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Structure-function Analysis of NRAGE: A Protein Involved in Developmental Neural Apoptosis

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STRUCTURE-FUNCTION ANALYSIS OF NRAGE: A PROTEIN INVOLVED IN DEVELOPMENTAL NEURAL APOPTOSIS

By

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STRUCTURE-FUNCTION ANALYSIS OF NRAGE: A PROTEIN INVOLVED IN DEVELOPMENTAL NEURAL APOPTOSIS

By Rebecca A. Cowling

Thesis Advisor: Dr. Joseph M. Verdi

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
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The Neurotrophins and Bone Morphogenic Proteins (BMP) have both been implicated in the maintenance of cellular proliferation and apoptosis in the developing nervous system. Downstream of both signaling pathways is NRAGE, a member of the Melanoma Antigen (MAGE) gene family and, under BMP stimulation, activates p38 leading to caspase 3 cleavage. NRAGE possesses a highly conserved MAGE Homology Domain (MHD) and a second, less well conserved MHD (denoted MHD2) as well as a unique 25 tandem WQXPXX hexapeptide repeat region. Binding partners for the MHD and hexapeptide repeat regions have been identified but their cellular consequences have not been defined. Therefore, a structure-function analysis of NRAGE was performed to identify whether it is the MHD or the repeat region that tranduces the apoptotic

signal. Nine NRAGE deletion mutants were built to isolate the identified regions and expressed in P19 cells, a neural development model system. Through Annexin V staining, it was found that the MHD is essential for apoptotic function and the hexapeptide repeat region does decrease the degree of cellular death. The MHD region also induces p38 activation and caspase 3 cleavage, as well as apoptosis. Overall, these studies ascribe yet another function to the highly conserved MHD and give further insight into the mechanisms governing neural progenitor cell apoptosis.

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LIST OF ABBREVIATIONS

(in order as they appear in the text)

NRAGE: Neurotrophin *R*eceptor Interacting M*AGE* protein

p75NTR: p75 *N*euro*t*rophin *R*eceptor

MAGE: *M*elanoma *A*nti*ge*n

BMP: *B*one *M*orphogenic *P*rotein

MHD: *M*AGE *H*omology *D*omain

Caspases: *C*ystienyl, *asp*artate specific prote*ases*

APAF-1: *A*poptosis *p*rotease *a*ctivating *f*actor

SMAC/Diablo: *S*econd *M*itochondrial *A*ctivator of *C*aspase/*D*irect *IA*P *b*inding protein with *lo*w pI

ATP: *A*denosine *Tri*phosphate

E (as in E14): *E*mbryonic

XIAP: *X* -linked *I*nhibitor of *ap*optosis

BrdU: *Br*omo*d*eoxy *U*ridine

CDK: *C*yclin *d*ependent *k*inase

Rb: *R*etino*b*lastoma

ICD: *I*ntracellular *d*omain

Dlxin-1: *Dlx In*teracting Protein

NF-68: *N*euro*f*ilament-68

GFAP: *G*lial *F*ibrilary *A*cidic *P*rotein

MAP2a/b: *M*icrotubule *A*ssociated *P*rotein

TAK: *T*GFβ *A*ctivated *K*inase

TAB: *T*AK1 *b*inding *p*rotein

RT-PCR: *R*everse *T*ranscriptase-*P*olymerase *C*hain *R*eaction

MHC: *M*yosin *H*eavy *C*hain or *M*ajor *H*istocompatibility *C*omplex

NGF: *N*erve *G*rowth *F*actor

BDNF: *B*rain *D*erived *N*eurotropic *F*actor

NT3 or NT4/5: *N*euro*t*rophin

Trk: *T*yrosine *R*eceptor *K*inase

JNK: *J*anus *N*-terminal *K*inase

RA: *R*etinoic *A*cid

TGFβ: *T*ransforming *G*rowth *F*actor

XAF: *X*IAP *A*ctivating *F*actor

IL-3: *I*nter*l*eukin-3

NADE: *N*eurotrophin *A*ssociated *D*eath *E*xecutor

EGFP: *E*nhanced *G*reen *F*luorescent *P*rotein

HEK: *H*uman *E*ndothelial *K*idney

N2A: *N*euroblastoma *2A*

DMEM: *D*ulbecco's *M*odified *E*ssential *M*edia

7AAD: 7 *A*mino-*A*ctinomycin *D*

BRCA: *Br*east *Ca*ncer

Chapter 1

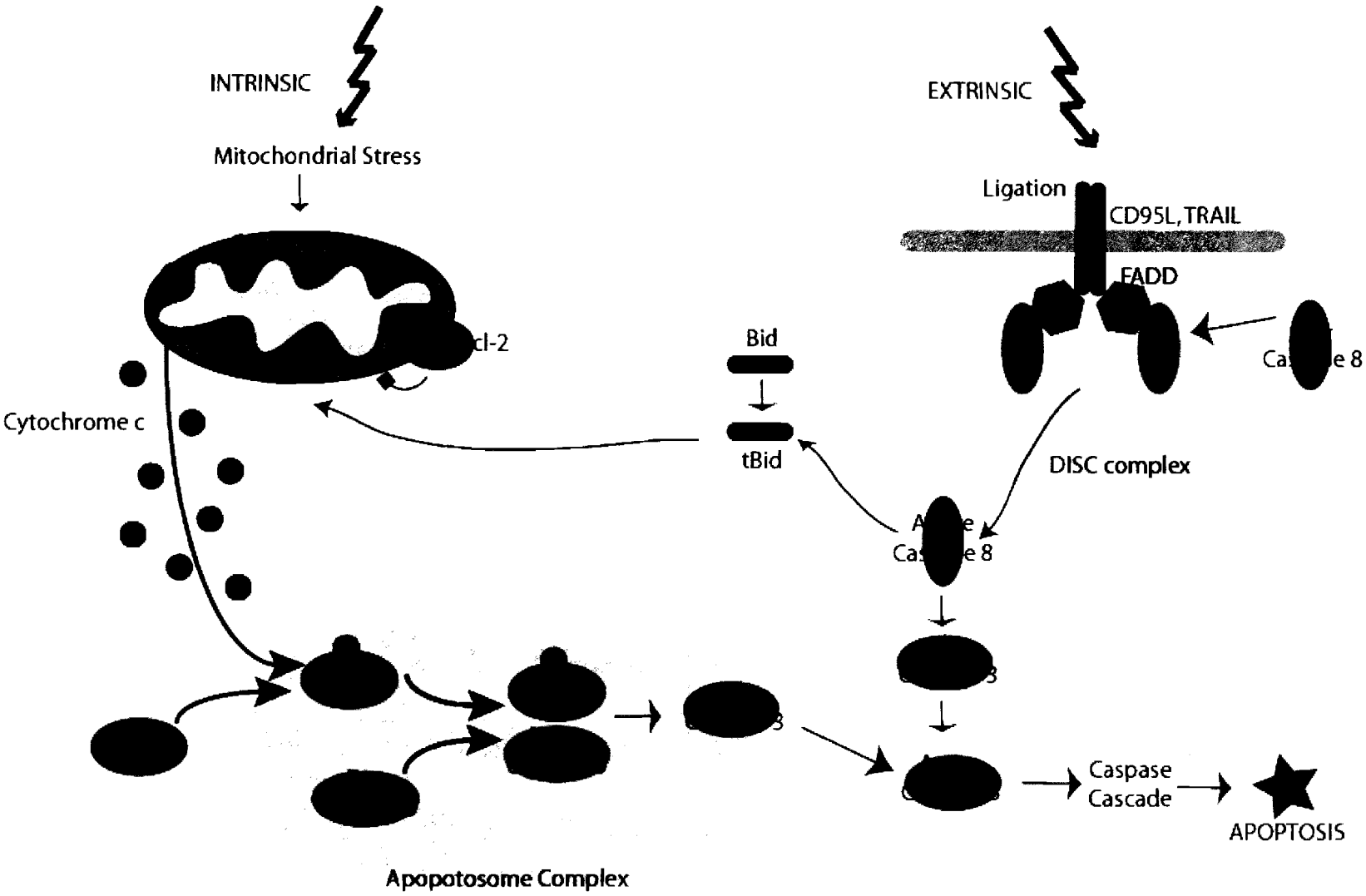
INTRODUCTION

Apoptosis is an Active and Selective Mechanism in Neural Development

The development of the mammalian nervous system begins in the early stages of embryogenesis and is not complete until the organism reaches adulthood. The brain and spinal cord are derived from the neural tube, a single layer of cells generated from the neuroepithelium. The cerebral cortex is generated through a series of events through which proliferation and diversification of a neural stem cell begins with its birth and its terminal function is defined by its birth date and the environment in which it receives its cues[1] (reviewed in [2]). There is a significant overproduction of neurons and, as a result, the brain is developed through a finely balanced mechanism of cell proliferation and apoptosis, or programmed cell death [3-5]. Too little proliferation or too much apoptosis in development can result in mental retardation and dementia later in life (reviewed in [6]). Conversely, too much proliferation or too little apoptosis can result in neuroblastomas and astrogliomas, central nervous system cancers with

very poor prognosis. Thus, an extremely significant method of both pruning the developing brain and maintaining tissue homeostasis is apoptosis.

Apoptosis is often the cells' response to genetic instability, working on the premise that a cell is better dead than damaged (reviewed in [7]). However, apoptosis or programmed cell death, [8] is also a normal developmental mechanism to control cell number and remodel tissues [9]. The molecules involved in apoptosis are evolutionarily conserved with a substantial expansion of gene families as the phylogenic tree diversifies [7]. Caspases (cystienyl, aspartate specific proteases) are major executioners of the apoptotic process and are subdivided into initiator and executioner molecules, both of which require dimerization and proteolytic cleavage to initiate downstream effects. Upon internally mediated mitochondrial permeabilization, cytochrome C is released and forms the apoptosome, an aggregate of APAF-1 (apoptosis protease activating factor), ATP, and cytochrome c (the intrinsic apoptotic pathway, reviewed in [7]). Herein the pro- initiator caspases are cleaved and these molecules can subsequently initiate the cleavage of executioner caspases (such as 3 and 7) which leads to cell death (figure 1). The extrinsic pathway works ultimately in a similar mechanism but is initiated through cell external cues. Upon appropriate ligand binding, the extrinsic pathway can utilize molecules such as SMAC/Diablo (Second Mitochondrial Activator of Caspase/Direct IAP binding protein with low pI) to release the inhibition that an Inhibitor of Apoptosis (IAP, XIAP being the prototypic molecule) family member has on caspase cleavage (Reviewed in [7]).


INTRINSIC
Mitochondrial Stress
EXTRINSIC
Ligation
CD95L, TRAIL
FADD
Bid
tBid
Cytochrome c
DISC complex
Caspase
Cascade
APOPTOSIS
Apoptosome Complex

Labeling of apoptotic cells by virtue of their fragmented DNA showed that apoptosis is substantial in the developing mouse cortex, beginning at around E (embryonic day) 12, peaking at E14 and decreasing to nearly negligible levels in the adult [4]. The majority of apoptotic cells in the developing brain are in the proliferative zones of the developing cortex such as the primoridal plexiform layer and the ventricular zone [4].

Up to 70% of cells are dying in the proliferative zones at E14 and this steadily decreased until about 1% of cells are actively apoptosing in the adult [4]. Furthermore, a large proportion of apoptotic cells are actively synthesizing DNA within an hour prior to death, as shown by BrdU staining [5]. This suggests that cells are active in the cell cycle as they are apoptosing, indicating that cell cycle activity is a significant event in initiating apoptosis. Although the dogma has been that post-mitotic neurons can not re-enter the cell cycle, current theories suggest that post-mitotic neurons can re-enter the cell cycle but this triggers apoptosis [10]. Several cell cycle molecules such as cyclin D-CKD and Rb/E2F are upregulated in cortical neurons and cell culture lines exposed to apoptosis inducing factors such as UV-irradiation [11]. Furthermore, G1/S checkpoint molecules, such as CDK4/6, Rb, p107 and p130, have all been shown to be critical for post-mitotic neuron survival, thus expression in post-mitotic neurons is often observed in cells undergoing apoptosis signaling events (reviewed in [12]). Overall, this demonstrates that post-mitotic neurons have a fail-safe mechanism; for when the cell cycle exit checkpoints are breached, apoptosis cascades are soon

triggered, preventing the cell from re-entering the cell cycle and possibly damage the cell and organ as a whole.

Identification of NRAGE and Regulation of NRAGE Expression and Activity

Using the $p75^{ICD}$ (Intracellular Domain) as bait, a single clone from an E13 (embryonic day 13) mouse library was isolated and named NRAGE for Neurotropin Receptor-Interacting MAGE Homolog [13]. In parallel, two similar proteins, MAGE-D1 [14] and Dlxin-1 [15] were cloned from human and mouse yeast-two hybrid screens respectively. These were subsequently found to be orthologs with 87% identity to the human MAGE-D1 and with 97% identity in the c-terminal 315 amino acids, including the hexapeptide repeat region [13]. NRAGE is encoded on the X-chromosome and contains a MAGE homology domain (MHD) as well as a 25 tandem WQXPXX hexapeptide repeat region (unique to any mammalian genome), characteristics which classify it as a MAGE-D family member [16] (figure 2). The MHD region is extremely well conserved in the MAGE-D subfamily and is partitioned on multiple exons, a unique feature of the MAGE-D genes and suggestive of these genes being evolutionarily the earliest MAGE genes [16]. There is also a second, less well conserved MHD region (denoted MHD2) which is only found in the MAGE-D family, E1 and L2 genes. NRAGE is a 775 amino acid protein with a molecular weight of about 87 kDa [13]. NRAGE co-immunoprecipitates with $p75^{NTR}$ when both proteins are endogenously- or over-expressed. NRAGE co-immunoprecipitates when the p75 ICD is aberrantly expressed, but NRAGE does not co-immunoprecipitate with the

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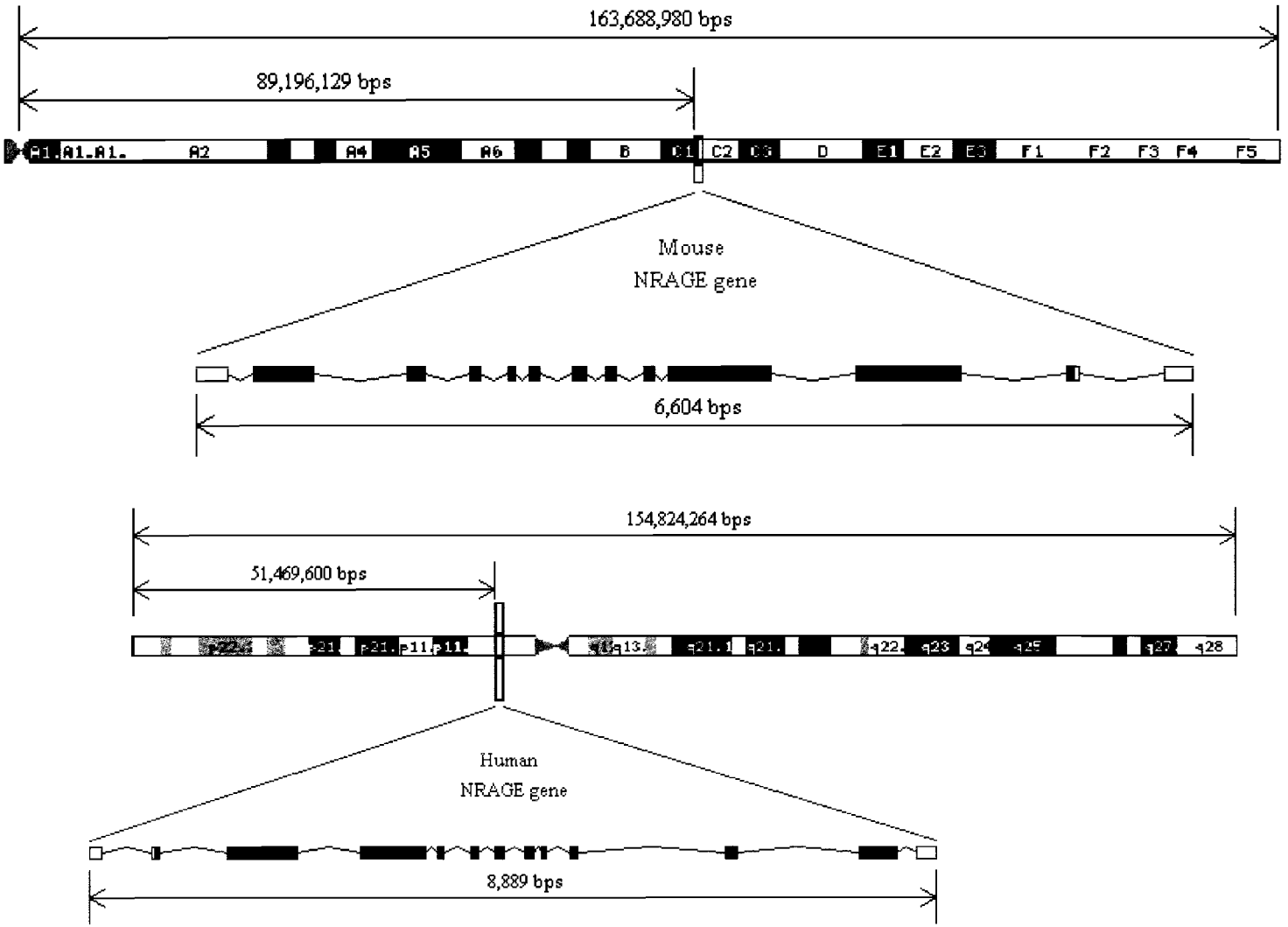
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A1.A1.A1.
A2
A4
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C1
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F3
F4
F5
Mouse
NRAGE gene
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51,469,600 bps
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p21
p21.3
p11.2
p11.1
q13.3
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q27.1
q28
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NRAGE gene
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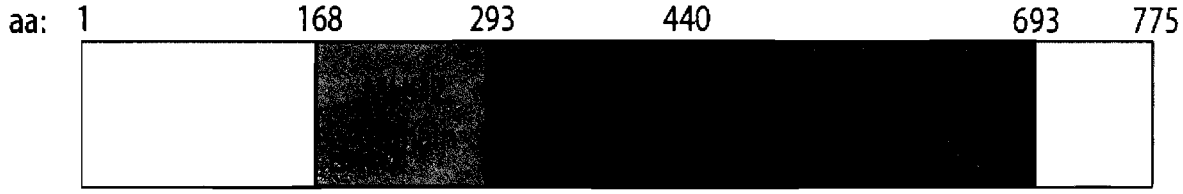

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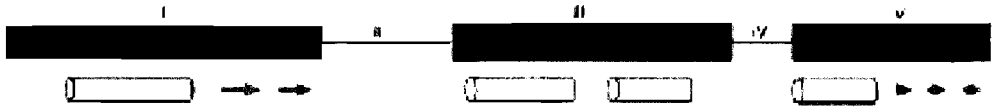


$p75^{NTR}$ C-terminal death domain on its own signifying that the death domain is not responsible for NRAGE interaction [13].

mRNA expression of NRAGE is ubiquitous, from the early embryo and extra embryonic tissues into adulthood. However, protein expression is highly regulated both temporally and spatially and is found predominantly in the developing brain [17]. Further inspection with antibodies against markers of neuronal differentiation found that NRAGE was co-expressed with cells reactive to neural and glial progenitor populations, as marked by NF-68 (neurofilament-68) and GFAP (Glial Fibrillary Acidic Protein) respectively, but did not co-localize with the late-neural marker MAP2a/b (Microtubule Associated Protein 2a/b). This demonstrates that NRAGE is originally expressed throughout the neuroepithelium while later in development, expression is found in committed but undifferentiated populations of cycling progenitors and is not expressed in post-mitotic neurons susceptible to changes in neurotrophin expression [17]. This temporally regulated expression is significant as neurotrophin-dependent survival of neurons begins at around E16 and continues into adulthood, suggesting that NRAGE expression can be an apoptosis effector of the neurotrophin pathway [13]. Notably, NRAGE was also expressed throughout the cerebral cortex where $p75^{NTR}$ expression was completely absent [18] suggesting that NRAGE has functions outside of $p75^{NTR}$ mediated signaling (further described below).

To clarify the downstream effects of NRAGE induced apoptosis, a series of biochemical assays were performed to determine how NRAGE confers its apoptotic effect. Upon NRAGE over-expression there are increased levels of

activated effector caspases 3 and 7 [19]. Using a tetracycline-inducible NRAGE adenovirus, cell death was rescued when broad spectrum caspase inhibitors BAF or zFAD-fmk were added, collectively signifying NRAGE mediates cell death through caspase activation [19]. Release of cytochrome c from the mitochondria, brought on by active caspase 9 and APAF-1 complexing with cytochrome c, is a key step in the apoptotic process[20,21]. In NRAGE-adenovirus infected cells, high levels of cytochrome c were detected in the cytoplasmic fraction indicating that NRAGE over-expression can contribute in the release of cytochrome c from the mitochondria. However this was not the result of activated caspase 9 because cells treated with the caspase inhibitor showed an accumulation of cytochrome c in the cytosolic fraction, indicating that cytochrome c is released from the mitochondria in the absence of caspase activation [19]. This means that mitochondrial membrane depolarization and the release of cytochrome c under the regulation of NRAGE are independent of the activation of caspase 9 and is not required for the apoptosome to form[19].

NRAGE's Role in Cell Proliferation and Differentiation

There is increasing evidence that misregulation of cell cycle molecules and breach of cell cycle checkpoints is one possible mechanism for neural progenitor and post-mitotic neuron- induced programmed cell death [22-25]. Through BrdU incorporation assays, it was found that NRAGE over-expression attenuates cell cycle progression in human endothelial kidney 293 cells [13, 26]. NRAGE over-expression induced p53 expression as early as 24 hours post transfection, and also induces activation of $p21^{waf1}$ and $p27^{kip1}$ within 24 hours,

maximally at 72 hours, indicating growth arrest at the G1-S transition within 72 hours of NRAGE over-expression [22, 26]. Apoptosis, as measured by Annexin V staining was greatly attenuated when p53 was knocked down in P19 cells treated with either Retinoic Acid (RA) or Bone Morphogenic Protein (BMP) suggesting that p53 is a downstream participant of NRAGE-mediated apoptosis through BMP activation [22]. Using p53-null mouse embryonic fibroblasts, it was shown that after 2 days of NRAGE over-expression, there is no change in cell cycle kinetics suggesting that p53 is downstream of NRAGE-induced activity [26]. Taken together, these experiments demonstrate that NRAGE can act upon the cell cycle by influencing the phosphorylation status of p53 which in turn mediates downstream $p21^{cip/waf}$ and $p27^{kip1}$ activities on the cell cycle [22,26].

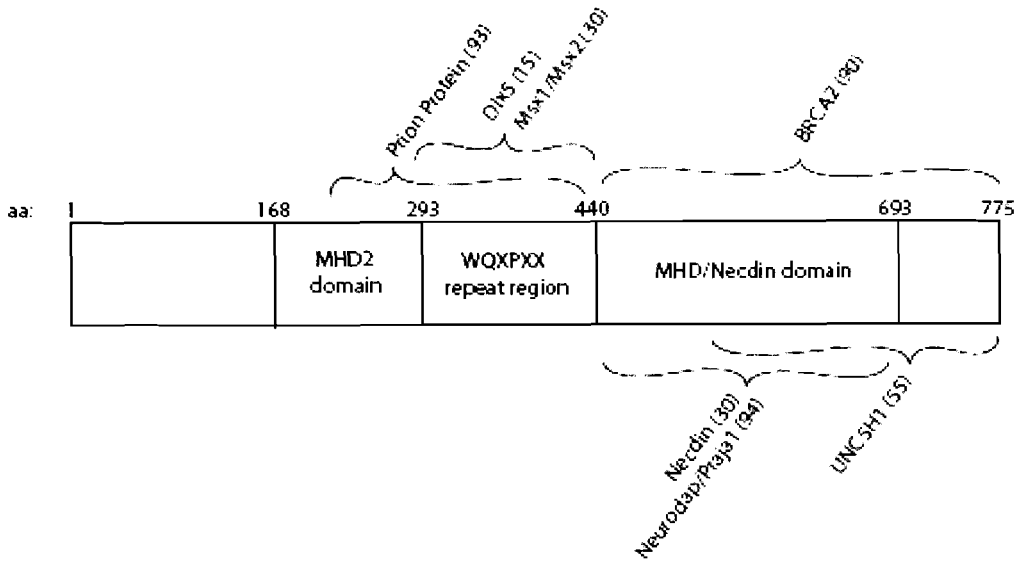
Through Western blot analysis it was also seen that the level of phosphorylated p38 was increased within the first 48 hours of NRAGE expression then decreased precipitously at 72 hours [22]. As p38 is a downstream mediator of TAK1-TAB1 (TAK1: TGFβ-Activated Kinase, TAB1: TAK1 binding protein) induced BMP signaling, this implies that NRAGE is an indirect mediator of p38 activation. NRAGE over-expressing cells at 24 hours showed an increase in the G2-M phase of the cycle, and at 48 hours most were either at G1 or in S phase which was maintained at 72 hours, signifying an increase in cell cycle kinetics [22]. However, when a pharmaceutical p38 inhibitor was used, TAK dominant negative construct overexpressed, or TAK morpholino expressed to knockdown expression, NRAGE over expressing cells incurred rapid cell cycle arrest at G1, and this was sustained over the 72 hour period. Furthermore, a dominant negative

SMAD5 was co-transfected with NRAGE and as predicted, there was a cell cycle arrest at G2, signifying a bipotential role of BMP-signaling through NRAGE [22].

Permanent withdrawal from the cell cycle is requisite for differentiation of cells to mature phenotypes. As NRAGE was shown to influence the cell cycle and over-expression can induce apoptosis, it is likely that NRAGE plays a role in differentiation. As NRAGE over-expression is often lethal to cells, a morpholino was utilized to knockdown NRAGE expression in P19 cells which were treated with retinoic acid to induce neuronal differentiation. Cells treated with the NRAGE morpholino showed a drastic reduction in the number of post-mitotic neurons as demonstrated by MAP2a/b staining while the number of GFAP+ glial cells remained constant. By contrast, the number of Nestin positive cells was increased in NRAGE morpholino treated cells, evidence of the cells' inability to terminally exit the cell cycle and differentiate into neurons [22]. Semi-quantitative RT-PCR showed that it was not the inability of the cells to produce pro-neural genes such as Mash1 in NRAGE knockdown cells, but rather there was a maintenance of stem-like gene expression such as Oct4/5 and neural differentiation antagonists Hes1 and Hes5 [22]. Overall, these data suggest that NRAGE is required for the terminal exit of the cell cycle in neural progenitor cells and a loss of cell cycle control can lead to NRAGE induced apoptosis through a BMP dependent pathway which includes the downstream activation of p38 [22].

Necdin

Necdin is a type II MAGE gene [27-34] and is the closest homolog to NRAGE [35]. Maternal Necdin is silenced, and loss of paternal Necdin, most often through abnormal imprinting, is associated with Prader-Willi syndrome, a neuro-behavioural disorder characterized by respiratory distress and hypotonia in neonates, and hyperphagia causing severe obesity, hypogonadism and behavioral problems in childhood [36]. Over-expression of Necdin strongly suppresses cellular proliferation by blocking entry into the cell cycle and can associate with cell cycle proteins p53 and E2F1 to inhibit their activity, inducing cell cycle arrest in progenitor cells. These interactions occur at Necdin's MAGE Homology Domain and it has further been shown that Necdin can form homodimers through its MHD. Because of these observations, the potential for interaction of Necdin and NRAGE through their respective MHD to induce cell cycle arrest and/or differentiation was investigated [30]. Full length NRAGE and deletion mutants which include the MHD were constructed and upon over-expression were show to immunoprecipitate with Necdin[30] (figure 3). NRAGE binds to Necdin at the MHD and NRAGE can also interact with Msx1/Msx2 at the repeat region, resulting in a trinary complex [30]. Both Msx1 and Msx2 co-immunoprecipitate with Necdin both in the presence and absence of NRAGE but could not associate with Necdin with a mutation that can not bind to NRAGE. This suggests that Msx1 or Msx2 can bind to Necdin only when Necdin is able to bind to NRAGE (likely a form of conformational change) [30].



XIAP: requires RING domain to interact with NRAGE (unknown NRAGE region required 67)
p75NTR: requires juxtamembrane region to interact with NRAGE (unknown NRAGE region required)
UNC5H1: requres Zu-5 and PEST region to interact with NRAGE MHD
Praja1: requires RING domain to interact with NRAGE MHD

Figure 3: Map of NRAGE with Corresponding Interactions with Adaptor Proteins.

Representative map of mouse NRAGE identifying the regions required for interactions with various proteins (identified as bracketed regions). Shown are the identified domains with the corresponding amino acid counts. References: Prion Protein[93], Dlx5[15], Msx1/Msx2[30], BRCA1[90], Necdin[30], Praja1/Neurodap-1[94], UNC5H1[55], XIAP[67] (MHD: MAGE Homology Domain).

To determine how this interaction affects cells, C2C12 muscle cells were utilized because Msx2 can repress transcriptional regulation of myogenic differentiation genes in these cells. NRAGE over-expression released the transcriptional repression effects of Msx2 potentially through a competitive binding mechanism[30]. Necdin over-expression alone increased the expression muscle differentiation genes MHC (myosin heavy chain) and MyoD each by a factor of three. However, when Necdin and NRAGE were both over expressed,

MHC expression increased nine-fold and MyoD expression increased five-fold. C2C12 cells naturally express NRAGE, so Necdin may augment the effects of NRAGE in sequestering of Msx2, thereby releasing inhibition of the differentiation of C2C12 cells [30]. However, a caveat to this data is that C2C12 cells do not express Necdin at detectable levels, and there is no normal tissue that has endogenously high levels of Msx and Necdin together, signifying that these results may be strictly interpreted in vitro. Having said that, Necdin and NRAGE may interact in other tissues as both are expressed in P19 cells, brain and skeletal muscle. It may be that Necdin's influence on the cell cycle can be monitored by NRAGE and that any inability of Necdin to prevent the cell from cycling may trigger NRAGE to induce differentiation.

NRAGE is a Key Mediator of the Neurotrophic Theory

The neurotrophic theory outlines the role of target-derived neurotrophic factors in regulation of cell survival during neural development [3] (reviewed in [37]). As a direct result, the theory defines the role of apoptosis in neural development as a necessary mitigating factor in the control of cell number and overall tissue architecture [9]. Neurotrophic factors are the survival signals produced by the target tissue (outlined below), and through their production, the target tissue regulates the neuronal input it receives. This regulation can be accomplished in three ways: regulation of production of neurotrophins; the regulation of their secretion; or the limited uptake by pre-synaptic terminals (reviewed in [3]).

The neurotrophins are a family of growth factors which include Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 4/5 (NT4/5) and Neurotrophin-3 (NT3). Apart from regulating survial, these growth factors also regulate dendritic and axonal growth as well as synaptic activity [38,39]. The neurotrophins also preferentially bind to pairs of growth factor receptors termed Trk (Tyrosine Receptor Kinases) and $p75^{NTR}$ (Neurotrophin Receptor). NGF preferentially binds to TrkA, BDNF and NT4/5 to TrkB and NT3 to TrkC, and each of these can bind to the $p75^{NTR}$ as shown by co-immunoprecipitation when the proteins are over expressed [40, 41]. The Trks have no sequence similarity to $p75^{NTR}$ and activate distinct neurotrophin-dependent pathways but $p75^{NTR}$ does cooperate to increase the binding affinity of the respective neurotropins to their receptors [42] and enhance NGF-mediated TrkA activation [42-44].

$p75^{NTR}$ has a significant role in cell death in a wide range of biological settings within and beyond the nervous system. In vivo studies have shown that over-expression of the intracellular domain results in widespread neuronal apoptosis in the developing central and peripheral nervous systems [45,46]. Accordingly, a lack of NGF, disruption of the association of NGF to $p75^{NTR}$ in cells, or mice lacking full length $p75^{NTR}$ (i.e. dominant negative receptor) results in decreased apoptosis in the retina and spinal cord [46-50]. Furthermore, the Janus N-terminal kinase (JNK) pathway is up-regulated upon initiation of NGF dependent apoptosis, and pharmaceutical inhibition of JNK signaling [51,52] or dominant-negative [53] expression of JNK attenuates NGF-dependent cell death.

The first investigation of NRAGE as a mediator of NGF-dependent death was conducted in MAH cells, an immortalized sympathoadrenal cell line. These cells naturally lack $p75^{NTR}$ and TrkA receptors and do not apopotose when stimulated with NGF even in the presence of exogenous expression of $p75^{NTR}$ [44]. Furthermore when NRAGE is over expressed, the cells show no increase in apoptosis when stimulated with NGF, demonstrating that NRAGE was not sufficient to mediate cell death in the absence of downstream receptor-mediated NGF activation [13]. However, when MAH cells stably expressing $p75^{NTR}$ were transfected with NRAGE, there was a significant reduction of cell survival when treated with NGF. Treatment with NGF also showed an increase of NRAGE at the membrane suggesting that recruitment of NRAGE to the membrane-

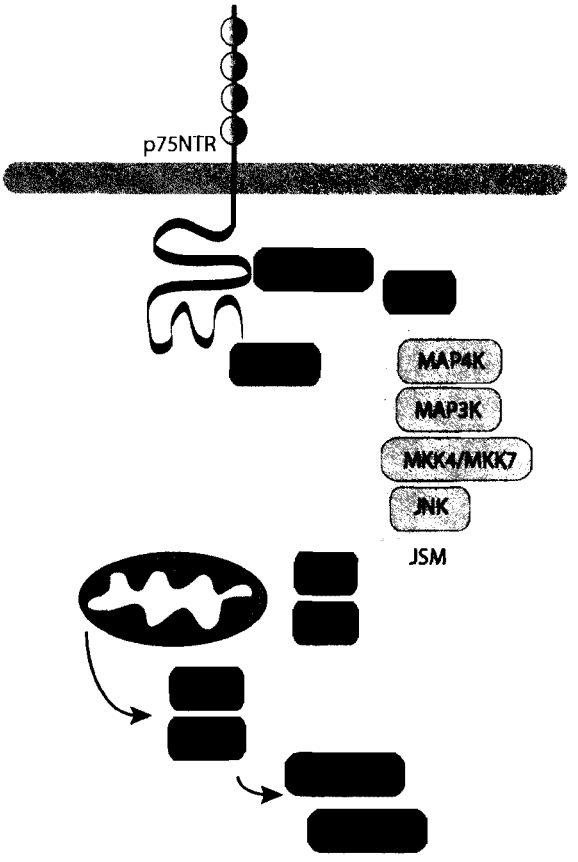


Figure 4: $p75^{NTR}$-Mediated Apoptotic Signaling Cascade. $P75^{NTR}$ mediates the apoptotic signaling cascade through NRAGE. Upon ligand activation, NRAGE signals through the MAP kinase pathway to phosphorylate JNK. Upon JNK activation there is a release of the apoptotic inhibitory molecules (such as BH3 molecules Bim, Bad, and SMAC/Diablo) which in turn, facilitates cytochrome c release and caspase activation, ultimately resulting in apoptosis (adapted from Barker, PA, 2004[95])

associated $p75^{NTR}$ is required for the initiation of the apoptotic cascade. This effect was specific to NGF, as neither BDNF nor NT3 induced cell death. Overall, this shows that NRAGE expression is requisite to NGF-mediated cell death through the $p75^{NTR}$ receptor [13]. Conversely, when MAH cells were exogenously expressing $p75^{NTR}$, TrkA and NRAGE, and treated with NGF, there was a rescue of cell survival, possibly through survival mechanisms mediated by NGF [13]. Induced NRAGE expression activated the JNK pathway and activated caspases 3, 7, and 9, as well as showing cytosolic accumulation of cytochrome c. Blockage of the pathway at JNK and c-Jun reversed the effects on caspase cleavage and rescued apoptosis [19]. Overall, these results demonstrate NRAGE is a mediator of $p75^{NTR}$ signaling through JNK resulting in caspase-mediated cell death (figure 4).

UNC5H1

UNC5H1 is a membrane associated receptor that is the closest family member to $p75^{NTR}$ [54] and is known to be involved in axon guidance and apoptosis. Williams et al [55] have shown that UNC5H1 can also associate with NRAGE. Through mutational analysis it was found that the C-terminal tail of NRAGE, including the MAGE domain is necessary to interact with UNC5H1 while the ZU-5 domain of UNC5H1 is required to interact with NRAGE specifically (figure 3). This interaction occurs predominantly at the cell membrane [55]. To understand the functional consequences of these interactions, PC12 cells were used to determine how these two proteins mediate neural differentiation. Native PC12 cells express

NRAGE at high levels but expression is significantly down-regulated upon differentiation, coincident with the inability of UNC5H1 to co-immunoprecipitate with NRAGE in differentiated PC12 cells. Over-expression of UNC5H1 in native PC12 cells induced massive apoptosis but the percentage of apoptotic cells diminished to neglible levels in differentiated cells. NRAGE transfection resulted in a mild increase in apoptosis in differentiated PC12 cells, partially rescued by over-expression of both constructs. Surprisingly, the over-expression of NRAGE in differentiated PC12 cells caused the cells to retract their processes, in the presence or absence of UNC5H1 suggesting multiple mechanisms of NRAGE activity in the development of neural architecture [55].

NRAGE's involvement in BMP-mediated Cell Death

Several reports have demonstrated that Bone Morphogenic Proteins (BMPs) and their receptors are expressed in the developing brain [23,56,57]. The temporal and spatial regulation of BMP signaling is highly regulated, as was shown in two elegant experiments using chick embryos and cortical progenitors [56,57]. In chick embryos, high levels of BMP4 expression in even numbered rhombomeres induces the expression of Msx2 in odd-numbered rhombomeres and increases the rate of apoptosis of neural crest cells [56]. In E13 rat cortical cultures, BMP at 10ng/mL shows substantial apoptosis. However, BMP treatment of E16 rat cortical cultures results in an increase in neuronal differentiation at the expense of glial differentiation while not altering the percentage of undifferentiated cells [57]. Delaying the addition of BMP4 to E16 cortical cells by three to four days

produces an increase in astrocytic differentiation, confirming the responses of the cell to BMP stimulation are time sensitive [57]. This suggests that at early stages of neural development, BMP signaling is involved in patterning as well as apoptosis and pruning of the neural precursor population, while at later stages it is involved in pro-neural differentiation. Noggin, the BMP receptor antagonist, added to E16 cortical progenitors results in an increase of oligodendrocytes at the expense of neurons, suggesting that BMP influence cortical cells by inhibiting oligodendrocyte differentiation and promoting neuronal differentiation. Importantly, BMP2 protein is abundant in the E16 cortex and in the neonate, and is present in the cortical plate and ependymal/subependymal region but absent in the white matter and corpus collosum. These reports illustrate that BMPs are essential in the developing brain to regulate brain cellular architecture through patterning effects of cellular differentiation and apoptosis [23].

NRAGE protein expression increases significantly upon treatment with RA and BMP4, a rise accompanied by an increase in activated caspase 3 [22]. Through the use of morpholinos to knock down NRAGE expression, the loss of NRAGE rescues the apoptotic effects of RA and BMP4 addition. Concurrently, the NRAGE knockdown stayed the increase in caspase activation, signifying that NRAGE is required for apoptosis in P19 and neural explants. NRAGE associated with all three BMP receptors, weakly with the SMAD proteins but very strongly with TAK1, TAB1 and XIAP, members of the non-canonical BMP pathway known to activate downstream p38 but only in the presence of BMP4. The phosphorylation status of p38 was increased in the presence of BMP4-stimulated

NRAGE, correlative with the level of TAK1, signifying that p38 activation is downstream of NRAGE and TAK1 and ultimately, BMP4 activation of receptor. Abrogation of p38 activity through the use of the chemical inhibitor SB203580 resulted in a decrease in apoptosis (as seen by flow cytometry). This was also confirmed though the use of a morpholino diminishing TAK1 levels and a SMAD5 dominant negative construct, demonstrating that both pathways are required for NRAGE-mediated BMP-induced apoptosis through the

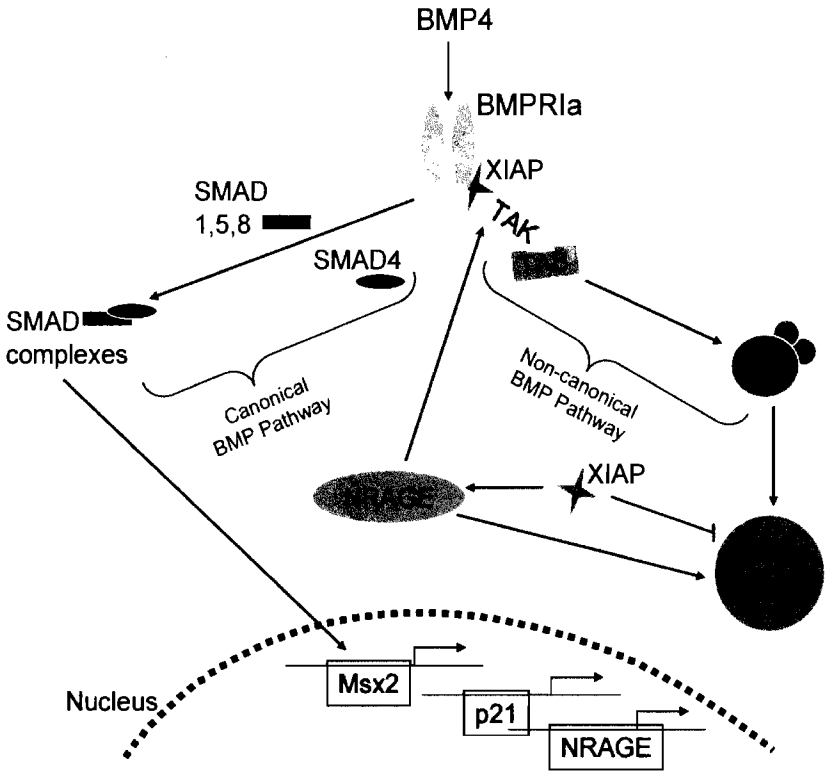


Figure 5: Canonical and Non-Canonical BMP Signaling. Schematic diagram of BMP signaling through the canonical SMAD adaptor proteins and the non-canonical pathway mediated by NRAGE. NRAGE is hypothesized to form a complex with XIAP, TAK1 and TAB1 to initiate p38 activation and downstream apoptotic responses (diagram courtesy J. Verdi)

phosphorylation of p38 [22].

Classical BMP signaling involves the members of the SMAD family of co-factors, which are shared with the TGFβ super family, of which the BMPs are a subfamily. The canonical pathway involves SMAD 1, 5 or 8 complexing with SMAD4, the co-factor, and the heterodimeric complex translocating to the nucleus where it activates transcription [58], [59]. SMADs 6 and 7 are inhibitors of the complex and generally are signaled through a separate but parallel mechanism to inhibit transcription. However, a second signaling pathway has been described which utilizes XIAP as the bridge between TAB1 and TAK1 and elicits downstream events in the JNK, NLK and MAPK pathways [60]. TAK1 is an activator of TAB1, a member of the MAPKKK family and is stimulated upon TGFβ1 or BMP4 activation [61] and upon over-expression can induce ventral mesoderm formation (see figure 5).

XIAP

NRAGE was also identified through a yeast two-hybrid screen as a binding partner for XIAP (X-linked Inhibitor of Apoptosis) [62]. As a member of the Inhibitor of Apoptosis (IAP) family, XIAP has an amino-terminal cystiene rich baculovirus IAP domain (BIR) and a RING zinc finger motif. XIAP was originally characterized as a protein that links the BMP receptors to downstream TAK1 and TAB1 and it had previously been shown that XIAP can bind to the BMP receptors through its RING domain and to TAB1 via its BIR domain [62]. XIAP can also interact with the nuclear protein XAF1, and Smac/DIABLO can sequester XIAP to prevent its anti-apoptotic activity [63-65]. Furthermore it has also

been implicated in JNK and NF-κB signaling [60,66]. NRAGE interacts with the RING domain and not the BIR domain of XIAP (figure 3). 32D cells are a promyeloid leukemic cell line, and a well established model for studying apoptosis by growth factor withdrawal as they undergo high rates of apoptosis upon Interleukin 3 (IL-3) withdrawal. When NRAGE is over-expressed, there is a significant increase in the number of apoptotic cells upon growth factor withdrawal, seen at a faster rate than with IL-3 withdrawal alone. Furthermore, the increase in apoptosis upon NRAGE over-expression is co-committant with an interaction of NRAGE with XIAP. Bcl-2 over-expression can rescue growth factor removal induced apoptosis in 32D cells, however, upon NRAGE overexposure, the effects of Bcl2 over-expression are completely abolished and cells undergo a similar rate of apoptosis upon IL-3 withdrawal [67]. This signifies that NRAGE and XIAP cooperate to induce apoptosis in 32D cells. However, the region to which XIAP interacts with NRAGE is not known, nor is it known whether this interaction is required for apoptosis during neural development. Understanding the region of NRAGE that is required for apoptosis, whether it is through a mechanism with XIAP, upstream or downstream molecules remains to be elucidated.

Msx/Dlx

The mouse transcript of NRAGE was discovered through an interaction with Dlx5, a member of the Dlx family of homeodomain proteins that are widely expressed in the developing forebrain, limbs and branchial arches during embryonic development [68,69] and is specifically expressed in developing bone and

induced in the process of bone fracture healing [68,70-73]. In this study NRAGE was identified in a yeast-two-hybrid screen and the 25 hexapeptide repeat of WQXPXX of was identified as the region most likely to bind with the N-terminal domain of Dlx5 as this sequence was overlapping in all the clones identified [15] (figure 3). Full length NRAGE and deletion mutants encoding the WQXPXX region could immunoprecipitate with Dlx5 as well as Dlx7 and Msx2, other members of the Dlx/Msx homeodomain protein family [30]. Both Dlx5 and Msx2 are induced by BMP-signaling and Msx proteins are involved in neural development [74] and in the BMP-mediated cell death pathway [23, 75]. Msx1 and Msx2 have been shown to be downstream regulators of BMP-mediated apoptosis, and in P19 cells Msx2 over-expression can rapidly induce apoptosis only when cells are in monolayer [75]. Importantly, BMP treatment of Msx2-expressing P19 cells did not augment apoptosis, signifying that over-expression was enough to induce apoptosis but addition of BMP to native P19 cells did result in an increase in Msx2 transcript [75], possibly because the BMP pathway was saturated by Msx2 over-expression. However, unpublished data by Masuda et al [15], indicate that BMP treatment of three cell lines including P19 did not alter the expression level of NRAGE. Overall, these results indicate that BMP-mediated apoptosis through Msx2 could be mediated by NRAGE. In situ data demonstrates that this may occur in the bone or developing limb as well as in the brain, as was originally described.

A Structure-Function Analysis of NRAGE is Warranted to Identify the Functional Domains of the Protein

Published literature demonstrates that NRAGE can bind to a number of varied binding partners and is associated with apoptosis in a number of developmental and cellular contexts (summarized in figure 3). The majority of these studies were borne of a yeast two-hybrid screen, which used a specific bait to isolate NRAGE. The regions of NRAGE involved in the binding of these proteins have not fully been characterized, especially in the case of XIAP and $p75^{NTR}$. Moreover the functional significance of these interactions have not been characterized with respect to their actions within and upon the cell. Surprisingly, the known interactors of NRAGE are also diverse in their own functions and these studies indicate that NRAGE has more functions than apoptosis alone. Thus, a structure-function investigation of the domains of NRAGE is warranted to identify how NRAGE can interact and carry out its functions. Unlike the other MAGE family members, MAGE-D genes have unique repeat regions as well as a second less well-conserved MAGE homology domain. NRAGE has been shown to interact with many molecules within apoptotic pathways but has since been shown to be important in other cellular mechanisms as well. The repeat region of NRAGE can associate with Dlx5 and Msx1/Msx2, proteins involved in BMP mediated apoptosis. The MHD of NRAGE can interact with Necdin and UNC5H1, both of which are involved in neural development however the functional significance of these interactions remains elusive. XIAP, a member of non-canonical BMP signaling pathway has also been shown to interact with

NRAGE, but like $p75^{NTR}$, the region responsible for these interactions has not been found (see figure 3 for a summary). Given the duality of NRAGE as a regulator of the mitotic index and apoptosis; we sought to ascribe specific NRAGE domains to these functions. We generated a series of deletion constructs that target each domain, similar to the classical structure-function studies of Shc [76], TrkA [77], and NADE [78]. To test these constructs in a neural differentiation paradigm, we utilized the P19 cell system to assess the roles of the individual regions in apoptosis. Both the WQXPXX region and MHD regions are known to interact with several proteins, thus our hypothesis is that these regions are essential to NRAGE functions. Furthermore it is hypothesized that this region is the region responsible for the downstream p38 activation and caspase cleavage previously described as essential to NRAGE-mediated apoptosis upon BMP stimulation. It was found that the repeat region is important but the MHD is essential for NRAGE mediated apoptosis. These actions are mediated by p38 and through which apoptosis is initiated through caspase 3 cleavage. Overall, these studies will help to clarify the role of the identified regions of NRAGE and ascribe functions to the regions and potentially to their binding partners as well.

Chapter 2

MATERIALS AND METHODS

NRAGE Deletion Clones

An adult ICR mouse brain was dissected free of meninges, washed twice with sterile PBS, minced, homogenized and RNA isolated using the Trizol reagent as per the manufacturer's protocol. cDNA was reverse transcribed using the BioRad iScript reverse transcriptase and the reaction product diluted in 80μL of nanopure water. Forward primers, where possible from the published sequence (NM 019791), incorporated an endogenous methionine as a start site for translation, a Xho restriction enzyme site, a Kozak sequence and start codon. The reverse primer incorporated a Sal1 restriction site (to facilitate subsequent cloning) after the removal of the termination codon, which would generate a fusion protein with eGFP in the pEGFP-N3 vector (Clontech). PCR reactions were performed with the Taq DNA polymerase using 3.5mM $MgCl_2$, (Promega) 0.2μM of each primer, 0.2μM each nucleotide and 1μl of the diluted cDNA. Primer sequence, extension times and melting temperatures are included in Table 1 and cloning strategy in Figure 6. Amplification products were run out on a 1% agarose gel stained with 100μg/ml ethidium bromide and excised from the gel (PCR products are shown in Figure 7a). Each respective NRAGE fragment was

Table 1: Primers generated to clone NRAGE fragments and for diagnostics.

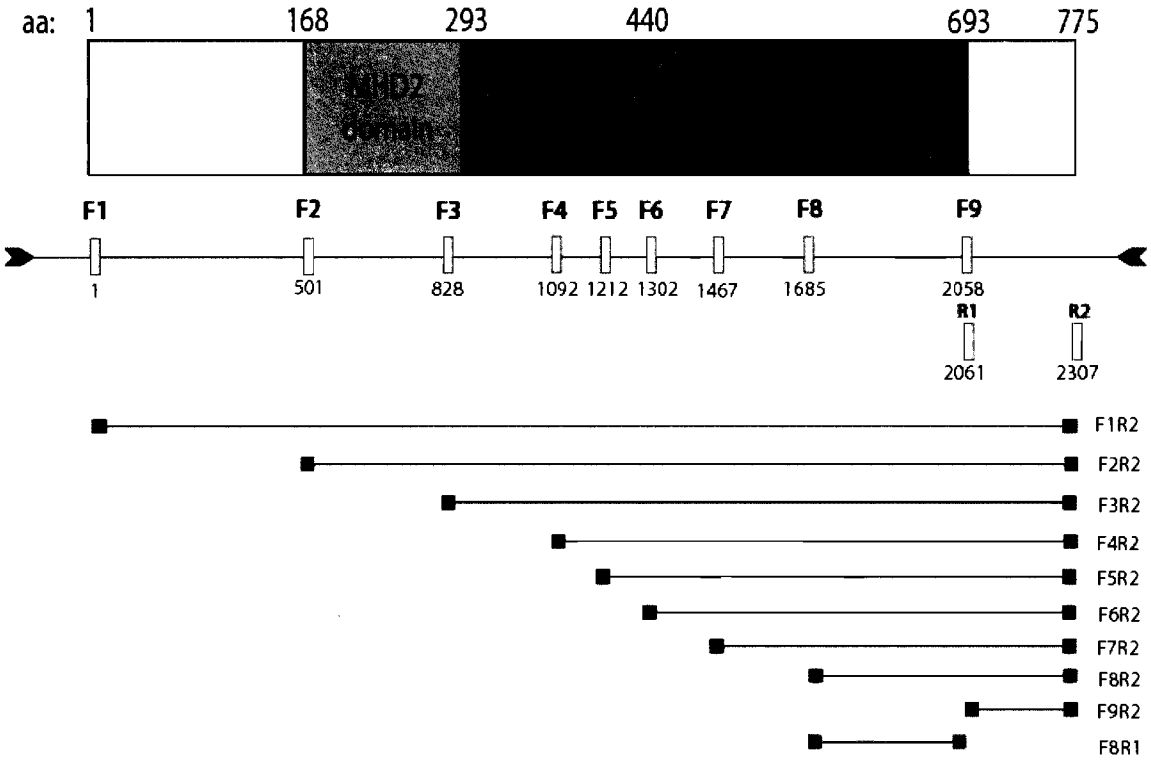
Melting temperatures of primers T_m, Annealing temperatures and extension times for PCR reactions and nucleotide and protein predicted sizes are included.

Name	Sequence	T_m (°C)	Annealing Temp with R2 (°C)	Extension Time	Fragment size with R2 bp (kDa incl.GFP)
F1	GAG ACG CTC GAG ATG GCT CAG AAA CCG	64	55	2.5 min	2328 (116)
F2	AGT GCC CTC GAG ATG CCA AAC AAC CAG	64	55	2 min	1810 (96)
F3	GTG ACC CTC GAG ATG GAC CCA CCT GGA GCA	69	55	2 min	1486 (84)
F4	TGG CCA CTC GAG ATG GCC TGG CAG AGT ACA	69	55	1.5 min	1220 (74)
F6	GAC TGG CTC GAG ATG GGA CCC TCA CCT AAT CTG	66	55	1.5 min	1010 (66)
F7	AAG CGC CTC GAG ATG GTG AGG GAT ATC ATC	64	55	1 min	845 (59)
F8	GGC ATT CTC GAG ATG GAT GGC AAC CGT GCC	67	55	1 min	820 (58)
F9	ATT GAG CTC GAG ATG GGA ATT GGA GAT GAG	61	55	30 sec	245 (37)
R1	CTC ATC GTC GAC TCC CAT GCG GTT TCT	60	-	-	-
R2	GAC AAT TTA GTC GAC CCA GAA GAA GCC	59	-	-	-
Actin F	TGT TAC CAA CTG GGA CGA CA	56	56	30	300
Actin R	CTC TCA GCT GTG GTG GTG A	57			
EGFP diag F	ATA AGC AGA GCT GGT TTA GTG	52	For sequencing purposes into the multiple cloning site		

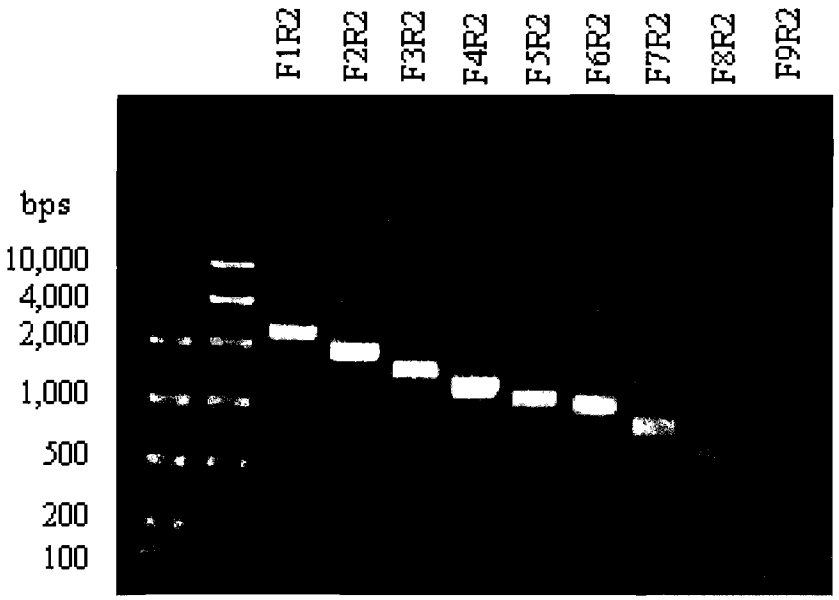
A:

(F1)MAQKPDGGAGLRGFQAEASVEDSALLVQTLMEAIQISEA
PPTSQATAAASGPNASPQSSQPPTANEKADTEVSAAAARPKTG
FKAQNATTKGPNDYSQARNAKEMPKNQSKAAFKSQNGTPKGPH
AASDFSQAAPTGKSAKKSEMAFKGQNSTKAGPGTTYNFPQSPS
(F2)ANEMTNNQPKTAKAWNDTTKVPGADAQTQNVNQAKMADV
GTSAGISEADGAAAQTSADGSQTQNVESRTIIRGKRTRKVNNL
NVEENNSGDQRRASLASGNWRSAPVPVTTQQ(F3)NPPGAPPN
VVWQTPLAWQNPSGWQNQTARQTPPAARQSPPARQTPSAWQNP
VAWQNPVIWPNPVIWQNPVIWPNPIVWPGPIVWPNPM(F4)AW
QSTPGWQSPPSWQAPPSWQSPQDWQGPPDWQVPPDWS(F5)MP
PDWSFPSDWPFPPDWIPADWPIPPDWQNL(F6)RPSPNLRSSS
NSRASQNQGPPQPRDVALLQERANKLVKYLMLKDYTKVPIKRS
E(F7)MLRDIIREYTDVYPEIIERACFVLEKKFGIQLKEIDKE
EHLYILISTPESLAGILGTTKDTPKLGLLLVILGIIF(F8)MN
GNRATEAVLWEALRKMGLRPGVRHPLLGDLRKLLTYEFVKQKY
LDYRRVPNSNPPEYEFLWGLRSYHETSKMKVLRFIAEVQKRDP
RDWTAQFMEAADEALDALDAAAAEAEARAEARNR(R1)M(F9)
GIGDEAVSGPWSWDDIEFELLTWDEEGDFGDPWSRIPFTFWAR
YHQNARSRFPQAFTGPIIGPSGTATANFAANFGAIGFFWVE(R
2)

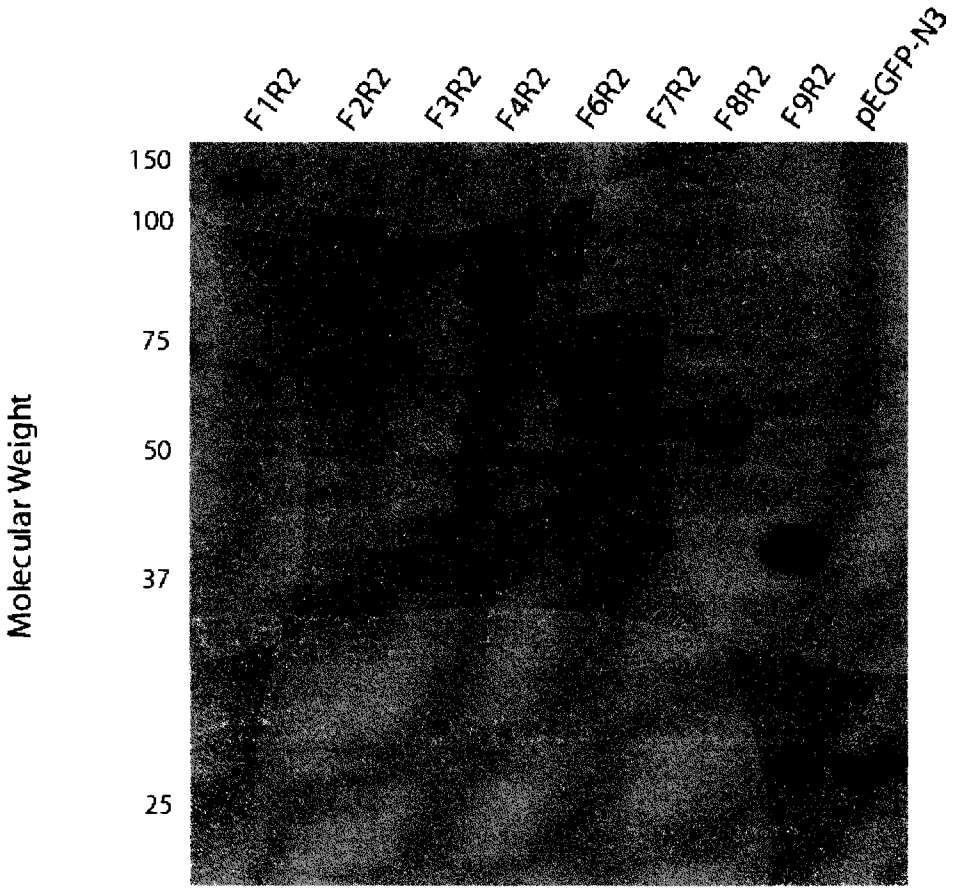
B:



A:


F1R2
F2R2
F3R2
F4R2
F5R2
F6R2
F7R2
F8R2
F9R2
bps
10,000
4,000
2,000
1,000
500
200
100

B:


Construct in pEGFP-N3
F1R2
F2R2
F3R2
F4R2
F6R2
F7R2
F8R2
F9R2
pEGFP-N3
Molecular Weight
150
100
75
50
37
25

cloned into the pCR-BluntII-TOPO vector using the TOPO isomerase ligation reaction, chemically transformed into Top10 supercompetent cells (as per manufacturer's instructions; Invitrogen) and plated onto LB-agar plates supplemented with 50μg/ml kanamycin. Random clones were grown overnight in LB media supplemented with kanamycin and plasmid extracted using the Eppendorf fast plasmid mini prep kit. 2μg of plasmid were digested with XhoI for validation and orientation of insert and grown in 125ml cultures for midi plasmid preps. Glycerol stocks were stored for future use. Nested PCR was also performed using the F(x+1) primer and R1 to determine whether the product inserted was able to be amplified using internal primer sites.

As there is a Xho site adjacent to the TOPO PCR product insertion site, we instead elected to use the SacI restriction enzyme which cut upstream of the PCR insertion site. 10μg of the NRAGE FxR2-TOPO vectors and pEGFP-N3 were digested sequentially with SacI then SalI, as recommended by New England Biolabs. Reaction fragments were isolated in a 1% agarose gel stained with 100μg/ml ethidium bromide and DNA excised from gel fragments by the QIAquick gel extraction kit (Qiagen – as per manufacturer's instructions). Ligation of the digested NRAGE fragments into the pEGFP vector was performed in 15μl reactions with 1μl of T4 DNA ligase (NEB) overnight at 14°C then electroporated into TOP10 electrocompetent bacteria and plated on LB-agar plates with 50μg/ml kanamycin. Random colonies were picked, plasmid isolated, and digested with SacI and SalI to determine correct integration. Representative constructs (F1, F2, F3, F4, F9/R2-GFP) were sequenced to determine proper

integration into the GFP vector and the framing of the NRAGE fragment into eGFP, using the pEGFP diag F primer to sequence into the multiple cloning site and the NRAGE F9 primer to sequence out of the NRAGE fragment and into the GFP vector.

Cell Culturing and Transfections

Human cell lines HEK 293 and N2A were maintained in DMEM (GibcoBRL) supplemented with fetal bovine serum (10% final concentration; Hyclone) and 1x antibiotic/antimycotic (100x stock solution; GibcoBRL). Murine P19 cells were grown in αMEM, supplemented with calf serum (7.5% final concentration), fetal bovine serum (2.5% final concentration) and antibiotic/antimycotic according to Jones-Villeneuve et al. [79]. All cells were grown at 37°C at 5% CO_2. P19 cells were fed every day and passaged at a 1:10 ratio every other day. 293 cells were fed every other day and passaged 1:10 every third or fourth day.

Transfections were performed in 6 well dishes at 70-80% confluency. In individual tubes, 8μg of DNA and 10μl of Lipofectamine 2000® (Invitrogen) were diluted in 250μl of serum free DMEM each for 5 minutes then the DNA added to the Lipofectamine mixtures and incubated to allow the DNA:liposome complexes to form. Cells were washed with serum free media then the transfection mixture added with 1.5 ml serum free media. Cells were incubated for 4-6 hours at 37°C before the removal of transfection mixture and the addition of serum-containing media. Expression was assayed 24 hours post transfection

using a Leica epifluorescence microscope at 50x magnification. Controls included the native pEGFP-N3 construct and mock transfections to assess efficiency. Efficiency was evaluated by estimating the number of fluorescent cells per field in comparison to the pEGFP-N3 transfections.

For P19 cell transfections, a confluent plate of cells were passaged 1:10 on day 1, transfection mixture was added as described above on the morning of day 2 and six hours later an equal volume of media containing twice the concentration of serum was added, without removing the transfection mixture. 24 hours later (48 hours post split) cells were washed and standard P19 media added and fluorescence analyzed by fluorescence microscopy before analysis by flow cytometry where appropriate. Transfection efficiencies were in the range of 30-60% with the longest NRAGE constructs giving consistently lower efficiencies, while expression intensity of the plasmids remained consistent across all constructs.

Cell Lysates and Western Blotting Procedure

Cells were aspirated free of media, washed with ice cold PBS, and mechanically detached from the plates. Cells were collected and spun at 1500rpm for 3 minutes before removing PBS and addition of 500µl RIPA buffer (150mM NaCl, 10mM Tris, pH 7.2, 0.1% SDS, 1% deoxycholate, 5mM EDTA) supplemented with 1µm sodium orthovanadate, 1µm sodium flouride and protease inhibitor cocktail (Sigma: 104mM AEBSF, 80µM aprotinin, 2mM leupeptin, 4mM bestatin, 1.5mM pepstatin A, 1.4mM E-64). Lysates were incubated on ice

for 20 minutes before relative concentrations determined using the BCA protein assay kit using a round bottom 96-well dish according to manufacturer's instructions (Promega). The lowest mean protein concentration sample was used for the largest volume with the equal amount calculated for subsequent samples. For a 12 well comb, 100µl was loaded (not including 2x loading buffer), a 15 well comb, 75µl of lysate and for a 20 well comb, 50µl of lysate.

Protein samples were run on a 10% acrylamide gel overnight at 50 volts and the gel transferred to nitrocellulose membrane (Amersham Biosciences). The transfer was performed in a glycine-based submersion buffer for 2 hours at 0.5A at 4°C. The transfer membrane was washed in TBS 1% Tween (TBST) then blocked in TBST with 5% BSA (BSA+TBST) (for phospho-specific detection) or non-fat dry milk for 1-2 hours. Antibodies were added at appropriate concentrations (see Table 2) in milk or BSA+TBST overnight at 4°C. Membranes were washed in TBST three times for 15 minutes each prior to the incubation of the appropriate HRP-conjugated secondary antibody at a 1:10,000 dilution (BioRad) and strep-tactin conjugated HRP at a 1:5000 dilution (for the Precision Plus Molecular Ladder, when appropriate) for 45 minutes at room temperature. The membrane was subsequently washed by at least three 15 minute incubations and drained of fluid. Equal parts of the enhanced chemiluminescent (ECL) substrate solutions were mixed and washed over the membrane for 1 minute then the membrane dried to remove puddles and developed using Sensitive Blue X-ray film (Pegasus Scientific) and the in-house X-ray developer. Molecular rulers were either the Precision Plus blue stained ladder or the broad range ladder (both

BioRad). NRAGE deletion constructs were immunoblotted for the GFP tag as the epitope for the commercially available NRAGE antibodies were removed in generating the constructs.

Table 2: Antibodies: their source, dilution and application.

Antibody, clone number	Company, catalog number	Dilution	Species	Application
GFP	Santa Cruz	1:1000	Rabbit	WB
P38	Cell Signaling 9212	1:1000	Rabbit	WB
p-p38	Cell Signaling 9211	1:1000	Rabbit	WB
Activated Caspase, Asp175 (5A1)	Cell Signaling 9964	1:1000	Rabbit	WB
Annexin V-PE (36)	BD Biosciences 51-65875	1:100	Preconjugated	FC
7AAD	BD Biosciences 51-68981	1:100	Preconjugated	FC
Actin	Sigma, A2228	1:1000	Mouse	WB

Legend: WB: Western blot, FC: flow cytometry

Flow Cytometry Analysis of Apoptosis

Three independent transfections were analyzed by Annexin V and 7AAD staining to determine the rate of apoptosis. At 48 hours post-transfection, cells were trypsinized to detach from the plate and spun at 1200 rpm for 3 minutes to pellet. 10µl of suspension was taken and cells counted using a hemocytometer to determine the number of cells per ml. Cells were diluted to 1 million per ml in Antibody Binding Buffer (0.1M Hepes/NaOH pH 7.4, 1.4M NaCl, 25mM $CaCl_2$ diluted 1:10 in sterile water) included in the apoptosis detection kit (BD Biosciences). For controls, 100µl of cells were placed in 5ml culture tubes with 5µl of either Annexin V-PE or 7AAD or both and incubated in the dark for at

least 20 minutes (see Table 2). 250µl of sample cells were incubated with 15µl of each apoptosis marker for at least 20 minutes in the dark. Upon completion of the incubation period, cells were diluted in 400µl (control) or 750µl (sample) of binding buffer and analyzed by flow cytometry using the Becton Dickinson FASC Calibur flow cytometer and Cell Quest software version 3.3. GFP+ cells were gated and cell incorporation of Annexin V and 7AAD determined in a sample size of 5000 cells. Gates were determined using untransfected and unstained cells. GFP and PE were excited by the Argon 488 laser and GFP read on the FL1 channel and PE on the FL2 channel. Compensation was set at 25.8% of the FL1 channel to reduce bleed-through of the GFP signal into the PE channel. All data was plotted in the log mode. Statistics were performed using Microsoft Excel Data Analysis Software. ANOVA was performed to determine the statistical validity of the series of constructs and two tailed t-test performed for the statistically significant pairings.

Chapter 3

RESULTS

The structure of NRAGE and the dissection of the protein

The domains of NRAGE were identified in the protein sequence and whenever possible native methoniones were used as start codons for the construct. Nine constructs were designed to be generated with two reverse primers. The forward primers (F1-F9) were designed upstream to the start of the region of interest while the R1 primer was designed to begin amplification at the end of the MAGE homology domain (MHD) and R2 to the end of the protein coding sequence. Fragments F1-F9/R2 were first cloned with constructs F1-F4/R2 and F6-F9/R2 successfully generated. F8R1 was also cloned for subsequent use. F1R2 encodes the complete NRAGE coding sequence while F2R2 takes off the N terminal sequence until the beginning of the MHD2. F3R2 encodes a protein which begins after the MHD2 region ends. F4R2 and F5R2 each remove sections of the WQXPXX domain while F6R2 removes almost the entire WQXPXX domain. F7R2 and F8R2 delete regions of the MAGE homology domain while F9R2 only encodes for the extreme C-terminal end of the protein with no identified regions. F8R1 encodes for the second half of the MHD (schematic diagrams in figure 6, PCR products in 7a and Western blot in figure 7b).

Cloning Strategy of NRAGE Constructs

The forward primers were designed with a Xho1 restriction digest site 5' to a designed Kozak sequence and start codon. Reverse primers deleted the stop codon and added a Sal1 restriction digest site which was all in frame to the pEGFP-N3 vector (Clontech). PCR products were generated from cDNA derived from adult mouse brain RNA. Products amplified matched predicted sequence sizes and were cloned into the TOPO cloning vector as per manufacturer's instructions. As the TOPO vector was not in the original cloning strategy it was subsequently discovered that there is a Xho1 site near the 3' PCR product insertion site so digestion of the constructs with Xho1 proved problematic. However, digestion with Xho1 allowed us to determine in which orientation the PCR product was inserted into the vector as the products were either the predicted fragment size or essentially linearized. Therefore, Xho1 was used as a diagnostic digest to 1) determine if the PCR product was in the vector and 2) which orientation the fragment inserted. Plasmids were digested and ligated into pEGFP-N3 and colonies digested to ensure correct fragment size. Representative constructs were also sequenced to ensure correct sequence. Constructs were transfected into 293 and N2A cells and showed expression by GFP and upon lysis and Western blotting, displayed immunoreactive protein at the predicted protein sizes (for fragment + GFP sizes, see table 2; Western blot, figure 8).

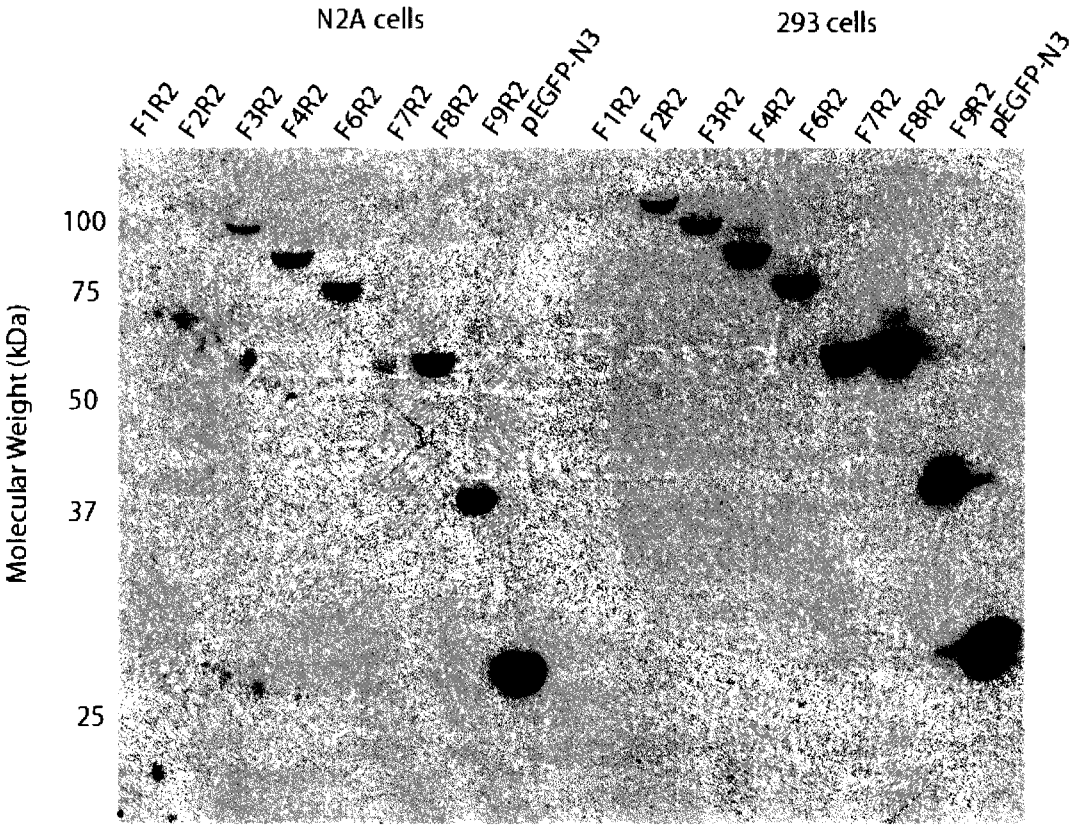


Figure 8: NRAGE Fragments are expressed in N2A and HEK293 Cells. N2A neuroblastoma cells and HEK293 cells were transfected with the NRAGE constructs and lysed after 24 hours. Shown are equivalent amounts of protein loaded on a 10% acrylamide SDS-PAGE gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blotted with an anti-GFP antibody and developed using X-ray film. F1R2 did not transfer. Molecular weight standards are on the Y-Axis.

Description of the P19 Cell Model System

P19 cells were used as the model system of this study for two main reasons. P19 cells are a murine embryonal carcinoma cell line isolated by McBurney and colleagues [79] that can produce a multitude of cell types under various differentiation conditions. Importantly, when cultured in suspension supplemented with 10^{-7}M retinoic acid for four days then grown on tissue culture treated plates, they can produce neurons within 4 days of plating, and smooth muscle cells and oligodendrocytes within another 4 days. Furthermore, these cells have also been shown to differentially express neurotrophins and have been used as a model system to study neurotrophin responsiveness in neural development [80]. Because of these properties, P19 cells have become an accepted cell line to study the development of the nervous system and more specifically the molecular regulation of neurogenesis. P19 cells were transfected with 0.4μg DNA and 1μl Lipofectamine 2000 per cm surface area of plate over a 24 hour period and transfection efficiency was determined by fluorescence of the GFP tag through fluorescence microscopy and Western blotting.

NRAGE Constructs Cause Differential Apoptosis

One goal was to identify the region of NRAGE responsible for the apoptotic events during neurogenesis. Deletion constructs generated above isolated the recognized domains of the protein as well as fragmented the regions in an effort to determine a minimal region necessary for apoptosis. Previous data in P19 cells show that full length NRAGE can induce apoptosis within 24 hours

and reproducibly at 48 hours [22]. Addition of BMP can accelerate this timeline to reproducibly at 24 hours, however we elected to wait the 48 hours to assay for apoptosis in order to demonstrate the true behaviour of NRAGE without supplementing with BMP. Thus, 48 hours after transfection, cell death was determined by Annexin V and 7AAD uptake by flow cytometry. Staining of three independent transfections showed a precipitous decrease in apoptotic cells transfected with Fragment 7, the fragment that does not contain any of the tryptophan-repeat region and part of the MHD (figure 9) and subsequent constructs F8R2 and F9R2 (ANOVA $p=5.2 \times 10^{-13}$).

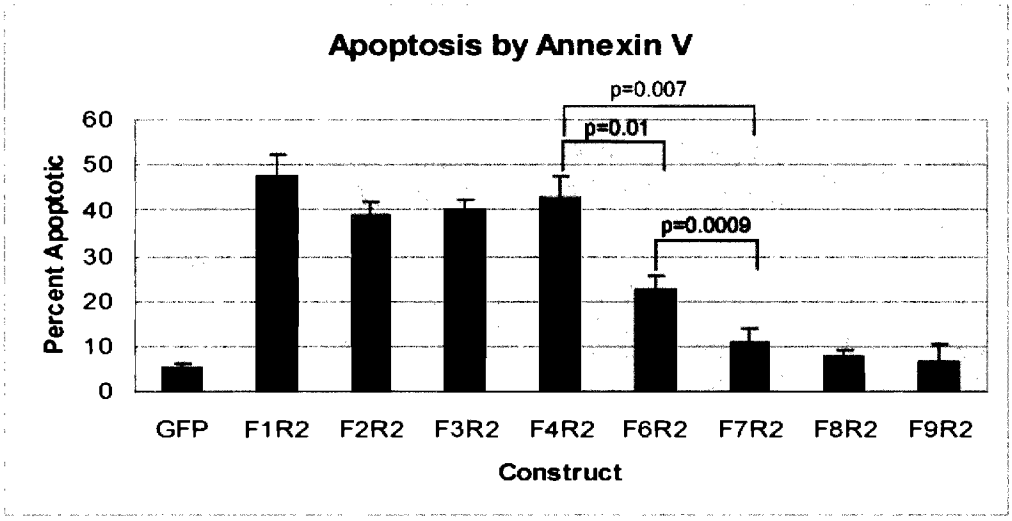


Figure 9: NRAGE constructs show differential apoptotic activity. Statistical analysis of the apoptotic potential of the NRAGE constructs in P19 cells. At three separate occasions, P19 cells were transfected with the NRAGE constructs, and 48 hours after transfection, were live stained with Annexin V-PE and analyzed by flow cytometry. Cells were gated for GFP+ cells and the presence of Annexin V-PE was read above a threshold determined by controls (single stained and unstained cells of GFP+ and untransfected cells). Statistical p-values are shown for the statistically significant construct pairings (further discussed in materials and methods and results).

There is, however, a significant increase (p=0.01) in cell viability upon the expression of F6R2 signifying that the end of the WQXPXX region and the beginning of the MHD does contain some apoptotic response. However, levels of cellular apoptosis returns to control levels with removal of the entire repeat region and some of the MHD as in construct F7R2 (F6R2-F7R2 p=0.0009; F4R2-F7R2 p=0.007).

NRAGE Deletions Show Altered Caspase and p38 Activation

Caspase 3 is a member of the executioner subfamily of caspases and as such is a broad-spectrum indicator of apoptosis. NRAGE has been shown to be involved in caspase-mediated activation and specifically caspase 3 cleavage, thus Western blot analysis was used to determine which fragments of NRAGE can mediate caspase activation. P19 cells transfected with the NRAGE constructs were lysed in RIPA buffer and subjected to Western blotting and immunodetection with an activated caspase 3 specific antibody. Presence of cleaved caspase 3 was detected in cells over expressing F1R2 and F2R2 constructs. Caspase activation remained constant but decreased by about half with constructs F3, F4, F6 and F7R2 while no activated caspase was detected with F8R2, F9R2, and F8R1 signifying that these constructs can not mediate caspase activation (figure 10a). This is in partial agreement with the Annexin V staining described above, crediting constructs F1 through F6 with the initiation of apoptosis. However, this is in opposition to flow cytometry data, as these data

show that F7R2 can initiate caspase cleavage however no appreciable level of apoptosis is seen by Annexin V.

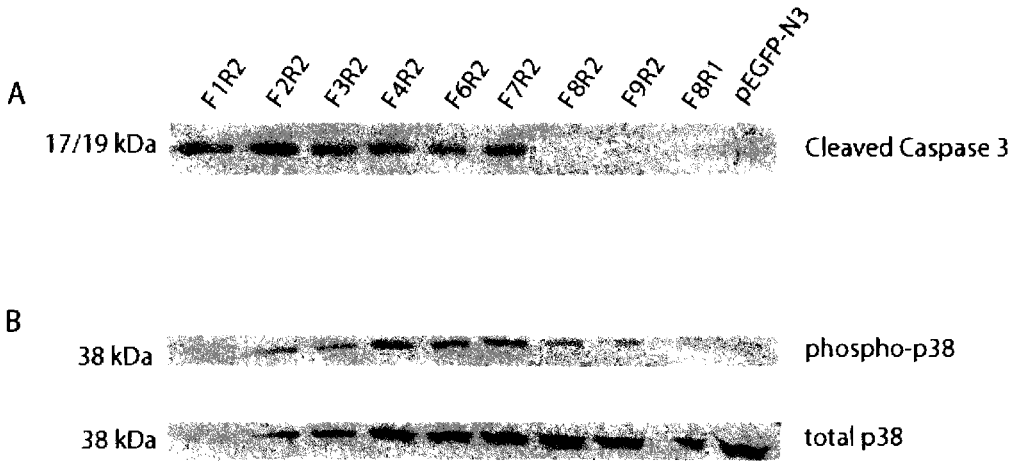


Figure 10: NRAGE deletion show altered caspase and p38 activity. A: P19 cells were transfected with the NRAGE constructs and lysed at 48 hours after transfection. Equal amounts of lysates were run on a 10% SDS-PAGE gel and immunoblotted to nitrocellulose membrane. Shown is an immunoblot analysis of cleaved caspase 3 from P19 cells. No cleaved caspase 2 could be deteced from F8R2 through pEGFP signifying a loss of caspase activity associated with apoptosis. **B:** Separate aliquots of cell lysates from the preparation described in figure 10A were simultaneously electrophoresed an immunoblotted with anti-p38 antibodies. Upper panel shows phospho-p38 staining with signal diminishing to control levels at F8R2 while total p38 (lower panel) remained consistent. Presented here is the best of two attempts, the left side of the membrane did not transfer efficiently.

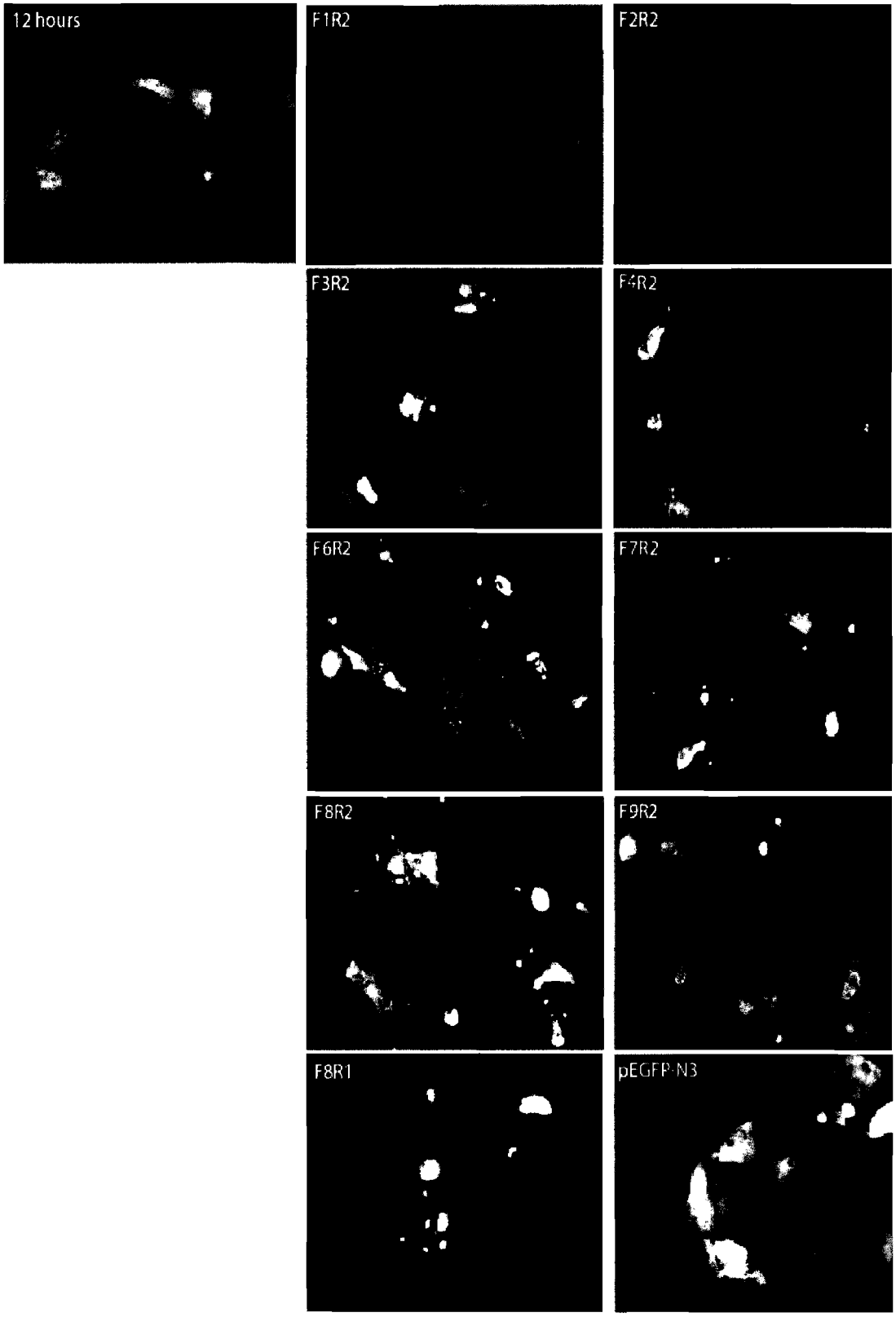
The phosphorylation of p38 is a significant event in the NRAGE mediated apoptosis pathway and has been shown to be activated in both the presence and absence of BMP. P19 cells were transfected with the NRAGE-GFP constructs and lysed for SDS-PAGE analysis. Through the use of phosphate specific antibodies, it was found that p38 is phosphorylated in constructs F1-F7 while

constructs F8R2, F9R2 and F8R1 show similar levels of phosphorylation to GFP control and in comparison to total p38. This signifies that the WQXPXX and the second half of the MHD are capable of downstream p38 activation, however the second half of the MHD is not sufficient to induce phosphorylation, as the F8R1 construct which encodes the C terminal portion of the MHD can not induce phosphorlyation on its own (figure 10b).

NRAGE Constructs Show Differential Sub-Cellular Localization

It is well documented that upon cleavage, MAGE protein localization can be altered, as the type I MAGE protein's MHD is transported to the cell surface to be the antigen of the major histocompatibility complex (MHC) [16]. A structure-function analysis of Necdin also showed subcellular localization differences in gene fragments not encoding regions of the MHD [81]. To further investigate whether our NRAGE fragments showed differences in subcellular localization, we transfected the GFP-tagged constructs into various cell types to discern differences in sub-cellular localization. Upon inspection of the fluorescent activity of the NRAGE-GFP constructs, it was found that the constructs displayed a wide variety of subcellular localization patterns that were consistent across cell types [HEK 293 cells, N2A cells, P19 cells, as well as mIMCD3 cells, a mouse kidney epithelial cell line (personal communication, G. Nikopoulos)]. Figure 11 shows the subcellular localizations of the fragments in P19 cells which were consistently bright at 12 hours but the largest constructs decreasing fluorescent activity significantly at 24 hours. Constructs F1R2, F2R2 show diffuse staining in the

cytoplasm while being excluded from the region of the nucleus. Constructs F3R2 through F8R2 and F8R1 show some cytoplasmic fluorescence but large focal points of fluorescence are observed in cytoplasmic regions, consistent with being in a subcellular structure such an aggresome or apopotosome. Fragment F9R2 appears to be diffusely detected in the cytoplasm with no focal expression. This suggests that the WQXPXX region and the MHD, the regions involved in protein-protein interactions permit intracellular aggregation while the MHD2 regions and the extreme N and C terminal portions oppose these abilities. Interestingly, no construct seemed to localize to the membrane, which is significant as the type I MAGEs are proteolytically processed and presented on the cell surface and NRAGE is known to interact with several membrane bound proteins ($p75^{NTR}$, UNC5H1, BMPR). This suggests that cell stimulation by appropriate ligands may be required for membrane and/or receptor localization.


12 hours
F1R2
F2R2
F3R2
F4R2
F6R2
F7R2
F8R2
F9R2
F8R1
pEGFP-N3

Chapter 4

DISCUSSION

This project was undertaken to give identity and function to the identifiable regions of the NRAGE protein. NRAGE is a member of the MAGE family of proteins, and as such has one highly conserved MAGE Homology Domain (MHD), one less-well conserved domain, denoted MHD2 and a unique 25 tandem WQXPXX tryptophan repeat region. The literature gives no definite function to the MHD of NRAGE however, several proteins have been shown to interact and have binding capacity to both the MHD and WQXPXX regions (see figure 3). It has also been demonstrated that repeat regions in general have possible binding capabilities. The trypotphan-rich region certainly has potential for binding as it is presumed to be 'sticky' and has a hypothetical binding-friendly confirmation. No NRAGE mutant mouse has been described (either transgenic or knockout) and previous cloning experiments have been restricted to the description of novel binding interactions. Moreover, it is only recently that NRAGE has been shown to interact with the non-canonical BMP-signaling pathway. Although NRAGE was originally cloned as a $p75^{NTR}$ intracellular domain interactor, the domain of NRAGE responsible for interactions with either $p75^{NTR}$ or the BMP receptors have not been characterized. Thus, an extensive

cloning strategy was designed to isolate the various regions of NRAGE and an analysis of the apoptotic cascade in reference to the mutant constructs performed to isolate the region responsible for apoptosis and confirm its effects on downstream signaling cascades.

The NRAGE MHD is Required for Apoptosis

The published literature shows that of the known molecules that interact with NRAGE, one third have been show to interact with the WQXPXX domain, one third to interact with the MHD and the interactions of the remaining molecules have not been characterized. Thus our hypothesis was that the region that was responsible for apoptosis was either the MHD or the hexapeptide repeat region. The MHD is the region most conserved across all MAGE genes yet very few of the MAGE genes have been functionally characterized; NRAGE and Necdin are notable exceptions. Mutations in the paternal necdin locus (the maternal gene is silenced) are thought to be at least partially responsible for Prader-Willi syndrome, a developmental disorder that affects the brain. Molecularly, Necdin is highly expressed in post mitotic neurons and is thought to play a major role in preventing the neuron from re-entering the cell cycle. The MHD of the type I MAGE genes is the region that is presented within the major histocompatibility complex (MHC) of tumor cells to signal a cytotoxic T cell response and subsequent tumor rejection. However, the intracellular processing of the protein for presentation in the MHC has not been identified and as such, little is known about the protein. Yet even with these discoveries, the MHD's

function(s) has not been conclusively documented, nor trends been seen. This is the first evidence of a definitive role of the MAGE domain in any cellular function.

The WQXPXX region was the other region that may have been involved in the apoptotic response. Repeat regions of proteins are important for protein-protein interactions and all MAGE-D genes have a repeat region, although there is no homology to one another. Proteins that have been shown to interact with this region of NRAGE have also been identified as downstream mediators of BMP signaling, thus it was reasonable to hypothesize that this region was required for downstream BMP mediated apoptosis. As all MAGE-D family members have a unique tandem repeat region, it may be that this region is important for interactions with partners rather than having a universal function shared among the MAGE-D family members. Although this region, through its binding interactions, could be requisite for NRAGE apoptotic functions, these experiments suggest that this is not the case.

Western blotting data confirmed the flow cytometry data that the MHD is essential for apoptotic function however these results showed that the first half of the domain can also induce caspase and p38 activation. Although a significant decrease in apoptosis was seen by Annexin V staining at F6R2 ($p=0.01$) and essentially no apoptosis detected with constructs F7R2 through F9R2, this only reiterates the necessity for the MHD in cellular apoptosis. XIAP is a member of the non-canonical BMP signaling pathway and is a highly conserved apoptotic molecule. Where XIAP binds to NRAGE has not been identified. It can be

surmised that NRAGE interacts with XIAP at the MHD as this region seems to be important in cell death.

The result of the MHD being required in apoptosis was somewhat surprising for two reasons. First, the MAGE homology domain is the most well conserved and thus is most likely to have an evolutionarily essential function (such as inducement of apoptosis). So far, the other MAGE genes have not shown to be involved in apoptosis, it seems unlikely that this region would be implicated in yet another function. Apoptosis is a highly dynamic process with many proteins participating in a highly conserved cascade. If it is true that the MAGE-D genes are the most evolutionarily conserved, it would be surprising that these genes and especially the MHD would have diverged to perform another task from cell cycle withdrawal or MHC. Secondly, the suspected adhesive properties of the tryptophan repeat region was hypothesized to be important for the recruitment of co-apoptotic molecules. Indeed, molecules shown to be downstream of one of the major NRAGE- mediated apoptotic pathways interact with the repeat region. Although there is a decrease in apoptosis when the majority of the repeat region is lost, reduction of apoptosis to basal levels only occurs once a significant portion of the MHD as well as the entire WQXPXX region is deleted. This begs for speculation to what the MHD does to or interacts with to be so critical for programmed cell death. One yeast-two-hybrid screen has been performed with the MHD which isolated two proteins, one of which is important for NRAGE turnover however a second one using a neural development-specific library may uncover new interactors to the MHD

specifically. Future studies currently underway will determine whether XIAP binding to NRAGE occurs at the tryptophan-repeat region or the MAGE homology domain, and where these interactions occur within the cell.

NRAGE Fragments are Differentially Localized Within the Cell

It was observed, upon inspection of GFP fluorescence, that the NRAGE fragments are differentially localized within the cell. This is consistent across cell types within the neural lineage (N2A, P19), across species (mouse P19, human N2A and HEK293) and across cell types (mIMCD3 epithelia, HEK293 endothelia, N2A neuroepithelia). Full length NRAGE is consistently detected in the cytoplasm while mutant constructs generated in this study that express regions of the WQXPXX and MHD regions are detected peri-nuclear and in subcellular compartments. F9R2, the construct missing the entire MHD, does not give such a punctate staining pattern. A Necdin structure-function analysis showed similar variant expression patterns, with protein generally detected both in the cytoplasm and abundantly in the nucleus [81]. Constructs missing portions of the MHD are not associated with the nucleus, rather are associated within the cytoplasm as speckles. Based on the observations of Taniura [81], it could be hypothesized that the MHD is the region of NRAGE that confers the sub-cellular distribution, however due to the design of our constructs, the only fragment that does not contain the MHD is F9R2.

The speckled appearance of certain NRAGE constructs raises questions as to why the protein is aggregated and with what it associates. The apoptosome is a

conglomerate of APAF1, ATP, and caspase 9 and cytochrome c when released from the mitochondria. XIAP is associated with the apoptosome to prevent abberant pro-caspase 9 cleavage and NRAGE can interact with XIAP. Although the kinetics and biochemistry needs to be tested, it could be that these specific NRAGE fragments show differential binding to the components of the apoptosome and as such, can aggregate. However this aggregation may not be within the apoptosome but as part of an aggregate in general. It does not seem like the fragments aggregate through the WQXPXX region, as constructs missing this region still show a punctate staining pattern and the C terminal portion of the protein (and the GFP tag alone do not self-aggregate) so it may be assumed that the MHD is involved in the accumulation of the protein. This is in agreement with Taniura [81] who also saw an aggregation of protein with constructs expressing the MHD. Little is known about the MHD, however further deconstruction of NRAGE by deleting the MHD while retaining the rest of the protein would confirm these results as well as further investigate the role of the MHD overall.

MAGE genes are cytoplasmically processed in the golgi and the MHD is presented as the antigen of the MHC. There is no published literature of NRAGE, or any of the other MAGE-D family members being cleaved and presented as the MHC, however this is theoretically possible. Thus, the pattern of fluorescence aggregation may be a result of the MHD being sequestered into lysosomes for proteolytic processing. This pattern is observed in cells with constructs lacking the N terminal portion of the MHD2. No function has been described for the less well conserved MHD2, which is present in all MAGE-D proteins and some other

type 2 MAGEs. It is possible that the MHD2, or a portion thereof is responsible for preventing the proteolytic processing that would present the MHD on the cell surface. However, it is important to note that there was no detectable expression at the cell surface signifying that the MHC may not be functional in these cells or that the proteolytic process was not completed, due to a lack of proteins involved or the functionality of NRAGE itself. These experiments were done with the use of an epifluorescence microscope, confocal microscopy should be used to confirm these results as well as more accurately determine if the proteins are aggregating at an organelle.

The C-terminal MHD is Essential for Caspase and p38 Signaling

Western blot analysis of the NRAGE constructs yielded surprising results when compared to the Annexin V flow cytometry data. As discussed above, Annexin V staining, corroborated by 7AAD staining (not shown) indicates that there is a precipitous decrease of apoptosis with the F6R2 construct which deletes the WQXPXX region but over-expression of the F7R2 construct, which deletes the first half of the MHD, restores cell survival. However, both caspase and p38 activation can occur in the absence of the N-terminal half of the MHD but only when the construct contains the extreme terminus of the protein. There are several reasons why these seemingly incompatible data can be explained. First, there may be an as of yet another unknown signaling pathway cooperating with the NRAGE-caspase 3 pathway to mediate the apoptosis cascade. In the absence of the first half of the MHD, caspase 3 can be activated, possibly through a p38

mediated response however apoptosis as measured by Annexin V is not apparent. This second signaling pathway may interact with the N-terminal portion of the MHD, thus when removed with F7R2, it can not interact to perform its function. Indeed, this may be the region to which XIAP interacts, however future experiments are being designed to address this. Second, data also shows that the caspase 3 and p38 activation response only occurs when the C-terminal portion of the MHD and its subsequent unconserved region remain intact. Although the Annexin V experiments did not include the F8R1 construct, it can be hypothesized that, based on Western blot data, the F8R1 construct would show no increase in apoptosis. This could be due to a conformational change in the folding of the MHD of F8R1, thus rendering it useless. However, this could also be due to incomplete binding of a protein which requires another protein to properly interact with NRAGE. NRAGE is widely accepted as an adaptor molecule for the various signaling pathway in which it interacts, this adaptor function may be as an accessory for docking. However, as stated above, significant further study needs to be completed to adequately explain why p38 and caspase activation does not structurally correlate with functional apoptosis assays.

NRAGE has been described as an apoptotic molecule in both BMP and NGF mediated signaling. The experiments reported here were completed in the presence of serum, which likely has some level of BMPs as well as neurotrophins. Considering the high levels of apoptosis in these pro-survival growth conditions, it is unlikely that the supplementation of the media of either growth factor would

dramatically alter the results presented here, other than to speed up the response. Thus the addition of neither BMP nor neurotrophins more accurately demonstrates the apoptotic potential of NRAGE. That being said, there is the possibility that the two cascades are inducing apoptosis through separate pathways. However, as the addition of BMP emphasizes the apoptotic response it is possible that under these culture conditions BMP treatment overrides the response to any other serum component. Serum free cultures that include the addition of individual growth factors may be done in the future to determine if and how the individual NRAGE protein domains initiate downstream cascades independently.

The NRAGE MHD is Required for Downstream BMP Activity

Although it has been well established that BMPs can evoke an apoptotic response, the role of NRAGE in this process has only recently been identified. BMP ligands and receptors have been shown to co-localize to areas of neuroepithelial apoptosis [23,25,57]. Studies have also shown in vitro that BMP signaling induces apoptosis in chick rhombomeres and neural explant cultures. Noggin, the BMP antagonist inhibits these responses and the over-expression of BMP receptors can have an effect on cell survival. However, until NRAGE was categorized as a downstream effector of BMP-mediated apoptosis, the Msx family of proteins has been the leading suspects in apoptosis. The Msx family encode homedomain genes well characterized as transcription factors involved in tooth, bone and skull formation. The Msx subfamily is extremely well conserved and

the three genes (Msx1, 2, 3) have 98% homology within the homeodomain region. In spite of this similarity, they show distinct functions in neural development, but can still compensate for one another in times of genetic crisis. Msx genes have been shown to be involved in BMP-induced apoptosis as over-expression of Msx2 in aggregated P19 cells in suspension results in marked apoptosis [75]. BMP promotes a similar level of apoptosis, however the two treatments do not show additive effects, signifying that Msx2 and BMP work through the same mechanism rather than parallel pathways [75].

NRAGE was identified as an interactor with Msx2 in a yeast two-hybrid screen, thus implicating NRAGE in the Msx2 apoptotic pathway. Although no studies have determined that NRAGE interacts with Msx2 in neural apoptosis, it may be an unlikely event as the over-expression of NRAGE induces massive apoptosis in P19 cells within 48 hours, regardless of the presence or absence of BMP. BMP accelerates apoptosis in NRAGE over-expressing P19 cells [22], but the effects of NRAGE-induced apoptosis is more rapid and context independent with respect to Msx2. The evidence that NRAGE and Msx2 interact is not in dispute, however it seems unlikely these two proteins work through a similar pathway to induce programmed cell death in neural development, especially as the region of NRAGE that Msx2 binds is not required for apoptosis (i.e. the WQXPXX repeat region). NRAGE-dependent apoptosis outside of the nervous system has not been well studied thus it is possible that the two proteins cooperate in a separate system (such as bone/limb development) to bring about a mutual response.

To date, NRAGE best fits as a member of the non-canonical BMP signaling pathway. NRAGE has been shown to co-immunoprecipitate with XIAP, TAK1 and TAB1 however none of these interactions have been localized on the NRAGE protein. XIAP has been shown to interact with both BMPR1a and NRAGE through its RING-finger domain. Moreover, TAB1 can interact with XIAP via its BIR domain and TAK1 can induce p38 phosphorylation in the presence of BMP stimulation. Western blotting shows that the first half of the MHD is required for p38 activation and this is likely a result of the loss of NRAGE association with XIAP. However, the region of NRAGE required to interact with XIAP has not yet been defined, although we may be able to hypothesize that the C-terminal portion of NRAGE is where XIAP binds, based on the apoptosis studies described above. Future studies are being designed to address these issues.

The MHD of NRAGE is Likely Responsible for its Function Within the Neurotrophic Theory

NRAGE was first described as an interactor with $p75^{NTR}$ and as such may play an important role in the neurotrophic theory. Importantly, NRAGE was found not to bind with the death domain region of $p75^{NTR}$ (the region to which NADE associates and confers its apoptotic potential) but rather the juxtamembrane region [13]. The neurotrophic theory is based on the premise that neurons which do not receive enough signal in the form of neurotrophins die due to the lack of survival cues. Although many interactors of $p75^{NTR}$ have been

identified, there has been a lack of clarity as to the mechanism which $p75^{NTR}$ can confer the apoptotic signal. It has been described that $p75^{NTR}$ can induce apoptosis both with and without ligand activation. This is likely cell context dependent as other studies have showed that $p75^{NTR}$ can induce apoptosis under the direction of NGF in cell types such as differentiated rat oligodendrocytes, Schwann cells, hepatic stellate cells, MAH cells, and many others (reviewed in [54]). However, in vivo data show that apoptosis is reduced when mouse embryos are exposed to antibodies to NGF or the extracellular domain of $p75^{NTR}$. There is also a hypomorphic knockout of the third exon of $p75^{NTR}$ which also shows reduced apoptosis [45]. Furthermore, transgenic over-expression of the intracellular domain of $p75^{NTR}$ show decreased cell number in the cortical, sympathetic and sensory neurons [45]. Studies have also shown that co-expression of $p75^{NTR}$ with Trk receptors can augment the affinity of neurotrophins to their respective receptors. Recently, a ligand for $p75^{NTR}$ has been described and is ironically, the uncleaved form of NGF. Pro-NGF appears to be a high affinity ligand for $p75^{NTR}$, can not bind with TrkA and can induce $p75^{NTR}$ mediated apoptosis in sympathetic neurons, oligodendrocytes and vascular smooth muscle cell lines [82,83]. Sortollin is a co-receptor necessary for Pro-NGF to signal upon $p75^{NTR}$. Overexpression of Sortollin in cells that do not normally express it renders these cells susceptible to the pro-apoptotic effects of pro-NGF on the $p75^{NTR}$ receptor [83]. It is possible, that in a severe shortage of neurotrophins (to act upon the heterodimeric receptor complexes), or in cells that do not display the Trk receptors, that $p75^{NTR}$ can

receive the NGF signal on its own or through pro-NGF stimulation with Sortollin, either of which evoke the apoptotic response.

NRAGE has not been shown to interact with any of the Trk receptors, nor Sortollin and over-expression of NRAGE can disrupt the binding of $p75^{NTR}$ to the Trk receptors. This competitive mechanism of $p75^{NTR}$ between Trk and NRAGE may be responsible for the apoptosis seen in the developing brain. As it has now been shown that the MHD is required for apoptosis in the developing brain, it seems likely that this region is responsible for interaction to the juxtamembrane region of $p75^{NTR}$, although this has not been confirmed biochemically. Furthermore, studies are needed to address the role of Sortollin in the pro-NGF initiated apoptosis response and the role NRAGE has in this mechanism. It is conceivable that NRAGE may function with $p75^{NTR}$ both in the presence and absence of Sortollin to confer apoptosis, and the role of Sortollin in this system may be to evoke a temporal- or spatial-sensitive response. However, Sortollin and NRAGE may have little to do with each other and instead the presence of Sortollin at the $p75^{NTR}$ may somehow inhibit the $p75^{NTR}$-NRAGE interaction.

NRAGE's Possible Activity as an Axon Guidance Molecule

UNC5H1 is a membrane-associated protein involved in axon guidance and apoptosis in the brain (reviewed in [55]). UNC5H1 is the closest related family member to p75NTR and it has been shown that the PEST and Zu-5 regions of UNC5H1 are important to interact with the MHD and downstream parts of NRAGE. Both $p75^{NTR}$ and UNC5H1 have been shown to be important in

neuronal apoptosis and both have a PEST sequence. Although this region is primarily associated in the rapid turnover of proteins through degradation machinery, it is interesting to note that these regions in UNC5H1 are juxtamembrane, the same site where NRAGE interacts with $p75^{NTR}$. Thus it is tempting to speculate that these two membrane receptors can mediate apoptosis through similar mechanisms and interactions with NRAGE. It is interesting to note that over-expression of UNC5H1 induced substantial apoptosis in native PC12 cells but not differentiated cells and the over-expression of NRAGE in differentiated PC12 cells induced the cells to retract their processes. Neurotrophins, the ligands of $p75^{NTR}$ and the Trks, are also involved in process outgrowth. Neurotrophins induce process outgrowth of neural cells as method of innervating the target tissue from which the trophin is produced. Processes are essential to this process, perhaps low levels of neurotrophic stimulation encourage the cell to send out processes but there is an insufficient level of survival stimulus at the target to sustain all responding cells. Thus the insufficiency of neurotrophin ends in neurotrophin-mediated apoptosis. It is known that NRAGE is present in the cell that produces processes and is available to receive the stimulus to apoptose. It is tempting to speculate that NRAGE is involved (or at least present) in the search for the neurotrophin-secreting target and that the lack of survival factor upon reaching the target triggers NRAGE-mediated apoptosis. The exact stimulus response mechanism is not clear, but future studies may be designed to investigate the role of NRAGE in this mechanism.

NRAGE's Involvement in Cancer

NRAGE is a member of the MAGE family of genes which were originally identified as tumor antigens, however, the antigenic function has only been attributed to type I MAGE clusters A, B, and C. The MAGE protein is cytoplasmically processed and the MAGE domain is presented in association with the antigens of the major histocompatability complex in a variety of tumor cells including melanoma, breast and prostate [84]. This presentation results in cytotoxic T cell response and subsequent tumor rejection. Although only expressed in a certain number of patients, this mechanism has been harnessed for therapeutic purposes, because when MAGE was experimentally over-expressed in cancer cells, there was regression when tumor-bearing mice were immunized with hMAGE-A3 [85-88]. In a clinical study, HLA-A1 patients who expressed MAGE-3 (previous nomenclature) on their tumors were administered MAGE-3 immunization, one quarter of these patients showed a regression of the metastases however there was no cytotoxic T cell response detected [84]. Currently, there are more than 6 clinical trials testing the efficacy of various MAGE gene treatments primarily in multiple melanoma and using MAGE-A genes [89].

BRCA2

Germline mutations in BRCA2 are responsible for predisposing women to breast and ovarian cancers, and rarely, sporadic mutations are found in breast tumors. BRCA2 also has many cellular responsibilities in normal biology including the stabilization of stalled DNA replication forks, cytokinesis,

gametogenesis, transcription regulation, suppression of cell proliferation and centrosome duplication (reviewed in [90]). BRCA2 has also been shown to inhibit the proliferation of the pancreatic cancer line Capan-1 [90]. Using the conserved region of the gene, it was found that BRCA2 can associate with the 116 C-terminal amino acid residues of NRAGE with high affinity (figure 4), and BRCA2 can increase the half life of NRAGE protein six-fold in the presence of cyclohexamide [90]. As many others have implicated NRAGE in the cell cycle, studies were conducted to determine the role of the BRCA2:NRAGE interaction. It was seen that both NRAGE and BRCA2 decrease the number of cells that incorporate BrdU, at a similar rate to p53, and this is an additive effect when both are transfected in combination [90]. The effects of over-expression of NRAGE and BRCA2 are similar in a mammary tumor cell line void of p53, suggesting that repression of the cell cycle is through a pathway independent of p53. However, growth retardation by BRCA2 is dependent on NRAGE as loss of NRAGE through RNA inhibition (RNAi) restores the growth potential [90]. The majority of breast tumor cell lines show low levels of NRAGE expression. However all the untransformed mammary epithelial cell lines and four of the more than twenty tumor lines show significant NRAGE expression. This signifies that NRAGE expression may be important in maintaining an normal state [90].

Recently there have been two studies which show an association of NRAGE and cancer. However, the revelation that NRAGE is associated with cancer is not surprising since apoptotic cascades have long been associated with

the disease as they are likely targets for mutations. NRAGE and BRCA2 together induce growth inhibition and some rare breast cancer cell lines (4 of more than 20 studied) show a lack of NRAGE protein. Furthermore, in a separate study, it was seen in rare subset of well differentiated yet highly invasive breast tumors, there are disproportionately high levels of BMPR1b and XIAP [91]. Although not directly studied or mentioned, it may be there is a loss of NRAGE activity associated with these two proteins, which can cooperatively associate to induce apoptosis. Although there is little data to back this up, normal BRCA2 may be able to extend the half life of NRAGE and thus repress the malignant growth of cells. Upon loss of or mutation of BRCA2, there is subsequent rapid degredation and loss of NRAGE protein which in turn leads to a decrease in apoptosis. This and over-expression of BMPR1b and XIAP could both contribute to breast cancer through similar or different mechanisms. There is much to study to confirm this, but if true, NRAGE could be involved in breast cancer progression.

Chapter 5

LIMITATIONS AND FUTURE STUDIES

Limitations of the Presented Study

This study utilized a cloning strategy that drastically reduced the NRAGE protein in order to isolate the recognized regions. In light of the Necdin structure-function study, in which the authors deleted only the region of interest without interfering with the upstream or downstream regions of the protein, it would be appropriate to deconstruct NRAGE in a similar manner to compare these sibling proteins. Furthermore, a less drastic manipulation of the protein would also more accurately demonstrate the true function of the region by minimizing the conformational changes and the ancillary functions of the upstream regions. However, by employing the cloning strategy outlined in this study, we may have discovered a function in the MHD2 region, as we have shown that the loss of the MHD2 changes the subcellular distribution only when the MHD is intact.

Technical difficulties are abundant when studying a protein involved in apoptosis, as it is difficult to maintain a population of expressing cells. Apoptosis as measured by flow cytometry was evaluated at 48 hours, however data not presented here demonstrated that at 24 hours we did show a similar pattern of degree of cellular death. Thus, Western blot and sub-cellular expression analysis

was performed at 24 hours as it was found that the protein was more abundantly expressed at this time point. However, it was still problematic to transfect the constructs, especially the longest ones and detect these proteins through western blot analysis. The longest fragment is ten times larger than the smallest fragment, thus transfection with equal weights revealed that the shorter fragments had consistently greater expression due to the number of plasmids integrated into the cell. However, pre-experimentation transfections revealed that altering the amount of plasmid transfected did not drastically alter the degree of expression in the cells. This is likely due in part to the optimization of the Lipofectamine transfection protocol, which 'maxes out' the plasmid:liposome ratio in the media.

Altered sub-cellular localization was shown with the NRAGE mutant constructs through epifluorescence microscopy however, it can not be definitely concluded that there was no localization to the membrane. To validate these conclusions, confocal microscopy using P19 cells expressing the NRAGE constructs is required. Furthermore co-staining with markers specific for the apoptosome (APAF1 or caspase 9) or the golgi/endoplasmic reticulum would further elucidate where in the cell these mutant proteins aggregate.

Future Studies

In order to fully characterize the structure-function analysis of NRAGE, the proliferation and differentiation aspects of NRAGE function need to be investigated. Previous reports have shown that NRAGE expression can influence the cell cycle through its actions on p53 activity[22]. Overexpression of NRAGE

attenuates BrdU incorporation which signifies an exit from the cell cycle. Therefore, BrdU incorporation assays, utilizing flow cytometry to read the percentage of GFP+ cells that incorporate BrdU, would identify changes in the cell cycle dynamics of cells expressing the NRAGE mutant constructs. It is expected that an intact MHD would abbreviate the cell cycle and induce a more rapid exit from the cell cycle, while the loss of the MHD would when the latter constructs are overexpressed would not alter the cell cycle kinetics.

It has also been demonstrated that the loss of NRAGE can alter the differentiation potential of P19 cells, as there is a reduction in the production of neurons at a result of the cell failing to cease the progenitor genetic program. A thorough analysis of the NRAGE mutants described here, as well as the GFP control, transfected into P19 cells then taken through the retinoic acid-mediated differentiation program would be warranted. At the termination of the program, cells would be stained for MAP2a/b (a neural marker), GFAP (a glial marker) and Mash1 (a neural stem cell marker) to determine the percentages of the cells in the various developmental stages. As Kendall et al. [22], showed that with a loss of NRAGE there is a gain of progenitors at the expense of neural populations (at 8 days of differentiation), it would be expected that the loss of the MHD would show similar results (i.e. F7, F8, and F9R2) as there would be no functional NRAGE signal. However, the overexpression of the constructs with the MHD intact would induce a rapid cell cycle exit which would allow the cells to terminally differentiate more quickly which would be seen by an increase in glial-positive cells.

We have demonstrated that the loss of portions of the NRAGE protein can alter the co-localization of the remaining protein to discrete regions of the cell upon over-expression. These results are consistent with the structure-function analysis of Necdin in which mutants that do not encode for the MHD or the N terminus are distributed uniformly in the cytoplasm. Although we have seen that some of the NRAGE mutants built may reside by or in the nucleus, in aggresomes or apoptosomes, confocal imaging with other markers is required to confirm nuclear localization or localization within aggregated structures. To elucidate whether the apoptosome is the where the construct concentrates, tagged molecules of the apoptosome such as a pro-caspase 9 or APAF1 construct will help define whether this structure is the apoptosome. However, NRAGE may also co-localize with other known interactors, such as TAK1 and TAB1, or p38 in the non-canonical BMP pathway, Msx/Dlx, or Necdin. Necdin deletion mutants can subcellularly localize, thus these two proteins may work in a similar mechanism to elicit downstream responses and their mutants behave in an analogous fashion. Indeed, future studies employing both NRAGE and Necdin may shed significant light on the role of the MHD in functions other than MHC-mediated cytotoxic T cell response.

XIAP is a known mediator of the non-canonical BMP signaling and also interacts with NRAGE. However, it not known with which region of NRAGE XIAP can interact, thus co-immunopreciptation of the NRAGE constructs with XIAP (as a XIAP-dsRed monomer construct or endogenously) will help to determine where XIAP interacts with NRAGE. Furthermore, it is still unknown

where XIAP and NRAGE meet within the cell to confer the apoptotic signaling. In light of the interesting result of the NRAGE cellular localizations, confocal imagery of the two proteins (as GFP and dsRed constructs, or YFP/CFP constructs which are currently being prepared) will help determine where in the cell the two proteins interact. These interaction studies may also shed light onto the interesting results seen in this study on the MHD. Several lines of evidence justify the hypothesis that XIAP interacts with the N-terminal half of the MHD. Caspase 3 and p38 activation occur in the absence of the N-terminal MHD whereas apoptosis is not apparent when the N-terminus of the MHD is intact. If XIAP does interact here, it stands to reason that XIAP inhibits NRAGE-mediated apoptosis even in the presence of caspase 3 activation. However, when binding of XIAP is lost, then the C-terminal portion of the MHD can signal apoptosis, as seen in the Annexin V data.

NRAGE was first identified as molecule interacting with the juxtamemembrane region of $p75^{NTR}$ however, little has been found as to the downstream activities of NRAGE after $p75^{NTR}$ interaction. Future studies must address this issue. Published yeast-two-hybrid screens have only uncovered the E3 ubiquitinating protein Praja1 as interacting with NRAGE. A second screen, focusing on the MHD may help elucidate downstream interactors of NRAGE under the control of $p75^{NTR}$. Furthermore, 2-dimensional gel analysis of neuroblasts and neurotrophin-stimulated neuroblasts may help elucidate changes in protein expression that could be attributed to apoptosis. On the other hand, the discovery of Sortollin and pro-NGF as the ligand and co-receptor of $p75^{NTR}$ may

also be of interest to future NRAGE-focused studies. As mentioned above, these molecules may play a role in the upstream mediated response of $p75^{NTR}$ and ultimately the fate of the cell itself.

Surprisingly, none of the mutant NRAGE molecules built appeared to show a sub-cellular localization to the membrane compartments. This is surprising as NRAGE is known to interact with at three membrane-bound receptors (p75NTR, BMPR, UNC5H1). Future studies using construct-bearing cells treated with either BMP or neurotrophins may show that certain constructs can move to the membrane-associated receptor upon appropriate stimulation. The NRAGE mutants were inserted into the pEGFP construct so as to detect its expression as all commercially available NRAGE antibodies have epitopes to the N-terminus. Antibodies generated to recognize the extreme C-terminus or the MHC of NRAGE would be useful to confirm these findings.

As stated above, these studies were all performed in the presence of serum, however, the addition of ligands may influence the translocation of the molecule in the cell. These studies will also shed light onto the requirements of the receptor-mediated apoptotic signaling cascade on NRAGE. This would clarify NRAGE is itself responsible for the downstream be through its own interaction with NRAGE or the necessary downstream cascades that NRAGE interacts with to confer its apoptotic potential. These studies will help determine whether the deletion of a specific region prevents apoptosis at the membrane level or downstream of NRAGE.

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