- mice versus wild-type littermate controls. I therefore conclude that NRIF is not a potent tumor suppressor.

The current data establish that NRIF is essential for p75 activated cell death and induces apoptosis in primary cells. However, much remains to be learned about this protein. Importantly, NRIF has domains indicating it may regulate transcription. Efforts are ongoing to determine the preferred DNA binding sequence of NRIF so that candidate genes can be found that NRIF may regulate. It is also unclear whether NRIF transcriptional control will play a role in both p75-mediated apoptosis and in cells that ectopically express NRIF and thus undergo cell death. It is possible that NRIF has multiple functions, depending on whether p75 is expressed. This is especially important considering the embryonic lethality of *nrif*-/- mice in the BL6 background. Since *p75* deletion is viable, this lethality strongly suggests that NRIF has a broader function in development. Understanding the reason for the lethality is paramount to further identifying the physiological importance of NRIF.

It is important to remember that NRIF is only one of a dozen or so p75 interacting proteins. These studies have focused solely on NRIF with little regard for the role of the other interacting protein. In the future, it will be important to determine the complex interplay of these different interacting protein in p75 signaling in different cellular contexts. Similarly, as NRIF is required for p75-mediated and p75-Sortilin-mediated apoptosis, it will be interesting to determine if NRIF is involved in other p75 signaling complexes, for example, in p75-Trk signaling or p75-Nogo receptor signaling.

Furthermore, what role NRIF might have in other TNF receptor signaling cascades still needs to be addressed.

Finally, the experiments presented here do not address the existence of NRIF2. As NRIF2 also interacts with p75, future experiments will need to address the role of NRIF2 in this paradigm. Although this homolog does not exist in humans, it may compensate for *nrif* deletion in mice (Benzel et al., 2001). Therefore, it will be interesting to examine p75-mediated apoptosis in the absence of both NRIF and NRIF2. It remains possible that deletion of both genes in mice will result in a novel or more dramatic phenotype.

The data presented in this dissertation demonstrate that NRIF is a proapoptotic molecule that is required for p75-mediated cell death. While numerous other p75-interacting proteins have been reported, none has been linked to established p75 signaling pathways. My data is the first to describe the outcome of p75 signaling in the absence of an interactor and provides valuable insight into NRIF function.

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