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# K-Ras, but Not H-Ras or N-Ras, Hyperactivation Regulates Brain Neural Stem Cell Proliferation in a Raf/Rb-dependent Manner

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by

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| | |
|---|---|
| APC | adenomatous polyposis coli protein |
| BFA | Brefeldin A |
| bHLH | basic helix-loop-helix transcription factor |
| BLBP | brain lipid binding protein |
| BMP | bone morphogenic protein |
| cAMP | cyclic adenosine monophosphate, cyclic AMP |
| CFC | Cardio-Facio-Cutaneous |
| CNS | central nevous system |
| CNTF | ciliary neurotrophic growth factor |
| CSC | cancer stem cell |
| EGF | epidermal growth factor |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinase |
| FGF | fibroblast growth factor |
| FTI | farnesyltransferase inhibitor |
| GAP | GTPase activating protein |
| GDP | guanosine diphosphate |
| GEF | guanine exchange factor |
| GEM | genetically-engineered mouse |
| GFAP | glial fibrillary acidic protein |
| GRPC | glial-restricted progenitor cell |
| GTP | guanosine triphosphate |
| H-RAS | Harvey-RAS |
| HVR | hypervariable region |
| ICMT | isoprenylcysteine carboxyl methyltransferase |
| JAK | Janus kinase |
| K-RAS | Kirsten-RAS |
| LIF | leukemia inhibitory factor |
| MBP | myelin basic protein |
| MeCP2 | methyl DNA binding protein |
| MEK | mitogen/extracellular signal-regulated kinase |
| mTOR | mammalian target of rapamycin |
| mTORC2 | mammalian target of rapamycin, complex 2 |
| N-RAS | Neuroblastoma-RAS |
| NF1 | Neurofibromatosis type 1 |
| NSC | neural stem cell |
| PDGF | platelet-derived growth factor |
| PDGFRα | platelet-derived growth factor receptor α |
| PDK1 | phosphoinositide-dependent kinase 1 |
| PH | pleckstrin homology |
| PI3K | phosphatidylinositol 3-kinase protein |

| | |
|---|---|
| RasGRP1 | RAS guanosine nucleotide releasing protein |
| Rb | retinoblastoma protein |
| RBD | Ras binding domain |
| RCE1 | RAS CAAX converting endopeptidase 1 |
| RHOGDI | RHO guanine nucleotide dissociation inhibitor |
| Shh | Sonic hedgehog |
| STAT | signal transducer and activator of transcription |
| SVZ | subventricular zone |

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**ABSTRACT OF THE DISSERTATION**

K-Ras, but Not H-Ras or N-Ras, Hyperactivation Regulates Brain Neural Stem Cell Proliferation in a Raf/Rb-dependent Manner

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Neural stem cells (NSCs) give rise to all the major cell types in the brain, including neurons, oligodendrocytes, and astrocytes. However, the intracellular signaling pathways that govern brain NSC proliferation and differentiation have been incompletely characterized to date. Since several neurodevelopmental brain disorders (i.e., Costello syndrome, Noonan syndrome) are caused by germline mutations in the *RAS* genes, Ras small GTPases are likely critical regulators of brain NSC function. In the mammalian brain, Ras exists as three distinct molecules (H-Ras, K-Ras, and N-Ras), each with different subcellular localizations, downstream signaling effectors, and biological effects. Leveraging a novel series of conditional Ras molecule-expressing genetically-engineered mouse strains, we demonstrate that K-Ras, but not H-Ras or N-Ras, hyperactivation increases brain NSC growth in a Raf-dependent, but Mek-independent, manner. Moreover, we show that K-Ras regulation of brain NSC proliferation requires Raf binding and suppression of retinoblastoma (Rb) function. Collectively, these observations establish tissue-specific differences in Ras molecule regulation of brain cell growth that operate through a non-canonical mechanism.

# CHAPTER 1

# INTRODUCTION

## DEVELOPMENT OF THE MAMMALIAN BRAIN

The gross cellular composition of the mammalian brain arises through sequential specification of three main types of cells. Neurons, the cells responsible for conducting neural signals in the mature brain, appear first, followed by the development of two types of neuronal support cells, astrocytes and oligodendrocytes. The temporal differentiation of individual cell types has been well characterized in rat models of brain development, which demonstrate that neurons first appear around embryonic day 12 (E12) and continue to develop through E18 (Figure 1.1) [1]. Following neuronal specification, astrocytes first appear at E18, followed closely by oligodendrocytes at postnatal day 1 (PN1). Both cell types continue to develop through early postnatal time points. While similar, the temporal sequences of rat and mouse embryonic brain development do not perfectly correlate. As indicated in Figure 1.1, mice begin to develop neurons at E10.5, astrocytes at E15.5, and oligodendrocytes at PN1 [2]. For simplicity, mouse development time points are used throughout this thesis document, unless otherwise noted. The timeline of cell specification is ultimately unsurprising; a neuronal network must be in place before the cells responsible for insulating (oligodendrocytes) and maintaining neurons (astrocytes) can establish themselves in appropriate proximity to the network. The temporal segregation of cell type-specific development requires a carefully orchestrated integration of temporal cues and cell-extrinsic/intrinsic signals to drive brain multipotent progenitors, neural stem cells (NSCs), towards the correct cell fates.

### *The Keystone of Brain Development: Neural Stem Cells*

NSCs give rise to all major cell types in the brain, including neurons, astrocytes, and oligodendrocytes, thereby underscoring the importance of these progenitor cells for mammalian brain development. In order to specify all three subtypes, these progenitor cells must arise early in embryonic development prior to neuronal fate specification. Multi-potent progenitor cells have been isolated from the rat spinal cord at rat E10.5, providing evidence for NSC populations that arise from the primitive neural tube before localizing throughout the developing central nervous system (CNS) [3, 4].

In order to characterize NSCs, a series of *in vitro* studies sought to isolate and define progenitor cell populations at specific time points throughout the course of embryonic brain development. Multipotent blast cells were first isolated during mid-embryogenesis in the developing rat brain (rat E13.5,

E14.5) using *in vitro c*lonal culture techniques [5]. While the majority of the resulting clones gave rise to either neurons or glia alone, a subset (22%) of these clones contained both neuronal and non-neuronal cell types. These findings represented the first isolation of a rudimentary progenitor cell, and demonstrated that neural progenitor cells have the capacity to generate both neuronal and glial cell lineages. Subsequently, progenitor cell populations were isolated from the E14 mouse striatum and could be cultured in the presence of epidermal growth factor (EGF). In culture, these cells spontaneously form spheres of proliferating cells (the field would later term these "neurospheres") that express the neurofilament protein Nestin, a marker previously found exclusively in progenitor cells from the neuroepithelium. Moreover, EGF-responsive neurospheres possess many of the hallmarks of progenitor cell populations in that these cells are capable of self-renewal over multiple passages and can undergo multi-lineage differentiation into neurons, astrocytes, and oligodendrocytes in culture [6, 7]. Later work also isolated NSCs earlier in embryonic development using the closely related fibroblast growth factor (FGF) [8]. As with EGF-responsive NSCs, FGF-responsive cells also demonstrated a capacity to adopt all three terminally differentiated cell fates [9]. Taken together, these studies not only define a temporal window during which NSCs appear, but also establish a foundation for *in vitro* culture studies of these progenitors for careful evaluation of their cell-autonomous properties.

The maintenance of NSCs during development requires several extracellular signals, including Notch and leukemia inhibitory factor (LIF) [10-12]. In NSCs, the Notch ligand activates several downstream effector components, including Janus kinase (JAK) and signal transducer and activator of transcription (STAT) transcription factors to promote NSC survival [11]. A role for LIF in maintaining NSC identity is apparent upon overexpression in the adult brain, which reduces neurogenesis and promotes NSC self-renewal [10]. Given its role in pro-glial signaling later in brain development (discussed below), it is possible that LIF primarily functions in inhibiting neuronal cell fate decisions while driving NSC self-renewal early in development and promoting gliogenesis later in development.

The differentiation of NSCs towards the three cell lineages depends partially on temporal regulatory restrictions. Using clonal differentiation assays, Qian, et al., elegantly demonstrated that Nestin$^{+}$ NSCs maintained *in vitro* first develop into neurons, as revealed by expression of the neuronal marker Tuj1 around the *in vitro* equivalent of E11 [13]. Glial differentiation occurred shortly thereafter,

evidenced by glial fibrillary acidic protein (GFAP) expression at E16 (astrocytes) and O4 expression around P3-P4 (oligodendrocytes). Early studies suggested that NSC lineage specification depends solely on a small subset of growth regulatory proteins: neurons by platelet-derived growth factor (PDGF), astrocytes by ciliary neurotrophic factor (CNTF), and oligodendrocytes by thyroid hormone T3 [14]. While fundamentally consistent, later studies would demonstrate that NSC fate specification is dependent on a more expansive list of coordinated signaling molecules to specify each cell fate. These signals merit further discussion in the context of each cell type.

Since most studies focused on defining NSC populations in the embryonic brain, it was thought that few, if any, progenitor cells persisted in the postnatal brain. This led to the false conclusion that an exhausted NSC pool limited the capacity of the brain to regenerate after birth and into adulthood. Subsequent studies have identified concentrated populations of NSCs within the neurogenic regions of the adult brain, including the hippocampal subventricular zone (SVZ), the third ventricular zone, and the fourth ventricular zone [15-17]. Perhaps the most well-characterized of these regions, the hippocampal SVZ, contains a hierarchy of precursor cells that include glial-like progenitor cells (Type B cells), which generate Type C transit-amplifying cells. These type C cells ultimately give rise to Type A neuronal precursor cells [16, 18]. Although Type B cells have the capacity to form multi-potent neurospheres *in vitro*, *in vivo* programming directs these cells to replace only neurons within the olfactory bulb [18, 19]. Progenitors within the third ventricle can also give rise to neurons, but not other cell types, suggesting that postnatal NSCs are directed most often towards neuron cell fates [15]. The overall plasticity of these progenitor cell populations may also be regionally-restricted, such that progenitors in different regions only give rise to certain neuronal subtypes [20]. Over the course of aging in the adult brain, the neurogenerative capacity of these cells is reduced. Within the SVZ, NSCs undergo increased asymmetric divisions that favor astrocyte over neuron formation and deplete the existing NSC pool [21]. Taken together, these studies demonstrate that a large population of embryonic NSCs readily exhibits a capacity for high proliferation and multi-lineage differentiation. This capacity allows for a high number of diverse cell types to be generated in a relatively brief period of time during brain development. In contrast, the adult brain contains a much smaller pool of NSCs that primarily function in maintaining existing neurons within the brain.

*Building a Neuronal Network*

To initiate brain development, NSCs first differentiate into the neuronal lineage. This cell fate is specified by a number of cell-intrinsic and cell-extrinsic factors which promote neurogenesis and simultaneously suppressing gliogenesis (Figure 1.2). Several basic helix-loop-helix (bHLH) transcription factors, including Neurogenin 1 and 2 (Ngn1, Ngn2), mammalian achaete-scute homologue (Mash1), and Math3, function to drive progenitor cells toward neuronal lineages [22, 23]. Genetic knockout studies of the individual *Mash1* and *Math3* genes has a minimal effect on neurogenesis *in vivo*; however, double knockout mice exhibit a significant loss of hindbrain neurons [23]. Progenitor cells in these mice that are normally committed to become neurons instead adopt a glial cell fate. This finding reveals a significant collaboration between Mash1 and Math3 to simultaneously promote neurogenesis and suppress gliogenesis. The same is also true for Ngn1, which promotes neurogenesis as a transcriptional activator. Ngn1 simultaneously inhibits astrogliogenesis by sequestering the CBP-Smad 1 signaling compounds while inhibiting STAT transcription factor function, which promotes astrocyte differentiation later in brain development (see below). Introduction of pro-neuronal bHLH genes at postnatal time points no longer promotes neurogenesis or blocks gliogenesis, demonstrating that their pro-neuronal signals are temporally restricted to embryonic tissues [22].

Extrinsically, ligands of the bone morphogenic protein (BMP) family promote NSC differentiation into neurons during the neurogenic period of brain development. Early studies demonstrated that the addition of the BMP4 ligand to explant cultures promotes expression of the Tuj1 neuronal marker in cells within the ventricular zone [24]. Moreover, a dominant negative BMP receptor (interfering with normal BMP-receptor signaling) blocked neuronal migration from this ventricular zone. This demonstrates that BMP is required for the regulation of neuronal cell fates in ventricular zone progenitor cells. Characterization of BMP2 in cultured rat cortical progenitor cells identified a temporally-restricted role for these ligands in promoting neuronal differentiation [25]. At the beginning of the neurogenic period (rat E13), addition of BMP2 inhibits proliferation and suppresses both neurogenesis and gliogenesis. However, the addition of moderate doses of BMP2 later in the neurogenic period (rat E16) drives neuronal differentiation, identifying a time-dependent role for BMP-mediated neurogenesis in the developing brain. Mechanistically, BMP ligands bind TGF-β membrane receptors to activate the Smad

family of transcription factors and drive cell fate decisions. To date, the exact mechanism of BMP-mediated promotion of neurogenesis is unclear, although in the adult, neurogenesis relies on the Smad4 protein [26]. Taken together, these data demonstrate a time-dependent, pro-neuronal role for BMP ligands, a fact that has additional relevance during the gliogenic period, as discussed below.

The mitogen/extracellular signaling-regulated kinase (MEK) and extracellular signaling-regulated kinase (ERK) effector proteins may also mediate extracellular signals that promote progenitor cell neurogenesis [27, 28]. In cultured cortical progenitor cells, inhibition of either Mek or its downstream effector protein, Erk5, inhibits neurogenesis, indicating a direct requirement for both effector proteins in mediating pro-neurogenic signals [28, 29]. The downstream C/EBP family of transcription factors is also required for normal neurogenesis, defining a Mek- and C/EBP-mediated pro-neurogenic regulatory pathway. Mechanistically, Mek phosphorylates (activates) C/EBP. In fact, C/EBP alone is sufficient to promote neurogenesis, as demonstrated by expression of a constitutively active member of the C/EBP family (C/EBPβ) in cortical progenitor cells [27]. C/EBP may also function in repressing pro-glial fate decisions in these same progenitor cells. When directed towards a glial cell fate using the CNTF cytokine, cortical progenitors differentiate into more astrocytes when C/EBP activity is inhibited [28].

The combination of these cell-intrinsic and cell-extrinsic factors is critical to direct NSCs towards neurogenic cell fates during a defined period of brain development. Subsequent brain development requires a switch to inhibit neurogenesis and instead direct NSCs towards glial cell fates to support the newly formed neuronal network.

*Supporting the Neuronal Network: Astrocytes*

In the adult brain, astrocytes function in neuronal maintenance by providing structural support, modulating blood flow, facilitating nutrient delivery, and absorbing waste and excess neurotransmitters [30]. To facilitate the formation of these astrocytes, formerly pro-neurogenic signals are inhibited and pro-astroglial signals are activated in NSCs around E15.5 (Figure 1.3). One regulatory mechanism underlying this switch relies on temporally-restricted methylation at two CpG dinucleotide sites upstream of the astrocyte-specific genes, glial fibrillary acidic protein (GFAP) and S100β [31, 32]. Within the promoter of the astrocytic *Gfap* gene, CpG methylation obstructs the binding of the STAT3 transcription

factor and prevents *Gfap* transcription [32]. Alternatively, CpG methylation within the promoter for a second astrocyte gene, *S100β,* facilitates binding of the methyl DNA binding protein (MeCP2), which enables inhibitory histone deacetylateses and other co-repressor proteins to bind and inhibit *S100β* expression [31]. During the neurogenic period at E11.5, methylation at both sites inhibits expression of either gene and can not be initiated even upon overexpression of the STAT3 transcription factor [32]. These promoters are only demethylated at the start of the gliogenic period (E14.5), thereby allowing transcription factor binding and subsequent *Gfap* and *S100β* expression [31, 32].

In addition to these cell-intrinsic regulatory mechanisms, extracellular pro-gliogenic signals increase at the start of the gliogenic period [33]. Many of these signals operate through growth factor binding to the gp130 family of membrane receptors. The importance of these receptors for gliogenesis apparent in mice deficient for gp130 receptors, which have increased neuronal cell death and reduced formation of $GFAP^+$ astrocytes in the brain [34]. This demonstrates a dual role for the gp130 receptors (and their associated growth factor ligands) in both promoting neuron survival and driving astrocyte differentiation. The gp130 receptors signal by homo- and hetero-dimerization of individual receptor family members, which transduces signals from a variety of cytokines, including the pro-astrocytic factors cardiotrophin-1 (CT-1), CNTF, and LIF [35, 36]. The pro-astrocytic growth factor CT-1 is secreted by neighboring neurons to foster the development of astrocytes within proximity of newly developed axons and synapses. Loss of *CT-1* expression in the developing brain reduces astrocyte formation, demonstrating a partial requirement for this cytokine in driving astrocyte differentiation [37]. The fact that only some astrocytes don't develop in *CT-1-/-* brains indicates that other growth factors must be involved in facilitating astrocyte differentiation. *In vitro,* the CNTF growth factor induces cortical progenitor cells to become astrocytes, a process which continues even after CNTF is removed from the culture media. The persistence of CNTF-mediated pro-glial signals demonstrates that CNTF irreversibly specifies astrocyte cell fate decisions and suggests that this cytokine is involved in the permanent switch from pro-neurogenic to pro-astrocytic cell fate decisions [38]. Lastly, the loss of the receptor for the pro-glial cytokine LIF causes decreased astrocyte differentiation *in vivo* and *in vitro* [39]. As discussed below, LIF signaling specifies astrocyte differentiation in collaboration with BMP signaling, underscoring an important role for LIF in pro-astrocytic cell fate decisions. Together, these results demonstrate that coordinated

signaling from a diverse set of pro-astrocytic cytokines is required for development of the full complement of astrocytes in the mature brain. Despite the diversity of cytokines operating during the pro-gliogenic brain development phase, these cytokines collectively signal to the gp130 downstream JAK effector protein to activate the glial-promoting STAT3 transcription factor [37, 38, 40].

Operating independently of gp130 and its associated growth factor ligands, Notch has also been identified as an important driver of astrocyte differentiation. This aspect of Notch signaling was originally demonstrated in neural crest stem cells of the peripheral nervous system. Upon activation of Notch, neurogenesis of these stem cells was suppressed while astroglial cell fates were increased [41]. Shortly thereafter, Notch signaling was also identified as a pro-astrocytic signal for hippocampal stem cells in the developing CNS [42]. Since Notch signaling requires cell-cell contact, Notch-mediated astrogliogenesis may be triggered by neighboring neuroblasts, which direct these stem cells to adopt an astroglial cell fate [41]. Similar to some gp130 ligands (CNTF), Notch irreversibly commits progenitor cells to the astrocyte lineage, suggesting a role for Notch is specifying the pro-neuronal to pro-glial signaling switch during brain development [42].

In addition to its early role in pro-neuronal signaling, BMP also drives progenitor cells towards the astrocyte fate during the gliogenic phase of brain development [43]. During this period, BMP now suppresses neurogenesis and restricts oligodendrocyte fate decisions [25, 43]. This is partially mediated by BMP2-dependent increases in expression of the transcription factors Id2, Id3, and Hes-5 which inhibit the expression of the pro-neuronal factors Mash1 and Ngn1 [44, 45]. Moreover, BMP2 expression inhibits the expression of the progenitor gene *Nestin* and the *Map2* neuronal gene. BMP2 also increases expression of the *S100β* astrocyte gene, thereby indicating that BMP2 expression drives progenitor cell development towards astrocyte rather than neuron cell fates [44]. This presents an important question: *how can the same pro-neuronal signaling molecule (BMP) also promote astrocyte differentiation later in development?* The mechanism underlying this switch to pro-astroglial fate choice relies on synergistic signaling with the pro-astrocytic LIF cytokine. At this stage, activation of either BMP2 or LIF alone drives only limited astrocyte fate specification, underscoring the importance of coordinated signaling from both molecules for normal astrocyte development [40]. Operating downstream of both LIF and BMP, the transcriptional coactivator p300 acts as a bridge between these two pathways by binding STAT3 (the LIF-

mediated transcription factor) at its N-terminus and Smad1 (the BMP2-mediated transcription factor) at its C-terminus [40]. As a result, this bridge protein coordinates the input from both cytokines and allows BMP to signal in a pro-glial manner.

*Supporting the Neuronal Network: Oligodendrocytes*

Oligodendrocytes are the third and final cell type to differentiate from NSCs during brain development. Upon differentiation, these cells function in forming an insulating, lipid-rich myelin sheath around neuronal axons to enhance electrical conduction of the nerves [30]. Oligodendrocytes derive primarily from glial-restricted progenitor cells (GRPCs), which give rise to astrocytes and oligodendrocytes, but not neurons, in the brain and spinal cord [46, 47]. However, in the spinal cord alone, oligodendrocytes and motor neurons can also derive from a shared progenitor cell population [48, 49]. In the brain, the primary pool of oligodendrocyte precursors arises from the ventral forebrain, although additional sources have been identified within the ganglionic eminences and in the postnatal neocortex [50]. Genetic ablation of any single oligodendrocyte precursor population does not disrupt normal oligodendrocyte development, indicating that these pools can compensate for shortages of any of the original progenitor pools.

Regionally-restricted differentiation signals are thought to direct GRPCs to adopt either astrocyte or oligodendrocyte cell fates during CNS development. In the chick spinal cord, oligodendrocyte differentiation is induced by Sonic hedgehog (Shh) signaling and antagonized by BMP signaling [51, 52]. The expression of both ligands is regionally restricted—BMP from the dorsal portion of the spinal cord, Shh from the ventral floor plate—allowing the formation of a signaling gradient that provides positional cues for glial cell differentiation. In the brain, Shh is expressed along the ventral midline at the telecephalon/diencephalon border in an area containing oligodendrocyte precursor cells [53]. This Shh expression overlaps with the expression of two pro-oligodendroglial bHLH transcription factor genes, *Olig1* and *Olig2*. Shh is both necessary and sufficient for the expression of these genes in the developing CNS *in vivo* [53, 54]. Additionally, both *Olig1* and *Olig2* are required for oligodendrocyte differentiation as genetic knock-out of both genes results in decreased oligodendrocyte differentiation [48, 49].

The *Olig* genes promote oligodendrocyte cell fate decisions by increasing expression of the SoxE group of high-mobility-group (HMG)-transcription factors, which includes the Sox8, Sox9, and Sox10 transcription factors, in oligodendrocyte precursor cells. Despite expression of all three transcription factors in developing and mature oligodendrocytes, each has a functionally distinct role in oligodendrocyte cell fate specification. Genetic ablation of Sox10 in developing embryos results in the accumulation of neural progenitor cells and the loss of oligodendrocytes during embryogenesis, demonstrating a requirement for this transcription factor in the terminal differentiation of oligodendrocytes [55]. In contrast, targeted loss of Sox9 in NSCs leads to the formation of more astrocytes and fewer oligodendrocytes, indicating that this transcription factor is necessary for pro-glial cell fate specification [56]. Lastly, although Sox8 is expressed in developing and mature oligodendrocytes, genetic loss of this transcription factor does not alter the formation of oligodendrocytes in the CNS, indicating that Sox8 is dispensable for oligodendrocyte differentiation [57]. Functionally, these transcription factors are responsible for the expression of oligodendrocyte-specific genes. In zebrafish, Olig1 cooperatively binds to Sox10 in order to express myelin basic protein, a key component for myelin sheath formation [58]. Similarly, genetic loss of either Sox9 or Sox10 in oligodendrocyte progenitor cells results in decreased expression of the PDGF receptor alpha (PDGFRα), an oligodendrocyte protein responsible for cell survival and migration [59]. Overall, this establishes a clear regulatory mechanism whereby Shh expression specifies oligodendrocyte cell fate through Olig and SoxE transcription factor-mediated expression of oligodendrocyte-specific genes.

**BRAIN DEVELOPMENT GONE AWRY: THE RASOPATHIES**

Normal development of the CNS is disrupted in several neurodevelopment disorders, providing insight into the regulatory mechanisms most critical for normal brain development. In particular, the neuro-cardio-facio-cutaneous syndromes comprise a family of related neurodevelopmental disorders resulting from hyperactivating mutations that affect the RAS signaling pathway (Figure 1.4). These disorders are more commonly referred to as RASopathies, underscoring the importance of the RAS pathway in regulating normal neural development. Several shared clinical features characterize the RASopathies, including neurodevelopmental defects (mental retardation, learning deficits, neurocognitive

delay), congenital heart defects, abnormal growth patterns (short stature, macroencephaly), and ectodermal defects (skin freckling, hyperkeratosis) [60]. A summary of these disorders, their causative mutations, and the CNS clinical features associated with each syndrome can be found in Table 1.1. Although the incidences of individual syndromes vary widely, as a whole the RASopathies occur in approximately 1 in 1,000 individuals [61]. Below is a brief summary of a subset of these disorders in the context of the CNS.

*Noonan Syndrome*

Noonan Syndrome affects approximately 1 in 1,000-2,500 individuals and is characterized by craniofacial deformities, short stature, and congenital cardiac defects [62, 63]. In the CNS, individuals with this disorder are most commonly affected by memory deficits, and learning, speech, and motor delays [64-66]. The causative mutations underlying Noonan Syndrome increase activity of several components within the Ras signaling pathway. A majority of individuals with Noonan Syndrome (>50%) harbor hyperactivating missense mutations in the *PTPN11* gene [67]. This gene encodes the protein tyrosine phosphatase protein SHP2 responsible for propagating receptor tyrosine kinase signals to the RAS-activating guanine exchange factor (GEF) protein SOS1. A smaller proportion of individuals (~20%) present with gain-of-function missense mutations in the *SOS1* gene itself [68, 69]. These mutations cluster within the autoinhibitory region of the protein, leading to hyperactivation of SOS1 and subsequent hyperactivation of RAS and its downstream effector ERK [68]. Activating mutations have also been identified in the K-RAS and N-RAS family members, although both mutations occur rarely in Noonan patients (<5%) [70, 71]. Similarly, a rare set of mutations occurs in the *RAF1* gene leading to the hyperactivation of the RAF1 effector protein operating downstream of RAS [72]. Taken together, these mutations aberrantly activate the RAS signaling pathway leading to disease progression and the CNS defects observed in individuals with Noonan Syndrome.

*Neurofibromatosis Type I*

Neurofibromatosis Type 1 (NF1) represents another commonly occurring RASopathy with an incidence of approximately 1 in 3,000 individuals [73]. The clinical features of NF1 include café-au-lait

spots, axillary freckling, iris Lisch nodules, and a predisposition to peripheral nerve sheath tumor formation (neurofibromas) [62]. In the CNS, learning deficits and behavioral abnormalities are the most common features of NF1 with various cognitive deficits reported to occur in 81% of the population [74]. Low-grade pilocytic astrocytomas often occur in children with NF1 and occur most frequently in the optic pathway, hypothalamus, and brainstem [75, 76]. The most common of these tumors, optic pathway gliomas, develop in approximately 15-20% of these individuals prior to age 10 [76, 77]. Unlike Noonan Syndrome, the NF1 disorder is caused by inactivating mutations within a single gene, *NF1*. This gene encodes the protein neurofibromin, a GTP-ase activating protein (GAP) that normally functions to inhibit RAS activity [78-81]. As a result, loss of the *NF1* gene leads to increased RAS activity [82].

*Costello Syndrome*

A relatively rare RASopathy, individuals with Costello Syndrome may exhibit neurodevelopmental delays, cardiac and musculoskeletal defects, and craniofacial abnormalities [62, 83]. Individuals affected by this disorder are also predisposed to the formation of a variety of malignant and benign tumors, the most common of which include rhabdomyosarcoma and neuroblastoma [84]. Similar to NF1, the causative mutations of Costello Syndrome are found within a single gene (*H-RAS*), with the vast majority of these mutations occurring within codon 12 of the gene [85-87]. These mutations inhibit H-RAS GTPase activity, locking the protein in a hyperactive state and leading to aberrant RAS pathway signaling. Moreover, preliminary phenotype-genotype correlational studies suggest that glycine to alanine mutations at this codon are associated with increased risk of malignant tumor formation relative to other Costello mutations [86]. Of the RASopathies, Costello Syndrome perhaps best demonstrates the consequence of hyperactivating mutations occurring within the RAS proteins themselves

*Legius Syndrome*

Individuals affected by Legius Syndrome, a rarely occurring RASopathy, come to medical attention with symptoms resembling those of Noonan Syndrome and NF1, including neurocognitive impairments, café-au-lait spots, axillary freckling, and macrocephaly [62]. Similar to NF1 and Costello Syndrome, Legius Syndrome results from inactivating mutations within a single gene, *SPRED1*, encoding

a regulatory protein that mediates RAS activity and its activation of the downstream MEK/ERK effector pathway [88, 89]. Loss-of-function mutations within the SPRED1 and related SPRY family of proteins have not been reported in other neurodevelopmental disorders, leading to the designation of Legius Syndrome as a distinct disorder within the RASopathy family [88].

*Cardio-Facio-Cutaneous Syndrome*

A final, rarely-occurring disorder in the RASopathy family, Cardio-Facio-Cutaneous (CFC) Syndrome has overlapping features with Noonan Syndrome, Costello Syndrome, and NF1. These include cardiac deficiencies, ectodermal and musculoskeletal abnormalities, and short stature [62]. In the CNS, CFC Syndrome patients may present with learning disabilities and motor and speech developmental delays. Unlike many of the disorders discussed already, CFC Syndrome is primarily caused by mutations affecting hyperactivation of RAS downstream effector proteins. The majority of these mutations lead to hyperactivation of the B-RAF protein, although mutations in K-RAS, MEK1, and MEK2 have also been reported in this patient population [90-92].

**THE RAS SIGNALING PATHWAY**

*Canonical Ras Signaling*

First identified in rat sarcoma cells, the 21 kDa small-GTPase protein RAS is the key transducer of growth factor signaling to downstream effector proteins. Specifically, RAS functions as a small-molecule switch by alternating between active, guanosine triphosphate (GTP)-bound and inactive, guanosine diphosphate (GDP)-bound states to transmit growth factor signals (Figure 1.4). RAS is activated by the exchange of bound GDP with a new GTP molecule, which causes conformational changes in the RAS protein. This process is facilitated by a family of GEF proteins that facilitate GDP release and GTP binding. Although one GEF protein, SOS, is most commonly implicated in RAS protein activation at the plasma membrane, other GEFs such as RAS guanosine nucleotide releasing protein (RasGRP1) can similarly activate RAS at locations distinct from the plasma membrane. RAS inactivation occurs by hydrolysis of the bound GTP to GDP, which remains bound to RAS. Although RAS has a slow

intrinsic GTPase activity on its own, this activity is greatly enhanced in the presence of GTPase-activating proteins (GAPs), such as p120GAP and neurofibromin, which inactivate RAS.

One mechanism of activating the RAS signaling pathway requires extracellular growth factor binding to receptor tyrosine kinase (RTK) proteins. A variety of growth factors are known to bind RTKs, including EGF, FGF, and nerve growth factor (NGF). Upon binding at the RTK extracellular domain, these growth factors promote homodimerization of the RTK proteins, which leads to autophosphorylation of the RTK intracellular domains. Within the cell, several adapter proteins, such as SHC, SHP2, and GRB2, contain SH2 domains specific for binding to the phosphorylated RTK intracellular domains. Simultaneously, these adaptor proteins bind the RAS-GEF protein SOS which, when bound to the RTK complex, activates RAS. Once activated, RAS functions to regulates cell growth and differentiation via two canonical downstream pathways, including through the downstream effector proteins phosphatidylinositol 3-kinase (PI3K) and the RAF/MEK/ERK signaling cascade [93, 94].

*Structure of the RAS Genes*

The RAS superfamily of genes consists of related small GTPase proteins that share sequence homology with the RAS genes. This family is divided into several subgroups, including the RAS, RHO, ARF, RAN, and RAB subfamilies. Within the RAS subfamily, there are multiple related forms of RAS found in mammals (Figure 1.5). Three closely related variants of this subfamily – H-RAS, K-RAS, and N-RAS – are considered to be the classical RAS proteins and are the focus of this thesis. Other RAS subfamily members are evolutionarily diverged from these classical RAS proteins and include E-RAS (expressed in embryonic stem cells), R-RAS (endothelial cells, vascular smooth muscle cells), and M-RAS (muscle cells and brain hippocampus, cerebellum) [95-98]. Of the classical RAS genes, both H-RAS (full name: Harvey-RAS) and K-RAS (Kirsten-RAS) were initially identified as the causative effectors of tumor growth upon their discovery in rat sarcoma virus in the 1960's [99, 100]. K-RAS is expressed as two alternatively spliced variants (K-RAS4A, K-RAS4B), however in this thesis, K-RAS refers to the ubiquitously expressed K-RAS4B isoform. N-RAS (Neuroblastoma-RAS) was initially isolated from the SK-N-SH neuroblastoma cell line and was capable of transforming NIH 3T3 fibroblasts [101]. Subsequent genetic mapping studies revealed that these classical RAS genes reside on different

chromosomal loci in humans: 11p (H-RAS), 12p (K-RAS), and 1p (N-RAS) [102-105]. In mice, the RAS genes also reside on different chromosomes (H-RAS on chromosome 7, K-RAS on chromosome 6, N-RAS on chromosome 3), indicating that mouse and human Ras genes diverged from one another relatively recently, as demonstrated by orthologous gene sequence analysis [106]. Despite their divergent genetic locations, the RAS molecules share 85% nucleotide sequence homology, suggesting that these genes diverged from a single common Ras gene [107]. The conserved region of the RAS genes encodes the GTP-binding region (a phosphate-binding loop [P-loop]) and the switch I and switch II regions that direct RAS binding to effector proteins (Figure 1.6). The remaining 15% of the nucleotide sequence encodes a 25 amino acid, C-terminal hypervariable region (HVR) that differs between the RAS genes and is thought to underlie the differential post-translational processing, plasma membrane localization, and signaling capacity of the encoded RAS proteins.

*Post-Translational Processing and Trafficking of the Ras Molecules*

All RAS molecules undergo extensive, multi-step post-translational modification to correctly traffic and localize RAS to the inner leaflet of the plasma membrane and endomembranes such as the Golgi and the endoplasmic reticulum (ER) (Figure 1.7). Without these modifications, the RAS molecules are hydrophilic with little affinity for the membranes where they ultimately localize. A series of lipid modifications is therefore required to convert the HVR to a hydrophobic region capable of anchoring these molecules within lipid membranes. RAS processing is initiated at a CAAX (C=cysteine, A=aliphatic amino acid, X=any amino acid) motif present at the carboxy-terminal portion of the HVR. Following translation, a hydrophobic isoprenyl group is added to the cysteine of the CAAX motif (prenylation) via the farnesyltransferase enzyme. This modification causes RAS to localize at the cytosolic surface of the ER for further modification steps [108]. Once at the ER, the RAS converting CAAX endopeptidase 1 (RCE1) enzyme cleaves the AAX tripeptide from the CAAX motif, allowing for subsequent methylation of the newly exposed α-carboxyl group (C186) by the isoprenylcysteine carboxyl methyltransferase (ICMT) enzyme [109-111]. Here, the RAS molecules begin to diverge in their processing. In this regard, K-RAS is more efficiently methylated by ICMT than its H-RAS and N-RAS counterparts [112].

The remaining upstream HVR sequence provides secondary signals that direct additional differential processing and trafficking of the Ras molecules (Figure 1.6) [113]. Two cysteines on H-RAS, C181 and C184, are the target of a covalent addition of fatty acids (palmitoylation) by the RAS palmitoyltransferase enzyme. N-RAS is similarly palmitoylated, but only at C181 [111]. Based on site mutagenesis studies, palmitoylation at these cysteines is required for trafficking of these proteins from the ER to the plasma membrane [112]. Using GFP-tagged molecules of H-RAS or N-RAS, two studies observed that these RAS molecules accumulate at both the Golgi and the plasma membrane, suggesting that H-RAS and N-RAS traffic to the plasma membrane via the Golgi apparatus [112, 114]. In the presence of Brefeldin A (BFA), a chemical agent that disrupts the Golgi membrane, there was significant loss of H-RAS-GFP or N-RAS-GFP localization at the plasma membrane. This confirms that both H-RAS and N-RAS transit from the ER to the plasma membrane in a Golgi-dependent manner.

In contrast, K-RAS is not palmitoylated but instead contains a polybasic, lysine-rich sequence within the HVR that confers a net positive charge at its C-terminus and targets K-RAS directly to the plasma membrane (Figure 1.7) [115]. Unlike H-RAS and N-RAS, GFP-tagged K-RAS is found at the plasma membrane but not at the Golgi, suggesting that K-RAS traffics to the plasma membrane without further processing within the Golgi. In support of this hypothesis, BFA treatment of cells does not reduce K-RAS-GFP accumulation at the plasma membrane, indicating that K-RAS transits to the plasma membrane in a Golgi-independent manner [114].

The exact mechanism underlying K-RAS trafficking to the plasma membrane remains unclear, however, several possible mechanisms have been proposed. First, given the net charge difference between the positively charged K-RAS HVR and the negatively charged plasma membrane, it is possible that plasma membrane trafficking occurs down an electrostatic gradient. Evidence in support of this theory arises from mutational studies of the K-RAS HVR that converted subsets of lysine residues to glutamine residues in order to alter the amphipathic and charge properties of this portion of the K-RAS molecule [116]. Mutations that maintained the +6 positive charge of normal K-RAS and the amphipathic properties of the HVR were most successful at correctly localizing GFP to the plasma membrane. Further evidence for charge-driven trafficking is found in the presence of the anionic lipid-binding agent neomycin, which reduces K-RAS-GFP association with the plasma membrane. This suggests that the

positively-charged K-RAS HVR requires negatively charged lipids within the membrane to direct its movement from the ER to the plasma membrane. A second mechanism may function by trafficking K-RAS to the plasma membrane in a microtubule-dependent manner. Support for this theory is based on the high affinity of prenylated and methylated K-RAS molecules for microtubules within the cell [117, 118]. In the presence of the chemical agent taxol, which stabilizes the otherwise dynamic microtubule structures, K-RAS localization to the plasma membrane is greatly reduced [114, 118]. Taxol administration has no effect on the localization of H-RAS-GFP at the plasma membrane, indicating that K-RAS trafficking alone occurs in a microtubule-dependent manner. A third and final potential mechanism suggests that K-RAS trafficking to the plasma membrane could be facilitated by a chaperone protein. This mechanism is well established for the RAS-related RHO GTPase protein which is trafficked to the plasma membrane by the RHO guanine nucleotide dissociation inhibitor (RHOGDI) chaperone protein after RHO geranylgeranylation and methylation at the ER [119]. On its own, RHO is rapidly degraded by cytosolic proteasomes. However, RHOGDIs sequester RHO in the cytosol and traffic these proteins directly to the plasma membrane. The prenyl binding protein PDEδ is proposed to function similarly for K-RAS by chaperoning its transport from the ER to the plasma membrane and by ensuring the correct localization of K-RAS molecules within plasma membrane microdomains [120]. Given the high occurrence of oncogenic K-RAS signaling in tumor progression, the K-RAS-PDEδ interaction has recently become the subject of small molecule inhibitor development. Indeed, disruption of this interaction leads to the localization of K-RAS to endomembrane domains and disrupts downstream K-RAS signaling [121]. Taken together, it is clear that trafficking of K-RAS to the plasma membrane occurs in a more complex manner than that of H-Ras and N-RAS trafficking. These early differences in HVR processing and plasma membrane trafficking underscores the importance of these processes in conferring functional differences on each of the individual RAS molecules.

*Microlocalization within the Plasma Membrane*

One of the important consequences of differential RAS processing and trafficking is the capacity for the structurally similar RAS molecules to trigger distinct signaling cascades from unique domains within the plasma membrane. The plasma membrane consists of a heterogeneous mix of cholesterols,

lipids, and proteins arranged in lipid and non-lipid raft domains that laterally segregate the RAS molecules within the plasma membrane (discussed below). Growing evidence indicates that RAS molecule signaling in these domains occurs primarily from nanoclusters—signaling domains approximately 6-20nm in diameter and containing as few as 6-8 RAS proteins per cluster [122]. Nanocluster formation is dictated by several factors, including plasma membrane and cytoskeleton composition, RAS HVR interaction with the plasma membrane, and effector molecule recruitment to individual signaling domains. In this regard, RAS nanocluster formation is dependent on the cholesterol composition of the plasma membrane. Both H-RAS-GDP and N-RAS-GTP cluster formation is cholesterol-dependent while the formation of H-RAS-GTP, N-RAS-GDP, and K-RAS-GTP nanoclusters are all cholesterol-independent [123]. Moreover, immunogold labeling of active RAS proteins within intact plasma membrane sheets revealed a differential dependence on an intact actin cytoskeleton for K-RAS$^{G12V}$, but not H-RAS$^{G12V}$, nanoclusters indicating a role for cytoskeleton composition in nanocluster formation [122]. The structurally distinct HVR sequences of the RAS molecules also drive nanocluster formation. This is exemplified by the stabilization of active H-RAS signaling nanoclusters by the cytosolic lectin protein galectin-1. Upon activation at the plasma membrane, H-RAS signaling nanoclusters are stabilized by galectin-1 binding at the farnesyl moiety within the H-RAS HVR [124]. Apart from simply stabilizing these signaling domains, H-RAS-galectin-1 interactions also direct H-RAS signaling to Raf-1 but not PI3K downstream effector proteins [125]. Similarly, the Sur-8 and SPRED1 proteins have also been reported to modulate RAS signaling to downstream effector proteins at the plasma membrane, suggesting an important role for these proteins in facilitating the formation of transient RAS-effector signaling complexes [89, 126]. Recent findings demonstrate that nanocluster formation can occur in a manner directly proportional to growth factor input signals, indicating that functionally, the formation of RAS nanoclusters ensures the fidelity of ligand input signaling to the downstream signaling cascades [127]. Taken together, the differential signaling capacity of the RAS molecules is therefore dependent upon lateral segregation of the RAS molecules and multiple layers specifying the membrane and effector composition of nanocluster signaling domains within the plasma membrane. This merits further discussion in the context of how the individual RAS molecules themselves segregate within the plasma membrane prior to nanocluster formation.

Comparison of H-RAS and K-RAS membrane localization indicates that these molecules occupy distinct portions of the membrane. Of the three RAS molecules, the localization of H-RAS within the plasma membrane has been best characterized using GFP-tagged H-RAS molecules and immunogold electron microscopy techniques. Early work demonstrated that H-RAS localization is dynamic, occupying both lipid raft and non-lipid raft portions of the plasma membrane [128, 129]. Trafficking between these domains is dependent on the activation state of H-RAS. Inactive, GDP-bound H-RAS is associated with lipid raft portions of the plasma membrane, while active, GTP-bound H-RAS transits to non-lipid raft portions of the membrane [129]. The underlying mechanism of this transition relies on three key regions of the H-RAS protein. The CAAX motif and the two palmitoyl sites (cysteines 181, 184) within the HVR have a high affinity for lipid raft domains, thereby localizing H-RAS-GDP to these domains by default [130]. However, a linker domain between the HVR and N-terminal portion of the protein exhibits affinity for non-lipid raft portions of the membrane. Once GTP-bound, the N-terminal domain of H-RAS exerts a repulsive force on lipid rafts which, in combination with the linker domain, triggers trafficking to non-lipid raft portions of the membrane. Here, the lectin protein galectin-1 stabilizes H-RAS-GTP in the non-lipid raft portion of the membrane and facilitates formation of H-RAS signaling nanoclusters [128]. Increasing amounts of galectin-1 increases the stability of these H-RAS signaling clusters and can increase the signaling output from these clusters [131].

In contrast, K-RAS localizes exclusively in non-lipid raft domains of the plasma membrane, although in areas distinct from those occupied by active H-RAS [128, 129, 132]. Owing to the poly-lysine sequence within the HVR, K-RAS localization requires anionic lipids to associate within the plasma membrane, as the C-terminal region of K-RAS has a low affinity for neutrally-charged lipids in the membrane [133]. Despite this dependence, K-RAS does not appear to associate with any specific anionic lipids. In order to form signaling nanoclusters, K-RAS relies on an intact actin cytoskeleton, the loss of which appears to be especially deleterious to activated K-RAS signaling [122]. K-RAS clustering also requires farnesylation of the HVR, as geranylgeranylation (an alternative prenylation step) does not allow for correct clustering of K-RAS in non-lipid raft portions of the membrane [128].

To date, few studies have focused on the membrane localization of N-RAS. Recent biochemical studies using a series of modified N-RAS lipid anchor motifs demonstrate that N-RAS occupies fluid-like,

non-lipid raft domains within the plasma membrane [134, 135]. Localization to the plasma membrane and subsequent activation of N-RAS requires that this molecule be palmitoylated at cysteine 181 within its HVR [136]. Following activation at the plasma membrane, recent studies suggest that this facilitates N-RAS clustering in lipid raft portions of the membrane, which precedes depalmitoylation and retrograde transport of N-RAS to the Golgi [137]. As such, this provides a mechanism by which active N-RAS may accumulate at both the plasma membrane and at endomembranes (Golgi and ER) as is discussed further in the next section.

*Differential Signaling of the RAS Molecules*

Activation of the RAS molecules occurs at multiple domains within the cell depending on the RAS family member and its subcellular localization. While the majority of Ras activation occurs at the plasma membrane, there is evidence that active H-Ras and N-Ras can also be found at the Golgi and the ER [138, 139]. This reflects the capacity for both molecules to transit from the plasma membrane to the Golgi by recycling endosomes upon HVR depalmitoylation [136, 140]. The differential activation of the Ras molecules in the cell may also reflect location-specific sensitivity to GEFs. While Ras is activated by a variety of GEFs at the plasma membrane, only RasGRP activates Ras at the Golgi [141, 142]. In combination, the differential localization and sensitivity to GEF activity provides mechanisms by which the Ras molecules can have divergent signaling capacities.

Two signaling pathways that operate downstream of active RAS have been especially well characterized in the literature to date. In the first pathway to be discovered, the Raf family of serine/threonine kinase proteins was demonstrated to directly bind to Ras in its active, GTP-bound form [143-146]. Although Ras itself cannot function as a kinase, Ras reduces Raf autoinhibition upon binding to the Ras binding domain (RBD) within the N-terminus of the Raf protein [147, 148]. Subsequently, Ras escorts Raf to the plasma membrane for subsequent activation by a number of kinases, including Src and protein kinase C (PKC) [94, 149]. There are three members of the RAF kinase family, RAF-1 (C-RAF), A-RAF, and B-RAF, all of which can be activated by the H-RAS, K-RAS, and N-RAS molecules [150]. However, only B-Raf activity is exclusively dependent on Ras-mediated activation as both C-Raf and A-Raf can be activated by Src in the absence of Ras [151]. Upon activation, Raf activates Mek1 and Mek2

by phosphorylating serines 217 and 221 on these proteins [152]. Subsequently, Mek1/2 activates the Erk1 and Erk2 effectors by phosphorylating threonine 202 and tyrosine 204 on these proteins [153]. This phosphorylation cascade is most often associated with canonical Ras signaling and is most often implicated in regulating cell growth, differentiation, and apoptosis.

Subsequent studies identified another downstream effector protein, phosphatidylinositol 3-kinase (PI3K), that binds active Ras in a Raf-independent manner [154]. Active PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate ($PIP_2$), converting the protein to the secondary messenger protein phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$). $PIP_3$ binds to proteins containing a pleckstrin homology (PH) domain such as phosphoinositide-dependent kinase 1 (PDK1) and Protein Kinase-B (AKT). Upon binding, $PIP_3$ facilitates PDK1/Akt trafficking to the plasma membrane, where Akt is subsequently activated by phosphorylation at threonine 308 [155, 156]. Once activated, Akt activates the mammalian target of rapamycin (mTOR) complex 1 (mTORC1), a key signaling complex in the regulation of gene expression, protein translation, and cell proliferation. To do so, mTORC1 increases protein translation by phosphorylating (activating) the p70 S6 kinase ribosomal protein and the eukaryotic initiation factor binding protein 4E-BP1 [157, 158].

These conventional pathways provide a framework by which to understand how RAS might regulate cell proliferation and differentiation functions. However, not every RAS molecule signals through these conventional pathways in the same way. As an example, K-Ras and H-Ras exhibit different capabilities of activating the Raf-1 and PI3K effector proteins at the plasma membrane. Relative to H-Ras, K-Ras is better able to recruit and subsequently activate Raf-1 at the plasma membrane [159]. In contrast, H-Ras is a more potent activator of PI3K than K-Ras. This differential capacity to signal to different downstream effector proteins is a direct result of different HVR sequences on the Ras molecules. Shortening of the H-Ras HVR to more closely resemble the K-Ras HVR results in improved Raf-1 recruitment to the plasma membrane by H-Ras. K-Ras also retains more Raf-1 within its signaling nanoclusters at the plasma membrane relative to H-Ras, thereby allowing K-Ras-GTP to induce Raf/Mek/Erk signaling more potently than H-Ras-GTP [160]. The location of individual Ras molecules is also critical to their differential signaling capacity. Activated forms of both H-Ras and N-Ras are found at the Golgi and ER, owing to accumulation during posttranslational processing and retrograde transport

from the plasma membrane [139, 161, 162]. Although H-Ras has a diminished ability to activate Raf-1 at the plasma membrane compared to K-Ras, H-Ras can activate Raf-1 and downstream Erk activity from the ER, leading to subsequent transformation of cells [139]. Taken together, these data suggest a model in which the biological effects of increased RAS signaling are dependent upon the specific RAS molecule in question as well as its location within the cell.

In addition to PI3K and RAF, RAS can also activate a variety of other signaling effector proteins including those involved in cell growth and function (PKC-ζ, RAC, β-catenin), cell fate decisions (PKC-γ, PDK1), and oncogenesis (JNK, RAN GTPase) [163-168]. It is likely that these effector proteins will also demonstrate differential susceptibility to activation by the individual Ras molecules. In fibroblasts, K-Ras, but not H-Ras, activation leads to increased activation of the small GTPase protein Rac, as demonstrated by increased Rac-GTP in these cells [167]. Together, the variety of downstream effector proteins coupled with the potential variability of individual RAS molecules to activate these effectors suggests a robust infrastructure by which RAS can regulate cell growth and function.

Despite a number of *in vitro* studies comparing the impact of Ras molecule activation on cell function, there are few studies comparing the consequence of Ras molecule hyperactivation *in vivo*. Recently, Haigis, et al., compared the consequences of activating individual Ras molecules in a mouse model of colon cancer [169]. Oncogenic mutations in K-RAS (K-RAS$^{G12D}$) occur in approximately 50% of colon cancers, whereas N-RAS mutations (N-RAS$^{G12D}$) occur only rarely (~5%). In order to determine how each hyperactivated Ras molecule impacts the progression of colon cancers, genetically engineered mice were generated which, in cells expressing Cre recombinase, express a hyperactivated copy of Ras that is transcribed under the control of the endogenous Ras promoter. These mice were also used in the current thesis and a full description of the constructs can be found in Chapter 2: Materials & Methods. Using a colon-specific promoter to drive Cre recombinase (and hyperactive Ras) expression in these cells, differential biological effects upon Ras molecule hyperactivation were observed. K-Ras hyperactivation led to hyperproliferation of the colon epithelium in a Mek-dependent manner. Conversely, N-Ras hyperactivation did not alter the growth of the epithelium but conferred increased resistance of these cells to programmed cell death (apoptosis). This study was the first to provide evidence of differential biological function of the Ras molecules *in vivo* and provides an important foundation for

studies comparing Ras molecule hyperactivation, as in the current thesis. Additionally, this work yielded an important tool for studying Ras molecule hyperactivation *in vivo* in a biological relevant, endogenous expression context.

*Activating Ras Mutations*

A relatively high proportion of cancers (30%) are known to result directly from activating RAS mutations [170]. Reflecting the differential capacities of the RAS molecules, mutations are not uniformly distributed across the individual RAS molecules. An analysis of the Catalog of Somatic Mutations in Cancer (COSMIC) database identifies K-RAS as the most frequently mutated in cancers (22% of all tumors), followed by N-RAS (8%) and H-RAS (3%) [170]. While this partially results from the differential signaling capacity of the RAS molecules, this may also reflect the differential contributions of each RAS molecule to total RAS expression. A previous analysis of multiple cancer cell lines determined that K-RAS is more highly expressed than either its N-RAS or H-RAS counterparts [161]. Nevertheless, the RAS molecules are commonly associated with certain families of cancers. K-RAS mutations are more commonly associated with cancers in tissues of endodermal origin, (lung, pancreas, colon) while N-RAS mutations occur most commonly in hematopoietic (neural crest-derived) disorders [171-174].

Aberrant RAS activation results from mutations at only three sites within the RAS genes: codons 12, 13, and 61. Although mutations at all three sites have been documented for each RAS molecule, the frequency of mutations at each site varies from molecule to molecule. For example, 80% of activating mutations in K-RAS occur at codon 12 while 60% of N-RAS mutations occur at codon 61 with only 35% occurring at codon 12 [170]. In contrast, activating mutations in H-RAS occur in relatively equal proportions at both codons. The frequency of activating mutations at these sites lies in the intrinsic requirement for the encoded amino acids in normal RAS-GAP function. The catalytic domain of RAS, N-terminal to the HVR and conserved between the RAS molecules, contains the amino acid sequences required for the intrinsic GTP-ase activity of RAS. Of particular functional importance is the role of the switch I (amino acids 25-40) and switch II (amino acids 57-75) regions within the catalytic domain which undergo significant conformational changes during RAS activation/inactivation (Figure 1.6) [175-177]. A resolved crystal structure of $AlF_3$-stabilized H-Ras in its transition state reveals the function of the mutated

sites in GTP hydrolysis. In its transition state, an arginine residue from the GAP protein (arginine 789 on $p120^{GAP}$) is inserted into the Ras active site to neutralize developing charges and stabilize the Ras switch II region [178]. This also stabilizes Ras glutamine 61, allowing the residue to participate in catalysis of the GTP γ-phosphate at the Ras active site. Of particular interest is the close proximity of Ras glycine 12 within Van der Waals distance of both Ras glutamine 61 and GAP arginine 789 within the Ras active site. This proximity precludes the addition of side chains, even one as small as alanine, without significant functional disruption to the Ras active site. Presumably, this constraint also applies to Ras glycine 13, which is found in equally close proximity to these residues in the active site.

The complexity of differential RAS signaling has frustrated the development of drug therapies aimed at reducing aberrant RAS pathway hyperactivation. Preliminary studies aimed at blocking Ras processing and subsequent localization to the plasma membrane suggested that farnesyltransferase inhibitors (FTIs) could therapeutically abrogate hyperactivation of all three RAS molecules [179, 180]. Despite a strong biological foundation in support of moving these inhibitors to clinical trials, phase I trials using FTIs in the context of NF1 plexiform neurofibromas yielded a limited tumor response to the drug [181]. While in theory all RAS molecules should be inhibited by FTIs, *in vitro* biochemical studies comparing the efficiency of FTI treatments revealed that RAS processing is differentially inhibited across the three RAS molecules [182]. In line with preclinical results, H-RAS processing is successfully inhibited in the presence of FTIs. However, both N-RAS and K-RAS can bypass this FTI inhibition by alternatively undergoing geranylgeranylation and subsequent processing and trafficking to the membrane. A second pharmacological agent, lovastatin, primarily inhibits cholesterol biosynthesis but also inhibits RAS prenylation [183, 184]. To date, preclinical and clinical trials of lovastatin have focused on correcting learning and behavioral deficits in individuals affected by NF1 with mixed success [185-187]. Given the differential processing, localization, and signaling capacity of the individual RAS molecules, future therapeutic treatments will need to consider taking a more directed approach to inhibit RAS pathway hyperactivation. In place of broad scale treatments such as FTIs and lovastatin, novel approaches will target individual downstream effector pathways as a more efficient and precise means by which to inhibit hyperactivation of the individual RAS molecules.

## SUMMARY AND SIGNIFICANCE

The specification of NSCs towards neuron, astrocyte, and oligodendrocyte cell fates is a closely regulated process necessary to ensure normal brain development. Disruptions in the signaling pathways described in this section, as demonstrated by genetic overexpression and knock-out studies, can radically skew NSC differentiation towards one lineage versus another to alter the overall composition of the brain. The RASopathy family of neurodevelopmental disorders further underscores the consequence of aberrant hyperactivation of a single regulatory pathway on normal brain development, resulting in abnormal neural functions, including learning deficits and behavioral abnormalities. Despite the clear importance of this signaling pathway in brain development, the mechanisms underlying RAS pathway hyperactivation and its impact on brain development are incompletely characterized. This is especially relevant to the function of NSCs, which give rise to the majority of cell types in the brain. Given the capacity for the three RAS molecules (H-RAS, K-RAS, N-RAS) to localize and signal differently upon activation, the contribution of each RAS molecule must be characterized in the context of brain development. In this thesis, we leverage a novel series of genetically-engineered mouse (GEM) strains to characterize the consequence of hyperactivation of the individual Ras molecules on brain development and the cell-intrinsic properties of NSCs. This work has important implications for the development of therapeutic approaches designed to target aberrant RAS pathway hyperactivation in the brain.

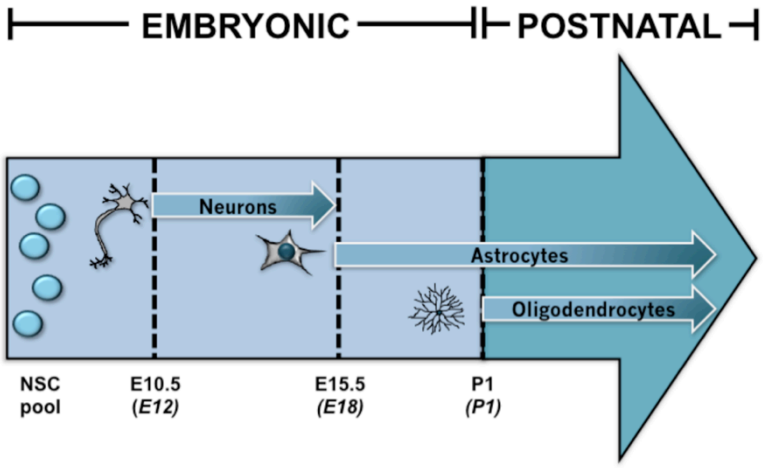


**Figure 1.1. Timeline of mammalian brain development.** The NSC pool differentiates into mature cell types during both embryonic and postnatal brain development in a temporally-regulated manner. Neurons develop first beginning at embryonic day 10.5 (E10.5) in the mouse. Astrocytes (E15.5) and oligodendrocytes (postnatal day 1 (P1)) develop afterward to support these newly formed neurons. The timeline of mammalian brain development was extensively characterized in the rat brain, correlating to the timepoints in parentheses.

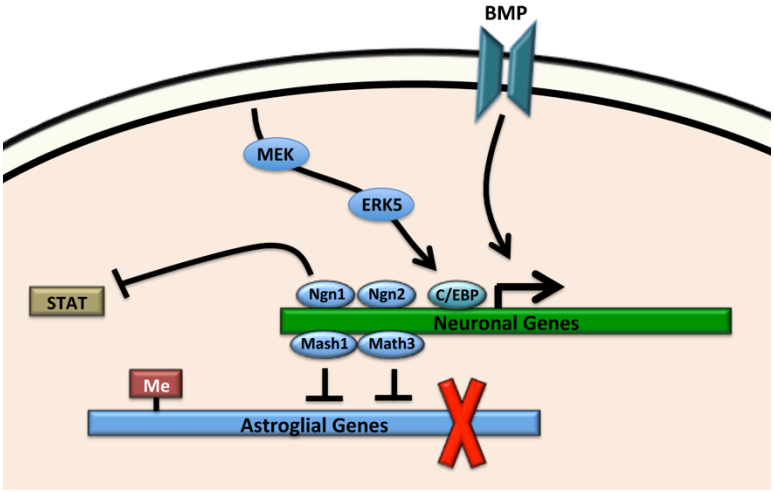


**Figure 1.2. Cell fate specification during the neurogenic period of brain development.** From E10.5-E15.5, neurogenesis is specified by both cell-intrinsic and cell-extrinsic factors. At the genetic level, the transcription factors Ngn1, Ngn2, Mash1, and Math3 promote transcription of neuronal genes. BMP signaling promotes neurogenesis directly, while MEK/ERK5 may mediate autocrine pro-neurogenic signals via the C/EBP transcription factor. At this stage, astrocyte cell fate decisions are inhibited by the pro-neuronal transcription factors Mash1 and Math3 and by CpG methylation (Me) in the promoter region of astrocyte-specific genes. Ngn1 also sequesters the pro-astrocytic STAT transcription factor from astrocytes-specific genes.

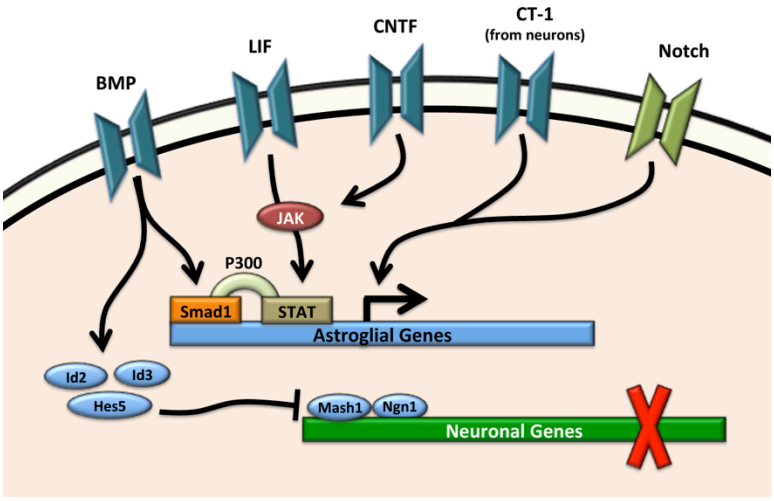


**Figure 1.3. Cell fate specification during the gliogenic period of brain development.** Gliogenic signals proceed predominantly through the gp130 family of membrane receptors (in turquoise). Cardiotrophin-1 (CT-1) and Notch promote gliogenesis based on signals from neurons and surrounding neuroblast progenitors, respectively. Leukemia inhibitory factor (LIF) and ciliary neurotophic factor (CNTF) activate STAT transcription factors via the Janus kinase (JAK) protein. BMP promotes Smad1 binding at astroglial gene promoters, which synergistically promotes astroglial gene transcription with STAT via the transcriptional coactivator protein P300. BMP also promotes expression of the Id2, Id3, and Hes5 transcription factors, which inhibit the action of the pro-neurogenic transcription factors Mash1 and Ngn1.

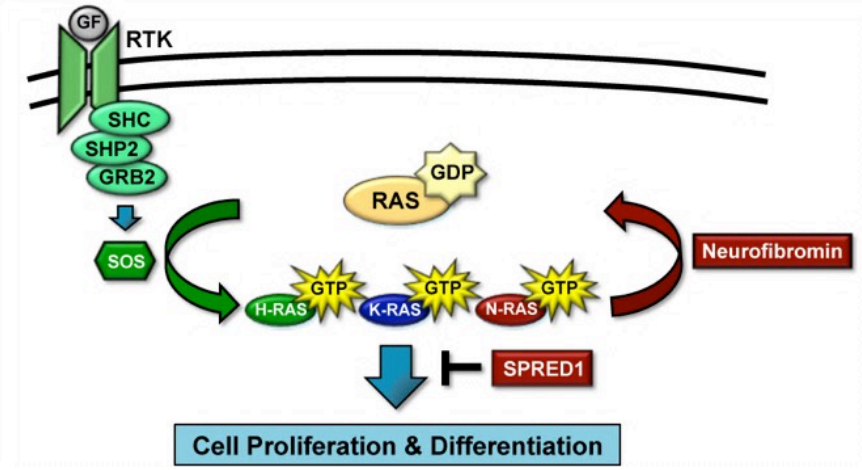


**Figure 1.4. The RAS signaling pathway.** The small G-protein RAS mediates downstream signaling initiated by growth factor (GF) binding at receptor tyrosine kinase (RTK) membrane proteins. RAS mediates these signals by transitioning between an active GTP-bound state and an inactive GDP-bound state, with activation assistance from the guanine exchange factor (GEF) SOS and inactivation facilitated by the GTPase activating protein (GAP) neurofibromin. There are three RAS molecules expressed in the mammalian brain that, when active, can promote cell proliferation and differentiation.

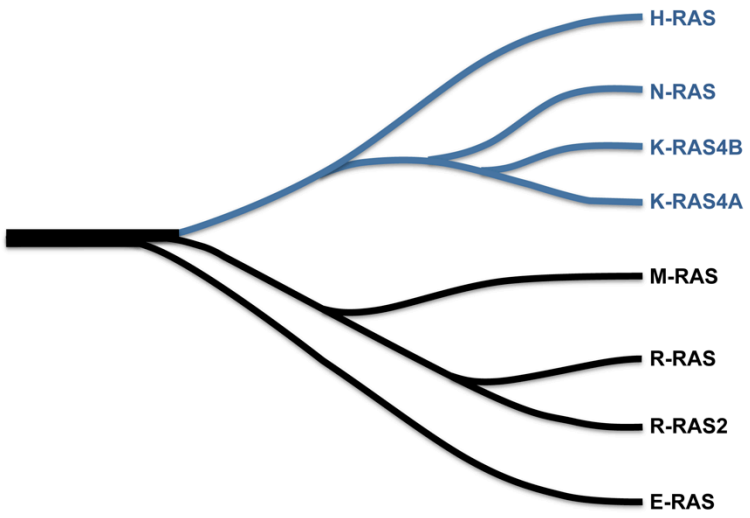


**Figure 1.5. Genetic evolution of the RAS subfamily of genes.** The RAS protein subfamily is composed of related GTPase proteins that share sequence homology with the classical RAS proteins (blue), H-RAS, K-RAS, and N-RAS, which are the focus of this thesis. As indicated by orthologous gene sequence analysis, the other members of this subfamily diverged from these classical RAS proteins and include E-RAS, expressed in embryonic stem cells, R-RAS, expressed in endothelial and smooth muscle cells, and M-RAS, expressed in muscle cells and some portions of the brain (hippocampus, cerebellum). Adapted from [188], points of genetic divergence are relative and not drawn to scale.

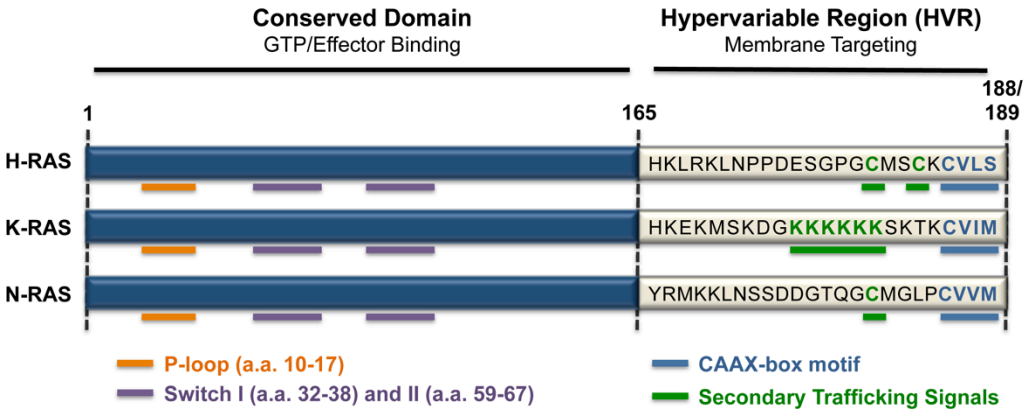


**Figure 1.6. Genetic structure of the H-RAS, K-RAS, and N-RAS genes.** In humans, the three related RAS genes are located on separate chromosomes: H-RAS on 11p, K-RAS on 12p, and N-RAS on 1p. The first 165 amino acids are conserved among the three genes and encode the GTP-binding (P-loop) and effector-binding (switch I, II) regions of the RAS proteins. The 25 amino acids at the C-terminus encode a hypervariable region (HVR) that underlies the differential processing, localization, and biological function of the RAS proteins. The HVR contains a CAAX (C=Cysteine, A=aliphatic amino acids, X=amino acid) motif and secondary signals for divergent RAS molecule post-translational processing. These secondary signals consist of a fatty acid addition (palmitoylation) at cysteines in the H-RAS (C181, C184) and N-RAS (C181) HVRs. In contrast, the K-RAS HVR contains a poly-lysine (K) sequence that acts as a secondary signal. Adapted from [189] and [188], numbers at the top indicate amino acid number (H-RAS and N-RAS are 189 amino acids in length, K-RAS is 188 amino acids in length).

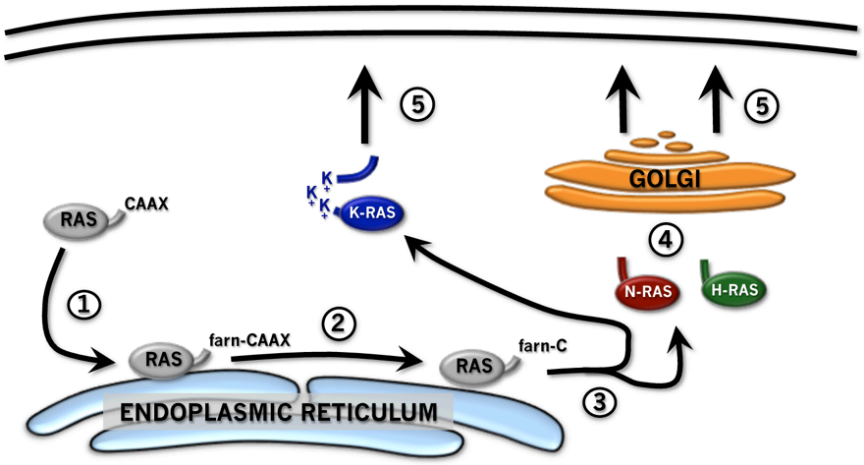


**Figure 1.7. Differential post-translational processing of the RAS molecules.** Upon translation, RAS molecules must undergo post-translational modifications to increase the affinity of the carboxy-terminus HVR for membrane localization. Immediately following translation, all RAS molecules are located in the cytosol. (1) To begin post-translational processing, the RAS molecules are farnesylated at the cysteine present within the CAAX motif at the C-terminus, localizing the protein to the endoplasmic reticulum where (2) the –AAX tripeptide is removed. (3) The three RAS molecules undergo differential methylation by the isoprenylcysteine methyltransferase (ICMT) enzyme. (4) H-RAS and N-RAS are trafficked through the Golgi apparatus where they undergo fatty acid modifications (palmitoylation). (5) Following Golgi processing, H-RAS and N-RAS are trafficked to the plasma membrane while K-RAS is transported to the plasma membrane by an unknown, Golgi-independent mechanism.

**Table 1.1. Summary of neuro-cardio-facio-cutaneous syndromes.**

| Disorder | Incidence | Mutated Genes | Encoded Protein | Occurrence within Disorder | CNS Clinical Features |
|---|---|---|---|---|---|
| Noonan Syndrome | 1 in 1,000-2,500 | *PTPN11* | SHP2 | ~50% | memory deficits<br>learning deficits<br>language development delay<br>motor delay |
| | | *SOS1* | SOS1 | ~20% | |
| | | *K-RAS* | K-RAS | <5% | |
| | | *N-RAS* | N-RAS | <5% | |
| | | *RAF1* | RAF1 | rare | |
| Neurofibromatosis Type 1 | 1 in 3,000 | *NF1* | Neurofibromin | 100% | learning disabilities<br>behavioral abnormalities<br>tumor predisposition (glioma) |
| Costello Syndrome | rare | *H-RAS* | H-RAS | 100% | neurodevelopmental delay |
| Legius Syndrome | rare | *SPRED1* | SPRED1 | 100% | mild neurocognitive impairment |
| Cardio-Facio-Cutaneous Syndrome | rare | *B-RAF* | B-RAF | 75% | learning disabilities<br>motor delay<br>speech delay |
| | | *MAP2K1* | MEK1 | 25% | |
| | | *MAP2K1* | MEK2 | | |
| | | *K-RAS* | K-RAS | rare | |

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# CHAPTER 2

# RAS ACTIVATION IN THE MAMMALIAN BRAIN

## INTRODUCTION

The formation of the mammalian brain is a carefully orchestrated process in which the sequential development of mature cell types ensures the formation of the functional neuronal signaling networks required for adult brain function. In the mouse, brain development is initiated by the specification of neurons beginning at embryonic day 10.5 (E10.5), with a neuronal network forming through E15.5. In order to support this newly formed neuronal network, glial astrocytes develop beginning at E15.5, followed shortly thereafter by the formation of insulating oligodendrocytes at birth (postnatal day 1 (PN1)). The close association between glial cells and neurons is necessary to ensure the health, viability, and signaling capacity of these neuronal networks [1]. Despite their divergent functions in the mature brain, all three cell types (neurons, astrocytes, and oligodendrocytes) arise from a single population of multipotent neural stem cells (NSCs). The specification of these diverse cell fates requires targeted inputs to direct NSCs to develop into one mature cell type versus another. This suggests an extensive array of signaling mechanisms is required to drive NSCs towards the correct cell fates at the correct developmental time point. Indeed, at developmentally-restricted time points, cell-extrinsic signaling factors (cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), bone morphogenic protein (BMP)) and cell-intrinsic factors (Mash1, Math3, Ngn and Olig transcription factors) cooperate to induce NSC adoption of neuronal, astrocytic, and oligodendroglial cell fates [2-10].

Despite research aimed at dissecting the cell type-specific and time-specific regulation of cell fate specification in the brain, the importance of the RAS signaling pathway in NSCs during brain development is often overlooked. The critical function of this pathway is most apparent in a family of neuro-cardio-facio-cutaneous neurodevelopmental disorders that are characterized by abnormal RAS pathway signaling. In the central nervous system (CNS), these disorders often manifest in the form of learning difficulties, behavioral abnormalities, and tumor predisposition [11]. In normal cells, the small GTP-ase protein RAS functions as a molecular switch by binding GTP in its active state and retaining GDP in its inactive state. When activated, RAS-GTP functions in mediating growth factor signaling and promoting cell proliferation and differentiation. In the neuro-cardio-facio-cutaneous syndromes, commonly termed RASopathies, normal RAS signaling is altered by germline mutations that lead to aberrant hyperactivation of RAS pathway signaling. In the mammalian brain, there are three RAS molecules expressed, H-RAS,

K-RAS, and N-RAS. Despite significant overlap in their genetic and amino acid sequences, variation at the C-terminal hypervariable region (HVR) directs differential post-translational processing, localization, and downstream effector protein signaling of the three RAS molecules [12-17]. These differences ultimately underlie differential biological functions of these molecules *in vivo* [18].

In this chapter, we hypothesized that hyperactivation of the RAS molecules at different developmental time points might have differential effects on progenitor cell growth and the resulting cellular composition of the adult brain. To test this hypothesis, we used Cre-driver strains (BLBP-Cre and GFAP-Cre) to drive hyperactivated Ras expression in progenitor cells at two distinct developmental time points. Our laboratory has previously developed and characterized a BLBP-Cre mouse to drive Cre recombinase expression in NSCs beginning at E9.5 [19]. Cre-expressing cells overlap with cells positive for the NSC marker brain lipid binding protein (BLBP) in the brain ventricular regions (a location of resident NSCs in the brain) and also express the BLBP marker when grown in neurospheres *in vitro*. Moreover, BLBP-Cre-expressing cells can undergo multi-lineage differentiation to give rise to neurons, astrocytes, and oligodendrocytes *in vivo* and *in vitro.* Together, these data are consistent with the conclusion that BLBP-Cre is expressed in the brain NSC population. In contrast, the GFAP-Cre mouse expresses Cre recombinase under a human promoter for glial fibrillary acidic protein (GFAP), a protein expressed in neuroglial progenitor cells and mature astrocytes. Our laboratory has previously demonstrated that Cre recombinase expression is initiated beginning at E14.5 in neuroglial progenitor cells which give rise to mature neurons, astrocytes, and oligodendrocytes [20, 21]. Both strains of mice were intercrossed with GEM strains containing a Cre-dependent construct to drive expression of a hyperactivated copy of the individual Ras molecules from their endogenous promoters in Cre-expressing cells. Here, we demonstrate that K-Ras, but not H-Ras or N-Ras, hyperactivation in NSCs at E9.5 leads to the formation of more astrocytes in the brainstem at approximately three weeks of age (PN18). In contrast, activating any of these Ras molecules in NSCs at E14.5 does not result in gross abnormalities in glial cell formation in the three week old brain. Characterization of the neuroglial progenitor cell population in *K-Ras*$^{BLBP}$ mice at early developmental timepoints (E12.5, PN1) revealed that *K-Ras*$^{BLBP}$ mice have more Sox2$^{+}$ NSCs in the embryonic hindbrain at E12.5 and have more Olig2$^{+}$ glial restricted progenitor cells in the brainstem of PN1 mice compared to littermate controls. Taken together, these data

indicate a temporally-restricted role for Ras molecule hyperactivation in altering NSC function such that more astrocytes are formed in the mature brain.

**MATERIALS AND METHODS**

***Mice.*** Activated H-Ras, K-Ras, or N-Ras allele expression was induced in NSCs *in vivo* by intercrossing BLBP-Cre [19] or GFAP-Cre [20] mice with mice containing *Lox-Stop-Lox (LSL)-H-Ras*$^{G12V}$ (Supplemental Figure S2.1; generated by Dr. Kevin Haigis), *LSL-K-Ras*$^{G12D}$ [22], or *LSL-N-Ras*$^{G12D}$ [18] constructs knocked into the respective *Ras* locus. All mice were maintained on a C57Bl/6 background under an approved animal studies protocol at Washington University.

***Immunohistochemistry.*** Brain tissues were collected at postnatal day 18 (P18). Prior to tissue harvesting, mice underwent intracardiac perfusion with Ringer's solution containing lidocaine and heparin followed by 4% paraformaldehyde. Tissues were subsequently post-fixed overnight in 4% paraformaldehyde, and then in 70% ethanol prior to tissue processing and embedding. Paraffin-embedded tissues were sectioned at 6μm thickness using a Leica RM2125 RTS microtome (Leica Microsystems Inc., Buffalo Grove, IL). Antigen retrieval and appropriate primary antibodies (Supplemental Table S2.1) were applied overnight at 4°C prior to the addition of species-appropriate horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA). Images of the pons (Supplemental Figure S2.2) were acquired on a Nikon Eclipse E600 light microscope (Nikon Instruments Inc., Melville, NY) equipped with a Leica EC3 camera. Six to ten mice were collected per genotype along with appropriate matched littermate controls.

***Immunofluorescence.*** Brain tissue was collected following perfusion and fixation in 4% paraformaldehyde as described above. After overnight fixation, these tissues were subsequently dehydrated in 30% sucrose for at least 24 hours prior to embedding in OCT mounting media (Sakura Finetek, Torrance, CA) and freezing at -80°C. All frozen tissues were sectioned on a Reichert-Jung Cryocut 1800 (Reichert Technologies, Depew, NY) cryostat into 10μm-thick sections. Primary antibodies (Supplemental Table S2.1) were applied overnight at 4°C followed by incubation with species-appropriate AlexaFluor® 488 or 568 secondary antibodies (Life Technologies) and counterstained with DAPI. For postnatal tissues, images of the pons (Supplemental Figure S2.2) were acquired using a Nikon Eclipse TE300 inverted fluorescent microscope with a Photometrics CoolSnap EZ camera (Photometrics, Tucson,

AZ) and compared to littermate controls. Marker-positive cells were quantified as a percentage of total $DAPI^+$ cells. For embryonic brain sections, images were acquired of the ventricular region of the hindbrain (the future site of the brainstem pons) using a Leica DFC 3000G camera. Since the high density of cells in these images precluded quantification of total $DAPI^+$ cells, marker-positive cells were normalized to the total tissue surface area in the images using Leica Application Suite Advanced Fluorescence software.

***Data Analysis.*** Unless noted otherwise above, tissue and cell staining images were quantitated using ImageJ image analysis software (U.S. National Institutes of Health). GraphPad Prism 5 software (GraphPad Software, La Jolla CA) was used for all statistical analyses in this study. Specifically, outlier values were determined using the Grubbs outlier test and statistical significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$) was determined using an unpaired, two-tailed Student's t-test.

## RESULTS

***K-Ras activation in early embryonic NSCs leads to increased astrogliogenesis in vivo.*** To define the impact of Ras hyperactivation on NSC function *in vivo*, we leveraged *BLBP-Cre* mice to target Cre recombinase expression (and Ras activation) to brain NSCs capable of multi-lineage differentiation beginning at E9.5 [19]. Expression of each individual activated Ras allele was accomplished using mice in which an oncogenic version was knocked into the endogenous *Ras* locus. Specifically, these $Ras^{LSL}$ (*H-*$Ras^{LSL\text{-}G12V}$, *K-*$Ras^{LSL\text{-}G12D}$, or *N-*$Ras^{LSL\text{-}G12D}$) mice contain a transcriptional stop element flanked by LoxP sites (Lox-Stop-Lox [LSL]) that prevents expression of the mutationally-activated *Ras* alleles in the absence of Cre expression. Following Cre-mediated recombination, mutationally-activated Ras is then expressed from its endogenous promoter.

At postnatal day 18 (PN18), there was a greater percentage of astrocytes in the brainstem of *K-*$Ras^{BLBP}$, but not *H-*$Ras^{BLBP}$ or *N-*$Ras^{BLBP}$, mice relative to littermate controls using the GFAP (1.8-fold) and S100β (1.2-fold) astrocyte markers (Figure 2.1A, 2.1B). In contrast, there were no differences in $APC^+$ oligodendrocytes (Figure 2.1C) or $NeuN^+$ neurons (Figure 2.1D) or differences in brain or body weights across the three Ras genotypes relative to controls (Supplemental Figure S2.1). These data demonstrate that K-Ras, but not H-Ras or N-Ras, activation in NSCs leads to the generation of more astrocytes *in vivo*.

***Ras activation in later embryonic NSCs does not alter glial cell formation in vivo.*** In order to determine if activating the individual Ras molecules in NSCs at a later brain developmental time point also increases gliogenesis in the brain, we intercrossed $Ras^{LSL}$ mice with *GFAP-Cre* mice. These mice express Cre recombinase in multipotent NSCs beginning at E14.5 [20, 21]. Upon Ras molecule activation in these NSCs, there was no difference in the percentage of brainstem $GFAP^+$ astrocytes or $APC^+$ oligodendrocytes in *H-*$Ras^{GFAP}$, *K-*$Ras^{GFAP}$, or *N-*$Ras^{GFAP}$ mice at P18 (Figure 2.2A, 2.2B). As with the $Ras^{BLBP}$ mice, there were also no differences in the body or brain weights across the three Ras genotypes when compared to littermate controls (Supplemental Figure S2.2). Taken together, these data demonstrate that K-Ras hyperactivation alone leads to increased astrocyte formation *in vivo.* Moreover, this increase in astrocytes is dependent on K-Ras hyperactivation in NSCs at E9.5, but not at E14.5, suggesting that K-Ras* alters NSC function within a limited developmental time frame.

***K-Ras*$^{BLBP}$ mice have more Sox2$^{+}$ and Olig2$^{+}$ progenitor cells in the developing embryonic and postnatal brain.** To determine how K-Ras hyperactivation at E9.5 alters the function of NSCs in the developing brain, we examined changes in two progenitor cell populations (Sox2$^{+}$ NSCs, Olig2$^{+}$ glial-restricted progenitor cells) during brain development. Specifically, we measured these progenitor cell populations at two developmental time points, shortly after *K-Ras*$^{BLBP}$ activation (E12.5) and at birth (PN1). At E12.5, the embryonic hindbrain represents the future location of the postnatal brainstem. In this region, there was a 2-fold increase in Sox2$^{+}$ NSCs per unit area (0.1mm$^{2}$) observed in *K-Ras*$^{BLBP}$ mice relative to littermate controls (Figure 2.3A, **p<0.01). At PN1, there was no longer a difference in the percentage of Sox2$^{+}$ NSCs in the brainstem of *K-Ras*$^{BLBP}$ and littermate control mice (Figure 2.3B). However, at this time point there was a 1.9-fold increase in the percentage of Olig2$^{+}$ glial-restricted progenitor cells (Figure 2.3C, ***p<0.001). These data demonstrate that K-Ras hyperactivation in NSCs at an early developmental time point causes a brief expansion in the population of progenitor cells, which likely underlies the increase in astrocytes observed in the *K-Ras*$^{BLBP}$ mouse brainstem at PN18.

**DISCUSSION**

The importance of RAS signaling during brain development is especially apparent in the context of the RASopathy family of neurodevelopmental disorders in which patients present with CNS defects such as neurocognitive deficits, learning difficulties, and behavioral abnormalities. While all of the RASopathies result from aberrant RAS pathway hyperactivation, the gain-of-function mutations occurring in this pathway vary widely between the individual disorders. Of particular interest to the current thesis, activating mutations do not occur uniformly within the RAS molecules. For example, Costello Syndrome is caused exclusively by hyperactivating mutations in the *H-RAS* gene while hyperactivating mutations in *K-RAS* or *N-RAS* cause Noonan Syndrome in a small proportion (<5% each) of patients affected by this disorder. This likely reflects the capacity of the individual RAS molecules to differentially alter cell growth and function during brain development. To this end, it remains unclear how each individual RAS molecule contributes to the regulation of normal brain development. Moreover, it is unknown if RAS pathway activation within different developmental time frames differentially disrupts normal brain development. To address these questions, we expressed activated forms of H-Ras, K-Ras, and N-Ras in NSCs at both early and later stages of brain development. This comparison allowed us to make several important observations about the Ras molecule-specific and time-specific consequences of Ras activation in the developing embryonic brain.

First, we have demonstrated that K-Ras, but not H-Ras or N-Ras, activation in NSCs increases astrocyte formation in the postnatal brain (PN18). Moreover, we have observed that this increase in K-Ras*-mediated astrocyte formation occurs only when K-Ras is activated in NSCs early in brain development (E9.5). When activated in NSCs later in brain development (E14.5), none of the three Ras molecules cause more astrocytes or oligodendrocytes to form in the postnatal brain. These results suggest a time dependent role for activated K-Ras in specifying glial cell fate decisions. Precedent for RAS-mediated regulation of cell fate decisions stems from several studies which examined the consequences of activation of Ras or its downstream Mek/Erk effectors on pro-neuronal and pro-glial fate decisions. In favor of pro-neuronal Ras signaling, the Ras downstream effector protein Mek is required for the phosphorylation (activation) of the pro-neuronal C/EBP transcription factor in *in vitro* cortical NSCs [23]. This transcription factor directs expression of the neuron-specific gene T$\alpha$1 $\alpha$-tubulin, causing these

NSCs to adopt a neuronal cell fate. *In vivo*, expression of the Ras effector protein Erk2 is required for cortical NSCs to adopt a neuronal cell fate. Conditional loss of the Erk2 gene in NSCs inhibits neuronal differentiation and instead results in increased astrocyte formation in the cortex, demonstrating that Erk2 is required for neuronal differentiation in the cortex [24]. While these studies clearly identify a pro-neurogenic role for Ras signaling in NSCs, other recent studies demonstrate a pro-gliogenic role for Ras signaling as well. Introduction of activated H-Ras in cortical progenitor cells by *in vivo* electroporation inhibits cortical neurogenesis and increases progenitor cell proliferation and the adoption of glial cell fates [25]. Strikingly, the Ras downstream effector proteins Mek1/2 are critical for astrocyte fate specification in NSCs as loss of both genes in E11.5 NSCs *in vivo* causes the complete loss of astrocytes in the adult brain. This loss of astrocytes can be rescued to wild-type levels by overexpression of Mek1 later in embryonic development (E15.5), indicating that Mek is both necessary and sufficient for astrocyte differentiation [26]. Although as a whole these studies suggest conflicting roles for Ras in either pro-neurogenic or pro-glial differentiation programs, a recent study demonstrates that Ras can direct both cell fate decisions within the same progenitor cell population. Upon expression of a constitutively activated form of Ras ($Ras^{G12V}$) in E12.5-E15.5 cortical progenitor cells *in vivo*, Ras inhibits expression of the pro-neuronal transcription factor Ngn2 and induces expression of the traditionally pro-neuronal Mash1 transcription factor in an Erk-dependent manner [27]. Subsequently, Mash1 is sufficient to specify both glial and neuronal cell fates in cortical progenitor cells in a Ras expression-dependent manner. In cells expressing high levels of Ras, more $Sox9^+$ proliferative glioblasts are formed, which differentiate into both astrocyte and oligodendrocyte precursor cells. In contrast, moderate Ras expression induces some progenitor cells to become $Dlx2^+$ GABAergic neurons. Collectively, these studies define the capacity of Ras signaling to specify multiple cells fates during brain development. Our data demonstrates that brain NSCs have a limited time frame in which they are sensitive to endogenously activated K-Ras signaling and suggests that K-Ras activation early in brain development might direct NSCs towards the glial (astrocyte) lineage rather than the neuronal lineage. However, *in vitro* studies on the NSC cell-autonomous consequences of Ras molecule activation (examined separately and discussed in Chapter 3) demonstrate that Ras does not alter NSC cell fate decisions *in vitro*. This suggests that K-Ras activation at E9.5 is sufficient to alter NSC growth such that more astrocytes are formed by PN18.

In support of this hypothesis, examination of $Sox2^+$ and $Olig2^+$ progenitor cells demonstrates that both cell populations are expanded at distinct time points during *K-Ras*$^{BLBP}$ mouse brain development. The increase in $Olig2^+$ restricted progenitor cells at an intermediate developmental time point (PN1) is significant as this transcription factor is found in astrocyte and oligodendrocyte progenitor cells and is required for their differentiation into mature glial cell types [28, 29]. It is important to note the similar fold-change differences in these cell types between *K-Ras*$^{BLBP}$ and littermate control mice at the different developmental time points. At E12.5, $Sox2^+$ NSCs are increased 2-fold in *K-Ras*$^{BLBP}$ mice, $Olig2^+$ progenitor cells at PN1 are increased 1.9-fold, and $GFAP^+$ astrocytes at PN18 are increased 1.8-fold. This remarkably consistent increase in each cell type suggests a model in which K-Ras activation initiates a single, brief expansion of $Sox2^+$ NSCs at E9.5. Subsequently, these NSCs give rise to and similarly expand the proportion of $Olig2^+$ restricted progenitor cells at PN1. At this time point, there is no difference in the percentage of $Sox2^+$ cells between *K-Ras*$^{BLBP}$ and wild-type mice, further reinforcing the idea that the initial $Sox2^+$ cells have all become $Olig2^+$ cells by PN1. Lastly, these glial restricted progenitor cells differentiate into an equal proportion of $GFAP^+$ astrocytes in the postnatal brain.

Taken together, this chapter defines a limited developmental window in which K-Ras activation alone impacts on the growth of NSCs in the developing brain. While many studies have explored the activation of Ras downstream effector proteins such as Mek and Erk, this study is the first to not only compare the impact of endogenously-expressed activated forms of the three Ras molecules but also compares their activation at both early and late developmental time points within the same progenitor cell population. Further studies are required to examine whether K-Ras activation alters NSC function in a cell-autonomous manner and the signaling mechanism by which Ras might alter NSC function. These studies and their experimental outcomes are discussed in detail in Chapter 3.

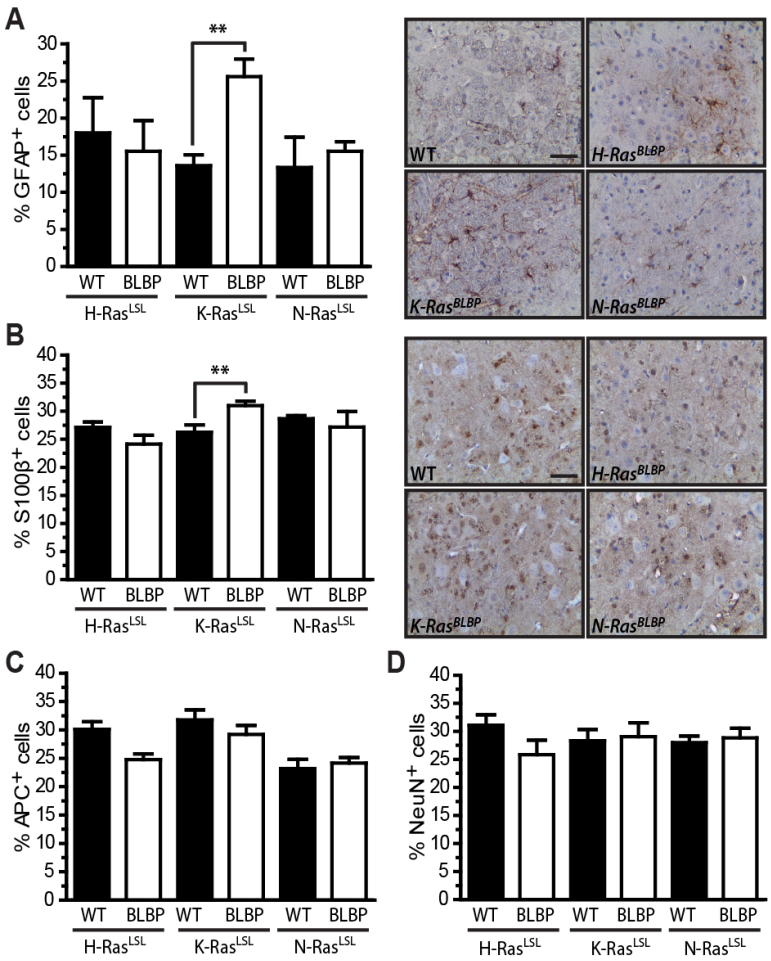



**Figure 2.1. K-Ras activation in E9.5 NSCs leads to increased gliogenesis *in vivo*.** At PN18, *K-Ras*$^{BLBP}$, but not *H-Ras*$^{BLBP}$ or *N-Ras*$^{BLBP}$, mice harbor an increased percentage of astrocytes in the brainstem quantified using the (**A**) GFAP (1.8-fold, **p<0.01) and (**B**) S100β (1.2-fold) astrocyte markers. Representative images appear on the right (20x, scale bar=50μm). There were no changes in the percentages of (**C**) APC$^+$ oligodendrocytes or (**D**) NeuN$^+$ neurons in the three *Ras*$^{BLBP}$ genotypes relative to littermate controls. Error bars represent the standard errors of the mean.

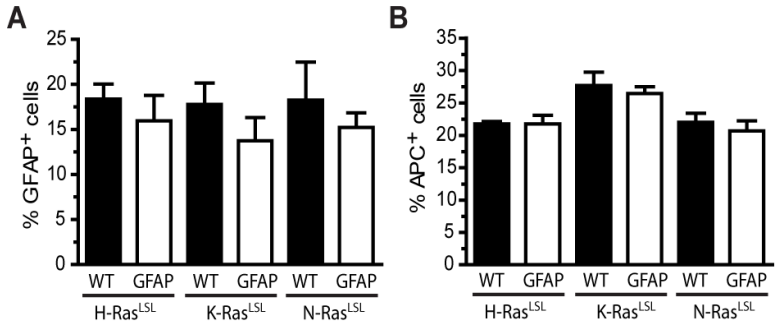



**Figure 2.2. Ras activation in E14.5 NSCs does not alter glial cell formation *in vivo.*** At PN18, *H-Ras*$^{GFAP}$, *K-Ras*$^{GFAP}$, and *N-Ras*$^{GFAP}$ mice do not harbor any differences in the percentage of brainstem (**A**) GFAP$^{+}$ astrocytes and (**B**) APC$^{+}$ oligodendrocytes relative to littermate controls. Error bars represent the standard errors of the mean.

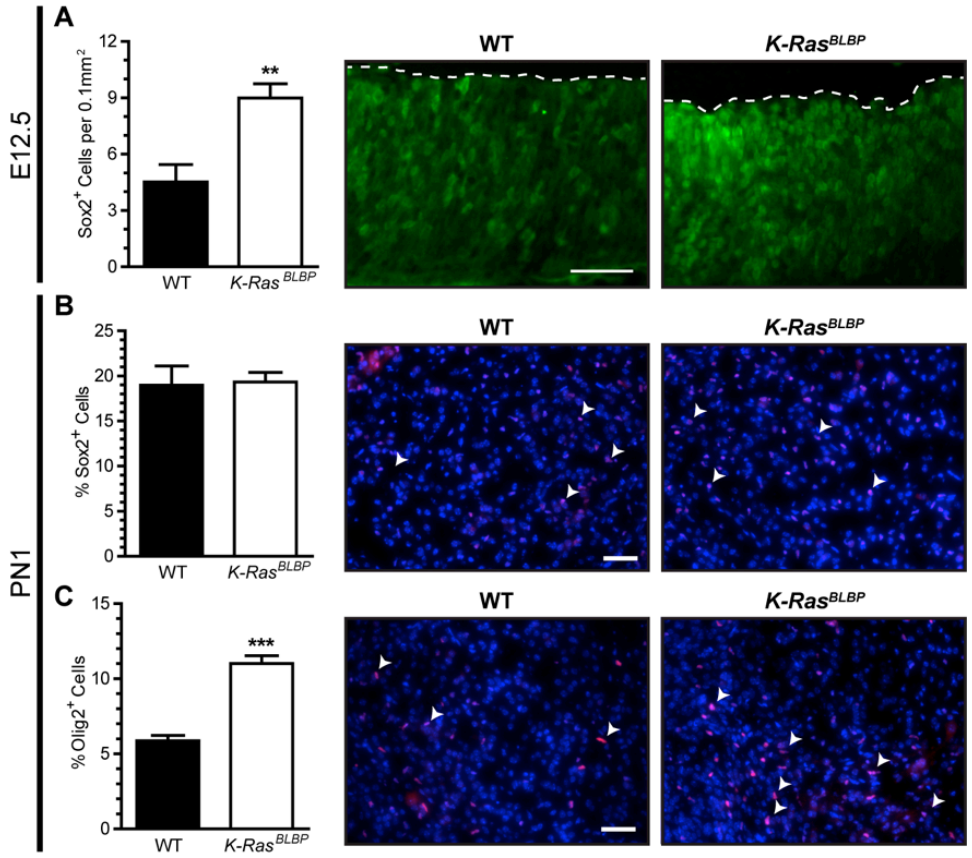
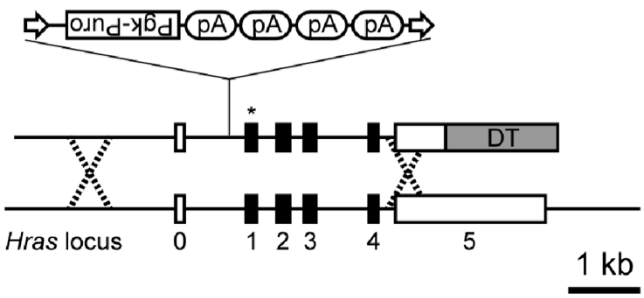
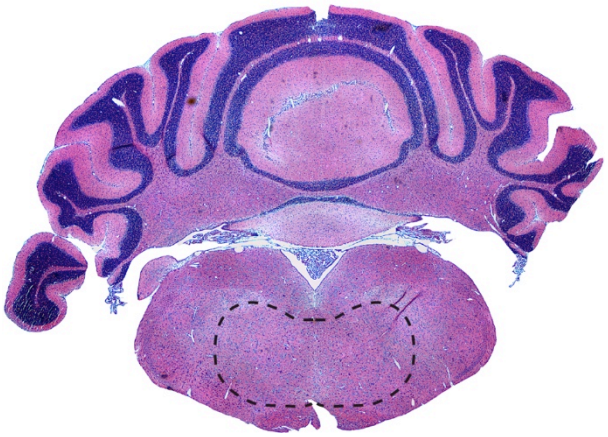


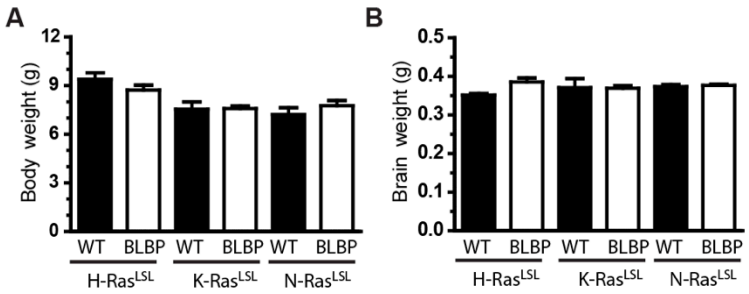

**Figure 2.3. *K-Ras*$^{BLBP}$ mice have more neuroglial progenitors during early brain development.** (**A**) At embryonic day 12.5 (E12.5), *K-Ras*$^{BLBP}$ mice have 2–fold more Sox2$^{+}$ NSCs per 0.1mm$^{2}$ in the developing hindbrain, the future site of the brainstem pons, compared to littermate controls (**p<0.01). Representative images appear to the right with Sox2$^{+}$ cells in green and the dashed line demarking the ventricular surface (20x, scale bar=50μm). (**B**) At postnatal day 1 (PN1), there is no difference in the percentage of Sox2$^{+}$ NSCs in the brainstem, however, (**C**) *K-Ras*$^{BLBP}$ mice have 1.9–fold more Olig2$^{+}$ glial progenitor cells compared to littermate controls (***p<0.001). Representative images appear to the right of their respective graphs with arrowheads identifying Sox2$^{+}$ or Olig2$^{+}$ nuclei (red), respectively. DAPI (blue) marks total cells (20x, scale bar=50μm).



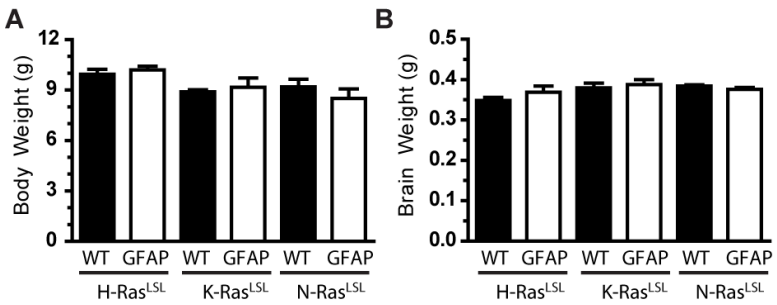

**Supplemental Figure S2.1. Targeting strategy for the *H-Ras*$^{LSL-G12V}$ mouse.** Schematic representation of *H-Ras*$^{LSL-G12V}$ mice at the endogenous H-Ras locus in murine embryonic stem cells. A transcriptional stop site flanked by LoxP sites (lox-stop-lox [LSL]) was inserted upstream of a G12V activating point mutation (*) in the first coding exon of H-Ras.



**Supplemental Figure S2.2. Imaging the postnatal brainstem pons.** Six - 0.1mm$^2$ images were acquired per postnatal brain within the reticular nucleus (dashed outline) of the brainstem**.**




**Supplemental Figure S2.3. Ras activation in BLBP-Cre-expressing NSCs does not alter body or brain weight.** Ras activation does not significantly change (**A**) body or (**B**) brain weight of *H-Ras*$^{BLBP}$, *K-Ras*$^{BLBP}$, or *N-Ras*$^{BLBP}$ mice relative to littermate controls.




**Supplemental Figure S2.4. Ras activation in GFAP-Cre-expressing NSCs does not alter body or brain weight.** Ras activation does not significantly change (**A**) body or (**B**) brain weight of *H-Ras*$^{GFAP}$, *K-Ras*$^{GFAP}$, or *N-Ras*$^{GFAP}$ mice relative to littermate controls.

**Table S2.1. Antibodies for immunohistochemistry (IHC) and immunofluorescence (IF).**

| Antibody | Species | Application | Antigen Retrieval | Source | Dilution |
|---|---|---|---|---|---|
| APC | mouse | IHC | 10mM Sodium Citrate | Millipore, Billerica, MA | 1:50 |
| GFAP | mouse | IHC | 10mM Sodium Citrate | Millipore | 1:500 |
| NeuN | mouse | IHC | 10mM Sodium Citrate | Millipore | 1:250 |
| Olig2 | rabbit | IF | --- | Millipore | 1:500 |
| S100β | rabbit | IHC | 10mM sodium citrate + 1mM EDTA | Sigma-Aldrich, St. Louis, MO | 1:1,000 |
| Sox2 | rabbit | IF | --- | Millipore | 1:1,000 |
| Sox2 | mouse | IF | --- | Abcam | 1:1,000 |

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# CHAPTER 3

# RAS ACTIVATION IN NEURAL STEM CELLS *IN VITRO*

## INTRODUCTION

Neural stem cells (NSCs) represent a self-renewing population of cells found in the neurogenic regions of the embryonic and adult brain [1, 2]. In addition to forming new NSCs (symmetric division, self-renewal, and proliferation), these stem cells can also give rise to all the major cell types in the brain, including neurons, oligodendrocytes, and astrocytes, through multi-lineage differentiation (asymmetric division) [3-6]. Each of these cell fate decisions (self-renewal versus differentiation) requires the activation of signaling pathways and transcriptional programs [3, 7-11]. In this regard, previous studies have identified numerous potential regulatory mechanisms involving p53, bmi1, sonic hedgehog (SHH), Notch, p27, REST/NSRF, epidermal growth factor receptor, and others [12-23]. While many of these signaling molecules may be responsible for NSC growth and differentiation, it is not clear which of the many effectors are important for the pathogenesis of diseases characterized by abnormal brain NSC function.

A number of neurodevelopmental disorders, including neurofibromatosis type 1 (NF1), Costello syndrome, and Noonan syndrome, are caused by germline mutations in genes that lead to hyperactivation of the Ras proto-oncogene [24-27]. Small GTPase proteins, like Ras, act as molecular switches by alternating between active, GTP-bound and inactive, GDP-bound states [28]. In each of the above neurogenetic conditions, there is more Ras in the active GTP-bound form, leading to increased Ras pathway signaling. The critical importance of Ras to brain development is underscored by mouse genetic knockout studies, in which de-regulated Ras signaling is associated with brain developmental defects or embryonic lethality [29-33].

Ras exists as three separate molecules (H-Ras, K-Ras, and N-Ras) in the mammalian brain. Despite 85% similarity in amino acid sequence across all of the Ras molecules, Ras molecule-specific function is dictated by unique hypervariable regions (HVRs) encoded by distinct C-terminal 25-amino acid sequences. While the HVR of all Ras molecules contains a CAAX-box motif which is isoprenylated to allow for proper targeting of Ras to the plasma membrane (PM) [34], differential palmitoylation directs their trafficking to the PM via the Golgi (H-Ras, N-Ras) or another, unknown mechanism (K-Ras) [35, 36]. This differential processing directs the different Ras molecules to distinct domains within the PM [37], and is thought to underlie their differential capacities to signal to downstream effector proteins in different cellular contexts [34, 38, 39].

Based on these potential differences, we hypothesized that H-Ras, K-Ras, and N-Ras may differentially regulate brain NSC growth and differentiation. Using *in vitro* approaches, we demonstrate that hyperactivation of K-Ras, but not H-Ras or N-Ras, increases brain NSC proliferation without altering multi-lineage differentiation. Moreover, we report that K-Ras controls brain NSC growth in a Raf-dependent, but Mek-independent manner, through binding and inhibition of retinoblastoma protein (Rb) function. Collectively, these results demonstrate that the Ras molecules have distinct biological effects on brain NSC function.

## MATERIALS AND METHODS

***Mice.*** NSCs for *in vitro* culture were collected from the brainstem of mice containing *Lox-Stop-Lox (LSL)-H-Ras*$^{G12V}$ (Supplemental Figure S2.1; generated by Dr. Kevin Haigis), *LSL-K-Ras*$^{G12D}$ [40], or *LSL-N-Ras*$^{G12D}$ [41] constructs knocked into the respective *Ras* locus. All mice were maintained on a C57Bl/6 background in accordance with approved animal studies protocols at Washington University.

***Neural Stem Cell (NSC) Culture.*** NSCs were generated from brainstems of PN1 mice as previously reported [42]. Briefly, dissociated brainstem tissue was cultured for five days in NSC-selective media supplemented with 1% N2 (Life Technologies, Grand Island NY), 2% B27 (Life Technologies), 20ng/mL fibroblast growth factor (FGF) (Sigma-Aldrich, St. Louis, MO) and 20ng/mL epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN) in ultra-low attachment dishes (Corning, Corning, NY). The expression of activated H-Ras, K-Ras, or N-Ras molecules was induced in NSCs following Ad5-Cre (University of Iowa Gene Transfer Vector Core, Iowa City, IA) infection. Ad5-LacZ-infected NSCs were used as controls. Ras activity was quantified using a Raf1-Ras binding domain (RBD) affinity chromatography assay kit (Millipore, Billerica, MA). NSC growth was assessed by direct cell counting following the seeding of 5,000 NSCs (in triplicate) in 24-well ultra-low attachment dishes containing NSC media (Corning). Cells were typsinized and counted using a hemocytometer after five days in culture. For all NSC growth and differentiation assays, experiments were performed on NSCs maintained in culture for fewer than three passages.

***Immunofluorescence.*** Neurospheres were collected after five days in culture, washed twice in PBS, and fixed for 30 minutes in 4% paraformaldehyde. Following fixation, neurospheres were dehydrated in 30% sucrose for at least 24 hours prior to embedding in OCT mounting media (Sakura Finetek, Torrance, CA) and freezing at -80°C. All neurospheres were sectioned on a Reichert-Jung Cryocut 1800 (Reichert Technologies, Depew, NY) cryostat into 10μm-thick sections. Primary antibodies (Supplemental Table S3.1) were applied overnight at 4°C followed by incubation with species-appropriate AlexaFluor® 488 or 568 secondary antibodies (Life Technologies) and counterstained with DAPI. At least eight neurospheres of similar diameter and cell number were imaged per sample using a Nikon Eclipse TE300 inverted

fluorescent microscope with a Photometrics CoolSnap EZ camera (Photometrics, Tucson, AZ) and compared to LacZ-infected control cells.

***Immunocytochemistry.*** Differentiation was assessed using methods previously published [17]. Briefly, 150,000 NSCs were plated in triplicate and allowed to adhere onto 24-well plates coated with 50µg/mL poly-D-lysine (Sigma-Aldrich) and 10µg/mL fibronectin (Life Technologies) in NSC media supplemented with 1% N2, 2% B27, and 1% fetal bovine serum. After six days in culture, adherent cells were fixed for 15 minutes in 4% paraformaldehyde and stained using primary antibodies listed in Supplemental Table S1. Cells were incubated with species-appropriate AlexaFluor® 488 or 568 secondary antibodies (Life Technologies) and counterstained with DAPI. For the EdU studies, 20µM 5-ethynyl-2'-deoxyuridine (EdU; Life Technologies) was added to the culture media 3 hours prior to fixation. EdU was detected using the Click-IT® EdU Assay Kit (Life Technologies) according to manufacturer's protocol prior to immunocytochemistry staining. Each experiment was repeated a minimum of three times using NSCs harvested from three independently-generated litters.

***Immunoprecipitation.*** Raf-1 immunoprecipitation was performed on whole cell lysates in a binding buffer containing 20mM HEPES (pH 7.9), 40mM KCl, 1mM $MgCl_2$, 0.1mM EGTA, 0.1mM EDTA, 0.1mM dithiothreitol, 0.1 NaF, 0.1mM $Na_3VO_4$, 0.5% IGEPAL, and 3mg/mL BSA. Total protein (500µg) in 400µl of binding buffer was pre-cleared with 30µl agarose protein G beads (Cell Signaling) for 2 hours at 4°C on a rotor. After removing the beads, lysates were incubated with Raf-1 monoclonal antibodies (Millipore) overnight at 4°C on a rotor. Agarose beads were added to these lysates, and incubated for 2 hours on a rotor at 4°C. The beads were washed three times in 500µl binding buffer, boiled in 20µl 4x Laemmli buffer, and separated on an 8% polyacrylamide gel for Western blotting. Raf-1 pull-down was confirmed by blotting with a different Raf-1 antibody (Cell Signaling). Each experiment was repeated a minimum of three times using NSCs harvested from three independently-generated litters.

***Flow Cytometry Analysis.*** Neurospheres were trypsinized and then fixed overnight in 50% ethanol in PBS containing 0.02% IGEPAL (Sigma-Aldrich). Following fixation, cells were washed three times in PBS

prior to trypsinization ((0.003% trypsin type-II (Sigma-Aldrich) in DNA analysis solution (0.1% IGEPAL, 0.05% spermine tetrahydrochloride (Sigma-Aldrich) in 3.9mM sodium citrate buffer)) for 10 minutes at room temperature. Cells were subsequently incubated for 10 minutes at room temperature in trypsinization inhibitor solution (0.05% trypsin inhibitor type-O (Sigma-Aldrich), 0.001% RNase A (Sigma-Aldrich) in DNA analysis solution) prior to staining (0.02% propidium iodide (Sigma-Aldrich), 0.015% spermine tetrachloride in DNA analysis solution) for 10 minutes at room temperature.

Apoptosis was measured using trypsinized neurospheres. Live cells were stained for annexin V and propidium iodide using an Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, MA) according to manufacturer's protocol. All samples were quantitated on a Beckton Dickinson FACScan machine (Beckton Dickinson, Franklin Lakes, NJ) using FlowJo acquisition and analysis software (TreeStar Inc., Ashland, OR).

***Western Blotting.*** Western blotting was performed as previously described [43] with appropriate primary antibodies (Supplemental Table S3.2). Detection was achieved using species-appropriate HRP-linked secondary antibodies (Cell Signaling, Danvers, MA) and enhanced chemiluminescence imaging (BioRad) using the ChemiDoc-It Imaging System (UVP, Upland, CA).

***Pharmacological Inhibition Studies.*** Optimal drug concentrations for all inhibitor studies were experimentally determined by dose escalation (data not shown). Following optimization, NSCs were treated with either 6.5μM GGTI-286 (Millipore), 200nM AZ628 (Selleck Chemicals, Houston TX), 2nM PD0325901 (Selleck Chemicals), 50nM AZD0530 (Selleck Chemicals), 20μM SB203580 (Selleck Chemicals), or 5 μM RRD-251 (Sigma-Aldrich) for five days in culture. Control cells were treated with DMSO.

***Data Analysis.*** Tissue and cell staining images were quantitated using ImageJ image analysis software (U.S. National Institutes of Health). The Grubbs outlier test was used to determine statistical outliers and statistical significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$) was determined using an unpaired, two-tailed Student's t-test and GraphPad Prism 5 software (GraphPad Software, La Jolla CA).

## RESULTS

***K-Ras activation does not alter NSC astrocyte differentiation.*** Based on the observed increase in astrogliogenesis *in vivo* described in Chapter 2, we employed neurosphere cultures generated from the brainstems of PN1 pups infected with adenovirus expressing either LacZ (control cells, CTL) or Cre recombinase (activated H-Ras, K-Ras, or N-Ras, denoted H-Ras*, K-Ras*, and N-Ras*, respectively) to determine whether K-Ras activation results in a change in brain NSC astrocyte differentiation. Active Ras-GTP pull-down by Raf1-RBD affinity chromatography confirmed that each of the Ras molecules was comparably activated following Ad5-Cre infection (Figure 3.1A). Upon *in vitro* differentiation of these NSCs over six days in adherent culture, only K-Ras*-expressing NSCs exhibited increased numbers of $GFAP^+$ astrocytes (1.8-fold) relative to controls (Figure 3.1B). Importantly, K-Ras*-expressing NSC cultures also showed an increase in the total number of $DAPI^+$ cells (1.8-fold) compared to control cells (Figure 3.1C). The increase in $GFAP^+$ cells proportional to total $DAPI^+$ cells resulted in no change in the percentage of $GFAP^+$ cells compared to controls (Figure 3.1D), indicating that K-Ras activation does not directly change NSC astrocyte differentiation.

Since we observed no change in astrocyte differentiation, we next sought to determine whether the increase in astrocyte numbers following K-Ras* expression reflected an increase in the NSC pool during differentiation. This was assayed by EdU incorporation in the six-day differentiation assay, observing differentiating cells at both early (one day post-plating) and late (five days post-plating) time points in the assay (Figure 3.2A). In addition to EdU incorporation, markers for NSCs (Sox2) and astrocytes (GFAP) were used to measure changes in both populations throughout the differentiation assay. After one day in culture, K-Ras*-expressing cells were proliferating 7.4-fold more than their control counterparts (Figure 3.2B). These proliferating cells were exclusively $Sox2^+$ NSCs, and not $GFAP^+$ astrocytes (Figure 3.2C). At this time point, there was no difference in the percentage of control or K-Ras* cells positive for Sox2, however, a significantly smaller percentage of K-Ras*-expressing cells were $GFAP^+$ compared to control cells (Figure 3.2D). After five days in culture, the K-Ras*-expressing cells were still proliferating 2.2-fold more than controls (Figure 3.2E), however, there was no difference in proliferating NSCs or astrocytes (Figure 3.2F). Importantly, a significantly higher percentage (1.6-fold) of K-Ras*-expressing cells were $Sox2^+$ NSCs relative to control cells, while there was no difference in the

percentage of $GFAP^+$ astrocytes in either K-Ras* or control cell populations (Figure 3.2G). Taken together, these data indicate that K-Ras activation increases the NSC pool, rather than driving NSCs towards astrocyte differentiation.

***K-Ras activation increases NSC proliferation in vitro.*** To determine whether the individual Ras* molecules differentially increase NSC growth *in vitro*, we directly counted NSCs after five days in culture. Similar to the previous *in vitro* data, K-Ras*-, but not H-Ras*- or N-Ras*-, expressing NSCs grew 2.3-fold more than controls (Figure 3.3A). Immunofluorescence staining of sectioned neurospheres after five days in neurosphere culture confirmed that K-Ras*-, but not H-Ras*- or N-Ras*-, expressing neurospheres have a 1.6-fold increase in the percentage of $Ki67^+$ proliferating cells (Figure 3.3B). To determine whether increased K-Ras* NSC growth results from increased proliferation or decreased cell death, we utilized flow cytometry to analyze both potential etiologies in the same NSC samples. Using propidium iodide to quantitatively stain cellular DNA content, fewer K-Ras*-expressing NSCs were found in the pre-cycling $G_0/G_1$ phase (-4.6%) of the cell cycle relative to controls, while more K-Ras*-expressing NSCs were found in S phase (+3.6%) and $G_2$/M phase (+2.6%) (Figure 3.3C, 3.3D). Using annexinV staining to identify cells undergoing programmed cell death (apoptosis), we found no difference between live and apoptotic K-Ras*-expressing and control NSCs (Figure 3.3E, 3.3F). These data demonstrate that K-Ras activation enhances NSC growth by increasing proliferation rather than by decreasing apoptosis. Together, these *in vitro* assays are consistent with a model that K-Ras hyperactivation promotes an approximately 1.7-fold increase in NSC proliferation, expanding the NSC pool, and resulting in a proportional increase (1.8-fold) in astrocyte formation upon differentiation (Figure 3.3G).

***K-Ras increases NSC proliferation in a Raf-dependent, but Mek-independent, manner.*** To define the signaling pathway responsible for activated K-Ras control of NSC proliferation, we performed a series of pharmacological inhibitor studies. First, we confirmed that K-Ras activation is required for K-Ras*-induced NSC hyperproliferation using the GGTI-286 geranylgeranyltransferase inhibitor. Following GGTI-286 (6.5μM) treatment, the hyperproliferation of K-Ras* NSCs was reduced to that of controls, as measured by direct cell counting (Figure 3.4A). Second, following inhibition with the pan-Raf inhibitor AZ628

(200nM), K-Ras*-induced NSC hyperproliferation was also attenuated to that of control cells (Figure 3.4B). Consistent with Ras- and Raf-mediated hyperactivation, increased Erk activation was observed in K-Ras*-expressing NSCs (Figure 3.4C). Third, to determine whether Raf/Mek signaling was responsible for K-Ras*-induced NSC hyperproliferation, K-Ras*-expressing NSCs were treated with the PD0325901 (PD901; 2nM) Mek inhibitor. Despite reduction of Erk activity to control cell levels, PD901 treatment did not reduce K-Ras*-mediated NSC hyperactivation to control cell levels (Figure 3.4D).

This unexpected result prompted an examination of potential Raf effector proteins. While we observed no change in Akt, Jnk, PKC-γ, PKC-ζ, Ran, β-catenin, and Yap activity, Src and p38MAPK were hyperactivated (increased $Src^{Y416}$ and $p38MAPK^{T180/Y182}$ phosphorylation, respectively) in K-Ras*-expressing NSCs compared to controls (Supplemental Figure S3.1A, S3.1B). However, pharmacologic inhibition of Src (50nM AZD0530) or p38MAPK (20μM SB203580) activity did not reduce K-Ras*-induced NSC hyperproliferation (Supplemental Figure S3.1C, S3.1D). Similarly, neither GGTI-286 (Ras inhibitor) nor AZ628 (Raf inhibitor) treatment reduced Src and p38MAPK hyperactivation (Supplemental Figure S3.1E). Collectively, these data demonstrate that K-Ras* expression increases NSC proliferation in a Raf-dependent, but Mek-, Src-, and p38MAPK-independent, manner.

***Raf-1 inhibition of Rb is responsible for K-Ras-induced NSC proliferation.*** To determine how K-Ras* expression regulates NSC proliferation, we focused on potential Raf-dependent mechanisms. Previous studies have shown that Raf-1 (c-Raf) directly binds the retinoblastoma tumor suppressor protein (Rb) to relieve Rb-mediated suppression of cell cycle progression [44]. We found that endogenous Rb bound to Raf-1 in K-Ras*-expressing NSCs as assessed by Raf-1 immunoprecipitation (Figure 3.5A). Based on these findings, we employed a small peptide inhibitor, RRD-251, previously shown to disrupt the Raf-1/Rb interaction [45, 46], to demonstrate that this interaction is required for K-Ras*-mediated NSC hyperproliferation. Treatment of K-Ras*-expressing NSCs with RRD-251 (5 μM) reduced K-Ras*-induced NSC hyperproliferation to control levels (Figure 3.5B). Together, these data establish a new model for K-Ras* regulation of NSC proliferation, in which K-Ras-GTP activation of Raf leads to Rb binding and inhibition, and leads to increased $G_0/G_1$-S transition and proliferation (Figure 3.5C).

**DISCUSSION**

Despite the central role of Ras in regulating embryonic development, the three Ras molecules do not equally impact on this process. For example, bi-allelic *K-Ras* deletion in mice results in lethality between 12 and 14 days of gestation, resulting from brainstem defects, hematopoietic abnormalities, and organ failure [29, 30]. In striking contrast, *H-Ras* or *N-Ras* deletion, either separately or in combination, does not interfere with normal embryonic development and results in healthy adult mice, indistinguishable from wild-type littermates [47, 48]. These differential effects are further highlighted by observations made in human neurogenetic disorders characterized by germline mutations in genes whose protein products regulate Ras activity (collectively termed "RASopathies"). In these RASopathies, which include Costello syndrome, Noonan syndrome, and neurofibromatosis type 1 (NF1), Ras activation is abnormally elevated. However, the causative RAS mutations are different: In Costello syndrome, *H-RAS* mutations [26] have been reported, whereas Noonan syndrome patients harbor *K-RAS* mutations [27], each with varying neurocognitive delays and learning disabilities. Similarly, in NF1, loss of *Nf1* gene expression in mouse brain NSCs from the third ventricle, lateral ventricle, and brainstem all lead to Ras hyperactivation [17, 42, 49]. While it is not known which Ras molecules are hyperactivated in these *Nf1*-deficient NSCs, only K-Ras activity is increased following *Nf1* loss in astrocytes [50]. This differential Ras activation is underscored by the finding of oncogenic *K-RAS*, but not *H-RAS* or *N-RAS*, mutations in pediatric brain tumors [51, 52], and prompted us to investigate the impact of each individual Ras molecule on brain NSC function. In this chapter, we make several important observations.

Herein, we demonstrate that activated K-Ras, but not H-Ras or N-Ras, increases the proliferation of brain NSCs. While no prior studies have systematically compared the three Ras molecules in the brain, previous work in other tissue types revealed divergent functions of these Ras molecules. In neural crest-derived hematopoietic cells, activated N-Ras is the primary regulator of cell self-renewal, proliferation, and cell fate decisions [53-56], whereas activated K-Ras primarily drives cell growth in tissues arising from the endoderm (lungs, colon) [40, 57]. Similarly, in endoderm-derived stem cells, overexpression of active *H-Ras*$^{G12V}$ promotes cell differentiation and suppresses cell growth, in contrast to *K-Ras*$^{G12V}$ overexpression that conversely promotes stem cell proliferation and inhibits cell differentiation [58]. Moreover, *K-Ras*$^{G12D}$ expression increases the proliferation of an intestinal stem cell population, whereas

*N-Ras*$^{G12D}$ expression increases the resistance of these cells to apoptosis without changing cell proliferation [41]. Lastly, *K-Ras*$^{G12D}$ expression has also been reported to cause bronchioalveolar stem cell hyperproliferation in the lung [57]. Taken together, these studies identify K-Ras* as a potent driver of progenitor cell proliferation, which is consistent with our finding that only K-Ras activation promotes brain NSC proliferation.

Given the high level of sequence similarity between the three Ras molecules and their capacity to signal to a similar subset of downstream effector proteins, it seems counterintuitive that these molecules should have such divergent functions. However, these differences are conferred by unique 25-amino acid, C-terminal HVRs. During post-translational processing, the Ras molecules are first prenylated by farnesyltransferase, which allows for trafficking to the endoplasmic reticulum [59, 60]. After this step, Ras molecule post-translational processing diverges: K-Ras becomes more highly methylated than its H-Ras and N-Ras counterparts [35]. H-Ras and N-Ras are then palmitoylated and trafficked to the plasma membrane through the Golgi apparatus while methylated K-Ras is trafficked to the plasma membrane independent of the Golgi [36, 61]. While the exact mechanism of K-Ras trafficking is unknown, its transport is dependent on a polybasic, lysine-rich portion of the K-Ras HVR not found within the HVRs of H-Ras or N-Ras [62]. Following its localization at the plasma membrane, each Ras molecule occupies a different microdomain: N-Ras is found in lipid raft domains, while K-Ras localizes to non-lipid raft portions of the membrane [37, 63]. H-Ras occupies lipid raft domains when inactive, but translocates to non-lipid raft membrane upon activation (in areas distinct from those occupied by K-Ras) [37, 64]. In addition, H-Ras and N-Ras are also capable of signaling to downstream effectors from the Golgi, a capability not shared by K-Ras [65, 66]. Collectively, the divergent processing and localization of the Ras molecules likely account for their capacities to signal to different subsets of downstream proteins to generate different biological outcomes [38, 39].

Ras/Raf signal transduction most commonly operates through Raf activation of Mek1/2 [67-69]. In striking contrast to this canonical signaling mechanism, we demonstrate that K-Ras* regulates brain NSC proliferation in a Raf-dependent manner that operates independent of Mek. Precedent for Mek-independent Ras/Raf growth control derives from several studies performed in numerous different cell types. First, Raf-1 overexpression has been shown to increase cell survival by inhibiting ASK-1 function

in the presence of the Mek pharmacological inhibitors PD98059 and U0126 [70]. Second, K-Ras*-expressing DLD-1 cells continue to proliferate despite treatment with the Mek inhibitor CI-1040. Proliferation of these cells was only inhibited in the presence of the Raf inhibitor AZ628 [41]. Third, protein binding discovery studies have revealed several other potential Raf binding partners, including the 14-3-3-zeta protein, which modulates Raf signaling during cell stress through its association with keratin 8/18 [71, 72]. Fourth, both B-Raf and Raf-1 associate with PKC-theta to inactivate the pro-apoptotic Bcl-2 family protein BAD and increase cell survival [73]. While these studies collectively identify a role for Raf in cell growth, we found that K-Ras-dependent brain NSC hyperproliferation depends on the productive interaction between Raf-1 and the retinoblastoma (Rb) cell cycle regulator [44].

The Rb tumor suppressor protein regulates $G_0/G_1$ to S phase cell cycle progression. In quiescent cells, Rb prevents cell cycle progression by inhibiting the expression of the E2F transcription factors to prevent DNA synthesis. Upon growth factor stimulation, this inhibition is relieved, allowing cells to progress through the cell cycle. Using genetic and biochemical techniques to alter Ras expression in the presence or absence of Rb expression, early work established a hierarchy for Ras/Rb/cell cycle regulation, whereby Ras inhibition of Rb function led to increased cell cycle progression (proliferation) [74, 75]. The mechanism underlying this Ras/Rb connection was identified when direct binding between Raf-1 and Rb was revealed [44]. Interrupting this interaction with the RRD-251 small peptide has been shown to block tumor cell growth in numerous model systems [44-46, 76-78]. Consistent with these findings, we demonstrate that K-Ras*-induced brain NSC hyperproliferation is dependent on Raf/Rb binding and inhibition of Rb function.

Taken together, using a novel collection of conditional Ras GEM strains and derivative brain NSCs, we establish differential effects of the three Ras molecules on brain NSC proliferation. Moreover, the elucidation of a non-conventional mechanism underlying Ras/Raf-mediated brain NSC growth control suggests additional determinants relevant to the study of diseases characterized by de-regulated RAS function. Future studies characterizing differences in Ras molecule function in disease-pertinent models will be critical to further defining and ultimately treating these disorders.

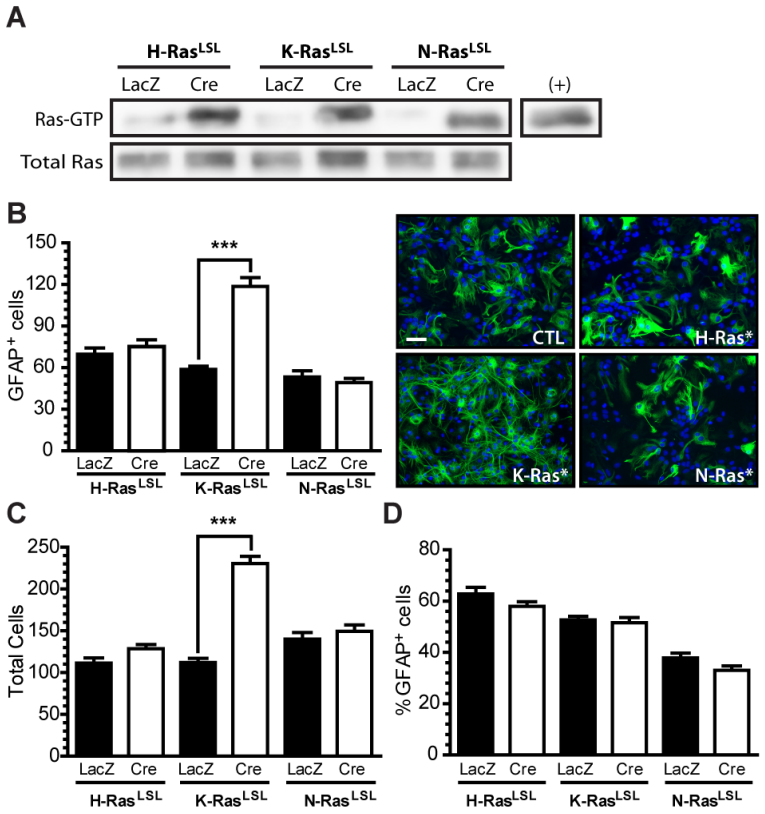



**Figure 3.1. K-Ras activation does not affect NSC multi-lineage differentiation.** (**A**) Ras activation (Ras-GTP) is increased in all three Ras* NSC populations after Cre expression. (**B**) K-Ras*-, but not H-Ras*- or N-Ras*-, expressing NSCs have a greater number of GFAP$^+$ astrocytes compared to controls (1.8-fold, ***p<0.001). Graphs of representative experiments are shown with representative images included (GFAP$^+$ (green) and DAPI$^+$ (blue), 20x, scale bar=50μm). (**C**) K-Ras*, but not H-Ras* or N-Ras*, -expressing NSCs also give rise to more total DAPI$^+$ cells compared to control cells (1.8-fold, ***p<0.001). (**D**) K-Ras*-, H-Ras*-, and N-Ras*-expressing NSCs do not have significantly different percentages of GFAP$^+$ astrocytes compared to controls. Error bars represent the standard errors of the mean.

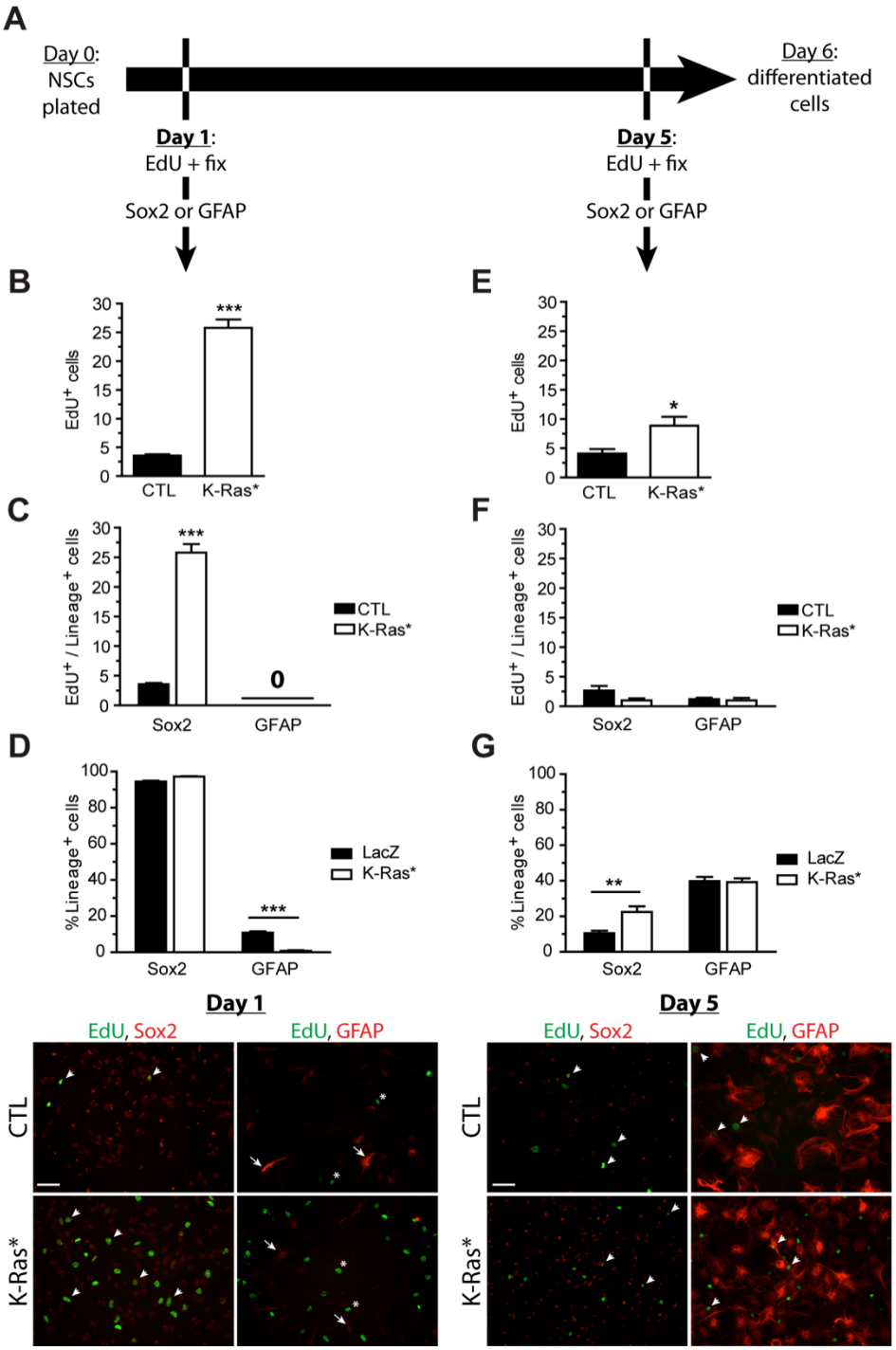



**Figure 3.2. K-Ras activation increases NSC proliferation during differentiation.** (**A**) EdU labeling at day 1 and day 5 post-plating was employed to determine whether $Sox2^+$ NSCs or $GFAP^+$ astrocytes proliferate during NSC differentiation. (**B**) K-Ras*-expressing cells proliferate 7.2-fold more than control cells at day 1 (***$p<0.001$). (**C**) These $EdU^+$ proliferating cells are exclusively $Sox2^+$ NSCs, and not $GFAP^+$ astrocytes. (**D**) At day 1, there is no significant difference in the percentage of $Sox2^+$ NSCs

between K-Ras* and control cell populations, however, there is a significant decrease in $GFAP^+$ astrocytes in K-Ras*-expressing cells relative to controls. (**E**) At day 5, K-Ras*-expressing cells proliferate 2.2-fold more than controls (*$p<0.05$). (**F**) There was no difference between proliferating NSCs ($Sox2^+$) and astrocytes ($GFAP^+$) in K-Ras*-expressing versus control cells. (**G**) At day 5, K-Ras*-expressing cells have a 1.6-fold increase in the percentage of $Sox2^+$ NSCs compared to control cells, while there is no difference in the percentage of $GFAP^+$ astrocytes between the two cell populations (**$p<0.01$). All graphs are representative experiments; error bars represent the standard errors of the mean. Representative images for (**D**) and (**G**) appear below their respective graphs (20x, scale bar=50μm). Arrowheads mark representative $EdU^+$/$Lineage^+$ double-positive cells, arrows mark cells positive for GFAP alone, and asterisks (*) mark cells positive for EdU alone.

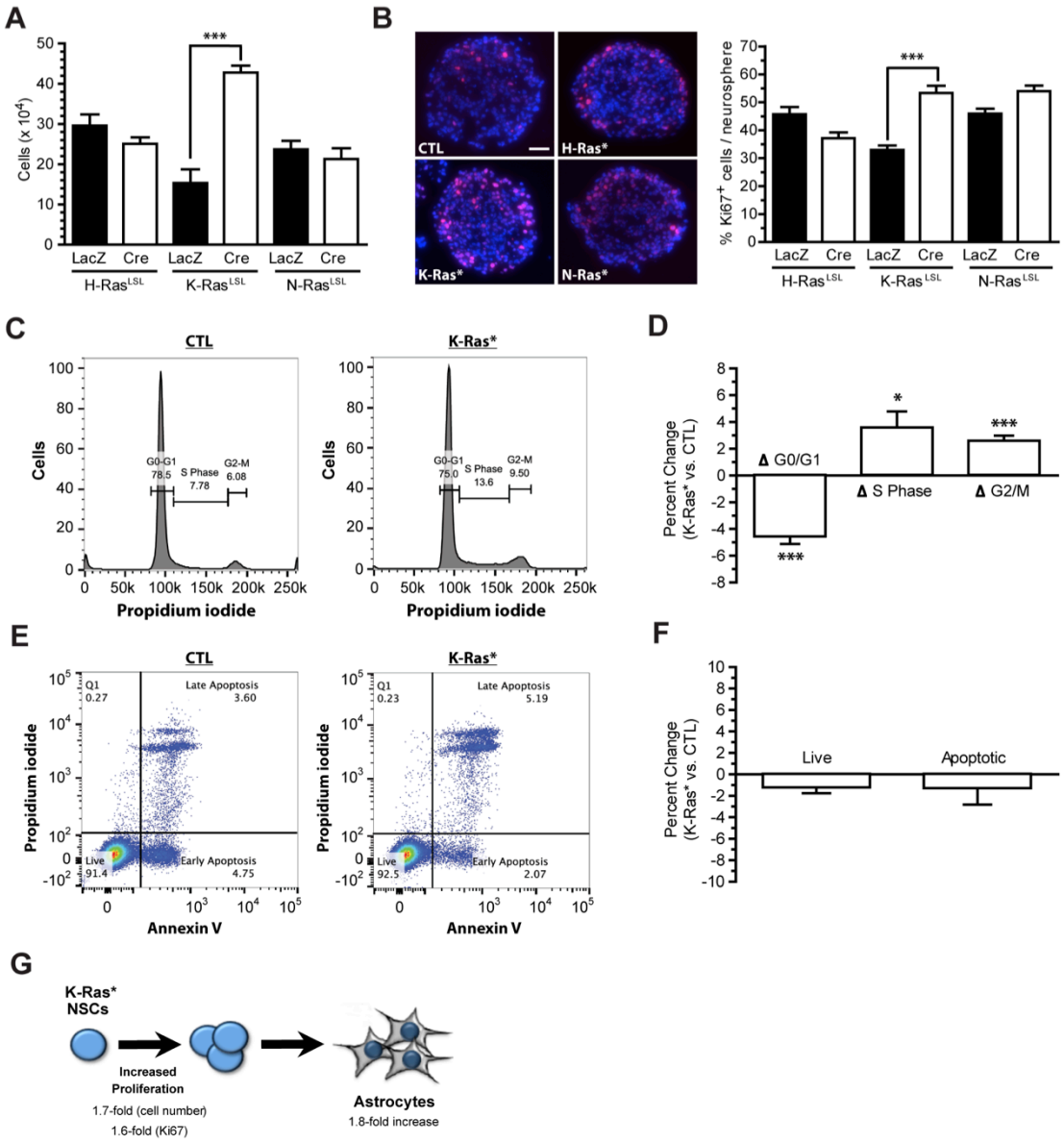



**Figure 3.3. K-Ras activation increases NSC proliferation *in vitro*.** (**A**) K-Ras*-, but not H-Ras*- or N-Ras*-, expressing NSCs have increased growth as measured by direct cell counting (2.3-fold, ***p<0.001). (**B**) K-Ras*-, but not H-Ras*- or N-Ras*-, expressing neurospheres have an increased percentage of Ki67$^{+}$ cells relative to controls (1.6-fold, ***p<0.001, 20x, scale bar=50μm). Graphs include representative experiments. (**C, D**) Flow cytometry of fixed propidium iodide (PI)-stained NSCs demonstrates an increased percentage of K-Ras*-expressing NSCs in S phase (+3.6%) and $G_2$/M phase (+2.6%) than in $G_0$/$G_1$ phase of the cell cycle (-4.6%) compared to control cells (*p<0.05. ***p<0.001). (**C**)

Shows one representative experiment, while (**D**) includes the data from all four experiments. (**E, F**) Flow cytometry of live NSCs shows no difference in the percentage of live (propidium iodide$^-$, annexin V$^-$) versus apoptotic (annexin V$^+$) cells. (**E**) Shows one representative experiment, while (**F**) includes the data from all four experiments. (**G**) Incorporating the data from all *in vitro* assays suggests a model in which K-Ras hyperactivation promotes NSC proliferation (1.7-fold) which leads to a proportional increase in astrocyte formation (1.8-fold).

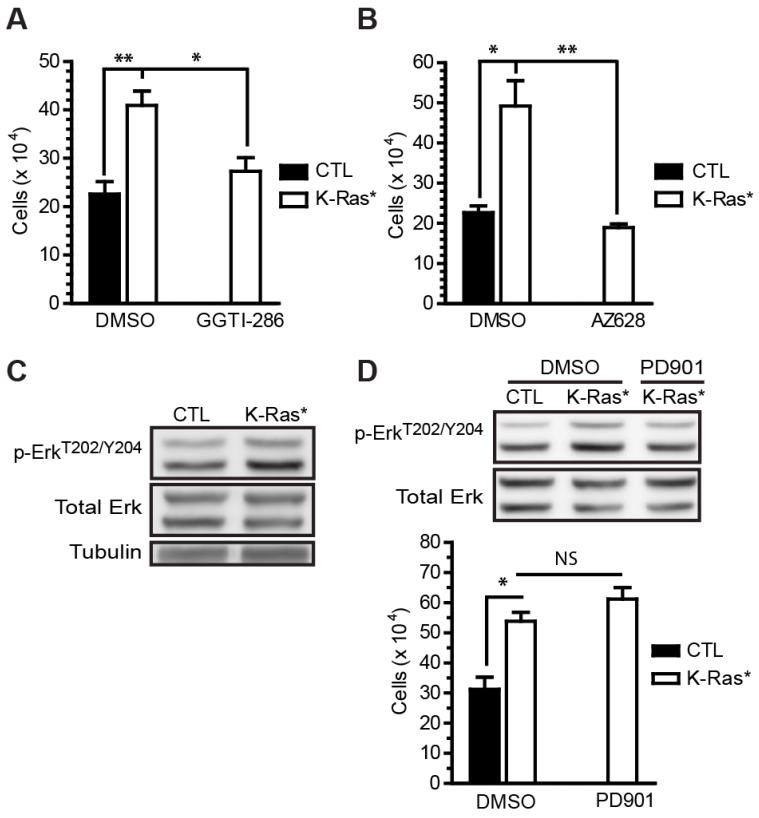



**Figure 3.4. K-Ras regulates NSC proliferation in a Raf-dependent, but Mek-independent, manner.** K-Ras*-induced NSC hyperproliferation is reduced to control levels following pharmacological inhibition of (**A**) Ras (GGTI-286, 6.5μM) (*p<0.05) and (**B**) Raf (AZ628, 200nM) (**p<0.01). (**C**) The downstream Ras effector, Erk1/2 (p-Erk$^{T202/Y204}$) is hyperactivated in K-Ras*-expressing NSCs relative to controls. (**D**) K-Ras*-induced NSC hyperproliferation following pharmacological inhibition with the Mek inhibitor PD0325901 is not reduced back to control cell levels (PD901, 2nM).

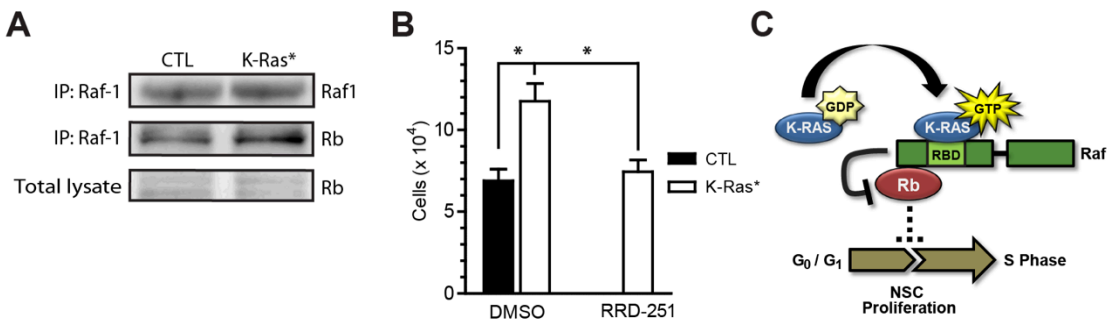
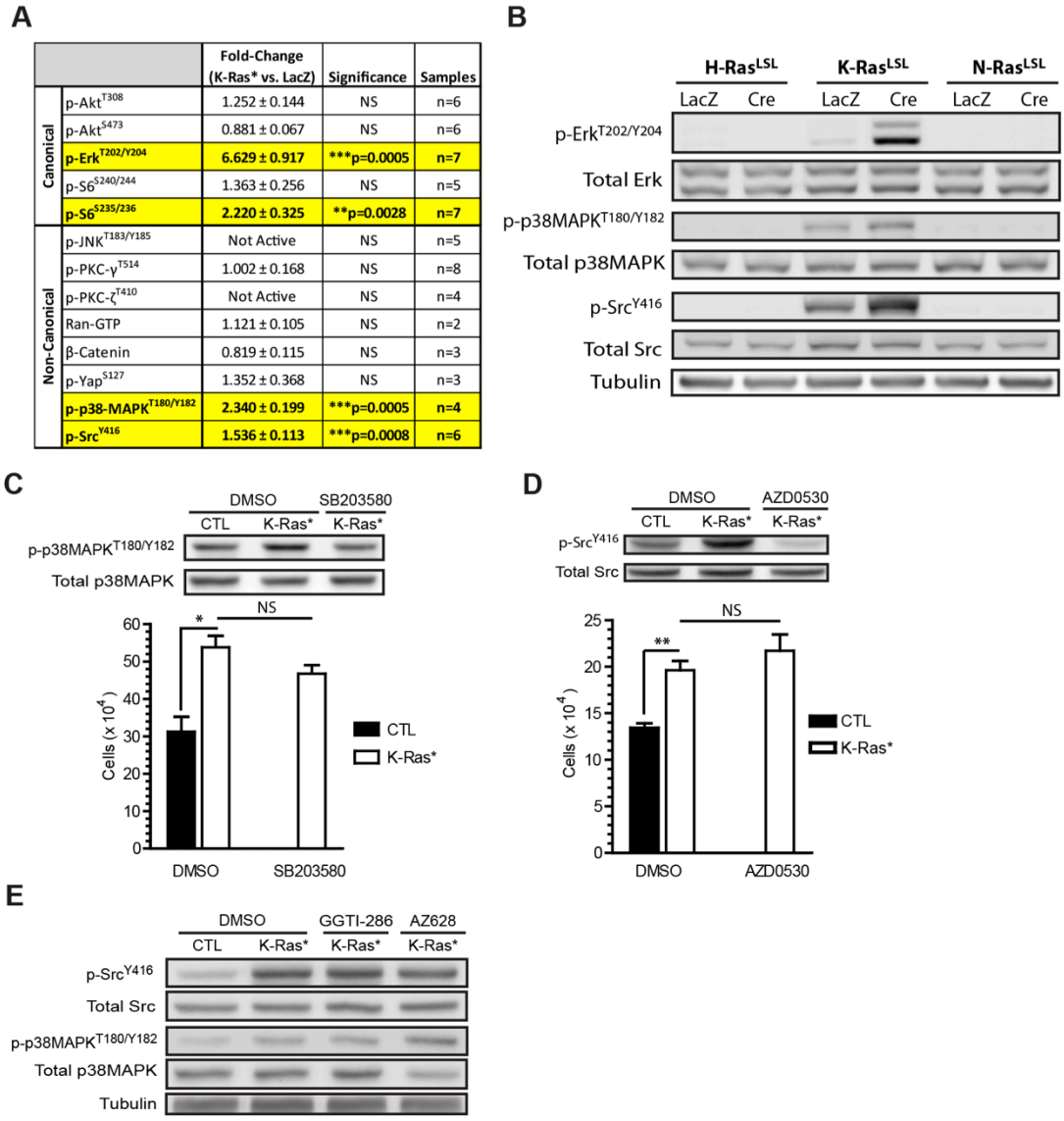



**Figure 3.5. K-Ras regulates NSC proliferation in a Raf- and Rb-dependent manner**. (**A**) Raf-1 immunoprecipitation reveals that Rb binds to Raf-1 in K-Ras*-expressing NSCs. (**B**) The small molecule inhibitor RRD-251 reduces K-Ras*-induced NSC hyperproliferation to control levels, as measured by direct cell counting (*$p<0.05$). **(C)** Proposed model for K-Ras*-mediated regulation of NSC proliferation. Active K-Ras-GTP activates Raf by binding to the Ras-binding domain (RBD). This leads to subsequent Rb binding to and inhibition of Rb activity, culminating in increased cell cycle progression (proliferation).




**Supplemental Figure S3.1. K-Ras*-, but not H-Ras*- or N-Ras*-, expressing NSCs exhibit increased Erk, p38MAPK, and Src activation.** (**A**) K-Ras activation in NSCs activates both canonical and non-canonical downstream effector proteins. (**B**) p-Erk$^{T202/Y204}$, p-p38MAPK$^{T180/Y182}$, and p-Src$^{Y416}$ expression are increased in K-Ras*, but not H-Ras* or N-Ras*, NSCs. However, pharmacologic inhibition of (**C**) Src (AZD0530, 50nM) and (**D**) p38MAPK (SB203580, 20μM) activity did not inhibit K-Ras*-mediated NSC hyperproliferation. (**E**) Western blotting of DMSO-, GGTI-286-, and AZ628-treated K-Ras*-expressing NSCs reveal no reduction in p-Src$^{Y416}$ and p-p38MAPK$^{T180/Y182}$ hyperactivation in K-Ras*-expressing NSCs.

**Table S3.1. Antibodies for immunocytochemistry (ICC), immunofluorescence (IF), and immunoprecipitation (IP).**

| Antibody | Species | Application | Source | Dilution |
|---|---|---|---|---|
| GFAP | mouse | ICC | Millipore, Billerica, MA | 1:1,000 |
| Ki67 | rabbit | ICC | Abcam, Cambridge, MA | 1:1,000 |
| Ki67 | mouse | IF | BD Biosciences, San Jose, CA | 1:500 |
| O4 | mouse | ICC | Millipore | 1:1,000 |
| Raf-1 | rabbit | IP | Millipore | 1:50 |
| Sox2 | rabbit | ICC | Millipore | 1:1,000 |

**Table S3.2. Antibodies for western blotting.**

| Antibody | Protein Size | Species | Source | Dilution |
|---|---|---|---|---|
| Akt | 60 kDa | rabbit | Cell Signaling, Danvers, MA | 1:5,000 |
| p-Akt$^{T308}$ | 60 kDa | rabbit | Cell Signaling | 1:500 |
| p-Akt$^{S473}$ | 60 kDa | rabbit | Cell Signaling | 1:5,000 |
| β-Catenin | 92 kDa | rabbit | Cell Signaling | 1:2,000 |
| Erk | 42 kDa | rabbit | Cell Signaling | 1:1,000 |
| p-Erk$^{T202/T204}$ | 42 kDa | rabbit | Cell Signaling | 1:1,000 |
| JNK | 46, 54 kDa | rabbit | Cell Signaling | 1:1,000 |
| p-JNK$^{T183/Y185}$ | 46, 54 kDa | rabbit | Cell Signaling | 1:1,000 |
| p38-MAPK | 43 kDA | rabbit | Cell Signaling | 1:1,000 |
| p-p38-MAPK$^{T180/Y182}$ | 43 kDA | rabbit | Cell Signaling | 1:500 |
| PKC-γ | 78 kDa | rabbit | Abcam, Cambridge, MA | 1:2,000 |
| p-PKC-γ$^{T514}$ | 78 kDa | rabbit | Abcam | 1:1,000 |
| PKC-ζ | 76 kDa | rabbit | Cell Signaling | 1:1,000 |
| PKC-ζ$^{T410}$ | 76 kDa | rabbit | Cell Signaling | 1:500 |
| Raf-1 | 65-75 kDa | rabbit | Cell Signaling | 1:500 |
| Ras | 21 kDa | mouse | Millipore, Billerica, MA | 1:2,000 |
| Rb | 110 kDa | mouse | Developmental Studies Hybridoma Bank, Iowa City, IA | 1:100 |
| S6 | 34 kDa | rabbit | Cell Signaling | 1:10,000 |
| p-S6$^{S240/244}$ | 34 kDa | rabbit | Cell Signaling | 1:5,000 |
| p-S6$^{S235/236}$ | 34 kDA | rabbit | Cell Signaling | 1:5,000 |
| Src | 60 kDA | rabbit | Cell Signaling | 1:2,000 |
| p-Src$^{Y416}$ | 60 kDA | rabbit | Cell Signaling | 1:500 |
| tubulin | 50 kDa | mouse | Sigma Aldrich, St. Louis, MO | 1:10,000 |
| Yap | 75 kDa | rabbit | Cell Signaling | 1:1,000 |
| p-Yap$^{S127}$ | 75 kDa | rabbit | Cell Signaling | 1:1,000 |

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# CHAPTER 4

# DISCUSSION AND FUTURE DIRECTIONS

Despite the significant similarity in their amino acid sequence, there is evidence that the three related Ras proteins examined here (H-Ras, K-Ras, N-Ras) function uniquely to alter cell growth and differentiation. In this thesis, I have established that constitutively-activated forms of these Ras proteins differentially impact on the function of NSCs when expressed in a biologically-relevant context from their endogenous promoters. First, I have demonstrated that only K-Ras, and not H-Ras or N-Ras, hyperactivation increases the proliferation of brainstem NSCs. Second, I have demonstrated that K-Ras hyperactivation does not alter the cell fate decisions of NSCs upon *in vitro* differentiation. Third, I determined that K-Ras*-mediated NSC hyperproliferation operates through a non-canonical downstream pathway that is dependent on the effector protein Raf and the cell cycle regulatory protein Rb. Taken together, these observations define differential functions and mechanisms of action of the Ras molecules in one important CNS cell type.

These findings complement and expand upon previous observations comparing the differential impact of Ras molecule activation *in vivo* and *in vitro*. Our laboratory has previously demonstrated that loss of *Nf1* gene expression in mature brain astrocytes leads to differential hyperactivation of the individual Ras molecules. In these studies, following *Nf1* gene inactivation in astrocytes, only the K-Ras molecule is hyperactivated [1]. Moreover, K-Ras hyperactivation in these cells increases astrocyte proliferation *in vitro* in a manner similar to loss of *Nf1* protein (neurofibromin) expression. In order to demonstrate that K-Ras hyperactivation can substitute for neurofibromin loss in the genesis of murine brain tumors (optic gliomas), *Nf1+/-* mice were generated in which K-Ras activation occurred in neuroglial progenitor cells. Consistent with the *in vitro* studies, *Nf1+/-* mice with K-Ras hyperactivation developed optic gliomas *in vivo.* In addition to this study in the brain, differential Ras molecule function has also been explored in a mouse model of colon cancer *in vivo* [2]. While expression of activated N-Ras in the colonic epithelium inhibits cell apoptosis, activated K-Ras increases epithelial hyperplasia. Despite this increase in cell proliferation, K-Ras activation is not sufficient to initiate tumor formation in these mice on its own. In colon cancers, genetic mutations leading to the loss of the *APC* tumor suppressor gene occur early in cancer progression, suggesting that loss of this protein functions in colon tumor initiation. In the mouse, epithelial loss of the Apc protein in the colon causes adenocarcinoma formation. To determine if Ras molecule activation alters the progression of these tumors, genetically engineered mice were used

that both lacked Apc expression and expressed activated forms of either K-Ras or N-Ras in the colonic epithelium. In the context of *Apc* loss, K-Ras, but not N-Ras, activation led to high-grade dysplasia within the colonic epithelium. This result indicates that K-Ras activation alone increases cell proliferation and, in a tumor environment, promotes colon cancer progression. Taken together, these findings establish that the individual Ras molecules have different biological functions *in vivo* and that K-Ras is critical for regulating cell proliferation.

In addition to the functional heterogeneity introduced by the distinct Ras molecules, other factors play important roles in determining how Ras functions in specific organs during life. These critical variables include how the various Ras molecules operate in specific cell types, different regions within the same organ, and during the course of various diseases.

**CELL-TYPE SPECIFICITY OF RAS SIGNALING**

The ability of RAS to regulate the growth of discrete cell populations within the same organ may rely on the differential utilization of unique effector pathways operating downstream of RAS. This cell type specificity can be generated by using different RAS molecules or changing the interaction of effector proteins with the same activated RAS molecule. Of these potential RAS downstream signaling pathways, the canonical RAF/MEK/ERK effector pathway is the predominant signaling cascade important for growth regulation in leukemic cells, hematopoietic cells, osteoblasts, and some populations of neural progenitor cells [3-6]. Nevertheless, recent data, including those presented in this thesis, suggest that RAS signaling may occur through other effector pathways in a cell type-specific manner. Here, I have elucidated one such pathway required for hyperactivated K-Ras-mediated increased NSC proliferation. Other cell types in the mammalian brain, including neurons and astrocytes, also rely on different non-canonical RAS signaling mechanisms to regulate their behavior.

Recent studies from our laboratory have identified and characterized non-conventional RAS effector pathways which regulate neuronal function. In the context of NF1, the Ras-GAP protein neurofibromin was originally thought to regulate glial and neuronal cell biology through Ras-dependent and Ras-independent signaling pathways, respectively [7]. Upon heterozygous *Nf1* gene inactivation, there is a reduction in neuronal intracellular cyclic AMP (cAMP) levels, leading to decreased axon

lengths, growth cone areas, and survival *in vitro*. The importance of cAMP to these neuronal phenotypes is underscored by the finding that pharmacological agents that inhibit cAMP degradation or increase cAMP production restore *Nf1*+/- axonal lengths, growth cone areas, and survival to wild-type levels. The idea that cAMP production by neurofibromin operated in a Ras-independent manner derived from experiments in which pharmacological inhibition of Mek or PI3K/Akt signaling had no effect on these defective *Nf1*+/- phenotypes. However, recent work demonstrated that cAMP homeostasis is actually Ras-dependent. In neurons, neurofibromin regulates cAMP through inhibition of atypical PKC (PKCζ), leading to $G\alpha_s$ and adenylyl cyclase activation [8]. Upon reduced expression of neurofibromin in *Nf1*+/- neurons, the activity of both Ras and PKCζ is increased. This subsequently inhibits $G\alpha_s$ and adenylyl cyclase activity and causes depletion of cAMP. Genetic and pharmacologic inhibition of Ras activity in *Nf1*+/- neurons restores cAMP levels and neuronal axon lengths to those of wild-type neurons. These results demonstrate that neurofibromin regulates cAMP levels in a Ras-dependent manner. This pathway is unique to brain neurons, as PKCζ is not expressed in brainstem NSCs (Supplemental Figure S3.1), precluding this pathway from regulating NSC growth and underscoring cell type-dependent signaling differences.

Similar to NSCs and neurons, astrocyte growth is also regulated by Ras, but in a manner dissimilar to neurons and NSCs. As outlined above, K-Ras hyperactivation increases astrocyte proliferation and is sufficient to form optic gliomas in *Nf1*+/- mice [1]. In *Nf1*-deficient astrocytes, Ras hyperactivation leads to greater cell proliferation through hyperactivation of the mammalian target of rapamycin (mTOR) complex [9]. As such, the increased proliferation of *Nf1-/-* astrocytes is reduced to wild-type levels following treatment with the mTOR inhibitor rapamycin. While the precise mechanisms responsible for mTOR activation in astrocytes remain to be completely elucidated, recent studies have demonstrated that neurofibromin regulation of brainstem astrocyte proliferation *in vitro* and optic glioma growth *in vivo* involves mTOR activation mediated by both the PI3K/AKT and RAF/MEK RAS effector pathways [10]. Moreover, unlike other cell types, neurofibromin mTOR activation does not depend on the tuberous sclerosis complex proteins tuberin and hamartin [11]. Tuberin can be phosphorylated by MEK or AKT, leading to hyperactivation of the RAS homolog enriched in brain (Rheb) molecule [12-15]; however, Rheb activation does not result in increased astrocyte growth *in vitro* or optic glioma formation

in *Nf1*+/- mice *in vivo* [11]. Similarly, genetic silencing of Rheb has no effect on *Nf1*-deficient astrocyte growth. Taken together, these studies demonstrate that neurofibromin loss leads to K-Ras hyperactivation, which subsequently activates mTOR through either the Mek or Akt effector proteins to increase astrocyte proliferation.

A potential mechanism for cell-type specificity of Ras function is suggested by another recent study from our laboratory. Although *Nf1*-associated gliomas (pilocytic astrocytomas) are caused by inactivation of both *Nf1* alleles, sporadic pilocytic astrocytomas are caused by mutations at the *B-RAF* gene locus. In these mutations, the genetic sequence encoding the B-RAF kinase domain is fused to a portion of a gene of unknown function, KIAA1549. Expression of this *B-RAF:KIAA1549* fusion gene (*f-BRAF*) leads to increased activation of the downstream effector proteins Mek and Erk in both cerebellar astrocytes and NSCs [16]. However, only f-BRAF-expressing NSCs, but not astrocytes, demonstrate increased proliferation relative to wild-type cells. An analysis of downstream effector proteins revealed that mTOR activity is increased in f-BRAF-expressing NSCs, but not in astrocytes. Additionally, the protein tuberin, which normally inhibits mTOR activity, is more highly phosphorylated (inactivated) in these NSCs compared to the astrocytes. Mechanistically, this cell type-specific phosphorylation of tuberin results from the differential subcellular localization of this protein in NSCs and astrocytes. In astrocytes, cell fractionation reveals that tuberin is localized to subcellular membranes whereas in NSCs, tuberin is found exclusively in the cytosolic fraction. It is thought that the cytosolic localization of tuberin in NSCs may allow this protein to be phosphorylated by Erk, such that mTOR activity and cell proliferation is increased. In contrast, tuberin in astrocytes is sequestered at the membranes, thereby precluding the tuberin-Erk interaction from occurring. This study provides evidence that the subcellular localization of effector proteins can account for differential signaling in different cell types.

Collectively, these observations demonstrate that activated RAS can differentially control the growth and function of individual cell types through a diverse number of cell type-specific downstream effector pathways. Moreover, this third study suggests a mechanism by which RAS interacts with different effector proteins such that activation of the same protein activates different effector pathways in a cell type-specific manner.

**REGIONAL HETEROGENEITY OF RAS SIGNALING**

In addition to cell type-specific responses to RAS signaling, NSCs isolated from different brain regions also respond uniquely following RAS activation. In this regard, *Nf1-/-* NSCs isolated from the brainstem proliferate faster and undergo more gliogenesis *in vitro* and *in vivo* relative to *Nf1-/-* NSCs isolated from the brain cortex. An analysis of downstream effector molecules responsible for transmitting the Ras growth-promoting signal in these two NSC populations reveals that NSC proliferation and differentiation is dependent on mTOR complex 2 (mTORC2)-mediated activation [17]. This mTOR complex is characterized by the presence of the Rictor protein, which allows mTOR to function as an activator of AKT through serine 473 phosphorylation. The differential capacity of brainstem NSCs to increase their proliferation and glial differentiation following neurofibromin loss and mTOR activation results from three-fold more expression of Rictor in these NSCs relative to those derived from the cortex. Silencing of Rictor expression reduced the hyperproliferation and gliogenesis observed in *Nf1-/-* brainstem NSCs, establishing differential expression of one downstream effector as a key determinant in dictating brain region-specific signaling and cellular function. In support of these data, brain region heterogeneity is also observed upon expression of the f-BRAF protein in NSCs isolated from the cerebellum and cortex. Similar to the results above, f-BRAF expression increases the proliferation of cerebellar NSCs, but not cortical NSCs [16].

In conjunction with the data presented in this thesis, recent work suggests that the individual RAS molecules may also signal in a region-specific manner. When expressed in cortical progenitor cells *in vitro*, two separate hyperactivated H-Ras constructs (H-Ras$^{G12V}$, H-Ras$^{G12S}$) inhibit neurogenesis, induce neural progenitor cell proliferation, and promote glial cell differentiation [18]. Moreover, introduction of the same constructs via *in vivo* electroporation during the embryonic neurogenic period (E13/E14) induces cell proliferation and premature gliogenesis. These data highlight an important role for H-Ras* function in specifying glial versus neuronal cell fates in the developing neocortex. In this thesis, activation of H-Ras (H-Ras$^{G12V}$) at a similar time point (E9.5) in the brainstem did not cause changes in glial or neuronal cell formation. Moreover, we have examined the impact of H-Ras activation in brainstem NSCs *in vitro* and found that this is insufficient to change NSC proliferation or alter NSC cell fate decisions relative to wild-type NSCs.

Taken together, these studies suggest that the regional specificity of RAS signaling may be conferred by differential expression of RAS downstream effector proteins or through regionally-restricted roles of the individual RAS molecules themselves. To the second point, we have not yet explored whether the individual RAS molecules function differently by brain region. If this proves to be accurate, further work will be necessary to determine the mechanistic basis by which region-specific, molecule-specific RAS signaling could occur in the brain.

**RAS SIGNALING IN DISEASES OF THE CENTRAL NERVOUS SYSTEM**

Additional insights into differential RAS signaling function can be realized by studying the consequences of RAS hyperactivation in the context of various brain disorders. In NF1, affected individuals can develop a wide variety of neurological problems that reflect abnormalities in neuronal (learning disabilities and seizures) and astrocytic (optic gliomas) biology. This highlights a particular vulnerability of multipotent NSCs to changes in *NF1* gene function, which could impact on the development and function of both differentiated cell types. In agreement with previous studies from our laboratory, I have demonstrated that *Nf1-/-* NSCs proliferate faster and give rise to more astrocytes upon differentiation relative to wild-type NSCs *in vitro* (Figure 4.1). Moreover, *Nf1* gene inactivation in BLBP-Cre-expressing NSCs *in vivo* leads to increased astrogliogenesis in the brainstem of PN18 mice relative to littermate controls (Figure 4.2). While the behavior of these *Nf1*-deficient NSCs is similar to that of K-Ras* NSCs described in this thesis, the mechanisms underlying their altered NSC function differs. Here, I have demonstrated that K-Ras hyperactivation increases NSC proliferation in a Raf/Rb-dependent manner. However, using NSCs from the same brain region and time of development, we have previously demonstrated that *Nf1*-deficient NSCs undergo increased proliferation in an mTOR/Rictor-dependent manner to activate the downstream effector protein Akt [17]. To determine if this pathway functions upon K-Ras activation, I examined the activity of both Akt (p-Akt$^{S473}$) and S6 kinase (p-S6$^{240/244}$) in K-Ras* NSCs and found that neither protein was hyperactivated in K-Ras* NSCs relative to wild-type NSCs (Supplementary Figure S3.1A). As a negative regulator of Ras, the loss of neurofibromin expression is known to increase RAS activity. Therefore, it stands to reason that hyperactive Ras could impact NSC growth in a manner similar to *Nf1* gene inactivation. However, these results demonstrate that this is not

the case and instead indicate that neurofibromin and K-RAS regulate the growth of the same cell population differently.

RAS also functions differently in different types of cancers. Of all cancers, RAS pathway dysregulation is an important step in cancer initiation as approximately 30% of all cancers arise from hyperactivating mutations in RAS [19]. Despite the widespread importance of RAS to tumorigenesis, the individual RAS molecules do not contribute equally to this process. For example, *K-RAS* mutations are found in 22% of all cancers, whereas *H-RAS* mutations are observed in only 8% of malignancies [19]. *N-RAS* mutations occur even more rarely, occurring in approximately 3% of all cancers. Some of this discrepancy can be accounted for by the developmental origin of the tissues in which these cancers develop. In tissues of endodermal origin (lung, pancreas, colon), cancers are most commonly associated with *K-RAS* mutations, while *N-RAS* mutations are often associated with cancers of the hematopoietic system [20-23]. It is interesting to note that progenitor cells in these tissue populations have been shown to be especially susceptible to activating RAS mutations, providing a cellular basis for tumor formation and suggesting a potential target for anti-cancer therapies [2, 21, 24]. These studies indicate that there is inherent heterogeneity in RAS pathway hyperactivation underlying the formation of different tumors.

Future studies could address this differential contribution of each hyperactivated RAS molecule to brain tumorigenesis using the endogenous $RAS^{LSL}$ mice used in this thesis. It is clear that K-Ras hyperactivation in terminally differentiated astrocytes is sufficient for tumor formation in the optic nerve, a result that merits further exploration on the tumorigenic consequence of K-Ras hyperactivation in NSCs [1]. Moreover, as K-Ras activation alone increases the growth of NSCs, we attempted to examine whether CNS tumors arise in aged $K\text{-}Ras^{BLBP}$ mice. Unfortunately, efforts to evaluate tumor susceptibility in these mice were not possible as aging beyond 18 days of age caused these mice to form an apparent rectal prolapse between 5-8 weeks of age (n=4), which required subsequent euthanasia. Follow-up necropsy results revealed squamous cell papilloma of the vagina and/or anus yet there were no indications of CNS-related tumors at this time point. Although this result limits the utility of $K\text{-}RAS^{BLBP}$ mice in evaluating the tumorigenic potential of hyperactivated K-RAS in NSCs, future studies might consider exploring this question using an alternative, NSC-specific Cre recombinase driver.

Taken together, these examples indicate that the context in which the RAS pathway is activated

varies in different diseases. As an example, in cancers and in the NSCs examined in this thesis, RAS pathway hyperactivation is the result of oncogenic RAS mutations, which may result in different levels of RAS activity than occurs upon the loss of a RAS regulatory protein such as neurofibromin. This has potential implications in how the RAS pathway might be targeted in different diseases.

**SUMMARY AND SIGNIFICANCE**

The requirement for RAS signaling in normal cell growth complicates efforts to inhibit RAS pathway hyperactivation in a specific manner and without the potential for off-target effects. This emphasizes a need for therapeutic options that more specifically target aberrant RAS activity in specific cell types, specific organ regions, and in specific diseases. The H-RAS, K-RAS, and N-RAS molecules described in this thesis are ubiquitously expressed across different mammalian cell types. However, here we have described mechanisms that confer specificity on RAS molecule signaling. First, the individual RAS molecules can be activated differently in various cell and tissue types. Second, the expression and/or localization of effector proteins differentially alters the interactions between RAS and its downstream signaling pathways. Third, activation of the same RAS molecule in the same cell type can alter cell function differently depending on how RAS is activated, such as by an oncogenic mutation within RAS or by gain-of-function/loss-of-function mutations to other components of the RAS signaling pathway. These mechanisms provide a potential means by which to inform the development of targeted therapies that reduce RAS pathway hyperactivity in specific tissues, cells, and in the various RASopathies.

It is possible that therapies could be developed that inhibit RAS pathway activity in a tissue-specific manner. While studies have implicated active K-Ras as the driver of cell proliferation in both the brain and colon, the Ras effector pathways operating in these tissues are not equivalent. This provides an opportunity to inhibit K-Ras-mediated astrocyte proliferation in brain tumors by targeting the mTOR complex or, conversely, targeting colon hyperplasia by inhibiting Mek/Erk activity. Elucidation of additional Ras tissue-specific pathways may afford additional opportunities to target RAS hyperactivation in individual tissues.

Within the same tissue, there is growing evidence that RAS can regulate the function of individual cell types through the use of cell type-specific effector pathways. In the brain, this represents a

remarkable opportunity to target RAS signaling in individual cell types rather than by globally inhibiting the RAS pathway. Moreover, this development is especially promising for the treatment of disorders that are characterized by defective regulation of multiple cell types, such as in individuals with NF1. In this disorder, one might envision that targeting of the RAS/PKCζ/Gαs pathway in neurons could effectively treat children presenting with learning and memory disabilities. In theory, these treatments could restore cAMP levels to those found in normal neurons. Alternatively, in children affected by *Nf1*-associated optic gliomas, therapies that target RAS/mTOR activity may prove valuable in inhibiting the proliferation of astrocytes that make up the bulk of these tumors. Additionally, the differential localization and expression of effector proteins in different cell types suggests that drugs that modulate the behavior and localization of effector proteins could allow for specific targeting of RAS signaling. Hypothetically, these cell type-specific approaches may demonstrate increased efficacy over conventional treatments targeting overall RAS signaling (i.e. lovastatin for learning disabilities) although more work is required before they can translate to the clinic.

Lastly, disease-specific mechanism by which the RAS pathway is activated in individual disorders may alter how therapeutic approaches reduce aberrant RAS pathway hyperactivation. Despite identifying RAS as the critical operator underlying the neuro-cardio-facio-cutaneous disorders, the nuances of the individual disorders likely result from the occurrence of disorder-specific mutations in different components of the RAS pathway. In NF1, NSCs are especially sensitive to *NF1* gene inactivation in order to account for defects in the function of both neurons and astrocytes in patients with this disorder. Nevertheless, it is unknown how *Nf1* loss in NSCs may result in the differential activation of the three Ras molecules relative to one another. While the current thesis is an important first step in understanding how RAS alters NSC function on its own, valuable follow-up studies will apply this knowledge to activating the Ras molecules in a manner consistent with *Nf1* gene inactivation.

The studies discussed here demonstrate that RAS signaling extends far beyond the RAF/MEK/ERK and PI3K/AKT/mTOR signaling pathways that are traditionally accepted as the primary RAS effector pathways. Indeed, a great deal of complexity and specificity is conferred upon RAS signaling through the utilization of other, non-conventional effector pathways. While this thesis has focused on the differential function of RAS signaling, there is undoubtedly similar utilization of non-

conventional pathways downstream of other developmental pathways, such as WNT, Notch, TGF-β, and hedgehog signaling. This cautions against the assumption that certain proteins signal via only a limited subset of effector pathways. Clarification of the effector pathways that confer specificity on these developmental pathways will facilitate more targeted approaches to regulate aberrant pathway signaling.

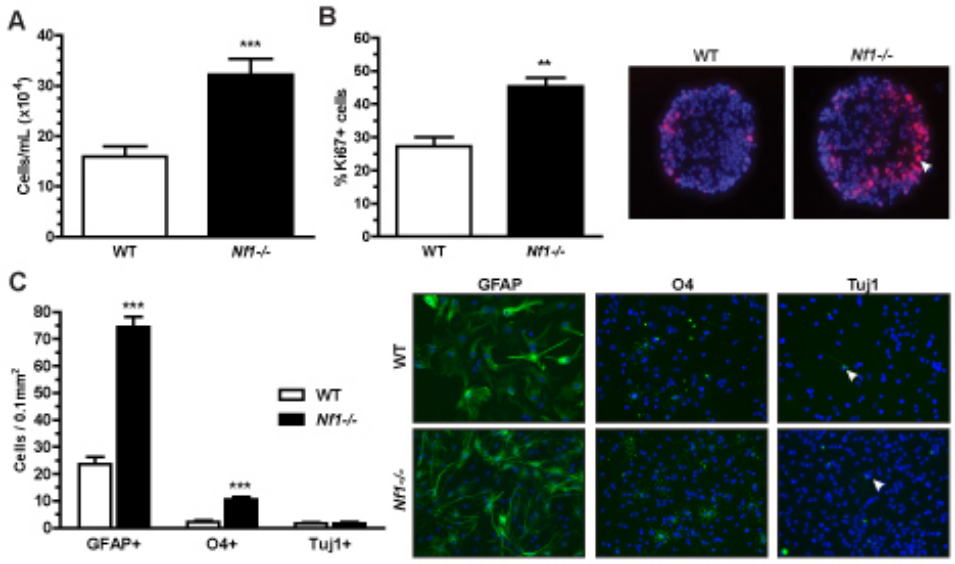


**Figure 4.1. *Nf1* loss results in increased NSC proliferation and glial cell differentiation *in vitro.*** *Nf1-/-* NSCs proliferate faster than WT controls as measured by (**A**) direct cell counting ($p<0.001$) and (**B**) the percentage of Ki67+ proliferating cells (arrowhead) in Nf1-/- neurospheres ($p<0.01$). (**C**) *Nf1-/-* NSCs give rise to increase numbers of GFAP+ astrocytes and O4+ oligodendrocytes ($p<0.001$) but not Tuj1+ neurons.

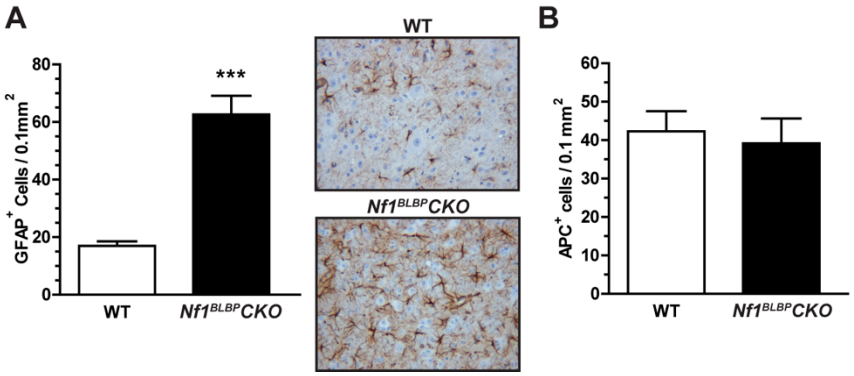


**Figure 4.2. *Nf1* loss in NSCs leads to increased gliogenesis *in vivo*.** (**A**) *Nf1* loss in *BLBP-Cre*-expressing NSCs (*Nf1*$^{BLBP}$*CKO*) leads to a 3.7-fold increase in brainstem GFAP$^{+}$ astrocytes (***p<0.001). (**B**) *Nf1*$^{BLBP}$*CKO* mice do not exhibit altered numbers of APC$^{+}$ oligodendrocytes.

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20. Li Q, Haigis KM, McDaniel A et al. Hematopoiesis and leukemogenesis in mice expressing oncogenic NrasG12D from the endogenous locus. Blood. 2011;117:2022-2032.

21. Kim CF, Jackson EL, Woolfenden AE et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell. 2005;121:823-835.

22. Shen SW, Dolnikov A, Passioura T et al. Mutant N-ras preferentially drives human CD34+ hematopoietic progenitor cells into myeloid differentiation and proliferation both in vitro and in the NOD/SCID mouse. Experimental hematology. 2004;32:852-860.

23. Tuveson DA, Shaw AT, Willis NA et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell. 2004;5:375-387.

24. Quinlan MP, Quatela SE, Philips MR et al. Activated Kras, but not Hras or Nras, may initiate tumors of endodermal origin via stem cell expansion. Mol Cell Biol. 2008;28:2659-2674.

# R. Hugh F. Bender

*Curriculum Vitae*

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## EDUCATION

**Washington University in St. Louis**, St. Louis, MO 2015
Doctor of Philosophy Candidate: Developmental, Regenerative, and Stem Cell Biology

**Wake Forest University**, Winston-Salem, NC 2009
Master of Science: Cell Biology

**Wake Forest University**, Winston-Salem, NC 2007
Bachelor of Science: Biology with Honors

## HONORS & AWARDS

**Young Investigator Award,** *Children's Tumor Foundation* 2012-2014

**Cell and Molecular Biology Institutional Training Grant**, *Washington University* 2010-2012

**Honors in Biology**, *Wake Forest University* 2007

## RESEARCH EXPERIENCE

**Washington University,** Ph.D. Thesis—Laboratory of David H. Gutmann, MD, Ph.D. 2010-2015
*Thesis: K-Ras, but not H-Ras or N-Ras, hyperactivation regulates brain neural stem cell proliferation in a Raf/Rb-dependent manner.*

To understand the regulation of neural stem cell (NSC) function in brain development, I utilized genetically engineered mouse models to test whether hyperactivation of the individual Ras molecules (H-Ras, K-Ras, N-Ras) differentially alters NSC proliferation and differentiation. Using complementary *in vitro* and *in vivo* approaches, I determined that K-Ras, but not H-Ras or N-Ras, regulates NSC proliferation via a non-canonical Ras effector pathway.

**Wake Forest University,** Master's Thesis—Laboratory of James F. Curran, Ph.D. 2007-2009
*Thesis: A method for identifying ribosomal pause sites in mRNA through new sequencing techniques.*

Designed and conducted experiments to develop a method for isolating ribosome-protected mRNA fragments from adenovirus-infected HeLa cells and refined existing protocols to convert mRNA fragments to dsDNA with appropriate adapters for high-throughput sequencing.

**Wake Forest University***,* Honors Thesis Research—Laboratory of Jacquelyn S. Fetrow, Ph.D. 2007
*Thesis: The relationship between the function and connectedness of protein drug targets in protein interaction networks.*

Worked with an interdisciplinary team to design and produce a software tool (FurBall) for analyzing and visualizing yeast two-hybrid data as a protein-protein interaction network. Subsequently utilized this tool to complete an independent thesis project aimed at characterizing the function and connectedness of drug target proteins in these interaction networks.

**Wake Forest University,** Undergraduate Research—Laboratory of James F. Curran, Ph.D. 2006

Assisted a graduate student in identifying the important role of the ribosomal E site in promoting frameshifting in *E. coli* during protein translation.

## PUBLICATIONS

2015 **Bender RHF**, KM Haigis, DH Gutmann. *Activated K-Ras, but not H-Ras or N-Ras, regulates brain neural stem cell proliferation in a Raf/Rb-dependent manner.* Stem Cells (*in press*).

2011 Link DC, LG Schuettpelz, D Shen, J Wang, MJ Walter, S Kulkarni, JE Payton, J Ivanovich, PJ Goodfellow, M Le Beau, DC Koboldt, DJ Dooling, RS Fulton, **RHF Bender**, LL Fulton, KD Delehaunty, CC Fronick, EL Appelbaum, H Schmidt, R Abbott, M O'Laughlin, K Chen, MD McLellan, N Varghese, R Nagarajan, S Heath, TA Graubert, L Ding, TJ Ley, GP Zambetti, RK Wilson, ER Mardis. *Identification of a novel TP53 cancer susceptibility mutation through whole-genome sequencing of a patient with therapy-related AML*. JAMA. 305(15):1568-1576.

## PROFESSIONAL PRESENTATIONS & INVITED TALKS

2014 **Bender RHF**, Haigis KM, Gutmann DH. The role of Ras molecule hyperactivation in neural stem cell function. Poster. *2014 Annual Neurofibromatosis Conference.* Washington, D.C.

**Bender RHF.** Hyperactive K-Ras in the regulation of neural stem cell function. *23rd Annual Developmental Biology Retreat*. Research Talk. Washington University, St. Louis, MO.

2013 **Bender RHF**, Haigis KM, Gutmann DH. The role of Ras molecule hyperactivation in neural stem cell function. Poster. *2013 Annual Neurofibromatosis Conference.* Monterey, CA.

**Bender RHF.** Dissecting the role of Ras hyperactivation in neural stem cell function. *22nd Annual Developmental Biology Retreat*. Research Talk. Washington University, St. Louis, MO.

**Bender RHF.** Dissecting the role of Ras hyperactivation in neural stem cell function. *Incoming Ph.D. Student Recruitment.* Research Talk. Washington University, St. Louis, MO.

2012 **Bender RHF.** Neurofibromin regulation of neural stem cells: a Ras who-dun-it. Research talk. *21st Annual Developmental Biology Retreat*. Washington University, St. Louis, MO.

2010 **Bender RHF.** The Ethics of Stem Cell Research. Invited Seminar Speaker. *Tower Hill School Forum Series: Morality vs. The Public Interest*. Tower Hill School, Wilmington, DE.

**Bender RHF**, LG Schuettpelz, DC Link. Validating therapy-related AML (tAML) mutations identified through whole-genome sequencing. Poster. *19th Annual Developmental Biology Retreat*. Washington University, St. Louis, MO.

2008 **Bender RHF** and JF Curran. Identifying translational control regions in mRNA through new sequencing technology. Poster. *24th Annual Perspectives in Biology Symposium*. Wake Forest University, Winston-Salem, NC.

## PROFESSIONAL SERVICE

**Washington University Board of Trustees,** *Graduate Student Representative* 2013-2014
Responsible for providing dialogue between University graduate students and the Board of Trustees. Provided graduate student perspective at quarterly Board meetings and on the Research-Graduate Affairs and Medical Finance Committees. Presented a year-end report ("Preparing graduate & professional students for non-traditional career opportunities") to the Board.

**Washington University BioEntrepreneurship Core**, *President* 2012-2013
The BioEntrepreneurship Core (BEC) is a graduate student-run group aimed at promoting innovation and entrepreneurship at Washington University through education and training opportunities for all University affiliates. During my term as President, the group's key accomplishments included: creation of a unified branding scheme to increase the visibility of the BEC, membership expansion, and directed efforts to broaden the BEC's reach as a boundary-spanning organization connecting the University and the St. Louis biotechnology communities.

**Washington University BioEntrepreneurship Core**, *Vice President* 2011-2012, 2013-2014

Assisted the President with the organization and execution of events, and developing new relationships with local entrepreneurs and biotechnology representatives. In my second term, I advised the succeeding President to provide continuity from the 2012-13 curriculum.

## RELATED EXPERIENCE

**Washington University**, Neurophysiology Course, *Graduate Teaching Assistant* 2010

**Wake Forest University,** Comparative Physiology Course, *Graduate Teaching Assistant* 2008-2009

**StemLife Sdn. Bhd.***, Intern—Kuala Lumpur, Malaysia* 2005

Participated in business conferences and event planning aimed at promoting umbilical cord blood stem cell collection services to both clients and their doctors. Also created a limited database system designed to compile and analyze the productivity of doctor stem cell collection procedures.