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The Role of p53 and ASPP2 in Neurodegenerative Disease

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Abstract

Two cellular processes of central importance to cancer and neurodegeneration are apoptosis and cellular senescence. Both are a means of cellular suicide that are utilized in time- and context-dependent manners and have important evolutionary purposes. However, they are also the drivers of many deleterious processes underlying cancer and neurodegeneration. Many of the cellular responses to aging and age-related diseases, including apoptosis and senescence, converge on the tumor suppressor pathway, p53.

Here I examine the molecular basis for loss of cell polarity and accelerated cell death mediated by apoptosis stimulating protein of p53 2 (ASPP2) in neurodegeneration. In this study we find that ASPP2 mediates STAT1-induced apoptosis. Lipopolysaccharide (LPS) induces ASPP2 mRNA expression *in vitro*. Also, LPS induces nuclear ASPP2 *in vivo* at the blood-cerebral spinal fluid-barrier (BCSFB), the brain's barrier to inflammation. Consistent with ASPP2's role as a gatekeeper to inflammation, ASPP2 mutant mouse brains possess enhanced neuroinflammation. Elevated ASPP2 expression is also observed in mouse models and human neuroinflammatory disease tissue in astrocytes. The identification of ASPP2 as a novel transcriptional target of STAT1 and the observed increase in ASPP2 expression in both mouse and human neuroinflammatory disorders, suggests that the identified STAT1/ASPP2 pathway may connect tumor suppression and cell polarity to neuroinflammation.

Additionally, I investigate the regulation of cellular senescence by p53 isoforms as a means to enhance neuroprotection of astrocytes in chronic neurodegenerative diseases, Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). Here we report that p53 isoforms, Δ133p53 and p53β, are endogenous regulators of cellular senescence in the central nervous system (CNS). Δ133p53 functions as a dominant-negative regulator of full-length (FL)-p53 and represses senescence, while p53β as a co-activator of FL-p53, promotes senescence. In neurodegenerative disease brain tissue, FL-53 and p53β are upregulated while Δ133p53 is downregulated. We demonstrate that Δ133p53 and p53β directly regulate astrocyte senescence, including the release of key neurotoxic pro-inflammatory cytokines such as IL-6 and IL-1β. Also, we show that the p53 isoform switch that occurs during aging and neurodegeneration promotes neuronal toxicity using co-culture experiments with human iPSC-derived motor neurons and human astrocytes. We also demonstrate that astrocyte senescence can be rescued through overexpression of Δ133p53, revealing a promising therapeutic approach to delay or inhibit the progression of neurodegeneration.

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Finally, a thank you those who have offered invaluable support, guidance, and love: Kieran Higgins and Coralie Violet.

Declaration

I, Casmir Turnquist, hereby declare that the work on which this thesis is based is my original work and that neither the whole work nor any part of it has been, is being or will be submitted for another degree in this or any other university. The work is original, except where listed by reference in the text or listed as follows: Western blots and qRT-PCR Figures 3.1.1A, 3.2.2B, 3.3C-D performed by Yihua Wang as published in (Turnquist et al. 2014). A complete list of publications resulting from this work are listed in Appendix B.

Signed:………………………………………………

Date:………………………………………………

Table of Contents

Title page.. i

Abstract.. ii

Acknowledgements.. iii

Declaration.. iv

Table of Contents.. v

List of Figures.. xiii

List of Tables.. xvi

Abbreviations.. xvii

Chapter 1 Introduction and Aim of Thesis.. 1

Chapter 2 Materials and Methods.. 51

Chapter 3 ASPP2 is Regulated by the STAT1 Inflammatory Signaling Pathway.. 62

Chapter 4 ASPP2 in p53-Mediated Apoptosis in Neurodegeneration.. 74

Chapter 5 p53 Isoforms Regulate Astrocyte Senescence in Brain Aging and Neurodegeneration.. 87

Chapter 6 Manipulation of Δ133p53 as a Therapeutic Strategy for Neurodegeneration.. 109

Chapter 7 Discussion and Concluding Remarks.. 123

References.. 141

Appendix A: Figure permissions.. 165

Appendix B: Published works.. 169

Chapter 1: Introduction

General Overview 1

PART I: Neurodegenerative Disease

1.1 Introduction 3

1.2 Multiple sclerosis 4

1.3 Viral encephalitis 5

1.4 Alzheimer's disease 6

1.5 Amyotrophic lateral sclerosis 9

PART II: Common Mechanisms Underlying Cancer and Neurodegeneration

1.6 Introduction 12

1.7 Inflammation 13

1.7.1 Toll-like receptors 16

1.7.2 Interferon signalling 18

1.8 Cell polarity 20

1.8.1 Brain barriers 20

1.8.2 Tight and adherents junctions 22

1.9 Cellular senescence 23

1.9.1 Senescence-associated secretory phenotype 23

1.9.2 Brain aging and neurodegeneration 26

1.10 Apoptosis 28

1.11 Autophagy 32

1.12 Therapeutic strategies and remaining challenges 35

PART III: Tumor Suppressor Pathway p53 37

1.13 Introduction 37

1.14 p53 isoforms 40

1.14.1 p53 isoforms in the CNS 44

1.15 ASPP Protein Family 44

1.15.1 ASPP2 in the CNS 46

Thesis Aims 50

Chapter 2: Materials and Methods

Materials

Table 2.1 Primary Antibodies 51

Table 2.2 Secondary Antibodies 52

Table 2.3 Plasmids/luciferase reporters 52

Table 2.4 Primers for qRT-PCR 53

Table 2.5 Retroviral and lentiviral vectors 54

Methods

2.1 Mouse colonies 54

2.2 Patient samples 54

2.3 MCAO stroke mouse model 55

2.4 TMEV infection mouse model 55

2.5 Maternal inflammation mouse model 56

2.6 IHC and IF 56

2.7 Immunobloting 57

2.8 RNA extraction and cDNA preparation 57

2.9 qRT-PCR 58

2.10 Cell culture 58

2.11 SA-β-gal staining 59

2.12 Neuronal differentiation of iPSC 59

2.13 Co-culture system 59

2.14 Quantification of neuronal apoptosis 60

2.15 Luciferase assay 60

2.16 Statistical analysis 60

Chapter 3: ASPP2 is regulated by the STAT1 inflammatory signaling pathway

Introduction 62

Results

3.1 ASPP2 is induced by LPS 63

3.2 ASPP2 is upregulated in inflammatory disorders using *in silico* microarray comparative analysis 68

3.3 LPS induced-ASPP2 expression is mediated by STAT1 70

Summary 72

Chapter 4: ASPP2 in p53-Mediated Apoptosis in Neurodegeneration

Introduction 74

Results

4.1 LPS induces nuclear ASPP2 expression *in vivo* in a mouse model of maternal inflammation 75

4.2 ASPP2 regulates IFN and LPS-induced apoptosis *in vivo* and *in vitro* 78

4.3 ASPP2 deficiency enhances neuroinflammation *in vivo* 80

4.4 ASPP2 is upregulated in reactive astrocytes in mouse neuroinflammation models and human neuroinflammatory disease 82

Summary 86

Chapter 5: p53 Isoforms in Brain Aging and Neurodegeneration

Introduction 87

Results

5.1 Cellular senescence of astrocytes in brain tissues from neurodegenerative disease patients 89

5.2. p53β is upregulated and Δ133p53 is downregulated in neurodegenerative disease brains and in aged astrocytes *in vitro* 94

5.3 Autophagic degradation of Δ133p53 and SRSF3-mediated regulation of p53β in astrocytes 98

5.4 Loss of Δ133p53 induces senescent phenotype in astrocytes 102

5.5 Increased p53β induces senescent phenotype in astrocytes 105

Summary 108

Chapter 6: Manipulation of Δ133p53 as a Therapeutic Strategy for Neurodegeneration

Introduction 109

Results

6.1 Increased neuronal death upon co-culture with Δ133p53-knocked-down or p53β-overexpressing astrocytes 112

6.2 Δ133p53 prevents astrocytes from senescence and enhances their neuroprotective function 117

Summary 122

Chapter 7: Discussion and Concluding Remarks

Summary 123

7.1. ASPP2-mediated apoptosis in neurodegeneration 125

7.2. Δ133p53 as a therapeutic strategy for neurodegeneration and other aging diseases 131

Conclusion 140

List of Figures

1.1	A common aging process?	13
1.2	Origin of CNS cells	15
1.3	Toll like receptors and ligands.	17
1.4	Canonical IFN Type I signalling pathway	19
1.5	Three distinct inducers of cellular senescence	25
1.6	Cellular senescence in neurodegeneration and physiological brain aging	27
1.7	The role of caspase-3 in apoptosis	31
1.8	Macroautophagy	33
1.9	Roles of p53 activation	39
1.10	Diagram of 12 known p53 isoforms	42
1.11	ASPP proteins	45
1.12	Phenotype of ASPP2-deficient mice	47
1.13	Histological abnormalities in the brain of ASPP2-deficient mice	47
1.14	The role of ASPP2 in cell polarity	49
3.1.1	ASPP2 is induced by LPS in macrophages	65
3.1.2	ASPP2 is induced by LPS in astrocytes and microglia	67
3.2	ASPP2 is upregulated in inflammatory disorders	69
3.3	ASPP2 induction is mediated by STAT1	71

4.1	LPS induces nuclear ASPP2 expression *in vivo*	77
4.2	ASPP2 regulates LPS-induced apoptosis	79
4.3	ASPP2-deficient mice posses neuroinflammation	81
4.4	ASPP2 upregulation in mouse models and human neuroinflammatory disorders.	84
5.1	Cellular senescence characteristics in neurodegenerative disease brain tissue	92
5.2	p53β is upregulated and Δ133p53 is downregulated in neurodegenerative disease and serial passaged primary astrocytes *in vitro*.	96
5.3	Autophagic degradation of Δ133p53 and SRSF3-mediated regulation of p53β	100
5.4	Knockdown of Δ133p53 induces senescent phenotype in astrocytes	103
5.5	Overexpression of p53β induces senescent phenotype in astrocytes	106
6.1.1	Generation of mature neurons from induced pluripotent stem cells (iPSC) and increased neurotoxicity by p53β overexpression in astrocytes	114
6.1.2	Increased motor neuron death upon co-culture with Δ133p53 knocked-down or p53β overexpressing astrocytes	115

6.2	Δ133p53 overexpression prevents astrocyte SASP and enhances neuroprotection	119
7.1	Proposed mechanism of ASPP2/STAT1-induced apoptosis in response to inflammatory stimuli	125
7.2	ASPP2 as a SMaRT factor	128
7.3	Proposed model of p53 isoform regulation of neurodegeneration and neuroprotection	139

List of Tables

1.1	Biological links between cancer and neurodegeneration	3
2.1	Primary antibodies	51
2.2	Secondary antibodies	52
2.3	Plasmids and luciferase reporters	52
2.4	Primers for qRT-PCR	53
2.5	Retroviral and lentiviral vectors	53
5.1	Human brain tissue	

Abbreviations

AD	Alzheimer's disease
AJC	Adherents junctional complex
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein-1
Apo	Apolipoprotein
APP	Amyloid precursor protein
ASPP	Apoptosis stimulating protein of p53
ATG	Autophagy related genes
Aβ	Amyloid beta
BBB	Blood brain barrier
BCSFB	Blood cerebral spinal fluid barrier
BrdU	Bromodeoxyuridine
BSCB	Blood-spinal cord barrier
C/EBP	CCAAT/enhancer binding protein
Caspase	Cysteine aspartate-specific proteases
CHX	Cycloheximide
CNS	Central nervous system
DAB	Diaminobenzene hydrochloride solution
DAPI	4',6-diamidino-2-phenylindol
DMEM	Dulbecco's Modified Eagle Medium
E	Embryonic day
EAAT2	Excitatory amino acid transporter-2
fALS	Familial amyotrophic lateral sclerosis
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded

fMRI	Functional MRI
GAPDH	Glyceraldehyde phosphate dehydrogenase
GAS	Gamma-activated sequences
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GLT1	Glutamate transporter 1
GSK-3	Glycogen synthase kinsase-3
GWAS	Genome-wide association studies
HAD	HIV-associated dementia
HD	Huntington's disease
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
iASPP	Inhibitory apoptosis stimulating protein of p53
ICC	Immunocytochemistry
IF	Immunofluorescence
IFN	Interferon
IFNAR	Interferon-α receptor
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
iPSC	Induced pluripotent stem cell
ISRE	Interferon-stimulated gene factor
JAK1	Janus kinase 1
LC3	Microtubule-associated protein 1 light chain 3
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion

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MDMA	Mouse double minute 2 homolog
MRI	Magnetic resonance imaging
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
ND	Non-disease
NeuN	Neuronal nuclei marker
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate receptor
NSC	Neural stem cells
NSL	Nuclear localization signal
OD	Oligomerization domain
P	Passage number
PAMP	Pathogen-associated molecular pattern
PB	Phosphate buffer
PBS-T	Phosphate buffered saline-Tween 20
PD	Parkinson’s disease
PS	Presenilin
qRT-PCR	Quantitative Real-Time Polymerase Chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT	Room temperature
SAMP	Senescence-accelerated prone mice
SASP	Senescence-associated secretory phenotype
SA-β-Gal	Senescence-associated β-galactosidase
SCID	Severe combined immunedeficient
SEM	Standard error of the mean
SH3	Src-homology 3 domain

siRNA	Small interfering RNA
SMA	Spinobulbar muscular atrophy
SOD1	Superoxide dismutase 1
STAT1	Signal transducer and activator of transcription
TA	Transactivation domain
TBS	Tris Buffered Saline
TLR	Toll like receptor
TMEV	Theiler's murine encephalomyelitis virus
TNF-α	Tumor necrosis factor-α
TYK2	Tyrosine kinase 2
WB	Western blot
ZO	Zona occludens

Chapter 1

Introduction

NEURODEGENERATION and cancer are two of the most devastating and costly illnesses of the 21st century. Alzheimer's disease (AD) is the most common form of dementia and is estimated to affect 36 million people, which will triple by 2050 (Wimo and Prince, 2010). The cost of AD to the USA is $172 billion and the world $604 billion in 2010 alone (Wimo and Prince, 2010). About 13.3 million new cases of cancer were diagnosed in 2010 and are estimated to have a worldwide burden of $290 billion, with cancers of the lung, bronchus, and trachea accounting for the largest burden on the global economy (Bloom et al., 2011; Brenner et al., 2011) .

Despite enormous scientific effort, only three USA Food and Drug Administration (FDA)-approved therapies have been developed for AD, four for Parkinson's disease (PD), and none for amyotrophic lateral sclerosis (ALS) in the past 10 years (CenterWatch (Firm), 2015). Although several therapies have been successful in

mouse models, they have failed to demonstrate efficacy in phase III clinical trials (Galimberti and Scarpini, 2011; Wilkins et al., 2011). Thus, it is clear that new approaches are required to address these complex and dynamic diseases. While neurodegeneration is characterized by accelerated cell death and cancer is characterized by excessive cell growth, increasing evidence suggests a molecular link.

Support for a molecular connection arises from epidemiological studies demonstrating an inverse relationship between susceptibility. Patients with a variety of neurodegenerative diseases including Huntington's disease (HD), spinobulbar muscular atrophy, hereditary ataxia, AD, and PD have a lower incidence of cancer than the general population, suggesting the presence of a common cancer protection mechanism (Bajaj et al., 2010; Coisne and Engelhardt, 2011; Ji et al., 2012; Roe et al., 2005). Another suggestion that cancer and neurodegeneration may share common biological programs comes from clinical trials of γ-secreatse inhibitors for AD, such as Semagacestat. Compounds that inhibit γ-secreatse the enzyme that generates β-amyloid (Aβ), were found to result in higher cancer incidence in a phase III trial (Doody et al., 2013). Indeed, the list of oncogenes and tumor suppressor genes that play key roles in the pathophysiology of neurodegeneration and carcinogenesis continues to expand (**Table 1.1**). Many of these molecules regulate cell cycle and self-renewal programs. In post-mitotic neurons this can result in apoptosis, while in a malignant cells, it can lead to uncontrolled proliferation.

Several lines of evidence suggest that these links may converge on the p53 signaling pathway, which regulates and is regulated by the γ-secretase complex

(Checler et al., 2007, 2010) and is a principal regulator of apoptosis and cell cycle arrest. Thus, investigation of the p53 signaling pathway in neurodegenerative diseases could contribute to our understanding of cancer resistance and also lead to the creation of new neurodegenerative disease therapies.

Here, I will review our current knowledge of several acute and chronic neurodegenerative diseases and remaining gaps in our understanding and therapeutic challenges. Then, I describe several key common features of cancer and neurodegeneration and finally the tumor suppressor genes, p53 and ASPP2, which become the focus of the remainder of the thesis.

Table 1 – Some biological links between cancer and neurodegeneration.

Gene or pathway	Role in cancer	Comments	Role in neurodegeneration	Comments
Cell cycle	Inappropriate entry → transformation	Any cycling cell has the potential to transform.	Inappropriate entry → apoptosis	Neurons have no potential to transform.
Apoptotic pathways	Loss of function → oncogenic	Cancer cells escape checkpoints and apoptosis.	Loss of function → neuroprotective	Death is only possible outcome for neurons committed to cell cycle
p53	Loss of function → oncogenic	Less p53 activity leads to higher risk of cancer.	Loss of function → neuroprotective	Less p53 activity leads to less degeneration.
Parkin	Loss of function → oncogenic	Deleted in breast, ovarian and adenocarcinoma	Loss of function → neuronal loss	Dysfunctional UPS → buildup of pro-mitotic proteins
ATM (ataxia–telangiectasia)	Loss of function → oncogenic	Deleted in breast and hematologic cancers	Loss of function → neuronal loss	Degeneration of certain cerebellar cells
Wnt cell survival pathway	Upregulation → oncogenic	Upregulated in colorectal, lung, breast and prostate	Loss of function → neuronal loss	Aβ-induced neurotoxicity in AD → loss of Wnt activity
Pin1	Upregulation → oncogenic	Upregulated in many human cancers	Loss of function → neuronal loss	Deficiency in AD→ dysfunctional tau and accumulation of Aβ
Ubiquitin proteasome system	Upregulation → oncogenic	Upregulated in myeloma and other cancers	Loss of function → neuronal loss	Dysfunctional in PD, AD and Huntington's disease
APP	Possible oncogene	Increased risk of leukemia in Trisomy 21	Precursor for Aβ → AD	Abnormal APP processing → AD

Table 1.1
Biological links between cancer and neurodegeneration.
Adapted from (Driver, 2012).

PART I: Neurodegenerative Disease

1.1 Introduction

Neurodegeneration is the inexorable loss of cells and function of the nervous system that occurs with both disease states and aging. While this thesis focuses on

general degenerative processes that are common to a variety of disease states, it is nevertheless important to appreciate the diverse etiologies and initiators of each disease with a focus on remaining therapeutic challenges. Four neurodegenerative diseases will be detailed that are relevant to Chapters 3-6, including multiple sclerosis (MS), viral encephalitis, AD, and ALS.

1.2 Multiple sclerosis

MS is a chronic autoimmune disease specifically targeting myelinated axons in the central nervous system (CNS) (Calabresi, 2004). The main pathophysiology includes demyelination and axon destruction that results in loss of nerve conduction and signaling. It is estimated that MS affects more than 2 million people worldwide (Compston and Coles, 2008; McFarland and Martin, 2007). Twice as many women are affected as men and it typically presents in adults between 20-45 years of age. Patients present with diverse symptoms and have varied levels of symptom manifestation, making it challenging to effectively treat. While the cause is unknown, it is thought to be a combination of genetic susceptibility and an environmental trigger, such as a virus or metabolic dysfunction that results in immune attacks on the CNS (Goldenberg, 2012). The dominant theory of MS is that it is mediated by the dysregulation of myelin-specific T-lymphocytes (Martin et al., 1992; McFarland and Martin, 2007). Demyelination in this theory is caused by the invasion of autoreactive T-cells into the brain that provoke a local immune response. Support for this theory comes from studies reporting an increase in myelin-specific T-cell reactivity in MS (Martin and McFarland, 1995). Although the symptoms of MS vary greatly among patients, general symptoms include, sensory and motor dysfunction, visual loss, gait disturbances, and tremors (Compston and Coles, 2008). Demyelination leads to

degradation of axons and lesions in gray and white matter (Evangelou et al., 2000). Invasion of inflammatory cells, including macrophages, T- and B-cells, are prominent features of MS (Lassmann et al., 2007).

In terms of treatment, before 1993 the primary therapy treated acute exacerbations of MS with corticosteroids, but no treatments were available to alter the course of the illness. Interferon injectables were released in the mid-1990s and have remained the main MS treatment for the past 20 years. While they reduce the frequency of relapses, they also incur several side effects (Walther and Hohlfeld, 1999). Since 2010 many new disease-modifying drugs have been developed, including 5 new agents and 1 dosage formulation (English and Aloi, 2015). While these therapies offer many advantages, they incur substantial cost and some adverse side effects. Natalizumab, a selective monoclonal antibody against the α4 integrin receptor on leukocytes that blocks their entry into the CNS, remains the most robust second-line treatment (Rudick et al., 2013). Fingolimod is another drug that alters the sphingosine-1-phosphate receptor lymphocytes and prevents them from escaping lymph nodes and crossing the blood-brain barrier (BBB) (Mandala et al., 2002). Alemtuzumab is a monoclonal antibody against CD52 found on mature lymphocytes and works to deplete lymphocytes and reduce replaces (Coles et al., 2012). Despite much progress in the development of these disease modifying therapies, they have limited efficacy in late stages of MS and do not improve structural recovery nor remyelination.

1.3 Viral encephalitis

Encephalitis is a general term for acute, typically diffuse, inflammation of the brain. Of the pathogens reported to cause encephalitis, viruses are the most

common. Acute viral infections, such as herpes simplex virus (HSV), human immunodeficiency virus (HIV), West Nile, and Nipah virus can prompt infections in the CNS resulting in encephalitis. HSV is the most common cause of encephalitis in the Western world (Kennedy, 2004). However, the etiology of encephalitis remains unknown in most patients. The distribution and frequency of various viruses that cause encephalitis varies considerably with geographical region. It is approximated that about 2,000 cases occur annually in the USA (Whitley et al., 1982) and worldwide viral encephalitis has an incidence of about one case per million per year (Johnston, 1998).

Prognosis and prophylaxis of encephalitis is often based on the identification of the specific causative agent. Often diagnosis is based on epidemiological clues such as the season of the year, prevalence of the disease in the local community, insect or animal contact, and immune status of the patient (Tunkel et al., 2008). Many of these viruses, including HIV, do not infect neurons, thus the CNS pathologies arise from astrocytes and microglia cells that produce neurotoxins, cytokines, and chemokines (Fischer-Smith and Rappaport, 2005). Although there is no definitive treatment in many cases of encephalitis, treatment options exist for specific viral agents. Acyclovir is the most effective anti-viral therapy that reduces mortality and morbidity for HSV infected patients (Chaudhuri, 2002). Corticosteroids and surgical intervention are other options that can be effective in particular circumstances (Chaudhuri, 2002).

1.4 Alzheimer's disease

AD is the most common form of dementia and is estimated to affect about 5 million in the USA and 44 million worldwide (Jorm et al., 2008). AD cost the world

$604 billion in 2010 alone (Wimo and Prince, 2010) and is characterized by increasing loss of memory and other cognitive functions. Atrophy of specific brain regions and neuronal subtypes have been identified, including the CA1 region of the hippocampus and the pyramidal cell of lamina II of the entorhinal cortex (Gómez-Isla et al., 1996; West et al., 1994). However, the majority of brain volume loss is due to shrinkage and atrophy of neuronal processes. Studies employing radiological imaging with magnetic resonance imaging (MRI) and functional MRI (fMRI) have revealed that neuronal function is not simply lost in AD, but neural network activity is aberrant and may underlie the disruption of learning, memory, and other cognitive functions (Palop and Mucke, 2010).

It is estimated that 95-99% of cases are sporadic due to a combination of genetic, epigenetic, and environmental factors, while the remaining cases comprise early-onset (<60 years) familial autosomal dominant AD which are attributed to mutations in the genes *amyloid precursor protein* (*APP*), *presenilin* (*PS*)-*1* and *PS-2* (Bertram et al., 2010). These mutations result in abnormal APP processing and altered production of Aβ peptides (Bertram et al., 2010). In support of the "amyloid cascade hypothesis," Down's syndrome patients possessing an extra copy of chromosome 21, on which the *APP* gene resides, develop early-onset dementia with characteristics of AD (Millan Sanchez et al., 2012). Additionally, individuals with duplication of the *APP* gene also possess early-onset AD and accumulation of Aβ peptides (Rovelet-Lecrux et al., 2006). The "amyloid cascade hypothesis" is also supported by evidence that Aβ accumulation results in a number of neuronal and astrocyte defects, including aberrant neuronal network activity and synaptic depression (Palop and Mucke, 2010).

In terms of late-onset (>60 years) AD genetics, apolipoprotein (apo) E4 is linked to AD in a gene-dose effect (Bertram et al., 2010). Genome-wide association studies (GWAS) found that apoE4 is the main gene associated with age-related cognitive decline in humans (Genin et al., 2011). Clusterin has also been identified as a second main susceptibility factor for AD (Harold et al., 2009; Lambert et al., 2009).

Neurofibrillary tangles are another pathological feature of AD consisting mainly of intracellular tau protein. Tau is a microtubule-binding protein that misfolds in AD and becomes abnormally phosphorylated (Goedert et al., 2006; Lee et al., 2001). While the number of neurofibrillary tangles correlates with the degree of dementia (Crystal et al., 1988), genetic studies suggest that tauopathy is downstream of the Aβ cascade (Hardy and Selkoe, 2002). Astrocyte dysfunction is also implicated in AD. An impairment in glutamate transporter, GLT1 results in insufficient glutamate uptake by astrocytes and neuron excitotoxicty (Huang and Mucke, 2012). Additionally, astrocyte dysfunction may contribute to initial neuronal deficits in AD as Aβ disrupts astrocyte calcium signaling and gliotramsmitter release (Vincent et al., 2010).

In terms of treatment, acetylcholine esterase inhibitors are currently the primary treatment for AD with transient and modest benefits. They function by increasing the levels of the neurotransmitter acetylcholine, which is depleted in AD brains. The inhibitors also antagonize glutamate receptors and prevent excess neuronal stimulation (Cummings, 2004). Unfortunately, there is little support that these drugs provide much benefit to AD patients beyond increasing the amount of time before patients require nursing home care if they are taken in combination with the NMDA receptor antagonist, memantine (Lopez et al., 2009). Pioneering clinical trials have sought to use therapies to increase Aβ clearance or decrease its production.

Inhibitors of γ-secretase, an enzyme that releases Aβ from its precursor have been developed. However, many of these trials have been halted due to efficacy and safety concerns, including the development of squamous cell carcinoma in the case of Semagacestat (Doody et al., 2013).

In addition to targeting Aβ, much effort has been spent to develop drugs that target tau pathology and apoE4. Tau phosphorylation is known to be induced by glycogen synthase kinsase-3 (GSK-3) and increasing evidence suggests that GSK-3 is involved in the amyloid cascade. GSK-3 was found to be upregulated in AD (Hye et al., 2005), suggesting it may be a useful biomarker. Interestingly, GSK-3 induces negative effects on learning and memory via impaired long-term potentiation (Hooper et al., 2007). Additionally, inhibition of GSK-3 recued Aβ pathology in a drosophila model of AD (Sofola et al., 2010). Thus, investigation of GSK-3 inhibitors have been the focus of clinical trials (Lovestone et al., 2014; Medina and Avila, 2010). Other approaches such as antioxidant therapy and anti-inflammatory drugs have been investigated to halt the oxidative damage and inflammatory components of AD. However, little success has been made in this area (Imbimbo et al., 2010). Additional promising strategies involve the use of therapies targeting modulators of aging including autophagy pathways (Harris and Rubinsztein, 2012), which is discussed further in Part II (Introduction 1.11).

1.5 Amyotrophic lateral sclerosis

ALS also referred to as Lou Gehrig's disease or “motor neuron disease” is a progressive neurodegenerative disorder characterized by the loss of upper and lower motor neurons initiating in mid-adult life. It is estimated to affect about 500,000 individuals in the USA and estimated costs are $256-$433 million per year

(Larkindale et al., 2014). Many hypotheses have been proposed regarding the cause of ALS, including chemical exposure, trauma, and physical activity. ALS affects white males aged >60 more than any other group (Mehta et al., 2014). Motor neurons from the brain stem, spinal cord, and cerebral cortex are primarily affected resulting in muscle paralysis and atrophy. Currently of unknown etiology, the disease is fatal within an average of 3-5 years after diagnosis typically due to respiratory failure (Shoesmith et al., 2007).

About 90-95% of cases are considered sporadic with the remainder identified as hereditary or familial (fALS). In 1993 a landmark discovery commenced the molecular era of ALS research by identifying mutations in the gene encoding superoxide dismutase 1 (SOD1) (Rosen et al., 1993). However, the strongest genetic risk factor for ALS to date is a hexanucleotide repeat within the noncoding region of the C9ORF72 gene and accounts for nearly 50% of fALS cases in Finland, 30% in Europe, and about 20% in the USA (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Missense mutations in SOD1 are implicated in around 20% of fALS cases (Bradley WG, 1995). The normal function of SOD1 is to convert highly reactive superoxide to hydrogen peroxide or oxygen. The SOD1 mutant mouse model recapitulates paralysis seen in human patients and has been useful for elucidating fALS disease mechanisms.

One of the most important findings arising from SOD1 mutant mouse model is that rather than reduced SOD1 activity, it is the accumulation of toxicities of the mutant protein that propagates symptoms (Turner and Talbot, 2008). Additionally, SOD1 mutants generally fail to fold properly suggesting the accumulation of misfolded SOD1 protein may mediate toxicity in ALS (Bruijn et al., 1997). However,

despite this success, much controversy still exists regarding the primary reason for mutant SOD1 toxicity. The most well supported hypotheses include the role of excitotoxic glutamate release, oxidative stress, protein misfolding and aggregation, neuroinflammation, and microhemmorrhages from spinal capillaries as main contributors (Ilieva et al., 2009). Many of these processes are regulated by astrocytes and several studies support astrocyte dysfunction as a direct contributor to non-cell autonomous neurotoxicity (Ilieva et al., 2009). Indeed, therapies targeting astrocytes have emerged as an important strategy to delay or inhibit the progression of ALS and as well as other neurodegenerative diseases (Colin et al., 2009; Furman et al., 2012; Pehar et al., 2014; Vargas et al., 2008; Verkhratsky et al., 2012). Currently the only FDA-approved drug for ALS providing modest benefit is Riluzole, which targets astrocyte glutamate transporter, excitatory amino acid transporter-2 (EAAT2) (Miller et al., 2012) and induces release of neurotrophic factors (Mizuta et al., 2001). In addition to the development of anti-gluatamatergic agents other approaches include agents targeting protein misfolding, autophagy, anti-apoptosis, antioxidants, and neurotrophic factors (Peters et al., 2015).

PART II: Common Mechanisms Underlying Cancer and Neurodegeneration

1.6 Introduction

To begin to untangle common mechanisms underlying cancer and neurodegeneration, one can view both diseases from the context of aging. The progressive decline in tissue and cellular function during aging is nearly a universal feature of biological organisms. What propels aging? Evolutionary theory postulates that aging is the result of the diminishing force of natural selection (Rose et al., 1991). A natural depletion in the older individuals in a population occurs due to the accumulation of extrinsic hazards, such as infection and starvation. As a consequence, there are generally fewer older individuals on which natural selection acts. Aging also increases susceptibility to a wide range of pathologies, which can be broadly categorized as hyperplasic or degenerative (Campisi et al., 2011). Hyperplasia encompasses gain-of-function diseases such as atherosclerosis and cancer, which involve the accumulation of cells and often additional cellular functions. Degeneration, by contrast, is characterized by a loss of tissue and cellular function and includes cardiovascular failure and neurodegenerative diseases (**Figure 1.1**).

An emergent view suggests that aging itself may be more than just a risk factor for these disorders, but may in fact be the underpinning driving force. This perspective adds an incentive to create therapies that delay or inhibit the progression of physiological aging processes to alleviate age-related conditions, including neurodegeneration. One central question in aging research is whether common biological processes underlie hyperplasic and degenerative pathologies

(**Figure 1.1**). Here I will review four key common mechanisms: inflammation, loss of cell polarity, apoptosis, cellular senescence, and autophagy. While these four processes involve dynamic interactions between multiple cell types, I will specifically focus on the role of astrocyte-neuron interactions in the CNS.

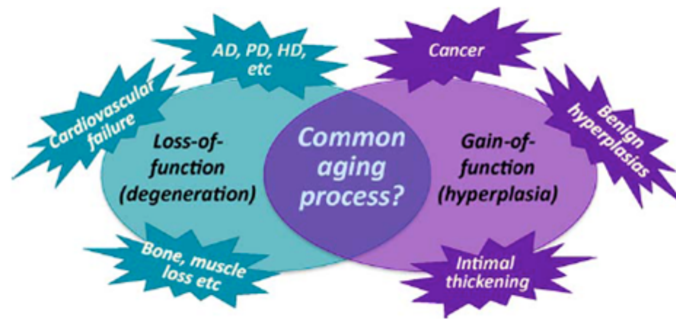


Figure 1.1
Is there a common aging process?
Schematic demonstrating the possible relationship among age-related diseases that are classified as either degenerative or hyperplasic. Adapted from (Campisi et al., 2011).

1.7 Inflammation

A low level of chronic inflammation is associated with aging in most tissues in the body including the CNS (Chung et al., 2009; Francesschi et al., 2007). While inflammation evolved to eliminate pathogens, damaged organelles, or invading foreign bodies, prolonged inflammation can have deleterious effects. Acute inflammation allows a short-lived burst of oxidative and nitrosative stress locally. However, chronic inflammation is often a long-lived and self-perpetuating response (Nathan and Ding, 2010). A key source of inflammation in the brain in both the context of aging and neurodegeneration are glia cells, which include astrocytes, microglia and oligodentrocytes. While astrocytes and oligodendrocytes originate

from neural progenitor cells, microglia are the resident macrophage population of the CNS and arise from myeloid progenitor cells (Ginhoux et al., 2013) (**Figure 1.2**).

Regardless of their origin, activation of glia underlies the neuroinflammatory response in acute CNS injury, stroke, MS, as well as chronic neurodegenerative diseases, such AD and ALS (Glass et al., 2010). Activation of glia is characterized by the release of inflammatory mediators such as chemokines and cytokines. Glia provide many types of support to neurons that are often disrupted in neurodegeneration, including neurotrophic and metabolic support, and regulation of glutamate and brain pH (Benarroch, 2005). Indeed the switch from neuroprotective to pro-inflammatory glia underlies neuronal loss in several neurodegenerative disorders (Colin et al., 2009; Furman et al., 2012; Pehar et al., 2014; Vargas et al., 2008; Verkhratsky et al., 2012). Next I will detail two specific types of inflammatory signaling in glia: toll-like receptor and interferon signaling.

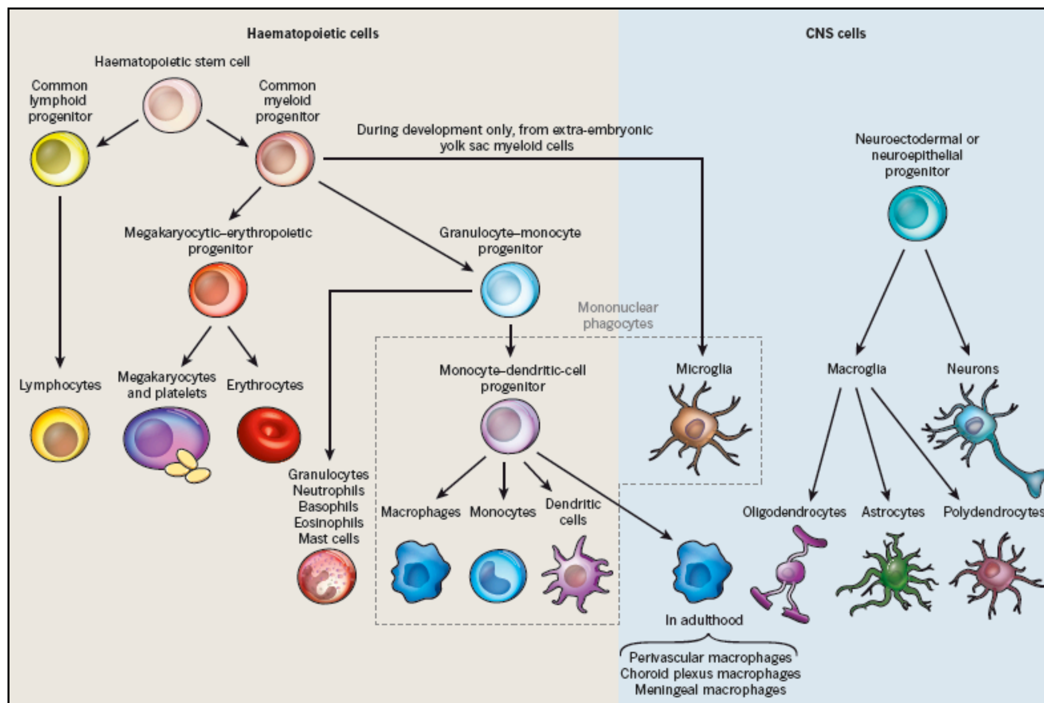


Figure 1.2
Origin of CNS glial cells.
Cells capable of inflammatory responses include astrocytes, oligodendrocytes, and microglia. Note that microglia are derived from myeloid progenitors and are the only hematopoietic cell type found in the parenchyma of the CNS.
Adapted from (Ransohoff and Cardona, 2010).

1.7.1 Toll like-receptors

Innate immunity is mediated by glial cells, particularly astrocytes and microglia, which are essential for the initial control of pathogen replication and activation of the adaptive immune system to promote pathogen recognition and clearance (Bailey et al., 2006). Glia regulate these processes in part through toll-like receptor (TLR) signaling. Mammalian TLRs are type I transmembrane receptors that recognize microbial pathogen-associated molecular patterns (PAMPs) of viruses, bacteria, yeast, parasites and fungi (**Figure 1.3**) (Akira and Takeda, 2004). At least 13 TLR genes have been identified in mammals.

Tight regulation of the TLR pathway is vital for maintaining homeostasis as TLR activation is linked to several infectious and inflammatory diseases. TLR signaling culminates in the activation of transcription factors such as nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT1), and activator protein (AP)-1, which regulate the induction of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), Interleukin(IL)-6 and IL-1β and recruitment of circulating leukocytes into the brain (Akira and Takeda, 2004). TLRs 1-9 are detected in the CNS under non-inflammatory conditions, but are upregulated by bacterial and viral infection (Bsibsi et al., 2002). While stimulation of TLRs on glial cells can activate essential functions for pathogen elimination, overactivation can lead to toxicity, especially in post-mitotic cells, such as neurons. In mouse models, systemic injection of TLR4 ligand, lipopolysaccharide (LPS), a component of gram-negative bacteria, leads to progressive neurodegeneration (Castaño et al., 1998; Qin et al., 2007). In humans increasing evidence implicates TLR4 in a number of neurodegenerative diseases and

CNS injury (Okun et al., 2009). For instance, TLR4 binds fibrillary Aβ and regulates glial activation (Reed-Geaghan et al., 2009).

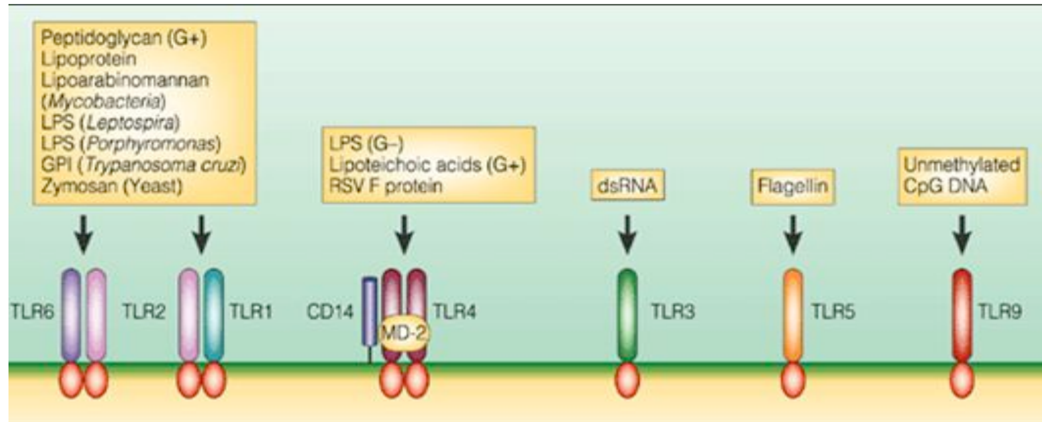


Figure 1.3
Toll-like receptors and ligands.
Toll-like receptors (TLRs) recognize a host of pathogen associated molecular patterns (PAMPs). Of particular note, a component of gram-negative (G-) bacteria, lipopolysaccharide (LPS) is recognized by TLR4 which requires two accessory proteins, CD14 and MD-2. Other ligands include double-stranded DNA (dsRNA) for TLR3, bacterial flagellin for TLR5, unmethylated CpGs found in bacterial DNA for TLR9. G+, Gram-positive; G–, Gram negative; GPI, glycophosphoinositol; RSV, respiratory syncytial virus. Adapted from (Medzhitov, 2001).

1.7.2 Interferon signaling

Another important component of the innate immune system in addition to TLRs, are interferons (IFNs), which are also associated with cognitive deficiencies that occur with aging and neurodegeneration (Dempsey, 2014). IFNs are specifically mediators of anti-viral immunity, but also have anti-proliferative and pro-apoptotic activities. Type I IFNs are produced as a first-line response to viral infection in almost every human cell type. Multiple family members (seven in human) act through a single IFN receptor (**Figure 1.4**) and converge on the transcriptional activation of an antiviral or inflammatory response through transcription factors, STAT1 and STAT3, which bind to gamma-activated sequences (GAS) or interferon-stimulated gene factor (ISRE) elements. The most well characterized family members in the CNS are IFN-α and –β. IFN-γ is the only member of the Type II IFNs. The role of viral infection and excessive IFN production in neurodegeneration is underscored by animal models of MS (Hemmer et al., 2002; van der Star et al., 2012), as well as IFN transgenic mouse models. For instance, transgenic mice producing IFN-α in glial fibrillary acidic protein (GFAP)-expressing astrocytes develop progressive neurodegeneration (Akwa et al., 1998). Also, a recent report showed that older mice display altered expression profiles of genes encoding IFNs in the choroid plexus and similar expression profiles were found in postmortem sections of aged human brains (Baruch et al., 2014), demonstrating that IFN signaling is important for CNS innate immunity in aging and neurodegeneration.

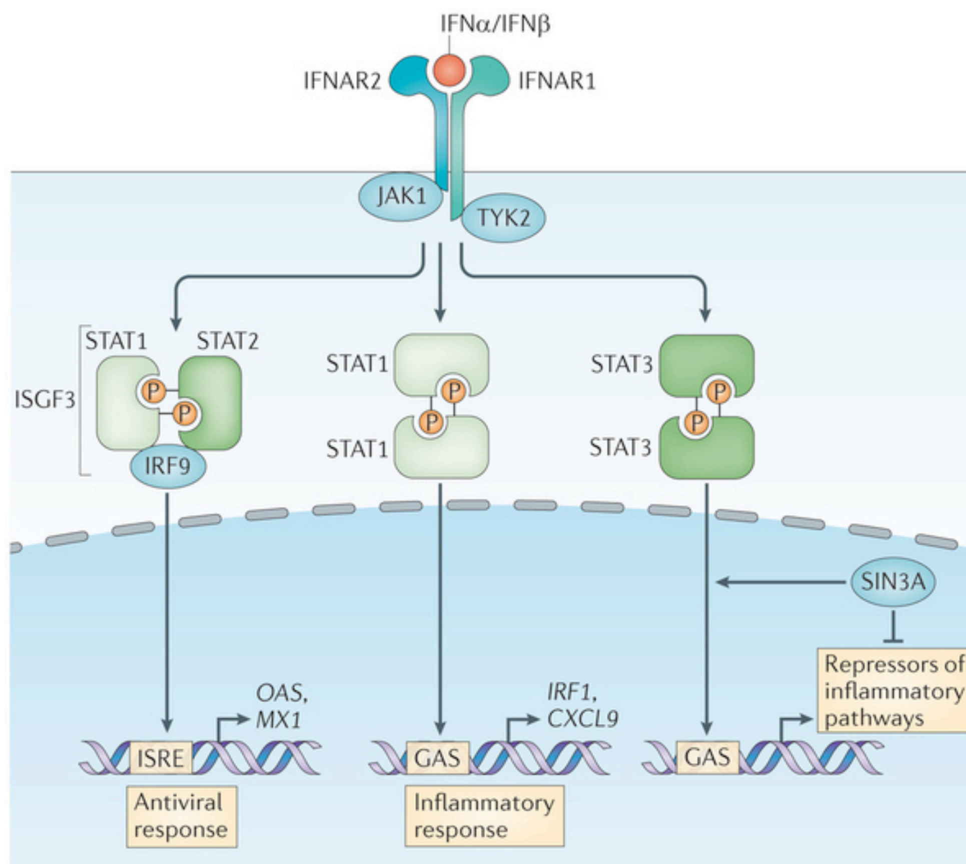


Figure 1.4
Canonical IFN Type 1 signaling pathway.
The interferon-α receptor is composed of 2 subunits (IFNAR1 and 2). Upon binding of IFN, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) are activated. Phosphorylation of the receptor by these kinases recruits signal transducer and activator of transcription (STAT) proteins to phosphorylate, dimerize, and translocate to the nucleus. There are three main STAT complexes that form and control separate gene-expression programs. The interferon-stimulated gene factor 3 (ISRE3) complex binds to IFN-stimulated response element (ISRE) to activate the antiviral response. Another complex made of STAT1 homodimers binds to gamma-activated sequences (GASs) to initiate the inflammatory response. Finally, STAT3 homodimers can indirectly suppress pro-inflammatory gene expression via an unknown mechanism. The STAT3 complex can bind SIN3A which suppresses STAT3 target genes. Adapted from (Ivashkiv and Donlin, 2014).

1.8 Cellular polarity

Cell polarity is the asymmetric distribution of proteins, RNA or lipids in a cell (Wolpert, 2013). The majority of animal cells are polarized. Polarization can be morphologically visible or only apparent at the functional and molecular level. For instance, the polarization of neurons can be appreciated by the presence of a single protruding axon from a cell body. In the case of progenitor cells, unequal partitioning of cell fate determinants occurs upon differentiation to dictate the function and identify of daughter cells. Given that cell polarity serves such vital importance to cell function and identity it is unsurprising that a loss of polarity is a hallmark of developmental defects and diseases states, including cancer and neurodegeneration. Loss of cell polarity represents an important link between neurodegeneration and cancer. In epithelial cancers, loss of cell polarity is a hallmark of cancer malignancy (Royer and Lu, 2011) and often associates with tumor-infiltrating lymphocytes and inflammation (Shaykhiev and Bals, 2007). Likewise, loss of brain barrier function prompted by neuroinflammation is linked to neurodegenerative disease onset and progression (Bednarczyk and Lukasiuk, 2011; Coisne and Engelhardt, 2011).

1.8.1 Brain barriers

The blood brain barrier (BBB) and the blood cerebral spinal fluid barrier (BCSFB) function to establish homeostasis in the CNS by limiting the influence of the blood stream. Importantly, the BBB and BCSFB are the brain's main barriers to infection (Coisne and Engelhardt, 2011). A third barrier, the blood-spinal cord barrier, (BSCB) is the morphological extension of the BBB into the spinal cord. The

BBB is composed of endothelial cells of microvessels within the CNS surrounded by perivascular macrophages and astrocyte end feet. The BCSFB is localized to epithelial cells of the choroid plexus.

The initial discovery of these brain barriers initially led to the concept that the brain is an immune-privileged site and that the barriers were impermeable. However, early transport studies demonstrating that the BBB regulated active transport of ions (Davson, 1976), glucose (Yudilevich and De Rose, 1971), and amino acids (Oldendorf, 1973), revealed the presence of diverse and highly specialized transport systems for particular substrates. Additionally, it is now known that migration of immune cells across the brain barrier is required for normal immune surveillance in the CNS (Engelhardt and Coisne, 2011).

While in the healthy brain, the BBB patrols entry of red blood cells, leukocytes, and plasma components, in disease and injury states including stroke, hemorrhage, and neurodegeneration, the permeability increases as the junctional complexes between adjacent cells are disrupted (Zlokovic, 2008). Previous studies have shown that a loss of cell polarity at these barriers prompts inflammatory changes, including the intrusion of immune cells and activation of microglia and astrocytes, which contribute to neurodegeneration (Bednarczyk and Lukasiuk, 2011; Coisne and Engelhardt, 2011). Alterations in the choroid plexus transcriptome (Marques et al., 2009) and BBB transcriptome (Rivest et al., 2000) in response to peripheral inflammatory stimuli demonstrates that the BBB and BCSFB themselves respond to disease states. Understanding the regulation of the brain barriers is thus essential for developing means to limit permeability in neurodegeneration and acute injury in which cerebral edema and increased intracranial pressure can be fatal.

1.8.2 Tight and adherents junctions

While the effect of polarization in different cell types can serve a variety of purposes, interestingly only a small set of factors determine polarity. Endothelial and epithelial cells of the BBB and BCSFB, similar to other highly polarized cell types, such as colon epithelial cells, respond to pathological stimuli through signaling at tight and adherens junctions. Tight junctions are cell-cell junctional complexes existing in the apical region of the cell membrane. Tight junctions of the BBB prevent the entry of large hydrophilic molecules including proteins across the BBB, but allow diffusion of small lipophilic molecules and small gas molecules, including oxygen and carbon dioxide (Abbott et al., 2006). Larger molecules that pass the BBB do so via specialized transporters on endothelial cell. Several transmembrane proteins compose tight junctions including, occluding, claudin, junctional adhesion molecules, and zona occludens (ZO), and the Par complex (Par3/Par6/aPKC) (Redzic, 2011). Loss of tight junction proteins is a hallmark of many neurodegenerative diseases (Zlokovic, 2008). For instance, in ALS the loss of occluding and claudin-5 within the capillary endothelial cells results in the disruption of the BSCB and promotes microhemorrhages within the spinal cord before the onset of disease. Additionally, in MS, the intrusion of activated immune cells in brain lesions is known to disrupt the BBB (Kirk et al., 2003). Thus, understanding the regulation of the tight and adherens junctions and identifying molecules that regulate these barriers remains an important challenge for the development of therapeutic targets.

1.9 Cellular senescence

Many features of aging derive from the inability of cells to adapt to stress and DNA damage. When damage begins to accumulate, mitotic cells from proliferative tissues undergo one of two mechanisms to avoid replication: cell cycle arrest (cellular senescence) or cell death programs. Cellular senescence, first discovered by Hayflick and Moorhead in 1961, was based on the observation that cells in culture could undergo a limited number of divisions, called the "Hayflick limit" (Hayflick and Moorhead, 1961). Beyond a loss of replicative ability, it has emerged that senescence also involves changes in gene expression, epigenetic regulation, and cell metabolism. In addition to replicative-induced senescence, stress-induced and oncogene-induced senescence have proven to be important biological processes underlying aging (**Figure 1.5**).

1.9.1 Senescence associated secretory phenotype (SASP)

Regardless of the senescence inducer, cells possess several characteristic phenotypes: an enlarged cytoplasm and flattened morphology, upregulation of pro-inflammatory cytokines, growth factors and proteases, which are collectively termed the senescence-associated secretory phenotype (SASP) (Coppe et al., 2010). Beyond SASP, hallmarks of senescence include upregulation of p53 target genes $p21^{WAF1}$ and miR-34a, β-galactosidase positivity, and γH2AX and HP1γ heterochromatin foci (Carnero et al., 2013). SASP is known to contribute to loss of tissue function that occurs with age. For instance, SASP can promote the following deleterious effects: epithelial-to-mesenchymal transition in normal and premalignant epithelium (Coppe et al., 2008, 2011), increase vascularization, and progression of tumors

(Coppe et al., 2010; Krtolica et al., 2001). SASP factors are generally upregulated at the transcript level as they are targets of several main inflammatory pathways including, NF-kB and CCAAT/enhancer binding protein (C/EBP) (Coppe et al., 2008; Freund et al., 2010). Also, DNA-damage response pathways are often also activated upstream of SASP factors, including p38 mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), and p53 (Coppe et al., 2008).

While deleterious roles of SASP have been described a variety of diseases including neurodegeneration, type 2 diabetes, and obesity, there are also several identified beneficial roles of cellular senescence (Muñoz-Espín and Serrano, 2014). For instance, "senescence surveillance "is a process by which chemokines and cytokines can facilitate the removal of tumor cells or communicate cellular damage to neighboring cells. Senescence induction has been recently reported to activate wound healing (Demaria et al., 2014; Krizhanovsky et al., 2008; Neves et al., 2015; Serrano, 2014). It is hypothesized that senescence may be a process that is beneficial in early life but detrimental during later stages, consistent with the evolutionary theory of antagonistic pleiotropy, (Williams, 1957). This theory is based on the idea that most organisms evolve in environments filled with hazards such as infection, starvation, and predation and thus aged individuals are rare and selected against. Age-associated disease may have escaped the force of natural selection. It follows that the processes that protect young organisms, such as tumor suppression, can be deleterious in old organisms.

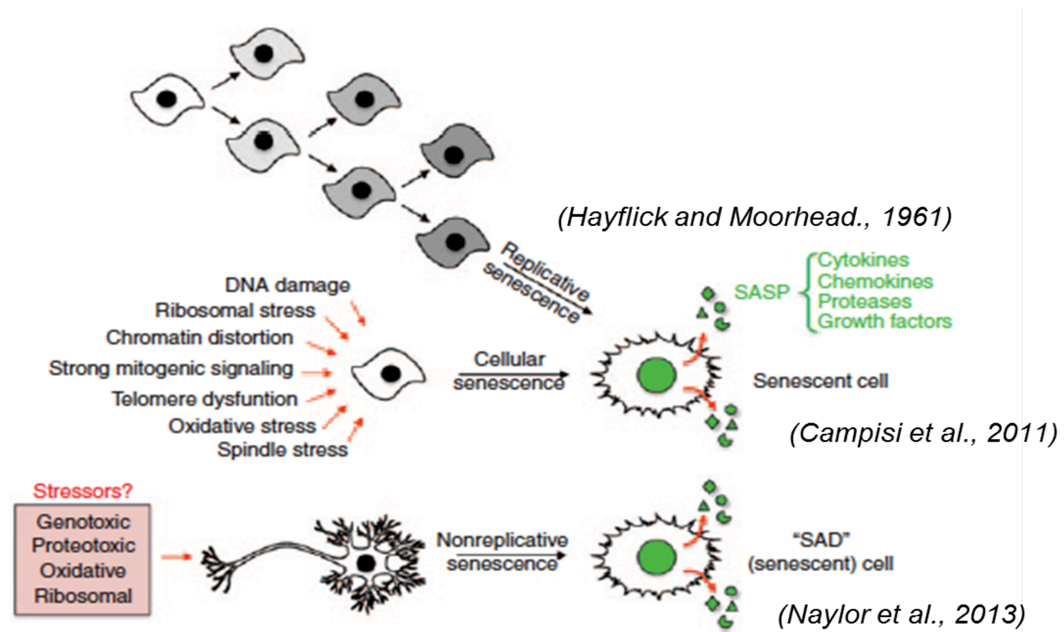


Figure 1.5
Three distinct inducers of cellular senescence.
Proliferative cells can undergo replicative and stress-induced senescence. Non-replicative senescence was recently identified and possible inducers are listed. Key papers identifying each type of senescence induction are indicated. Adapted from (Naylor et al., 2013).

1.9.2 Brain aging and neurodegeneration

Senescent cells are known to accumulate in a variety of tissues with age (Erusalimsky and Kurz, 2005; Dimri et al., 1995). In the brain, increased senescence is associated with both general aging and neurodegenerative disease (Tan et al., 2014; Salminen et al., 2011; Bhat et al., 2012; Campisi et al, 2011; Chinta et al., 2014) (**Figure 1.6**). While most evidence supports astrocyte senescence as a key feature of neurodegeneration, neuron senescence has also recently been reported as a type of non-replicative senescence (Jurk et al., 2012; Mattson et al., 2006; Naylor et al., 2013) (**Figure 1.5**).

Astrocytes exert both neuroprotective and neurodegenerative effects in a context-dependent manner, which are associated with either repression or induction of SASP, respectively. During aging and neurodegenerative disease, astrocytes switch from an anti-inflammatory, neuroprotective state to a pro-inflammatory, neurodegenerative state. Numerous studies demonstrate that astrocyte senescence originating either from ALS model SOD1 mutant mice (Christou et al., 2013; Das and Svendsen et al., 2014; Fritz et al., 2013; Nagai et al., 2007), senescence-accelerated prone mice (SAMP8) (Garcia-Matas et al., 2008), aged murine astrocytes *in vivo* and *in vitro* (Das and Svendsen et al., 2014; Pertusa et al., 2007; Papadopoulos et al., 1998; Gottfried et al., 2002), and those exposed to oxidative stress (Bitto et al., 2010) can promote non-cell autonomous neurotoxicity while simultaneously losing their neuroprotective functions.

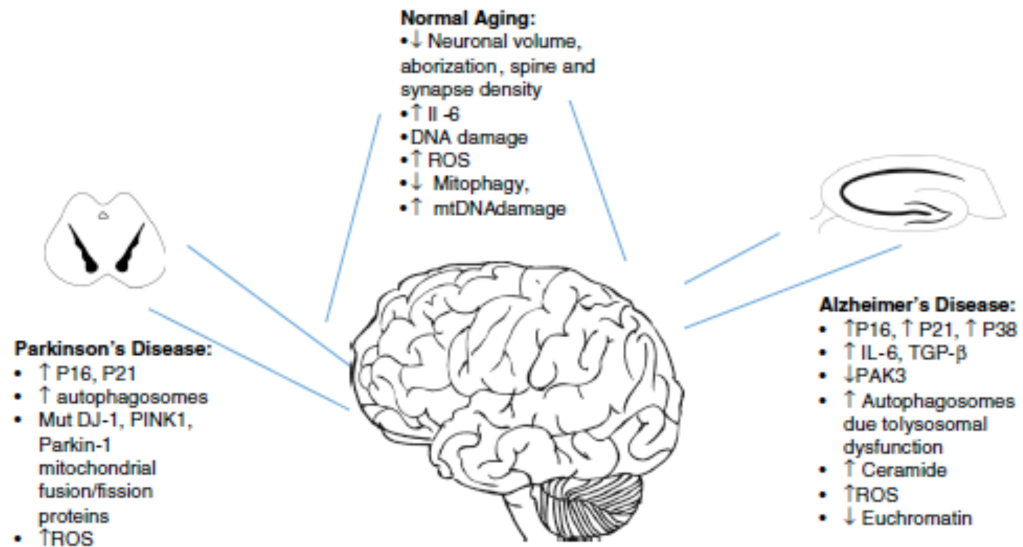


Figure 1.6
Cellular senescence in neurodegeneration and physiological brain aging.
Hallmarks of cellular senescence are increased in normal aging and neurodegenerative diseases, including AD and PD. Adapted from (Tan et al., 2014).

1.10 Apoptosis

The discovery of cell death pathways in the 20th century dispelled the idea that natural death is a passive process (Surova and Zhivotovsky, 2013). Cellular senescence (discussed in Introduction 1.9) and apoptosis are thought to be variants of the same process of cellular elimination with the evolutionary aim of increasing survival of an organism though controlled elimination of damaged or excess cells. In fact, the former is often referred to as “assisted cellular suicide” (Muñoz-Espín and Serrano, 2014). The initiation of both processes remains similar: in response to cellular damage, stress, oncogene signaling, cellular pruning, or during embryonic development, cells halt their proliferation machinery through the action of tumor suppressors, including p53. Programmed cell death, or apoptosis, is a physiologically important process in development, immunity, and tissue homeostasis. Apoptosis also plays essential roles during neurogenesis and CNS development (Li and Yuan, 1999). While too little apoptosis can promote cancer and autoimmune diseases; too much apoptosis can amplify ischemic conditions and drive neurodegeneration. Aberrant apoptosis is implicated in a number of neurodegenerative diseases (Okouchi et al., 2007) and human cancers (Hajra and Liu, 2004).

Generally, cell death is thought to be mediated by two distinct pathways: necrosis and apoptosis. However, variations of these pathways and others have been identified, such as necroptosis (Linkermann and Green, 2014; Re et al., 2014). Generally, apoptosis is a highly regulated mechanism of cell death characterized by chromatin condensation, DNA fragmentation, and cell shrinkage. While apoptosis is an energy-dependent process requiring ATP for the formation apoptosome, necrosis is cell death that occurs in the absence of ATP (Tsujimoto, 1997). Necrosis is

characterized by swelling and eventual disintegration of intracellular organelles, accompanied by disruption of the outer cellular membrane, and release of intracellular organelles into the intercellular space. Notably, apoptosis involves the activation of cysteine aspartate-specific proteases (caspase) cascades, whereas necrosis does not.

Caspases are responsible for the disintegration of a cell undergoing apoptosis (Thornberry and Lazebnik, 1998). They exist in the cytoplasm as an inactive zymogen form and are then activated by proteolysis (Ghavami et al., 2012). Two separate cascades have been identified as the extrinsic and intrinsic pathways (**Figure 1.7**). The extrinsic pathway is activated by a receptor-mediated pro-apoptotic signal, such as Fas. Following Fas binding caspase-8, the initiator caspase, is activated leading to the subsequent activation of caspase-3, the executioner caspase (Salvesen and Duckett, 2002). Caspase-3 has been implicated as an initiator of the cell death cascade and thus is typically used as a marker for cell entry into apoptosis. Its use as a apoptosis marker also arises from the fact that it is a convergence point for various apoptotic signaling cascades as it is activated by the upstream caspase-8 and caspase-9 (**Figure 1.7**) (Kujoth et al., 2005). The intrinsic or mitochondrial apoptotic pathway is known to be activated by stress and developmental apoptotic signals, which is initiated by the translocation of Bax and other Bcl-2 pro-apoptotic members. Following translocation, cytochrome c is released from the mitochondria to the cytosol and binds Apaf-1 to form the apoptosome (Cain et al., 1999). This formation activates caspase-9, which then also results in executioner caspase-3 activation (McIlwain et al., 2013).

The link between caspase activation and neurodegeneration comes from studies showing that genes known to increase susceptibility to early-onset AD, including *PS-1, PS-2, APP*, are caspase substrates (Wellington and Hayden, 2000). Indeed, caspase-3 cleavage of these proteins is thought to increase sensitivity to cell death in AD. Cleaved caspase-3 is upregulated in neurodegenerative disease, and thought to be the final effector in apoptosis of neurons (Hartmann et al., 2000; Zhao et al., 2003).

Astrocytes participate in non-cell autonomous neuronal cell death in neurodegeneration. Indeed, astrocyte cell death is emerging as a key component of neurodegeneration (Eddleston and Mucke, 1993). Signals that induce apoptosis in astrocytes *in vitro* and *in vivo* include oxidative stress, cytokines, and Aβ, glucose deprivation, and hypoxia, viral infection (Eddleston and Mucke, 1993). Astrocytes are essential to neuronal survival in several ways including astrocytic release of neurotrophins, regulation of extracellular ions and neurotransmitter levels, control of the blood-CNS interface, and repair of the extracellular matrix (Barres, 2008). Beyond these roles in the healthy CNS, astrocytes respond to injury and disease. While acute reactive astrocytosis at the early stage of CNS disease or infection can have a beneficial role on neurons by eradicating invading pathogens and providing trophic support, a chronic astrocytic response can contribute to neuronal loss (Lin et al., 1998).

Furthermore, astrocyte apoptosis can lead to the death of surrounding cells propagating neurodegeneration (Takuma et al., 2004). One mechanism by which astrocyte apoptosis is implicated in neurodegeneration is the loss of gap junctions between astrocytes and neurons. Gap junctions are composed of connexins subunits, which exchange glucose, inositol triphosphate, glutamate, and lactate. Preservation

of these junctions is essential to neuronal activity. Loss of gap junctions between neurons and astrocytes is associated with neuronal injury, hypoxia, glutamate insult. Also, blockade of astrocyte gap junctions increases neuronal vulnerability to oxidative stress (Takuma et al., 2004).

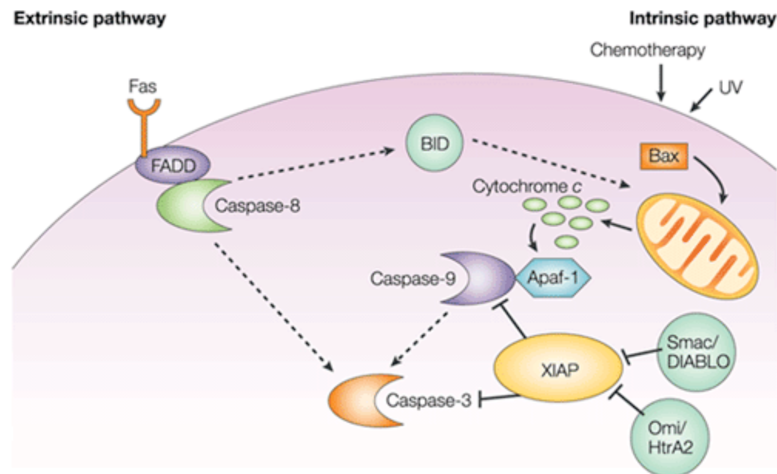


Figure 1.7
The role of caspase-3 in apoptosis.
The extrinsic and intrinsic cell death pathways show that both result in the activation of caspase-3 either through caspase-8 or caspase-9. FADD, Fas-associated death domain protein; UV, ultraviolet light; XIAP, X-linked IAP. Adapted from (Salvesen and Duckett, 2002).

1.11 Autophagy

Autophagy is a highly evolutionary conserved process used to recycle obsolete cellular components, eliminate protein aggregates, or damaged organelles (Mizushima et al., 2007). The term comes from the Greek words for "self" and "eating." As aging involves the loss of adaptive cellular mechanisms to eradicate stress, autophagic function is especially important to clear damaged cellular components. Three types of autophagy have been identified including macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy (or simply referred to as autophagy) (**Figure 1.8**) involves sequestering portions of the cytoplasm within a double-membrane vesicle, called an autophagosome. Autophagosomes are delivered to lysosomes for bulk degradation to renew cytoplasmic parts of the cell. The autophagy machinery involves a set of autophagy related (ATG) proteins, including ATG5, ATG7, and ATG12, Beclin-1, and microtubule-associated protein 1 light chain 3 (LC3), an autophagosome protein whose expression serves as an indicator of autophagic flux. Stages of macroautophagy include: 1) formation of the phagophore; 2) engulfment of cytoplasm by the phagophore; 3) eleongation of phagophore membrane; 4) fusion of phagophore membrane with the autophagosome to seal it; 5) Fusion of the autophagosome with the lysomsome to form autolysosome which prompts degradation of the internal membrane; and 6) release of breakdown products and recycling to the cytosol (**Figure 1.8**).

Autophagy has long been considered an essential but non-selective bulk degradation pathway. Recently, however selective autophagic elimination of

different cellular organelles or particular protein aggregates has been reported (Hubbard et al., 2012). Specific autophagy receptors are responsible for selective autophagy by attaching cargo to the location of autophagosome engulfment (Johansen and Lamark, 2011). Also selective autophagy targets a specific set of ubiquitinated substrates and serves as an alternative to proteasome-mediated degradation. p62/SQSTM1 binds LC3-II to enter the autophagosome and is then degraded as an autophagy substrate. p62/SQSTM1 also functions as a ubiquitein-binding receptor that is incorporated into autophagosomes and degraded via selective autophagy (Johansen and Lamark, 2011).

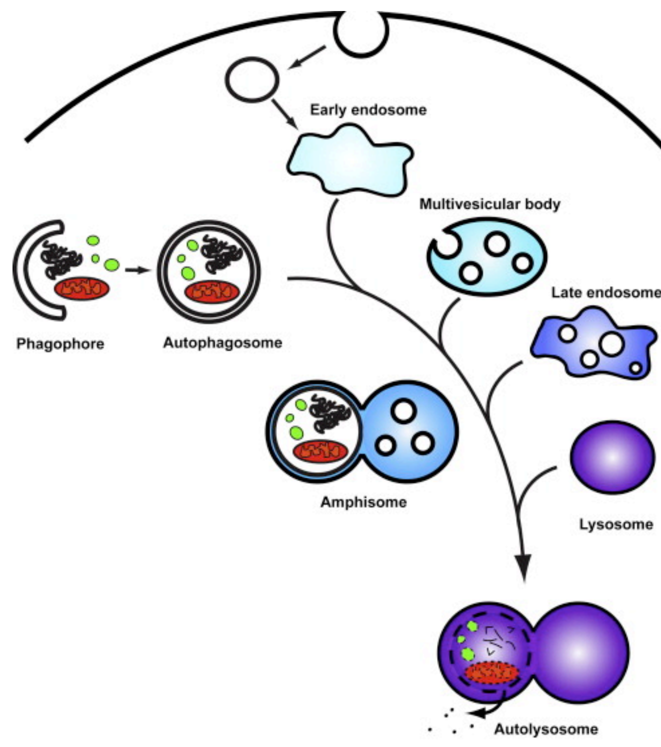


Figure 1.8
Macroautophagy.
Adapted from (Knaevelsrud and Simonsen, 2010)

Many neurodegenerative diseases are characterized by abnormal protein misfolding and aggregation of either diffusible oligomers, fibrils, or large protein aggregates, including AD, ALS, HD, and PD (Soto, 2003). The discovery that autophagy regulates intracytoplasmic aggregated proteins in neurodegeneration (Rubinsztein et al., 2012) lead to the investigation of autophagy as a treatment approach. Autophagy dysregulation including insufficient autophagy activation, reduced lysosome function, and autophagic stress related to pathological autophagy activation have been implicated in neurodegeneration (Cherra and Chu, 2008; Hochfeld et al., 2013). Autophagy inhibition is thought to impair flux through the ubiquitin-proteasome degradation pathway (Korolchuk et al., 2009). As autophagy is thought to be the main process by which protein aggregates are eliminated, methods to enhance autophagic degradation has been the focus of genetic and pharmacological studies to enhance this process in neurodegeneration (Harris and Rubinsztein, 2012; Rubinsztein et al., 2007). Stimulation of autophagy has shown to rescue some features of neurodegeneration in fly and mouse models (Menzies et al., 2010; Ravikumar et al., 2004). Although it is still unclear under what circumstances and stage of the disease autophagy stimulation would be optimal. Increasing autophagy may not always be beneficial. For instance, autophagy stimulation during the initiation phase upstream of Beclin-1 and ULK might worsen disease. Further work is needed to understand how precisely autophagy is disrupted in neurodegeneration. Also, clarifying how beneficial autophagy is converted to detrimental autophagy is the subject of intense research.

1.12 Therapeutic strategies and remaining challenges

While there are a common set of pathologies shared among disparate neurodegenerative diseases, both acute and chronic, there has been little success in formulating a unified theory of neurodegenerative pathology. This is in part due to the challenge of relating the various components. For example, enhanced oxidative stress can increase neuroinflammation and propagate protein aggregation. On the other hand, neuroinflammation might itself precede and produce other neurodegenerative disease pathologies. One conclusion to be made is that the etiology is a non-linear process involving many common starting points with certain processes (inflammation, loss of cell polarity, cellular senescence, apoptosis, and autophagy among others) interacting reciprocally and dynamically to produce a common neurodegenerative outcome (Hensley and Harris-White, 2015).

The development of useful animal models remains another major challenge in neurodegenerative disease research. Murine models of disease have long been recognized as a powerful tool to understand the genetics and pathophysiology of human disease (Justice et al., 2011). However, most neurodegenerative disease mouse models do not recapitulate all of the features of human disease, indicating that the underlying biology may differ between species. Importantly, in human neurodegenerative disease the process of aging is overlaid with a number of other mechanisms previously detailed. Indeed, diseases such as AD can manifest over decades. Most murine and cell culture models do not adequately capture this aging aspect due to the short life span of the species or the chronological "resetting" that occurs during reprogramming in the case of induced pluritpotent stem cell (iPSC) models (Garber, 2014).

The main advantage of murine models for neurodegeneration is the ease of manipulating the genome to create knockout and transgenic animals to elucidate the role of a particular gene in the onset of neurodegeneration. However, it still remains unclear how well these models capture the features of human disease pathology. Given that many clinical trials for neurodegenerative disease, particularly ALS, have failed in phase III trials after demonstrating efficacy in mouse models (Galimberti and Scarpini, 2011; Wilkins et al., 2011), it is still uncertain how useful the data obtained from these murine models is for the creation of new therapies. To improve the value of mouse models, it has been proposed to give further consideration to the heterogeneity and disease penetrance within a patient population. For example, if the disease is inherited in a Mendelian manner, the underlying genes causing the disease are known, and it is present with high penetrance then the value of a mouse model may be more promising (Lutz and Osborne, 2014).

Furthermore, the need to develop biomarkers of neurodegenerative disease is of particular importance from a clinical perspective for improved diagnosis and to monitor disease progression. To date, no solidly confirmed biomarkers for AD exist and MRI is the main tool for diagnostic and disease monitoring. Most of the early pre-clinical biomarkers that do exist require CSF extraction. While, CSF markers are a significant advance, problems with batch-to-batch assay variations continue (Mattsson et al., 2011) and stability of biomarkers is also a concern (Bateman et al., 2007). Much energy has focused on the use of blood based (Lovestone, 2014) and exosome-based biomarkers (Properzi et al., 2013). Promising plasma based AD signatures were recently identified from blood using 2 dimensional gel electrophoresis and mass spectrometry (Akuffo et al., 2008; Cutler et al., 2008; Hye

et al., 2006). Remaining challenges are the need for larger sample sets with accompanying clinical data, identification of true controls without pre-clinical pathology, and post-translational modifications of proteins (Lovestone, 2013). Nevertheless, plasma biomarkers are a promising strategy in combination with MRI and has already led to the identification of several key inflammatory and complement signature proteins (Furney et al., 2011; Ray et al., 2007).

PART III: Tumor Suppressor Pathway p53

1.13 Introduction

As the so-called "Guardian of the Genome" (Lane, DP, 1992), p53 remains one of the most well studied genes, with currently over 100,000 articles in PubMed. The interest in p53 in part is due to its ability to control a vast number of cellular processes, such as apoptosis, senescence, DNA repair, cell cycle arrest, and metabolism (Vogelstein et al., 2000; Levine and Oren, 2009) (**Figure 1.9**). Indeed, the activation of p53 is implicated in response to a wide variety of stressors and developmental processes, while p53 inactivation is an almost universal step in the development of human cancer. The most well characterized p53-mediated pathways are the cell death and cellular senescence pathways.

The p53 signaling pathway is emerging as a key regulator of both cancer and neurodegeneration. The association between mutations in *TP53* and cancer indicates that the inactivation of its function is linked to tumorigenesis (Symonds et al., 1994). By contrast, p53 is enhanced in the brains of AD patients (García-Ospina et al.; Kitamura et al., 1997; de la Monte et al., 1997; Ohyagi et al., 2005); and is thought to be associated with neuronal death (Cotman and Anderson, 1995; Miller et

al., 2000; Shimohama, 2000). Although the relationship between p53 and AD is not completely understood as few targets linking p53 transcriptional activation to AD pathology have been identified, several studies have associated Aβ production with p53 activity (Checler et al., 2010). Aβ increases the expression of p53 and its target, Bax. Also, p53 inhibitor, pifithrin-α, or knockdown of p53 abolishes the effects of Aβ, including activation of the apoptotic cascade and destabilization of the lysosomal membrane (Fogarty et al., 2010).

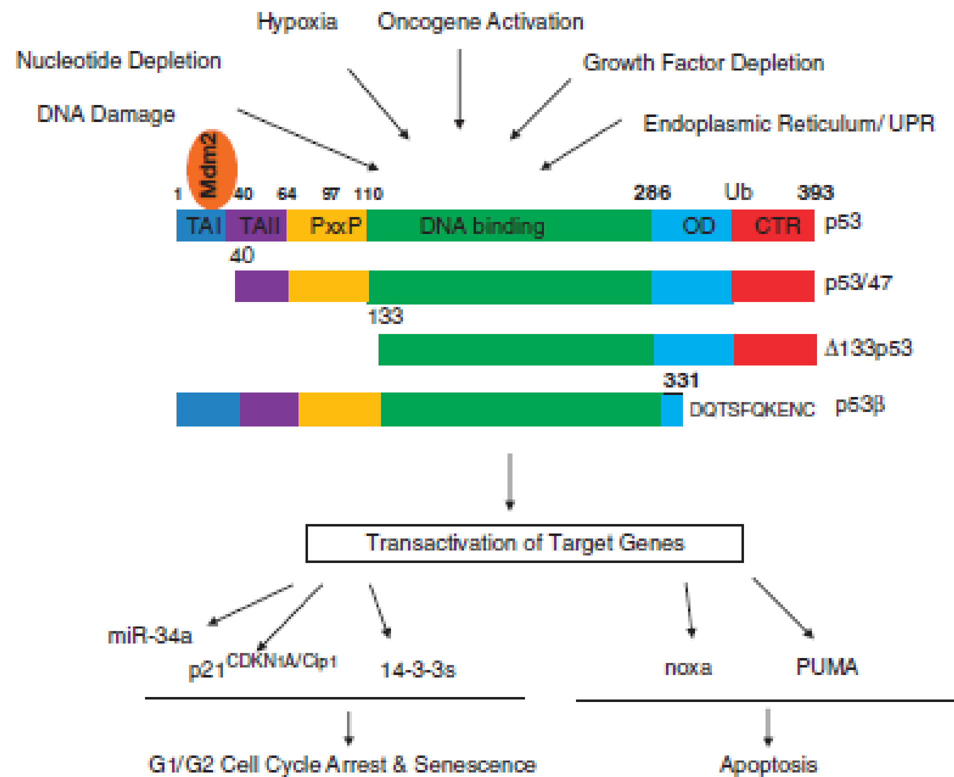


Figure 1.9
Roles of p53 activation.
Various types of stress and damage pathways converge on p53 leading to the transactivation of a host of gene products inducing apoptosis, cell cycle arrest, and senescence. p53 isoforms lack different domains of the full length protein, but all have an intact DNA binding domain. Of note, p53β possesses an alternative carboxyl-terminus that lacks most of the oligomerization domain (OD) and the carboxyl-terminal regulatory domain. Δ133p53 lacks the first 133 amino acids, including both transactivation domains (TAI and II) and the poly-proline-rich domain (PxxP). Adapted from (Olivares-Illana and Fåhraeus, 2010).

1.14 P53 isoforms

One aspect of p53 regulation that has eluded researchers in the field is understanding how cells carry out distinct cellular processes via a common p53 upstream activation pathway (Vousden and Prives, 2009). Some answers to this question have been gained after the identification of p53 isoforms, which are created through alternative splicing, alternative promoter usage, or alternative translation initiation sites (Bourdon JC, 2005). As these isoforms are truncated p53 proteins, their study has allowed the illumination of some of the most important questions plaguing p53 researchers. In addition to shedding light on how the diversity of responses is generated through a similar upstream activation, p53 isoforms have also elucidated the relationship between distinct p53 domains in terms of independent activities and biological functions. For instance, how p53 activation can selectively induce cellular senescence in a context-dependent manner was previously unknown. As cellular senescence is thought to a main mechanism by which p53 exerts its tumor suppression function, understanding how it is controlled is vital for the creation of cancer and other aging disease therapies.

The link between loss of senescence and tumor progression came from animal model studies demonstrating that halting senescence promoted more malignant tumor progression and also that senescent cells were found in pre-malignant tumor tissue that were progressively lost during tumorigenesis (Dankort et al., 2007; Collado et al., 2005; Braig et al., 2005). The link between p53 activation and cellular senescence arose from the finding that DNA damage agents induce senescence via the p53 and retinoblastoma (Rb) pathways. By contrast, senescence invoked by H-

Ras and p38MAPK activation is p53-independent and p16^{INC4}-dependent (Adams, 2009).

Within the p53-dependent senescence activation pathway, p53 isoforms, Δ133p53 and p53β, were identified as endogenous regulators of cellular senescence (Bourdon et al., 2005; Fujita et al., 2009; Mondal et al., 2013; Horikawa et al., 2014) (**Figure 1.10**). Δ133p53 is an N-terminal truncated isoform generated through alternative transcription initiation site in intron 4 of the p53 gene. Δ133p53 lacks the transactivation domain responsible for p53's interaction with mouse double minute 2 homolog (MDM2) (Picksley et al., 1994). Δ133p53 dominant-negatively inhibits FL-p53, and is a negative regulator of cellular senescence. While most isoforms are expressed in a tissue-specific manner Δ133p53 appears to be ubiquitously expressed (Bourdon et al., Genes Dev. 2005). However, aberrant Δ133p53 expression is found in a variety of tumors including breast (Bourdon et al., Genes Dev. 2005) and colon cancers (Fujita et al., 2009), suggesting that it may be implicated in tumor formation.

Δ133p53 is downregulated in response to p53-mediated replicative senescence, but not H-Ras-mediated oncogene-induced senescence (Fujita et al., 2009). Either knockdown or overexpression of the isoform regulates senescence through either inducing or downregulating the expression of p53 target genes, respectively. Δ133p53 downregulation during replicative senescence occurs only at the protein level and not at the mRNA level. Recent reports, suggest that Δ133p53 acts in a promoter-specific manner and independently of FL-p53. Most interestingly, overexpression of Δ133p53 extends the replicative lifespan of normal human fibroblasts, suggesting a possible means to reverse age-induced senescence (Fujita

et al., 2009). Although the mechanism for life extension is not completely understood, there are a few reports suggesting that it can function as transcription factor independently of FL-p53. In fact, a recent report showed that Δ133p53 initiates the transcription of DNA-repair genes such as Rad51 (Gong et al., 2015). Additionally, upregulation of Δ133p53 *in vitro* inhibits apoptosis and G1 cell cycle arrest mediated by FL-p53. However, G2 cell cycle arrest is not affected (Aoubala et al., 2011). Thus, it is clear that Δ133p53 dominate-negatively inhibits only select p53 activities. Another possibility is that Δ133p53 provides a proliferative advantage through its interaction with p63 and p73 (Stiewe et al., 2007).

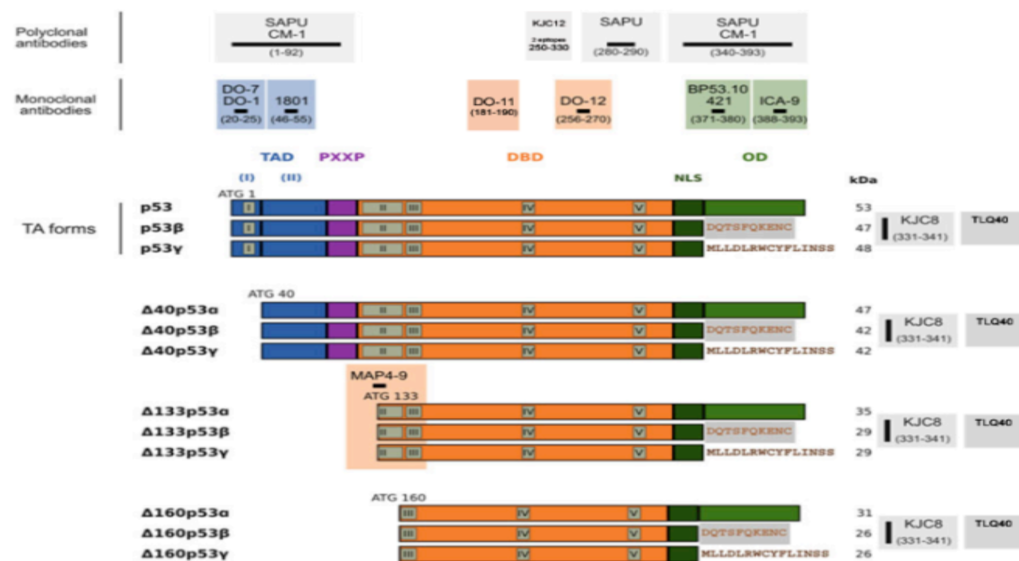


Figure 1.10
Diagram of 12 known p53 isoforms.
The antibodies used to detect specific isoforms and which antigen they detect. Adapted from (Marcel et al., 2011).

While Δ133p53 only exists in primates, equivalents of Δ133p53 have been identified to create zebrafish and mouse models with an N-terminal deletion mutant of p53 (Δ122p53). In these Δ122p53 mice, bone marrow derived cells show an increased S phase. Additionally, several organs examined including skin, pancreas and kidney, show elevated levels of bromodeoxyuridine (BrdU) positive cells together indicating that the mice have increased proliferative capacity (Slatter et al., 2011). Also, Δ122p53 mice display a variety of inflammatory conditions, including lymphocyte accumulation in liver and lung tissues and generalized inflammation including elevated levels of serum IL-6, TNF-α, and IFN-γ that precede the onset of malignancy. Additionally, mice have a reduced lifespan and altered tumor spectrum compared to p53 knockout mice. Most tumors are of B-cell origin, but about 20% are bone cancers and the remainder a variety of other tumor types (Slatter et al., 2011). The carboxyl-terminal truncated isoform, p53β, is a co-activator of FL-p53 and promotes senescence. The role of Δ133p53 and p53β in regulating cellular senescence has been shown in normal human fibroblasts, in aging- and tumor-associated $CD8^+$ T-lymphocytes, raising the hypothesis that the isoforms may play a role in the CNS (Chapters 5 and 6).

1.14.1 p53 isoforms in the CNS

There is only one report of expression of p53 isoforms, p53β and Δ133p53, in the CNS. Bourdon and colleagues reported that Δ133p53 is expressed in non-disease human brain tissue while p53β and p53γ have little to no expression (Bourdon et al., 2005). Δ40p53 is the most consistently expressed isoform in glioblastoma and is expressed in regenerative processes, such as neural progenitor cell proliferation (Takahashi et al., 2013).

1.15 ASPP protein family

The apoptosis stimulating protein of p53 (ASPP) protein family consists of three members: ASPP1, ASPP2 and iASPP. ASPP2 was initially identified as 53BP2 (p53-binding protein 2) in a yeast two-hybrid screen (Iwabuchi et al., 1994). The full-length ASPP2 protein was identified later (Samuels-Lev et al., 2001). The proteins share a highly conserved carboxyl-terminal end, which consists of three domains: ankyrin repeats, Src-homology 3 domain (SH3) and proline-rich region (Sullivan and Lu, 2007) (**Figure 1.11**). The carboxyl-terminus is the preferred binding site for a number of binding partners such as NF-kB p65 (Yang et al., 1999) and the p53 protein family (p53, p63 and p73) (Iwabuchi et al., 1994; Robinson et al., 2008).

The most well characterized role of the ASPP family of proteins is to modulate apoptosis mediated by p53 and its family members. The residues involved in the interactions between ASPP2 and p53 were identified through crystal structure analysis (Gorina and Pavletich, 1996). ASPP1 and ASPP2 enhance the ability of p53 to stimulate the expression of pro-apoptotic target genes, but not genes involved in cell-cycle arrest (Bergamaschi et al., 2004; Samuels-Lev et al., 2001).

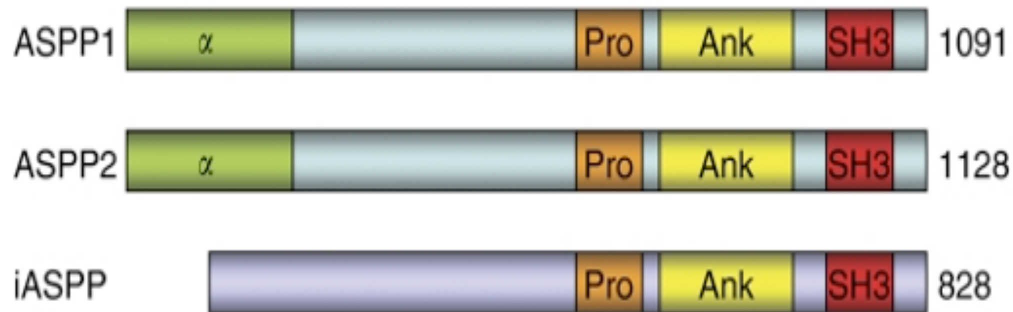


Figure 1.11
ASPP proteins.
The amino acid length is indicated on the right side. Each functional domain is given a separate color. Abbreviations: α – alpha helical domain; Pro – proline rich region; Ank –ankyrin repeats; SH3 – Src homology 3 domain.

The most evolutionary conserved member, iASPP, is the inhibitory form of the protein, which serves to prevent p53 from stimulating the expression of pro-apoptotic genes by competing for binding to p53 (Bergamaschi et al., 2003).

ASPP2 is the most well characterized member of the ASPP family. The SH3 domain and carboxyl-terminal ankyrin repeats of ASPP2 interact with a variety of proteins that are hubs for apoptosis regulation, including those involved in pro-apoptotic activities such as p53 and Bax and also anti-apoptotic Bcl-2 proteins (Katz et al., 2008; Rotem et al., 2007), viruses such as hepatitis C virus core protein (Cao et al., 2004), and inflammatory regulators, such as NF-kB p65 (Yang et al., 1999). Support for the role of ASPP2 as a tumor suppressor arises from studies showing that all contact residues between ASPP2 and p53 are mutated in human cancers (Gorina and Pavletich, 1996). In agreement with this finding, the ASPP2-p53 interaction stimulates only the apoptotic function of p53 and family members (Bergamaschi et al., 2004; Samuels-Lev et al., 2001) and not its senescence function.

This is accomplished through the binding of ASPP2 to the DNA-binding domain of p53, p73 and p63 and enhancing their transactivation functions on the promoters of apoptotic genes, Bax, Puma, and Pig3, but not senescence associated gene $p21^{WAF1}$. The biological importance of the ASPP2-p53 interaction was identified through the creation of ASPP-deficient mice, which revealed that heterozygosity of the ASPP2-null allele enhanced tumorigenesis including B-cell lymphomas, sarcomas, and squamous cell carcinoma (Vives et al., 2006).

1.15.1 ASPP2 in the CNS

After ASPP2 was identified as a tumor suppressor, the idea that it may also integrate a diverse range of signaling pathways to protect normal development was instigated through further examination of ASPP2-deficient mice. The phenotype of ASPP2-deficient mice revealed gross developmental anomalies in the CNS and heart, prompting further investigation of the role of ASPP2 in these tissue-specific developmental contexts. The majority of ASPP2 knockout mice died during weaning likely due to complications from CNS developmental abnormalities, including a 100% incidence of hydrocephalus (**Figure 1.12**). Thus, heterozygous ASPP2-null mice were created to study tumor susceptibility and other developmental functions. Histological analysis of the CNS revealed dilated ventricles and abnormal organization of subventricular structures (Sottocornola et al., 2010)(**Figure 1.13A**). While wild-type (ASPP2 +/+) mice had a columnar and pseudostratified ventricular zone, ASPP2 Δexon3 mice had disorganized and less columnar organization of the ventricular zone (**Figure 1.13B**). Given the observation of a disorganized neuroepithelium in the ventricular zone, it was hypothesized that ASPP2 might control cell polarity.

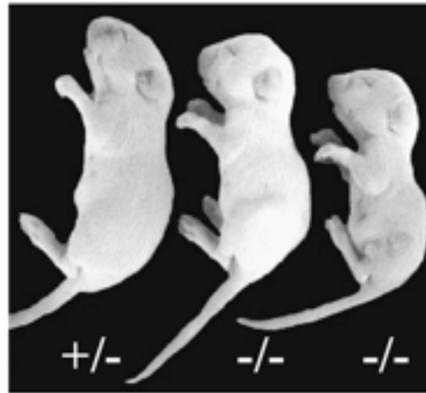


Figure 1.12
Phenotype of ASPP2-deficient mice.
Gross CNS abnormalities, including domed head and hydrocephalus, in ASPP2-deficient mice at P9. *ASPP2* genotypes are indicated below. Adapted from (Vives et al., 2006).

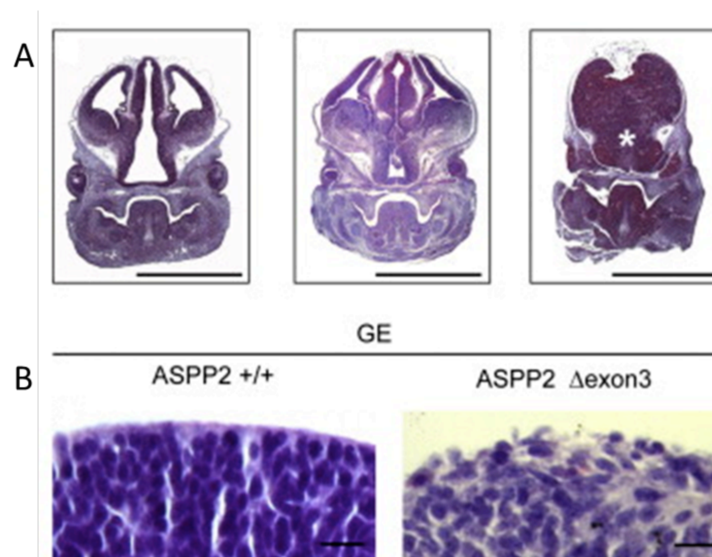


Figure 1.13
Histological abnormalities in the brain of ASPP2-deficient brain.
(a) Abnormal growth of the neuroepthelium in ASPP2 Δ3/Δ3 with differing severity. Asterisk indicates area of abnormal growth. (b) Loss of cellular polarity in ganglionic eminence in ASPP2-deficient mice. Adapted from (Sottocornola et al., 2010).

Epithelial cells in addition to other cell types, exhibit cell polarity characterized by the outward positioning of the apical cell surface and separation from the basolateral surface by the adherents junctional complex (AJC) (as discussed in Introduction 1.8). The Par complex (Par-3/aPKC/Par-6) is essential for maintenance of epithelial cell polarity. The observation that ASPP2 is localized at the apical surface of the neuroepithelia in the choroid plexus and co-localized with Par-3 and ZO-1, and partially with the adherens junction protein, β-catenin (**Figure 1.14G**), lead to the further investigation of ASPP2's function in cell polarity.

The tight/adherens junctions of the AJC serve to maintain the integrity of the choroid plexus composing the BCSFB, disruption of which can lead to hydrocephalus. After demonstrating that ASPP2 co-localizes with components of the AJC including Par-3 and ZO-1, the role of ASPP2 in the maintenance of AJC in the choroid plexus was investigated. When ASPP2 was lost, ZO-1 and Par-3 expression was disrupted in the choroid plexus (**Figure 1.14A-F**). The ultrastructure of the choroid plexus was next examined using transmission electron microscopy, which revealed that wild-type mice had cuboidal and tightly joined cells connected by the AJC. However, ASPP2-deficient mice demonstrated rounded choroid plexus cells, a disorganized epithelium, and tight/adherens junctions that failed to form tightly-joined electron-dense junctions (Sottocornola et al., 2010). These results confirmed that ASPP2 serves to maintain the integrity of tight/adherens junctions at the BCSFB (**Figure 1.14G**) and is a regulator of cell polarity.

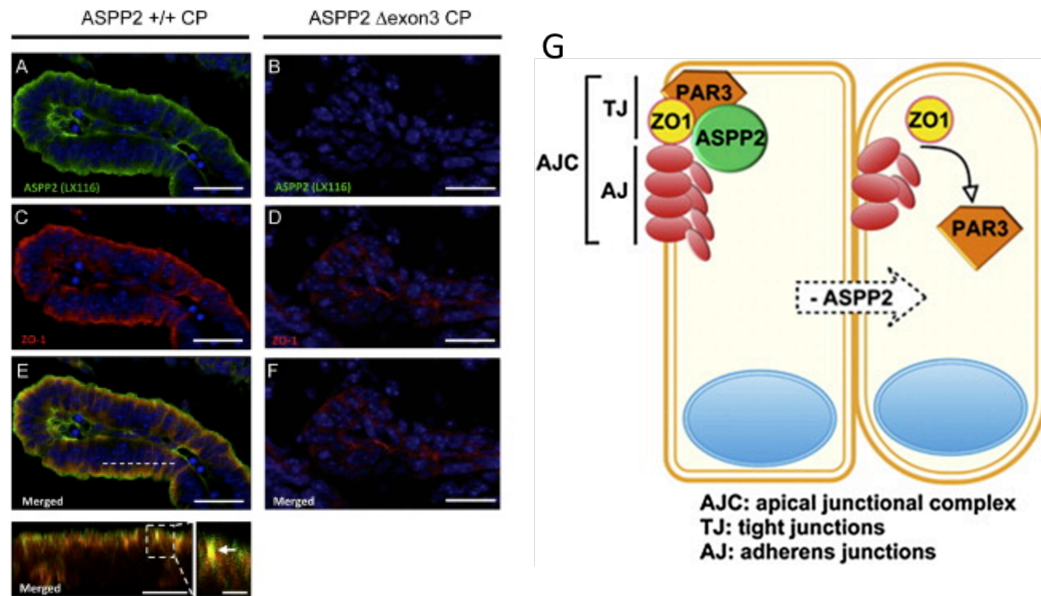


Figure 1.14
The role of ASPP2 in cell polarity.
(A, C, E) ASPP2 co-localizes with components of the AJC in the choroid plexus (CP) in wild-type (ASPP2 +/+) mice, while (B, D, F) tight junction proteins are disrupted in ASPP2-deficient (ASPP2 Δexon3) mice. (G) Schematic showing ASPP2 binding AJC complex proteins and how the loss of ASPP2 leads to the loss of the tight junction complex. Adapted from (Sottocornola et al., 2010).

Thesis Aims

Increasing evidence supports a molecular link between cancer and neurodegeneration. In epidemiological studies, AD and PD are associated with decreased cancer risk. Additionally, dysregulation of the cell cycle is a prominent feature of both diseases. While in the CNS this can result in apoptosis, in malignant cells the result is unhindered proliferation. Healthy aging requires a balance between regulators of self-renewal and cell cycle suppression. The p53 pathway is thus essential in maintaining this balance. The goal of this research is to investigate common p53-mediated mechanisms with the aim of identifying novel therapeutic targets for apoptotic and senescence programs in neurodegenerative diseases.

Apoptosis (Chapters 3 and 4):

1) Is ASPP2 is regulated by inflammatory signals in the brain?

2) Does ASPP2 play a role in cell death programs in neurodegeneration?

Senescence (Chapters 5 and 6):

1) Is there is a role for p53 isoforms , Δ133p53 and p53β, in the human CNS?

2) Are senescent cells in the CNS regulated by p53 isoforms in neurodegeneration and brain aging?

Chapter 2

Materials and Methods

Table 2.1 Primary antibodies

Antigen	Name	Source	Application
Actin	C-20	Santa Cruz	WB
Caspase 3	-	Cell Signalling	WB
Cleaved-caspase 3	-	Cell Signalling	WB
Δ133p53	MAP4	Borek (serum), (Fujita et al., 2009)	IF, WB
γH2AX	Ab22551	Abcam	IHC
GFAP	-	Abcam	IF, ICC
Iba-1	-	Wako	IF, IHC
LX116	ASPP2	Ascites	IHC
LX49.3	iASPP	Ascites	IF
MYD88	-	Abcam	WB
Nestin	-	Life Technologies	IF
NOS2	Ab129372	Abcam	IHC
$p16^{INK4}$	550834	BD Pharmingen	IF
p53	CM5	Leica	IHC
p53β	TLQ40	Borek (serum), (Fujita et al., 2009)	IF, WB

RELA/p65	A, F-6	Santa Cruz	ICC
RELA/p65	Active subunit, 12H11	Millipore	WB
S32	ASPP2	Serum	WB
SOX1	-	Life Technologies	IF
STAT1	M-23	Santa Cruz	IF, ICC
TLR4	-	Abnova	WB
β-tubulin	-	Abcam	WB

Table 2.2 Secondary antibodies

Antibody name	Host/type	Source	Applications
Alexa Fluor® 488 anti-rabbit IgG	goat	Invitrogen	IF, ICC
Alexa Fluor® 488 anti-mouse IgG	goat	Invitrogen	IF, ICC
Alexa Fluor® 546 anti-rabbit IgG	goat	Invitrogen	IF, ICC
Alexa Fluor® 546 anti-chicken IgG	goat	Invitrogen	IF
Alexa Fluor® 546 anti-mouse IgG	goat	Invitrogen	IF, ICC
Alexa Fluor® 647 anti-mouse IgG	donkey	Invitrogen	IF
Anti-Mouse Immunoglobulins/HRP	rabbit	Dako	WB
Anti-Rabbit Immunoglobulins/HRP	swine	Dako	WB
Biotinylated Anti-Mouse IgG	goat	Vector labs	IHC
Biotinylated Anti-Rabbit IgG	goat	Vector labs	IHC

Abbreviations – horseradish peroxidase (HRP), Immunohistochemistry (IHC), Immunocytochemistry (ICC), Western blot (WB), Immunofluorescence (IF).

Table 2.3 Plasmids/luciferase reporters

Name	Vector	Information	Source
ASPP2-Luc	pcDNA3.1	Human ASPP2 promoter	(Fogal et al., 2005)
E2F1	pcDNA3.1	Human E2F1	(Fogal et al., 2005)
Control plasmid	pcDNA3.1	Empty vector	Invitrogen

RELA/p65	pcDNA3.1	Human p65	Nancy Rice
kB-Luc	pcDNA3.1	Human kB	Ron Hay
IKKβ	pcDNA3.1	Human IKKβ	Ron Hay
STAT1	pcDNA3.1	Human STAT1	Stefan Constantinescu
STAT3	pcDNA3.1	Mouse STAT3	Stefan Constantinescu

Table 2.4 Primers for qRT-PCR

Transcript name	**Direction**	**Sequence (5' to 3')**
Δ133p53	Forward	TGACTTTCAACTCTGTCTCCTTCCT
	Reverse	GGCCAGACCATCGCTATCTG
TNFα	Forward	TCTCATCAGTTCTATGGCCC
	Reverse	GGGAGTAGACAAGGTACAAC
GFAP	Forward	ACACCAGCACTTCCCTTCCTTC
	Reverse	TCTGCTCATCTTTCCTCTTCCC
iNOS	Forward	CCCTTCCGAAGTTTCTGGCAGCAGC
	Reverse	GGCTGTCAGAGAGCCTCGTGGCTTTGG
IL1β	Forward	TTGACGGACCCCAAAAGATG
	Reverse	AGAAGGTGCTCATGTCCTCA
GAPDH	Forward	TGTCAGCAATGCATCCTGCA
	Reverse	TGTATGCAGGGATGATGTTC
p53β	Forward	GCGAGCACTGCCCAACA
	Reverse	GAAAGCTGGTCTGGTCCTGA
18S	Forward	GTAACCCGTTGAACCCCATT
	Reverse	CCATCCAATCGGTAGTAGCG

Primers in the table were purchased from Eurofins. Primers for mASPP2, miASPP, and mTLR4 were purchased from Qiagen (sequences available from Qiagen).

Table 2.5 Retroviral and lentiviral lectors

Name	Vector
Δ133p53-GFP	pLOC
p53β-GFP	pLOC
Control lentiviral vector-RFP/GFP	pLOC
Δ133p53-V5	pCQXIN
p53β-V5	pCQXIN
Control retroviral vector	pCQXIN

2.1 Mouse colonies

ASPP2 Δexon3 mutant mice were generated on a mixed C57BL/6Jx129SvJ background and backcrossed in a Balb/c background for 9 generations. *ASPP2* Δexon3 mutant mice were genotyped as described in previous studies (Vives et al., 2006), using the following primers: 5'-CTCCACCCCAGGAAATTACA-3' (intron 3), 5'-CGGTTTGGAAGTCAAAGGAA-3' (exon 3) and 5'-GGACCGCTATCAGGACATA-3' (neomycin resistance gene).

2.2 Patient samples

For chapters 2 and 3, human temporal cortex and aged- and region- matched inflammatory disease tissue was obtained from the Thomas Willis Oxford Brain Bank. For immunostaining experiments, 3 cases of subacute/chronic encephalitis of presumed viral etiology, 3 cases of cerebral infarct, and 3 region matched control cases were used. Experiments involving human tissue were carried out in accordance with the Human Tissue Act and the Codes of Practice from the Human Tissue Authority and received ethics approval under the project title: ASPP in normal and diseased brain (TWOBC

Request 74) (full ethics: Xin Lu: 09/0606/78). For chapters 4 and 5, human neurodegenerative (AD or ALS), aged- and region- matched non-disease (ND) control tissue was obtained from the Georgetown Brain Bank. Pediatric control tissue was obtained from the National Institute of Mental Health, NIH, Brain Bank. All human tissues were obtained in collaboration with Professor Brent T. Harris, Department of Neuropathology, Georgetown University.

2.3 MCAO Experimental stroke

The Middle Cerebral Artery Occlusion (MCAO) model was induced by 30 minutes of left-sided intraluminal filament occlusion as a model of transient focal cerebral ischemia as previously described (Engel et al., 2011).We inserted a surgical filament into the external carotid artery, and threaded it forward into the internal carotid artery until the tip occluded the origin of the MCA, resulting in a cessation of blood flow and subsequent brain infarction in the MCA territory. After 50 minutes, the filament was removed and the MCA re-perfused. Animals were recovered in heated incubators for 12 hours and body temperature kept at 35-36°C. Fluid balance was maintained by intraperitoneal 5% glucose normal saline injections of 0.4 mL daily for 7 days.

2.4 Theiler's murine encephalomyelitis virus (TMEV) infection

PFU BeAN 8386 virus was suspended in 0.03 mL sterile HBSS, and injected into the right posterior cortex through a 27 gauge needle. Injections were localized to a point half-way to midline at ear level, into the right posterior cerebellar cortex. Sham mice received injections of 0.03 mL HBSS. TMEV viral production and infection was

performed in accordance with previous studies (Tsunoda and Fujinami, 2010).

2.5 Maternal inflammation model

An intraperitoneal injection of 0.01 mg/kg lipopolysaccharide (LPS, *E. coli* 055:B5, Sigma-Aldrich, Dorset, UK) was made to female C57BL/6 mice at embryonic day (E)13.5 (Stolp et al., 2011). Age-matched control animals were injected with an equal volume of saline. Animals were sacrificed via cervical dislocation (dams) or decapitation (fetuses) 8 days after LPS injection. All animal procedures were conducted in line with UK Home Office approval (license number 30/2524).

2.6 Immunohistochemistry (IHC) and Immunofluorescence (IF)

Tissue sections were washed in PBS before blocking for 1 hour in PBS containing 0.1% Triton X and 10% donkey serum (Sigma). Donkey serum is used to block non-specific binding sites before incubation with primary antibody overnight at 4°C. Antigens were detected using the antibodies listed in **Table 2.1**. After overnight incubation they were washed in PBS 3 times for 10 minutes, before incubation with the appropriate conjugated secondary antibodies for 1 hour at room temperature (RT). The secondary antibody was conjugated to fluorophores: Alexa-488, -568 and -647 (Invitrogen, Paisley, Renfrewshire, UK; 1:400). After washing in PBS 3 times for 10 minutes, sections were incubated for 10 minutes in 4',6-diamidino-2-phenylindole (DAPI, 10ug/mL, Sigma-Aldrich) to counterstain the cell nuclei, and rinsed 3 times for 10 minutes in 0.1 M phosphate buffer (PB). Sections were mounted and slides coverslipped with FluorSave mounting medium (Chemicon). Omission of primary antibody was used as a negative control in all IF experiments. For IHC on paraffin sections, slides were heated to 65°C before immersion in histoclear and rehydration with graded alcohols. Sections

were blocked in 1% H_2O_2 in PBS-Tween 20 (PBS-T) and then in 5% normal goat serum in PBS-T. Antigens were detected using the antibodies listed in Table 1. Binding of the primary antibody was detected using a biotinylated secondary antibody listed in **Table 2.2** with an ABC standard kit (Vector Laboratories). Visualization was enabled using a 0.05% diaminobenzene hydrochloride solution (DAB; Sigma). Omission of primary antibody was used as negative controls in all IHC experiments.

2.7 Immunoblotting

Cell lines were grown in 100 cm dishes, washed with PBS, and lysed in urea buffer. Lysates were kept on ice for 30 minutes prior to sonication. Protein concentration was measured using the Bradford assay method. NuPAGE 4x loading buffer was added to all lysates and then boiled for 5 minutes. Then, 30-100µg of protein was loaded onto an SDS polyacrylamide gel for electrophoresis. Proteins were then transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk in Tris Buffered Saline (TBS, 125mM Tris and 200mM NaCl), containing 0.1% Tween-20. Membranes were incubated in the primary antibodies listed in Table 1 overnight at 4ºC, and washed 3 times in TBS-Tween-20. Membranes were then incubated in secondary antibody listed in Table 2 for 1 hour at RT and the signal visualized using enhanced chemiluminescent detection (ECL, Amersham Biosciences) using X-ray films (Fujifilm).

2.8 RNA extraction and cDNA preparation

mRNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. For mouse brain tissue, 10-20 mg of frozen cortical tissue was added to 300 µL lysis buffer containing 0.001% β-mercaptoethanol. Tissues or cells were homogenized and lysate mixed 1:1 with 70% ethanol and centrifuged

through an RNeasy Mini Spin column. The column was washed and treated with DNAse 1 for 15 minutes, before washing again to remove contaminants. RNA was eluted with RNase-free water. The abundance and quality of the resulting RNA was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). RNA samples were diluted so that 200ng total RNA could be used for a 25 μl- reverse-transcription reaction. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen).

2.9 Quantitative Real-Time Polymerase Chain reaction (qRT-PCR)

For the quantitative analysis of mRNA expression, the Tecan Sunrise 7500 real time PCR system (Applied Biosystem) was employed, with the DNA binding dye SYBR Green (Qiagen) for the detection of PCR products. Each reaction was performed in triplicate using 1μl of cDNA in a final volume of 25μl. The following thermal cycle was used for all samples: 10 minutes-95°C; 40 cycles of 30s-95°C, 40s-primer specific annealing temperatures, 40s-72°C. The melting points, optimal conditions, and specificities of the reactions were first determined using a standard procedure. qRT-PCR was performed using gene-specific primers listed in **Table 2.4**. The expression level of each target gene was analyzed based on the ΔΔCt method and the results expressed as relative expression corrected to the house-keeping gene glyceraldehyde phosphate dehydrogenase (GAPDH).

2.10 Cell culture

The murine macrophage cell line RAW267.4 and murine microglial cell line BV-2 and HEK 293T cells (ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Invitrogen, Paisley, UK) with 10% heat-inactivated fetal

bovine serum (FBS) (GIBCO), 5% penicillin, and 5% streptomycin. Primary human astrocytes (ScienCell) were maintained in astrocyte medium (ScienCell). Cells were plated at 1 x 10^5 cells/mL treated with LPS (1μg/mL) (LPS, *E. coli* 055:B5, Sigma-Aldrich, Dorset, UK) or left untreated at time points up to 24h after treatment. SV40 and Large T antigen immortalized astrocytes were obtained in collaboration with Dr. Eugene Major, NINDS/NIH and derived as previously described (Major et al., 1985; Ferenczy et al. 2013). Primary human fetal astrocytes were obtained from Sciencell.

2.11 SA-β-gal staining

SA-β-gal staining was performed with the Senescence-β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA).

2.12 Neuronal differentiation of induced pluripotent stem cells (iPSC)

NIH stem cell bank, i20 cell line was used with Gibco® Pluripotent Stem Cell Neural Induction (Life Technologies) as previously described (Yan et al., 2013). Motor neuron differentiation of iPSCs was performed in collaboration with Dr. Kenneth Fischbeck (Grunseich et al., 2014).

2.13 Co-culture system

Following differentiation to neural stem cells (NSC) iPSC, NSCs were plated at a density of 3×10^4 cells/cm^2 in a 4-well chamber slide coated with 20 μg/mL poly-L-ornithine and 10 μg/mL laminin. Media was changed to neural differentiation media (1X Neurobasal Medium, 2% B-27 Serum-Free Supplement, 2 mM GlutaMAX-1 Supplement, Life Technologies) after 2 days. Media was changed every 3 days and

NSCs were allowed to mature neurons for 1 month. Primary human astrocytes were plated on top of the mature neurons at a density of 3×10^4 cells/cm^2. Media was changed to a 1:1 ratio of astrocyte (Sciencell) and neuron differentiation medium, as previously described (Brennand et al., 2011; Muratore et al., 2014). After 48 hours cells were fixed with 4% paraformaldehyde.

2.14 Quantification of neuronal apoptosis

In co-culture experiments, the percent apoptosis was calculated by counting number of cleaved-caspase 3-positive, MAP2- or NeuN-positive neurons in 5 frames for each condition in 3 biological replicates. Additionally, the number of neurons remaining after the 48 hour co-culture period was also quantified with 5 frames per condition and 3 biological replicates and counting the number of NeuN-positive neurons.

2.15 Luciferase assay

HEK 293T Cells were plated in 24-well tissue culture plates and maintained in medium for 24 h. Cells were transfected using Lipofectamine 2000 (Invitrogen) with the indicated amounts of the plasmids or luciferase reporters listed in Table 3. All cells were also transfected with renilla control plasmid for normalizing transfection efficiencies. After transfection, cells were left overnight. The following day, cells were either left untreated or incubated with the indicated amount of ligand. After the indicated treatment period, cells were harvested in passive lysis buffer and assayed for luciferase activity per the manufacturer’s protocol.

2.16 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical comparisons were made using Student's *t*-test. Differences were considered significant at a value of * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$. ImageJ software was used to quantified by gel bands from immunoblots using densitometry.

Chapter 3

ASPP2 is Regulated by the STAT1 Inflammatory Signaling Pathway

Introduction

INFLAMMATION and cell polarity disruption represent an important link between neurodegeneration and cancer. In epithelial cancers, loss of cell polarity is a hallmark of cancer malignancy (Royer and Lu 2011), and often associates with tumor-infiltrating lymphocytes and inflammation (Shaykhiev and Bals 2007). Likewise, loss of brain barrier function prompted by neuroinflammation is linked to neurodegenerative disease onset and progression (Bednarczyk and Lukasiuk 2011). The BBB and BCSFB are the brain's main barriers to infection (Coisne and Engelhardt 2011). Previous studies have shown that a loss of cell polarity at these barriers

prompts inflammatory changes, including the intrusion of immune cells and activation of microglia and astrocytes, which contribute to neurodegeneration (Bednarczyk and Lukasiuk 2011). However, one central question is how is a loss of cell polarity at the brain barriers sensed by cell death machinery leading to the progression of neurodegeneration? We hypothesized that ASPP2, a haploinsufficient tumor suppressor, activator of p53, and apical polarity regulator may play a role in the communication between the brain barriers and cell fate determination in the nucleus. The aim of this chapter is to understand how ASPP2 might play a role in inflammatory signaling that occurs in neuroinflammatory and neurodegenerative disorders.

Results

3.1 LPS induces ASPP2 in macrophages, microglia and astrocytes *in vitro*

In the CNS, microglia and astrocytes, as well as infiltrating macrophages, are primarily responsible for the inflammatory signaling events leading to neurodegeneration. Recent reports support the role of TLR4 in a number of cerebral inflammatory disorders (Okun et al. 2009; Lehnardt et al. 2003). Thus, we used the TLR4 ligand LPS to examine whether ASPP2 is responsive to inflammatory signaling.

A mouse macrophage cell line RAW264.7, a mouse microglial cell line BV-2, a human monocyte cell line THP-1, and primary human astrocytes were treated with

1 μg/mL of LPS for 2 hours. The expression patterns of ASPP2 were detected by immunofluorescence staining using an antibody against ASPP2. As a positive control for LPS treatment, we also examined the expression level and localization of a major inflammatory signaling transcription factor NF-κB RELA/p65, which is known to translocate to the nucleus upon LPS stimulation (Chow et al. 1999). In untreated cells, ASPP2 was expressed at low levels (**Figure. 3.1.1A-B**), whereas expression of ASPP2 increased in all cell lines examined upon LPS treatment (**Figure 3.1.1 and 3.1.2**). In RAW264.7 cells, ASPP2 expression patterns were similar to those of RELA/p65, localized mainly in the cytoplasm and partially in the nucleus upon LPS treatment (**Figure. 3.1.1B**). Nuclear and cytoplasmic fractionation showed that ASPP2 was induced in both fractions in RAW264.7 cells (**Figure 3.1.1C**).

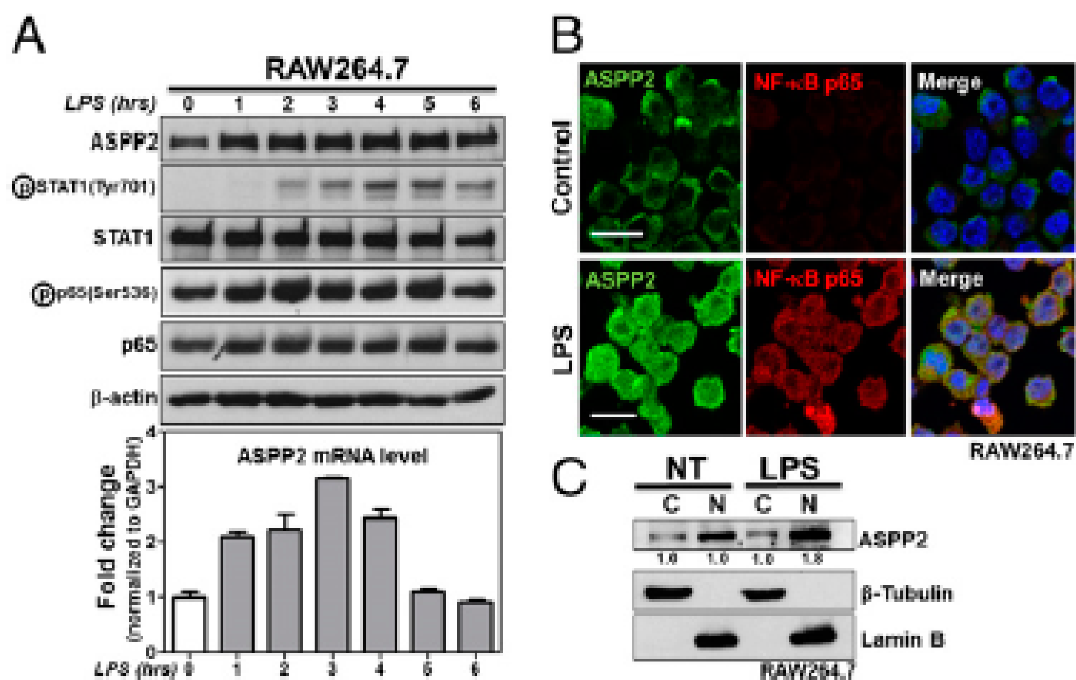


Figure 3.1.1
ASPP2 is induced by LPS.
(a) LPS time course showing increased ASPP2 expression at protein and mRNA levels in RAW264.7. Western blot performed by Yihua Wang. Expression levels of signaling pathways downstream of LPS were examined, including STAT1 and p65. (b*)* IF staining of ASPP2 and p65 after 2 hours LPS treatment in RAW264.7. Scale bars: 10μm. (c) Nuclear (N) and cytoplasmic (C) fractionations of ASPP2 upon LPS treatment in RAW264.7. Quantification was performed using densitometry analysis.

In primary human astrocytes, ASPP2 was expressed in the cytoplasm in a pattern overlapping that of the intermediate filament protein, GFAP (**Figure 3.1.2C**). The ability of LPS to induce ASPP2's expression was further confirmed by immunoblotting. In BV-2 cells, clear induction of ASPP2 was observed 2 hours after LPS treatment (**Figure 3.1.2A**).

To determine whether LPS regulates ASPP2 at the protein or mRNA level, we first examined whether LPS treatment affects ASPP2 protein stability using cycloheximide (CHX) to block protein synthesis. ASPP2 expression levels were measured in RAW264.7 cells using a CHX time course, alone or in combination with LPS. The presence of LPS had a minimal impact on the kinetics of ASPP2 reduction (**Figure 3.1.2E**). We next examined the effect of LPS treatment on ASPP2 mRNA levels using qRT-PCR. Upregulation of ASPP2 mRNA was observed in a time-dependent manner, peaking at 2 hours after LPS treatment in RAW264.7 (**Figure 3.1.1A**), BV-2 cells (**Figure 3.1.2A**) and THP-1 cells (**Figure 3.1.2B**). The ability of LPS to induce ASPP2 mRNA expression is specific since, under the same conditions, the mRNA level of iASPP, the inhibitory member of the ASPP family, was not altered (**Figure 3.1.2D**).

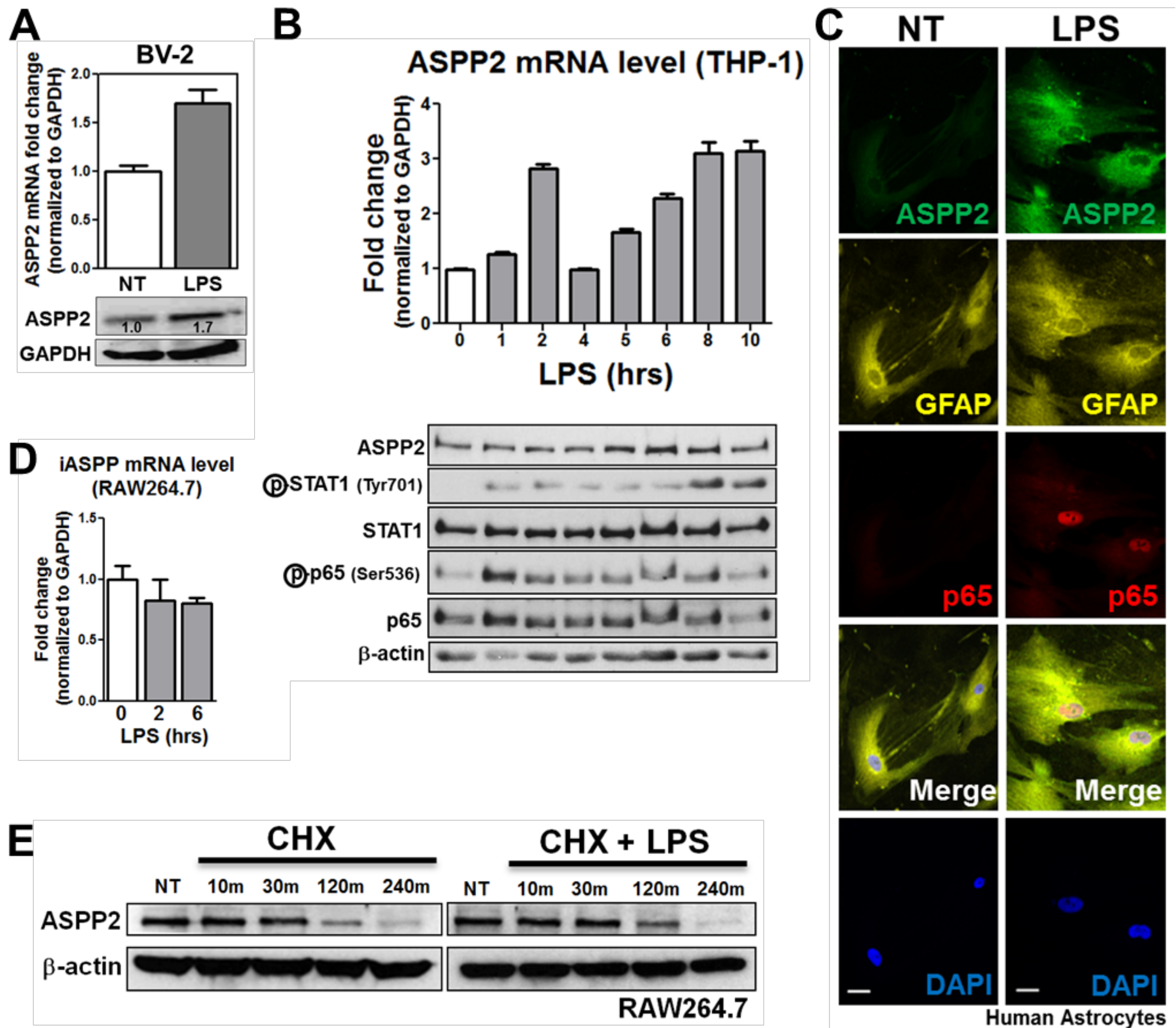


Figure 3.1.2.
ASPP2 is induced by LPS in astrocytes and microglia.
(a) ASPP2 mRNA and protein levels are induced in BV-2 cells after LPS treatment. (b) LPS time course showing increased ASPP2 expression at the protein and mRNA levels in THP-1. Expression levels of signaling pathways downstream of LPS were examined, including STAT1 and p65. Western blot performed by Yihua Wang. (c) ASPP2 is induced in primary human astrocytes in the cytoplasm in a pattern overlapping the intermediate filament glial fibrillary acidic protein (GFAP). Scale bar: 10μm. (d) iASPP mRNA is not induced following LPS treatment in RAW264.7 cells. (e) Cycloheximide (CHX) treatment with or without LPS treatment in RAW264.7 cells

3.2 ASPP2 is upregulated in inflammatory disorders using *in silico* microarray comparative analysis

Genome-wide analysis of LPS-regulated genes has been studied extensively. Knowing that LPS induces ASPP2 mRNA, we took advantage of gene array data available from NextBio database (Kupershmidt et al. 2010) and carried out bioinformatics analysis to investigate whether ASPP2 mRNA expression was altered in response to inflammation. In agreement with our findings, ASPP2 was induced in cells of human and mouse myeloid lineage after stimulation with LPS (**Figure 3.2A-B**). Additionally, we examined ASPP2 mRNA expression in a panel of paired diseased and control human macrophages. Compared to the control macrophages increased ASPP2 mRNA was detected in all macrophages derived from patients suffering from inflammatory disorders (**Figure 3.2C**). In addition, we observed increased ASPP2 expression in brain tissue from patients with neurodegenerative disorders including MS, PD, and HD (**Figure 3.2D**).

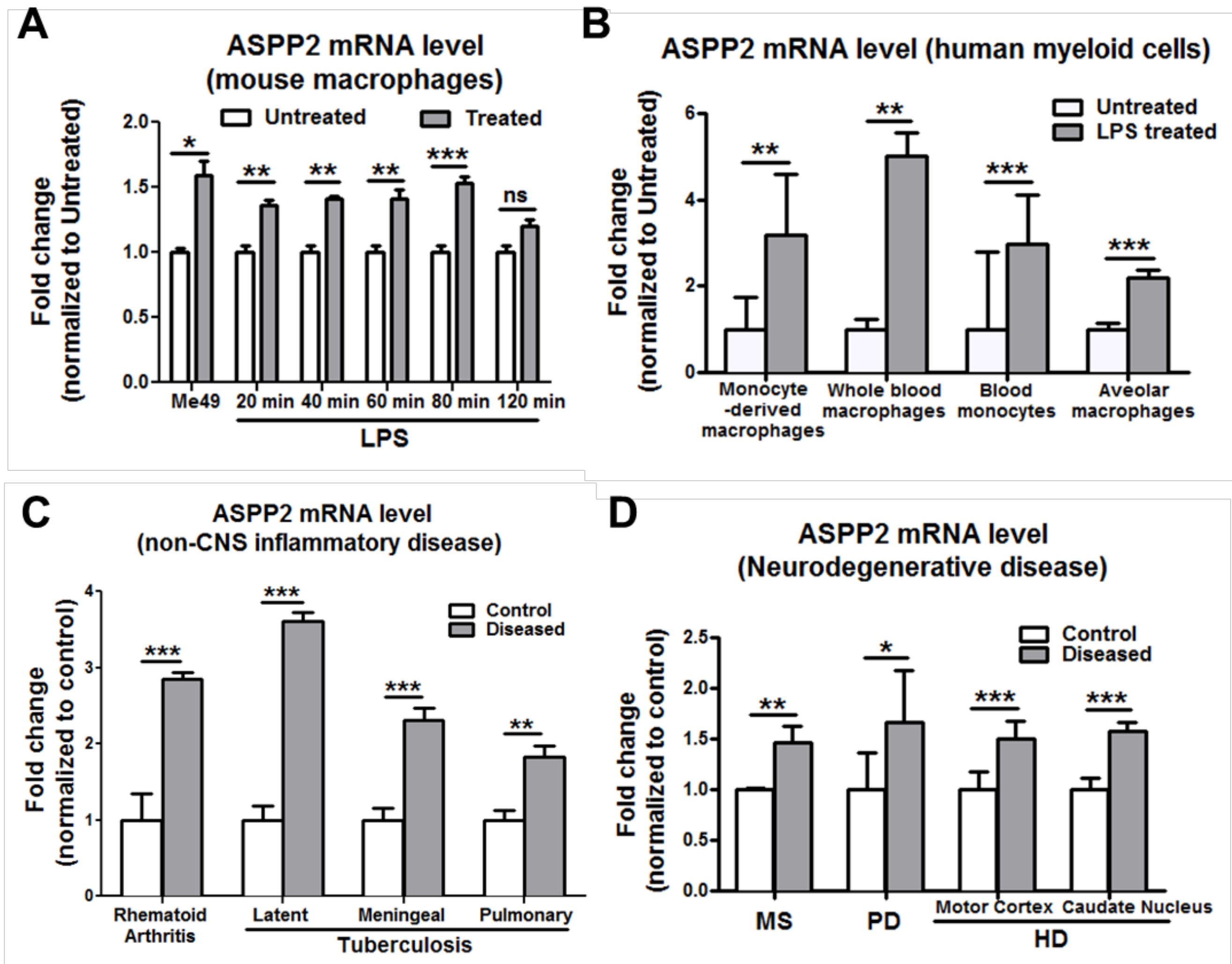


Figure 3.2.
ASPP2 is upregulated in inflammatory disorders.
Previously published gene array data showing ASPP2 mRNA expression levels in (a) mouse macrophages treated with Me49 (Jensen et al. 2011) and LPS at various time points compared to controls (Ramsey et al. 2008); (b) human monocyte-derived macrophages (Schroder et al. 2012), whole blood (Wurfel et al. 2005), blood monocytes (Dower et al. 2008), and alveolar macrophages (Reynier et al. 2012) treated with LPS compared to controls; (c) Inflamed/infected macrophages from patients with i) rheumatoid arthritis (Koulouvaris et al. 2008), ii) latent tuberculosis (Thuong et al. 2008), iii) meningeal tuberculosis (Thuong et al. 2008), iv) pulmonary tuberculosis (Thuong et al. 2008) compared to control macrophages; (d) Brain tissues from patients with neurodegenerative diseases: multiple sclerosis (MS) (Padden et al. 2007), Parkinson’s disease (PD) (Zhang et al. 2005), Huntington’s disease (motor cortex), Huntington's disease (caudate nucleus) (Hodges et al. 2006) compared to control brain tissue.

3.3 LPS induced-ASPP2 expression is mediated by STAT1

LPS requires TLR4 to initiate the activation of downstream transcription factors. Additionally, co-receptor MD-2 has shown to play an important role in cell surface recognition of LPS (Chow et al. 1999; Yang et al. 2000). We thus introduced TLR4 or MD-2 siRNA to RAW264.7 cells in the presence or absence of LPS. As expected, TLR4 siRNA almost prevented LPS from inducing ASPP2 in RAW264.7 cells (**Figure 3.3A**). MD-2 siRNA also dampened LPS-induced ASPP2 in RAW264.7 cells (**Figure 3.3B**). These data suggest that intact TLR4 signaling is required for LPS to induce ASPP2.

The downstream effectors of LPS/TLR4 are canonical MYD88-p65-dependent and non-canonical MYD88-p65-independent pathways. LPS/TLR4 activates a number of downstream transcription factors, including p65, STAT1, IRF-3 and AP-1. Analysis of the ASPP2 promoter region in conjunction with ENCODE transcription factor binding data (Bernstein et al. 2012) revealed that in addition to the previously identified E2F site (Fogal et al. 2005), both human and mouse ASPP2 promoters contain potential p65 and STAT1 binding sites, but do not contain IRF-3 and AP-1 sites. To test whether ASPP2 induction may be STAT1- or p65-dependent, siRNA was employed. In RAW264.7 cells, p65 and STAT1 siRNA reduced STAT1 and p65 expression with similar efficiency (**Figure 3.3D**). Interestingly only STAT1 RNAi, but not p65 RNAi diminished ASPP2 induction after 3 hours of LPS treatment (**Figure 3.3C**) Since IFN is upstream of the JAK/STAT1 pathway ASPP2 induction was examined with IFN treatment in RAW264.7. IFN-β was able to induce ASPP2 expression at the protein and mRNA levels in RAW264.7 cells (**Figure 3.3E**).

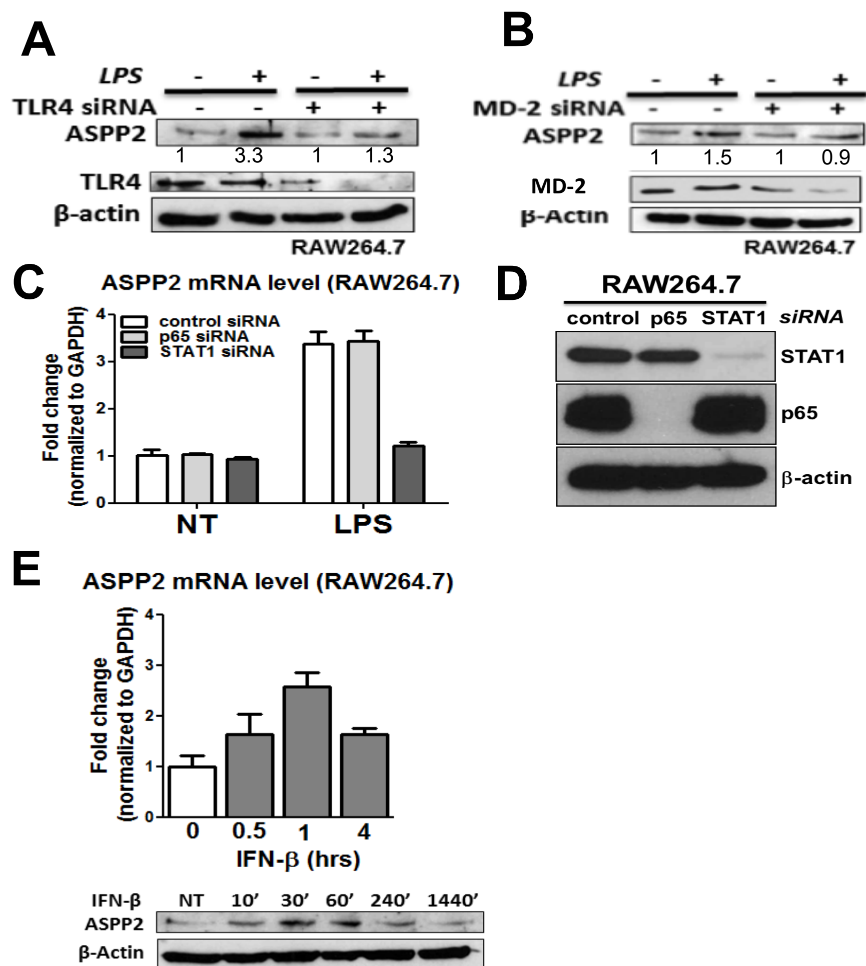


Figure 3.3.
ASPP2 induction is mediated by STAT1.
(a) TLR4 siRNA reduces ASPP2 induction following LPS treatment in RAW264.7 cells. (b) siRNA of MD-2 reduces ASPP2 induction following LPS treatment in RAW264.7 cells. Quantification was performed using densitometry analysis. (c) STAT1 siRNA, but not p65 siRNA, reduces ASPP2 induction following LPS treatment in RAW264.7 cells. (d) Western blot confirmation of p65 or STAT1 knockdown. (e) IFN time course showing increased ASPP2 expression at protein and mRNA levels.

Summary

The findings presented in this chapter suggest that ASPP2 acts as a novel sensor of infection. Under non-inflammatory conditions, ASPP2 is localized to the tight junctions of the BCSFB (Sottocornola et al. 2010), thus is in an ideal position to detect inflammatory stimuli at the apical cell membrane. ASPP2 is a scaffold protein with dynamic cellular localizations. These unique features place it in an ideal position to dictate the cell's response to infection and inflammation. When ASPP2 binds Par-3 via its N-terminus, it maintains the integrity of the apical polarity complex and TJs of the BCSFB. In this way, ASPP2 may act as a defender against infection and inflammation. The kinetics of ASPP2 induction by LPS (2 hours after treatment) suggests that it is likely to be involved in the early phase of infection. Further experiments included in our paper (Turnquist et al. 2014) (see Appendix B), but not included in this thesis revealed that ASPP2 is in fact a *bona fide* transcriptional target of STAT1. STAT1 binds the ASPP2 promoter/enhancer in an LPS/IFN-inducible manner. The finding that ASPP2 is a novel transcriptional target of STAT1 provides further support for the role of ASPP2 in sensing and dictating the cell's response to infection.

Bacterial and viral infections induce inflammatory cellular responses through TLRs. LPS and IFN are used as inflammatory stimuli to mimic infections induced by gram-negative bacteria sensed by TLR4 and viral RNA sensed by TLR3, respectively. LPS and IFN are known to have cell- and context-dependent pro- or anti-apoptotic functions. LPS is able to induce MYD88-mediated anti-apoptotic pathways though p65

(Lombardo et al. 2007), and LPS can also induce STAT1 activation through a MYD88-independent and IFN-dependent pathway. What determines whether LPS or IFN activation results in cell death or survival? The identification of ASPP2 as a transcriptional target of STAT1 could provide an explanation how a pro-apoptotic cell fate is determined. To test this hypothesis given that ASPP2's most well characterized role is promoting p53-mediated apoptosis, we next investigated if the induction of ASPP2 by inflammatory stimuli promotes its apoptotic function in neuroinflammatory and neurodegenerative diseases.

Chapter 4

ASPP2 in p53-Mediated Apoptosis in Neurodegeneration

Introduction

APOPTOSIS mediated by p53 is attributed to a number of acute and chronic neurodegenerative disorders including AD, PD, MS, and HD (Ladiwala, Li et al. 1999, Grison, Mantovani et al. 2011, Chang, Ghafouri et al. 2012). In the previous chapter, we established that ASPP2 is regulated by inflammatory stimuli converging on the STAT1 pathway. This chapter addresses the role of ASPP2 upon induction. Consistent with a pro-apoptotic role of p53 in CNS cells, inhibition of p53 by the chemical inhibitor pifithrin-α (Davenport, Sevastou et al. 2010), or deletion of the p53 gene in a mouse model of MS (Dower, Ellis et al. 2008), enhanced cell survival. These findings indicate that tumor

suppressor pathways involving p53 may be deregulated in neurodegenerative disorders. The ASPP family of proteins specifically activate p53 target genes involved in apoptosis. While ASPP1 and ASPP2 stimulate the apoptotic function of p53, iASPP is inhibitory (Trigiante and Lu, 2006). Building from the previous chapter in which we identified that ASPP2 is induced in a STAT-1 dependent manner and further work showed that ASPP2 as a novel transcriptional target of STAT1 (Turnquist et al., 2014), next we examined whether this pathway is relevant to neurological disease *in vivo*. We selected to investigate disorders in which STAT1 activation is implicated, such as MS, stroke and viral encephalitis (Lehnardt et al. 2003; Zhong et al. 1997). Additionally, we examined the role of ASPP2 in an LPS-induced maternal inflammation model (Stolp et al. 2011).

Results

4.1 LPS induces nuclear ASPP2 expression *in vivo* in a mouse model of maternal inflammation

To examine whether LPS-induced ASPP2 induction could be observed *in vivo*, and to understand the role of LPS-induced ASPP2 transcription in the context of cerebral inflammatory disease, ASPP2 expression was examined in an LPS model of maternal inflammation. In this model, pregnant mice were injected intraperitoneally with LPS at embryonic day (E) 13.5 (Stolp et al. 2011). Pup brains were then examined at postnatal (P) day 8. Under basal conditions, ASPP2 is known to be expressed in the TJs of choroid epithelial cells, where it binds Par-3 to maintain cell polarity (Sottocornola et al. 2010). We confirmed that ASPP2 was expressed at the junctions of the choroid plexus epithelial cells in the animals receiving saline injections as a

control (**Figure 4.1A-B**). In the LPS injection group, however, ASPP2 expression was upregulated and it accumulated in the nucleus rather than at the TJs (**Figure 4.1C**). Immunohistochemical staining with an antibody against p53 also revealed that ASPP2 was co-localized with nuclear p53 in the choroid plexus (**Figure 4.1D**). As ASPP2 is able to enhance the apoptotic function of p53, the observed LPS-induced nuclear accumulation of ASPP2 and p53 lead us to investigate whether ASPP2 plays a pro-apoptotic role in response to inflammatory stimuli.

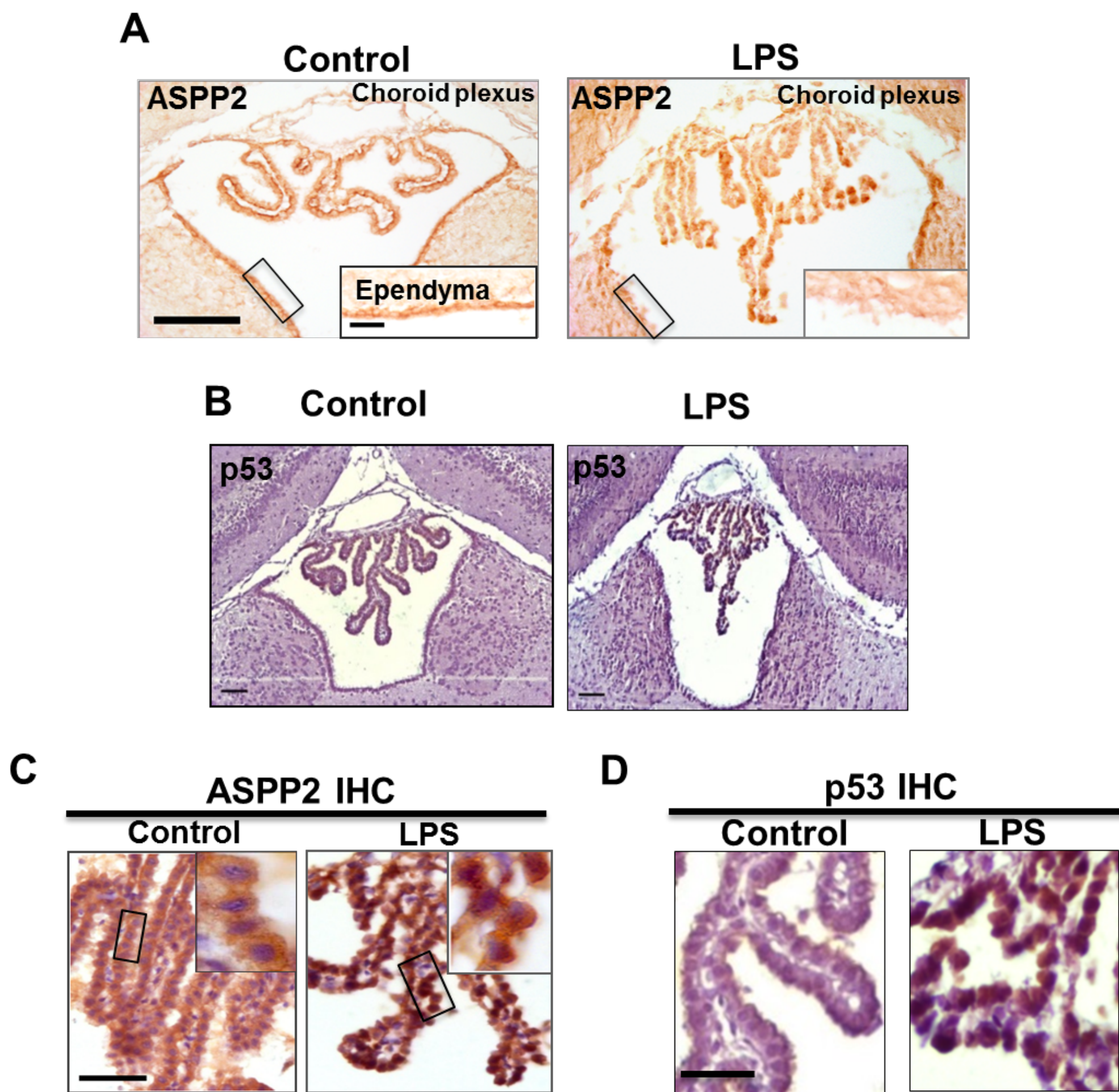


Figure 4.1.
LPS induces nuclear ASPP2 expression *in vivo*.
(a) In a model of maternal inflammation, ASPP2 is induced and relocalized to the nucleus from the tight junctions of choroid plexus epithelial cells, but not the ependymal cells lining the third ventricle. Scale bar: 100μm, 25μm (inset). (b) Following LPS injection, p53 appears in the nucleus. Scale bars: 50 μm. (c) Upon LPS injection, ASPP2 is disrupted from the TJs, relocalized to the nucleus of CP epithelial cells. Scale bar: 25μm. (d) Following LPS injection, p53 appears in the nucleus. Scale bar: 25μm.

4.2 ASPP2 regulates IFN/LPS-induced apoptosis *in vivo* and *in vitro*

To further examine the role of ASPP2 in apoptosis, ASPP2-deficient mice and wild type mice were injected with LPS or saline intraperitoneally. Previous studies have shown that systemic administration of LPS is capable of inducing apoptosis in the brain, particularly in the hippocampus (Semmler et al. 2005). Levels of cleaved-caspase 3 were compared in hippocampal brain sections from ASPP2 Δ3/Δ3 and wild type mice compared to the saline injected controls. Cleaved-caspase 3 positive cells were quantified in the hippocampus and found to be significantly less prevalent in the ASPP2 Δ3/Δ3 mice versus wild type mice injected with LPS (**Figure 4.2A-B**) supporting a pro-apoptotic role of ASPP2. Few cleaved-caspase 3 positive cells were found in mice receiving saline injections (**Figure 4.2B**)

Previous studies have shown that STAT1 can cooperate with p53 to induce apoptosis by selectively enhancing the transcriptional activity of p53 on p53 target gene promoters bearing an ASPP2 signature such as Bax (Soond et al. 2007) and Noxa (Townsend et al. 2004). Since LPS-induced STAT1 activity induces ASPP2 expression, we tested whether LPS-induced nuclear ASPP2 and p53 may play a pro-apoptotic role in response to inflammatory stimuli. To test this, ASPP2 was deleted in RAW264.7 cells, which express wild type p53. Upon treatment with LPS, the expression level of cleaved PARP diminished in the ASPP2-depleted cells but not control cells (**Figure 4.2C**). These data support the role of ASPP2 in mediating LPS-induced apoptosis in vitro and in vivo.

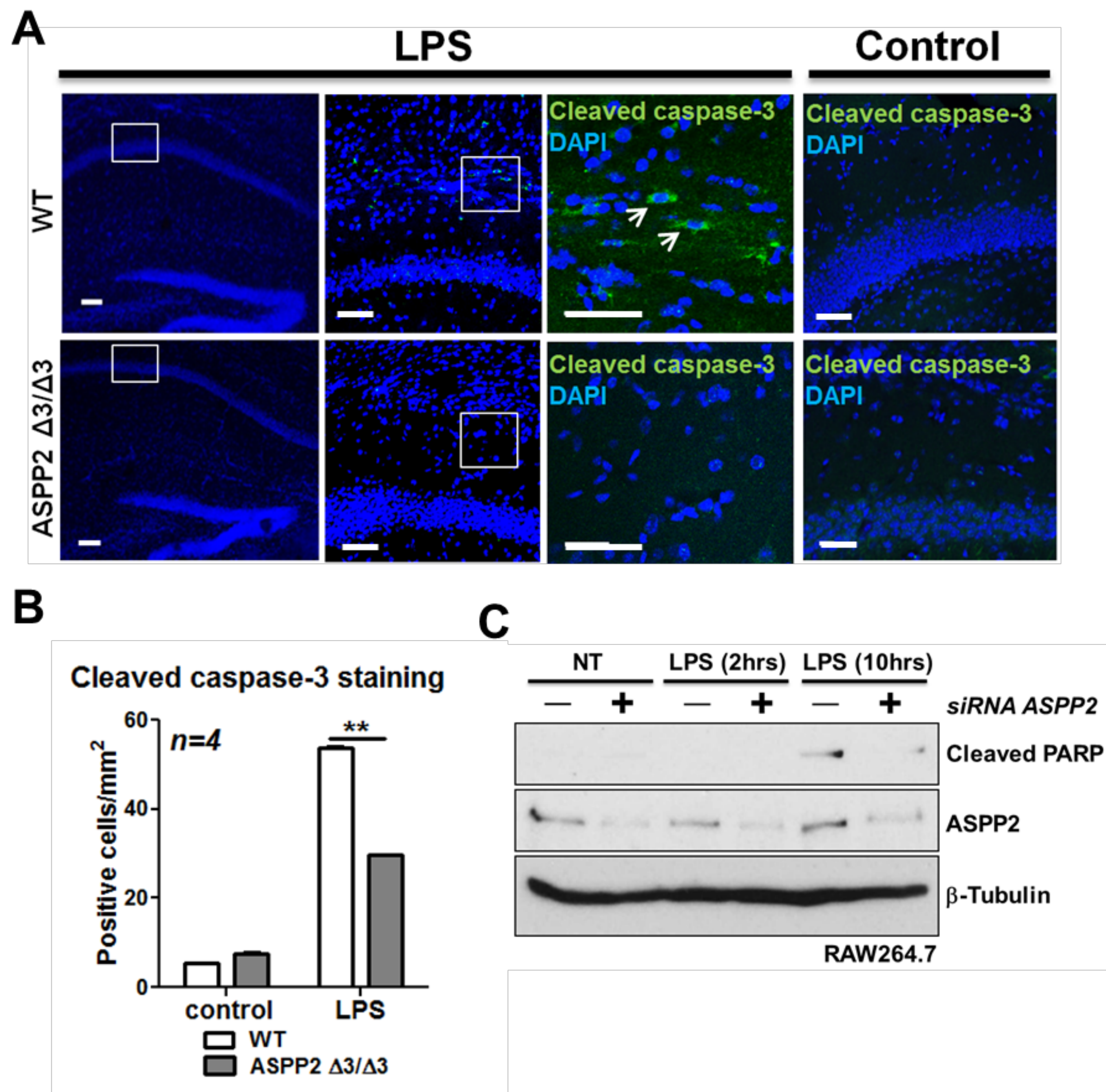


Figure 4.2.
ASPP2 regulates LPS-induced apoptosis.
(a) IF staining of cleaved caspse-3 in LPS-injected and control saline-injected WT and ASPP2 Δ3/Δ3 mice. Arrows indicate cleaved caspase-3-positive cells. Scale bars: 25μm. (b) Quantification of the number of cleaved caspase-3-positive cells in the hippocampus after LPS or saline injection (n=4). (c) ASPP2 knockdown reduces cleaved PARP expression after 10 hours' LPS treatment in RAW264.7 cells. NT: no treatment.

4.3 ASPP2 deficiency enhances neuroinflammation *in vivo*

As ASPP2 was previously shown to maintain the TJs between CP epithelial cells (Sottocornola et al. 2010), the brain's main barrier to inflammation, we examined whether loss of ASPP2 in ASPP2 Δ3/Δ3 mice results in increased neuroinflammation. IHC staining demonstrated that ASPP2 Δ3/Δ3 mice possess activated microglia and astrocytes throughout the parenchyma at E15.5 (**Figure 4.3A**) and P20 (**Figure 4.3C**). Using qRT-PCR with cortical tissue from ASPP2 Δ3/Δ3 and WT mice, we observed that ASPP2 Δ3/Δ3 mice displayed a significant increase in several pro-inflammatory cytokines, including TNFα at E15.5 (**Figure 4.3D**). The extent of neuroinflammation increased by P20 as the production of TNFα and IL1β in cortical brain tissues increased dramatically compared to age-matched WT mice (**Figure 4.3B**). Thus, the localization of ASPP2 at the TJs of the BCSFB under basal conditions, and the neuroinflammatory phenotype of ASPP2 Δ3/Δ3 mice, suggest that ASPP2 may act as a barrier to inflammation.

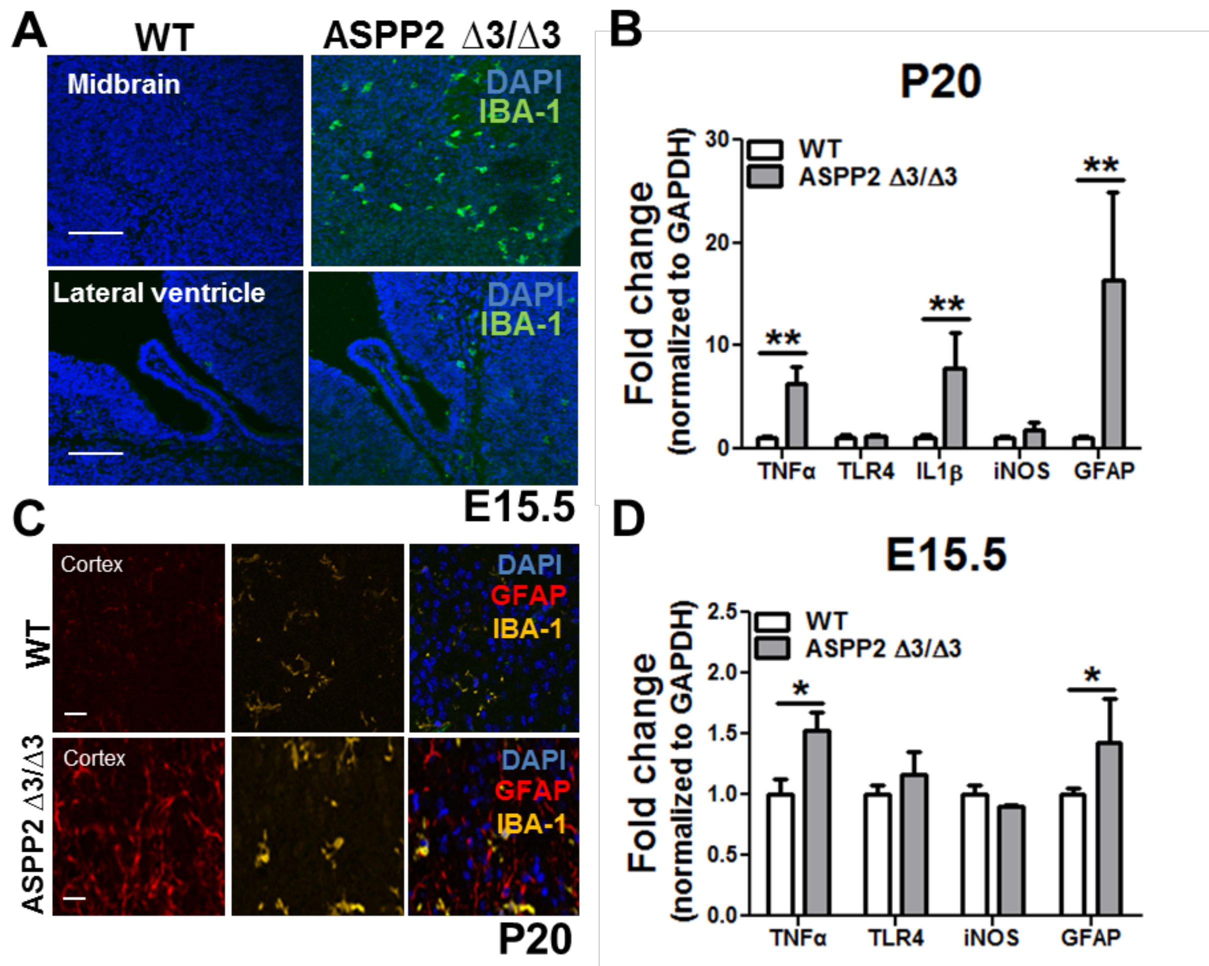


Figure 4.3.

ASPP2-deficient mice possess neuroinflammation.

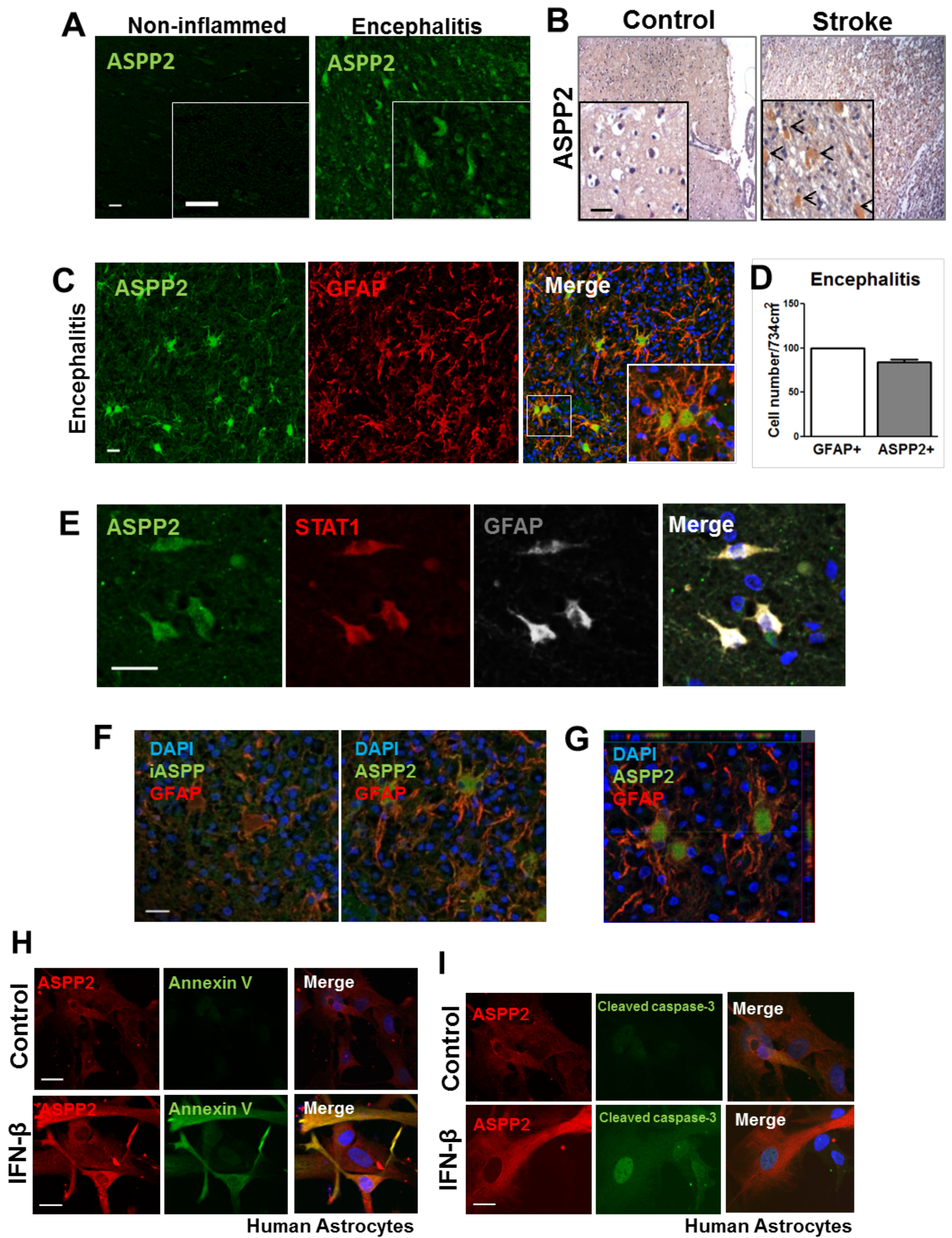
(a) Increased IBA1-positive microglia in ASPP2 Δ3/Δ3 mice at E15.5. Scale bars: 100μm. (b) Increased pro-inflammatory cytokines in cortical brain tissue of ASPP2 Δ3/Δ3 mice at P20. (c) Increased IBA1-positive microglia and GFAP-positive astrocytes in ASPP2 Δ3/Δ3 mice at P20. Scale bars: 10μm. (d) Increased pro-inflammatory cytokines in cortical brain tissue of ASPP2 Δ3/Δ3 mice at E15.5.

4.4 ASPP2 is highly expressed in reactive astrocytes in mouse neuroinflammation models and human neuroinflammatory disease

To test the hypothesis that ASPP2 is involved in neuroinflammatory disorders involving STAT1 signaling, we used mouse models of inflammatory disorders in which IFN activation is implicated in the disease pathology, including multiple sclerosis and stroke (Rubio, Palomo, and Alcami 2010; Takagi et al. 2002). Tissue sections were obtained from an animal model of experimental stroke, middle cerebral artery occlusion (MCAO), and the Theiler's murine encephalomyelitis virus (TMEV) infection model of MS. ASPP2 immunohistochemistry revealed marked upregulation in cells with astrocytic morphology found in the dentate gyrus of the hippocampus ipsilateral to the cerebral infarct in the MCAO model (**Figure 4.4K**), and in the cerebral cortex of the TMEV model (**Figure 4.4J**). ASPP2 was not as highly expressed on the hemisphere contralateral to the cerebral infarct, indicating that ASPP2 upregulation is specific to the areas of the brain in which major damage was present.

We next examined human tissue samples obtained from a variety of disorders associated with neuroinflammation. Immunohistochemical staining demonstrated low to no expression of ASPP2 in non-inflamed tissue (**Figure 4.4A-B**). However, high expression of ASPP2 was observed in cerebral infarct (**Figure 4.4B**) and in subacute/chronic encephalitis (**Figure 4.4A, C**). Morphological analysis suggested that reactive astrocytes were the dominant ASPP2-expressing cell type. To confirm this, we performed double immunofluorescence staining with anti-ASPP2 and anti-GFAP antibodies on biopsy tissue, which enabled superior ASPP2 antigen

preservation. ASPP2 was found to be highly expressed in the cytoplasm of reactive GFAP-positive astrocytes, particularly those with gemistocytic morphology (**Figure 4.4C-D**). The detected increase in ASPP2 expression is specific since iASPP, the inhibitory member of the ASPP family of proteins was not highly expressed in either encephalitis or control tissue (**Figure 4.4F**). The number of ASPP2-positive cells was quantified per cell subtype and revealed that ~83% of GFAP-positive cells had high expression of ASPP2 (**Figure 4.4D**). As encephalitis is known to arise from viral infection and IFN-α secretion in encephalitis is linked to neuronal dysfunction (Sas et al. 2009), we chose to examine whether STAT1-mediated ASPP2 induction was present in human tissue samples. In agreement with this hypothesis, ASPP2 was found to be upregulated and co-localized with STAT1 in GFAP-positive reactive astrocytes (**Figure 4.4E**). To further confirm the role of ASPP2 induction in astrocytes we treated primary human astrocytes with IFN-β for 24 hours, after which they began to undergo apoptosis, expressing Annexin V and cleaved-caspase 3, and also upregulating ASPP2 expression (**Figure 4.4H-I**). These findings are consistent with the notion that ASPP2 is a transcriptional target of STAT1 and it plays a pro-apoptotic role in response to inflammatory signals such as LPS and IFN.


A
Non-inflammed
Encephalitis
ASPP2
ASPP2
B
Control
Stroke
ASPP2
C
Encephalitis
ASPP2
GFAP
Merge
D
Encephalitis
Cell number/734cm²
150
100
50
0
GFAP+
ASPP2+
E
ASPP2
STAT1
GFAP
Merge
F
DAPI
iASPP
GFAP
DAPI
ASPP2
GFAP
G
DAPI
ASPP2
GFAP
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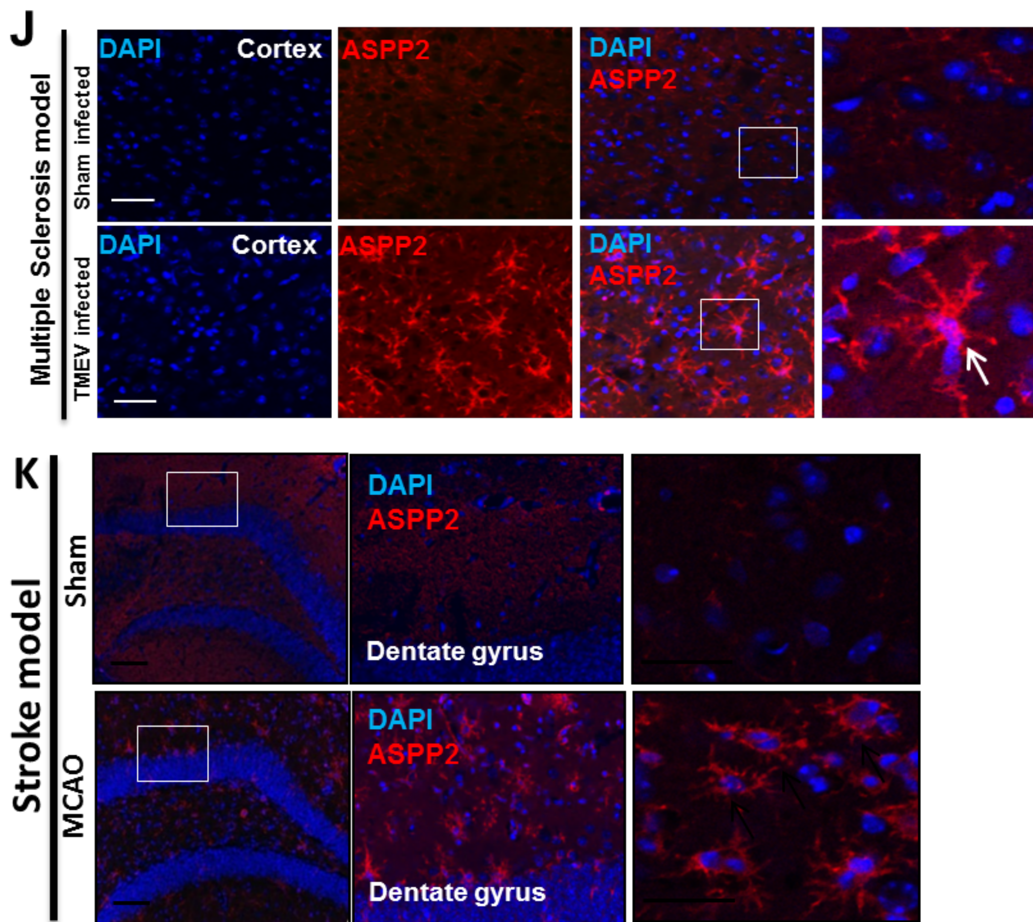


Figure 4.4.

ASPP2 upregulation in mouse models and human neuroinflammatory disorders. Increased ASPP2 expression in astrocytes in (a, c) encephalitis and (b) stroke. (d) About 85% of ASPP2-expressing cells also co-express GFAP. (e) ASPP2 is upregulated in GFAP-positive reactive astrocytes. STAT1 (red) and ASPP2 (green) co-expression in GFAP (white)-positive reactive astrocytes. Scale bars: 25μm. (f) iASPP is not highly expressed in astrocytes in encephalitis tissue. Scale bar: 10μm. (g) Orthogonal plane of ASPP2-expressing astrocytes in encephalitis tissue demonstrates ASPP2 is localized to the cytoplasm. Scale bar: 10μm. Annexin-V (h) or cleaved caspase-3 (i) immunocytochemistry staining of human astrocytes after 24 hours IFN-β treatment. Scale bars: 25μm. ASPP2 induction in an animal model of (j) MS and (k) stroke. Scale bars: 25μm.

Summary

The observed induction in ASPP2 expression in mouse neuroinflammation models and human neuroinflammatory disorders and the finding that the ASPP2 Δ3/Δ3 mouse hippocampus has reduced apoptosis in response to systemic LPS injection supports the importance of ASPP2 in sensing, integrating, and dictating the cellular response to inflammatory stimuli. ASPP2's upregulation in the LPS-induced maternal inflammation model demonstrates that inflammatory stimuli are capable of inducing ASPP2 *in vivo* at the location at which it serves as a barrier to inflammation. When the TJs of the BCSFB are disrupted, ASPP2 is displaced from the cell junctions and re-localizes to the nucleus where it is upregulated. Hence the dynamic nature of its cellular localization and diverse functions places ASPP2 in an ideal position sense inflammatory stimuli at the apical cell membrane, relay this information to transcriptional machinery in the nucleus by acting as a messenger, and determine cell fate as a reader, as a so-called "SMaRT Factor" (Lu, in preparation) (see Discussion 7.2).

Chapter 5

p53 Isoforms in Brain Aging and Neurodegeneration

Introduction

SENESCENT cells accumulate with brain aging and neurodegenerative diseases, such as AD and ALS (Bhat et al., 2012; Tan et al., 2014). While most evidence supports astrocyte senescence as a key feature of neurodegeneration, neuron senescence has also recently been reported (Jurk et al., 2012; Mattson and Magnus, 2006; Naylor et al., 2013). Numerous studies demonstrate that astrocyte senescence originating either from ALS model superoxide dismutase 1 (SOD1) mutant mice (Christou et al., 2013; Das and Svendsen, 2014; Fritz et al., 2013; Nagai et al., 2007), senescence-accelerated prone mice (SAMP8) (García-Matas et al., 2008), aged murine astrocytes *in vivo* and *in vitro* (Das and Svendsen, 2014; Gottfried et al., 2002; Papadopoulos et al., 1997;

Pertusa et al., 2007), and those exposed to oxidative stress (Bitto et al., 2010) can promote neurotoxicity while simultaneously resulting in the loss of their neuroprotective functions. Further support for the role of astrocyte senescence in AD pathology comes from a report that Aβ peptides, accelerate senescence and progresses neurodegeneration (He et al., 2013). Despite increasing evidence that astrocyte SASP promotes neurodegeneration, it remains unclear how it is regulated and if it can be delayed or inhibited as a therapeutic strategy.

A main regulator of cellular stress responses and senescence is the gene *TP53*. Recently, it was reported that human *TP53* encodes at least 12 natural isoforms through means of alternative splicing or alternative promoter usage (Bourdon et al., 2005). We have recently identified p53 isoforms, Δ133p53 and p53β, as endogenous regulators of cellular senescence (Fujita et al., 2009a; Horikawa et al., 2014; Mondal et al., 2013; Tang et al., 2013). Δ133p53, the N-terminal truncated isoform, dominant-negatively inhibits FL-p53, and is a negative regulator of cellular senescence. The C-terminal truncated isoform, p53β, is a co-activator of FL-p53 and promotes senescence. The role of Δ133p53 and p53β in regulating cellular senescence has been shown in normal human fibroblasts, in aging- and tumor-associated T lymphocytes, raising the hypothesis that the isoforms may play a role in the CNS.

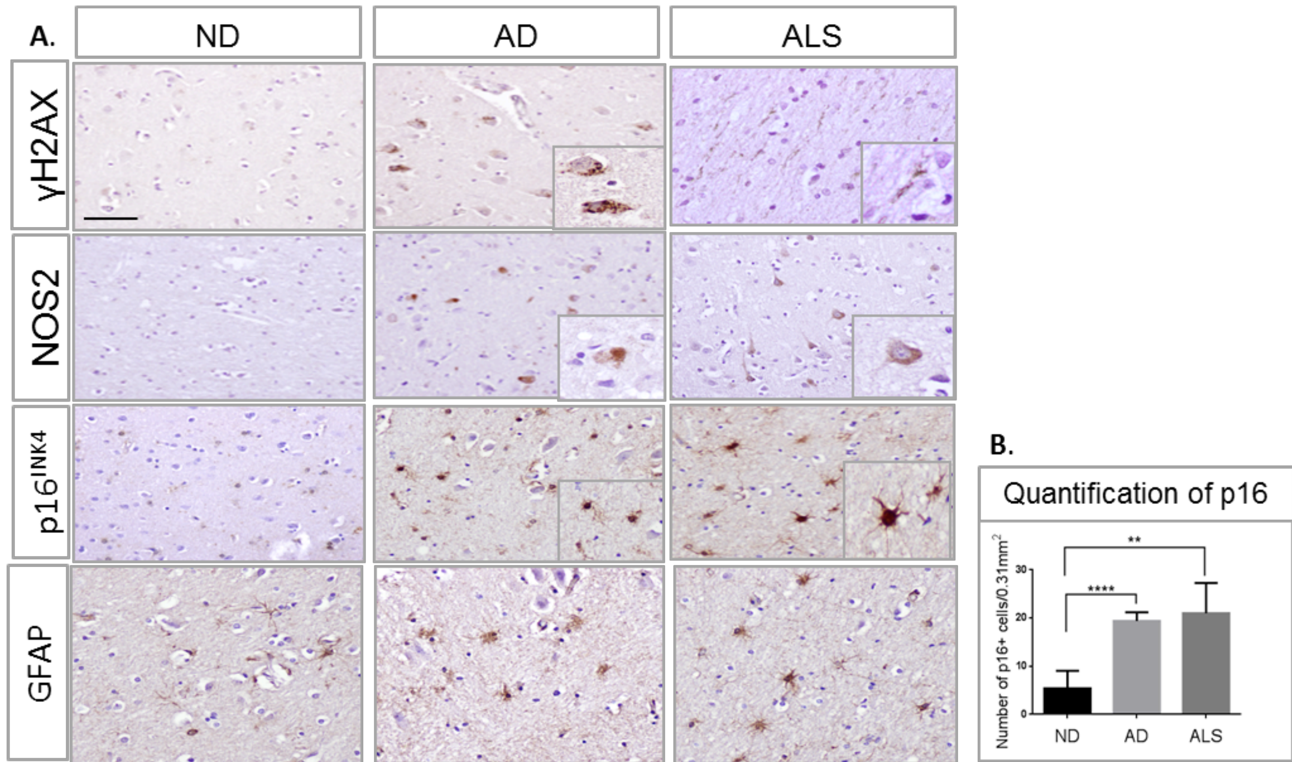
5.1 Cellular senescence of astrocytes in brain tissues from neurodegenerative disease patients

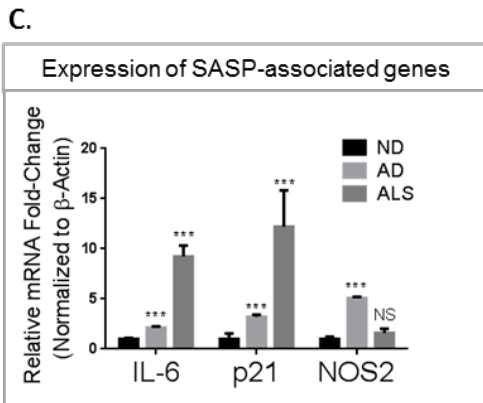
To examine whether neurodegenerative disease tissues have increased features of cellular senescence, patient tissue samples were obtained from the Georgetown University Brain Bank and the Human Brain Collection Core, National Institutes of Mental Health (NIMH), including AD, ALS, age-matched non-disease, and non-disease pediatric tissues (**Table 5.1**). Immunohistochemical staining was performed using antibodies to proteins known to be associated with cellular senescence, such as $p16^{INK4}$ (Rayess et al., 2012), NOS2 (Sohn et al., 2012), and γH2AX (Wang et al., 2009)(**Figure 5.1A**). An increase in the senescence-associated biomarkers was prominent in all neurodegenerative samples examined compared to controls (**Figure 5.1A**). Quantification of the number of $p16^{INK4}$-expressing cells revealed a significant increase in this senescence-associated gene in AD and ALS tissues (**Figure 5.1A-B**). Although both AD and ALS are associated with increased cellular senescence, qRT-PCR using brain tissue RNA samples showed that SASP cytokine, IL-6, and a p53-inducible senescence regulator, $p21^{WAF1}$, were upregulated more remarkably in ALS, while NOS2 upregulation was more evident in AD (**Figure 5.1C**), possibly reflecting the different disease pathologies. IL-6 and $p21^{WAF1}$ expression levels were much lower in non-disease pediatric brain tissues than in non-disease aged brain tissues (**Figure 5.1D**), indicating that these senescent changes not only are associated with neurodegenerative diseases, but also may occur during physiological brain aging. In agreement with astrocyte-like morphology of the senescent cells (**Figure 5.1A**),

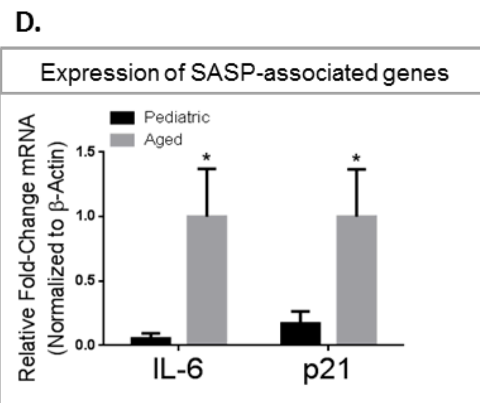
immunofluorescence co-staining of p16^{INK4A} and glial fibrillary acidic protein (GFAP) as an astrocyte marker showed that the senescent cells were astrocytes (**Figure 5.1E**). Furthermore, both immunohistochemical (**Figure 5.1A**) and immunofluorescence staining (**Figure 5.1F**) of GFAP showed the presence of astrocytes with enlarged and flattened cytoplasms, which is characteristic of senescent cells (Kuilman et al., 2010), in AD and ALS brain tissues, but not in non-disease control tissues.

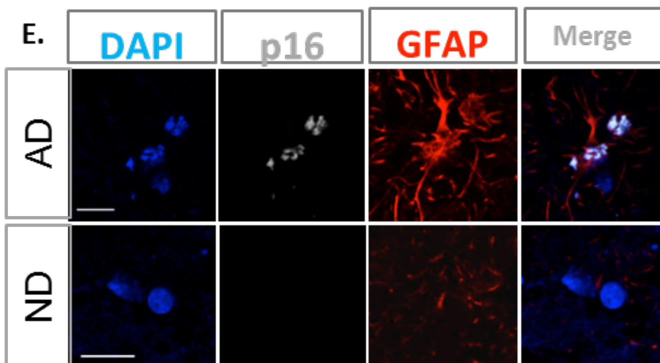
Tissue type	Case	Age, sex	Region	Sample type
AD	1	63, male	Frontal lobe	Frozen
	2	90, male	Frontal lobe	Frozen
	3	82, female	Frontal lobe	Frozen
	4	82, male	Frontal lobe	Frozen
	5	67, male	Hippocampus	FFPE
ALS	1	54, male	Motor cortex	Frozen
	2	55, male	Motor cortex	Frozen
	3	72, female	Motor cortex	Frozen
	4	55, male	Motor cortex	FFPE
Non-disease Aged	1	60, male	Frontal lobe	FFPE
	2	68, male	Frontal lobe	Frozen
	3	41, male	Frontal lobe	Frozen
	4	47, male	Frontal lobe	Frozen
	5	72, male	Motor cortex	FFPE
Non-disease Pediatric	1	1, female	Frontal lobe	Frozen
	2	0.5, female	Frontal lobe	Frozen
	3	0.1, female	Frontal lobe	Frozen
	4	0.1, female	Frontal lobe	Frozen

Table 5.1. Human brain tissues. Abbreviations – Formalin-fixed paraffin-embedded (FFPE); AD (AD); Amyotrophic lateral sclerosis (ALS).


A.
ND
AD
ALS
γH2AX
NOS2
$p16^{INK4}$
GFAP
B.
Quantification of p16
Number of p16+ cells/0.31mm^2
ND
AD
ALS


C.
Expression of SASP-associated genes
Relative mRNA Fold-Change (Normalized to β-Actin)
ND
AD
ALS
NS
IL-6
p21
NOS2


D.
Expression of SASP-associated genes
Relative Fold-Change mRNA (Normalized to β-Actin)
Pediatric
Aged
IL-6
p21


E.
DAPI
p16
GFAP
Merge
AD
ND

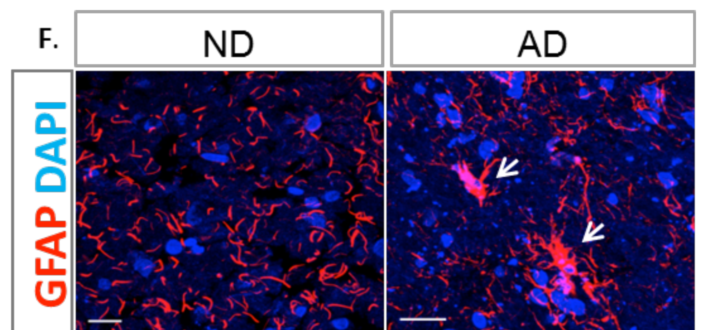

F.
ND
AD
GFAP DAPI

Figure 5.1: Cellular senescence characteristics in neurodegenerative disease brain tissue. (a) Increased expression of senescence-associated proteins, γH2AX, NOS2, and $p16^{INK4}$ and GFAP in Alzheimer's disease (AD) (Case 5), amyotrophic lateral sclerosis (ALS) (Case 4), and non-disease (ND) age-matched control tissues (Case 5), Scale bars = 50 μm. (b) Quantification of $p16^{INK4}$-positive cells in AD (Cases 1-4) and ALS (Cases 1-3) compared to ND (Cases 1-4). (c) Elevated SASP-associated genes in AD (Cases 1-4) and ALS (Cases 1-3) compared to ND (Cases 1-4). (d) Increased expression of IL-6 and $p21^{WAF1}$ mRNA in aged ND tissues (Cases 1-4) compared to pediatric brain tissues (Cases 1-4). (e) Increase in $p16^{INK4}$ and GFAP-positive cells in AD (Case 1) compared to ND (Case 1). Scale bars = 10 μm.

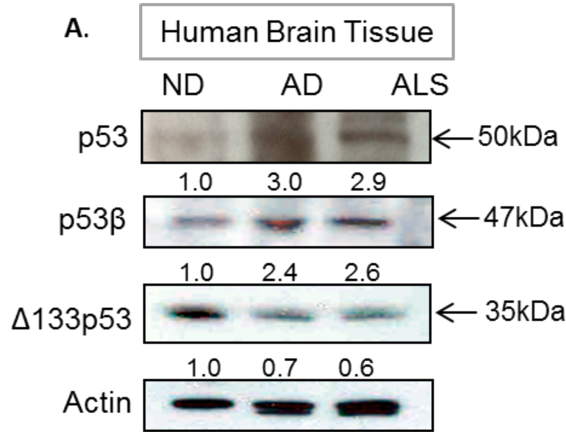
5.2 p53β is upregulated and Δ133p53 is downregulated in neurodegenerative disease brains and in aged astrocytes *in vitro*

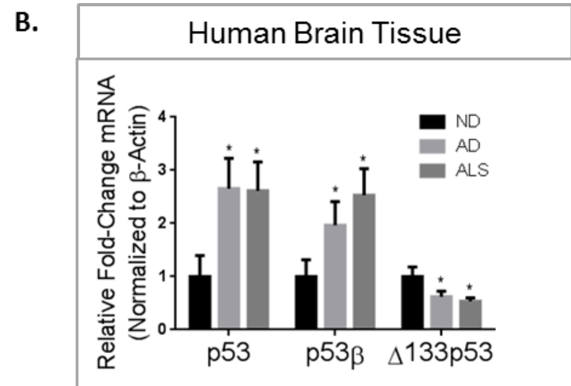
To examine the expression of the p53 isoforms, p53β and Δ133p53, in AD, ALS and age-matched ND brain tissues, western blot analysis was performed using the isoform-specific antibodies (Fujita et al., 2009a; Mondal et al., 2013), along with detection of full-length p53. We found that full-length p53 and p53β were upregulated, while Δ133p53 was downregulated, in AD and ALS tissues compared to non-disease tissues (**Figure 5.2A**). Quantitative densitometric analysis determined that the upregulation of full-length p53 and p53β was 2-3-fold and the downregulation of Δ133p53 was 0.5-0.6-fold (**Figure 5.2B**). These findings are consistent with a previous report of low levels of p53β and high levels of Δ133p53 mRNA expression in non-disease human brain tissues (Bourdon et al., 2005), and also agree with the expression profiles of p53β and Δ133p53 observed in other *in vivo* conditions associated with increased senescence, such as pre-malignant colon adenomas (Fujita et al., 2009a) and $CD8^+$ T-lymphocytes in the elderly (Mondal et al., 2013).

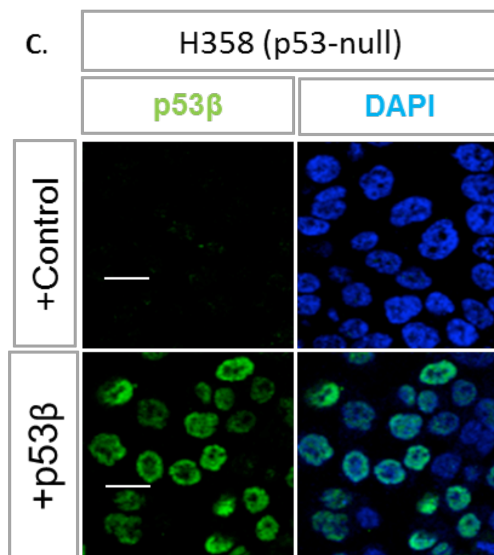
To examine which cell type(s) expresses the p53 isoforms, we co-stained human brain tissues with the isoform-specific antibodies and anti-GFAP antibody. The specificity of the isoform-specific antibodies in immunocytochemical staining was confirmed using a p53-null cell line with p53β or Δ133p53 expression vector (**Figure 5.2C-D**). Both p53β and Δ133p53 signals were detected in GFAP-positive cells and localized within DAPI-positive nuclei (**Figure 5.2E, arrows**). Nuclei of GFAP-negative cells did not express p53β or Δ133p53 (**Figure 5.2E, arrowheads**). Our findings

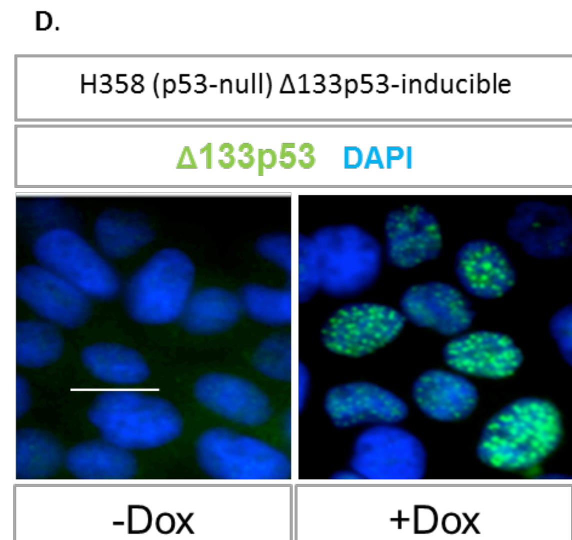
indicate that p53β and Δ133p53 are mainly localized in astrocyte nuclei, where full-length p53 is also localized in non-disease and AD tissues (Kitamura et al., 1997; Mendrysa et al., 2011).

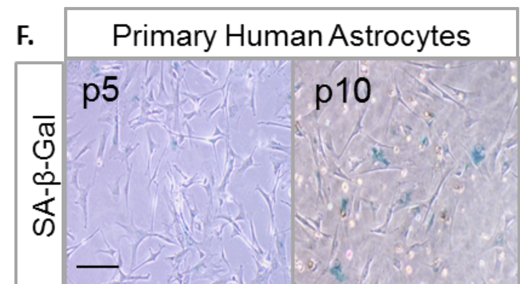
Because aged human CD8$^+$ T-lymphocytes undergo similar changes in p53 isoform expression both *in vivo* and *in vitro* (Mondal et al., 2013), we next examined whether *in vitro* cultured astrocytes at the end of their proliferative lifespan also show increased p53β and decreased Δ133p53 as observed in neurodegenerative states *in vivo*. Primary human astrocytes were serially passaged and monitored with SA-β-Gal staining (**Figure 5.2F**), until they became growth-arrested and fully senescent at passage number (P)15. Western blot analysis comparing early passage (P5) and near-senescent state (P13) showed that p53β was upregulated and Δ133p53 was downregulated in astrocytes approaching senescence (**Figure 5.2G**). These results indicate that aged astrocytes *in vitro* recapitulate the expression signature of p53β and Δ133p53 in astrocytes *in vivo* in neurodegenerative diseases.

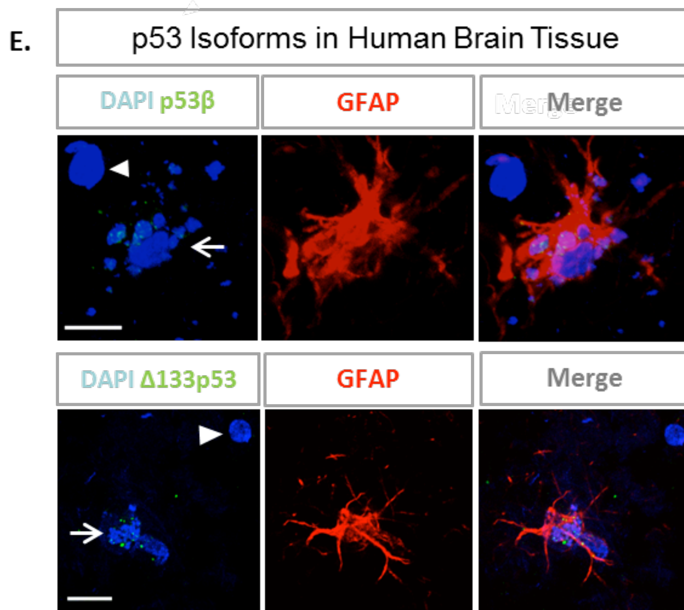

A.
Human Brain Tissue
ND AD ALS
p53
50kDa
1.0 3.0 2.9
p53β
47kDa
1.0 2.4 2.6
Δ133p53
35kDa
1.0 0.7 0.6
Actin


B.
Human Brain Tissue
Relative Fold-Change mRNA
(Normalized to β-Actin)
ND
AD
ALS
p53
p53β
Δ133p53


C.
H358 (p53-null)
p53β
DAPI
+Control
+p53β


D.
H358 (p53-null) Δ133p53-inducible
Δ133p53 DAPI
-Dox
+Dox


F.
Primary Human Astrocytes
SA-β-Gal
p5
p10


E.
p53 Isoforms in Human Brain Tissue
DAPI p53β
GFAP
Merge
DAPI Δ133p53
GFAP
Merge

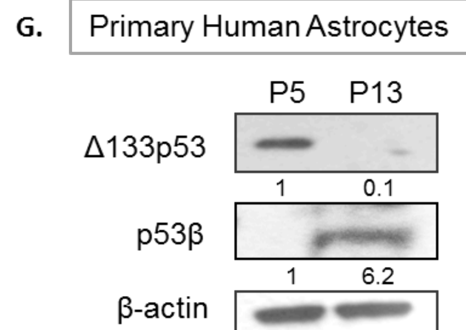

G.
Primary Human Astrocytes
P5 P13
Δ133p53
1 0.1
p53β
1 6.2
β-actin

Figure 5.2: p53β is upregulated and Δ133p53 is downregulated in neurodegenerative diseases and serial passaged primary astrocytes *in vitro*. (a) Representative Western blot showing elevated expression of full-length p53 and p53β and decreased Δ133p53 expression in Alzheimer's disease (AD) (Case 3) and amyotrophic lateral sclerosis (ALS) (Case 3) compared to non-disease (ND) age-matched control tissue (Case 2). Densitometric values are normalized to β-actin. (b) Summary of densitometric analyses of Western blots from AD (Cases 1-4), ALS (Cases 1-3), and ND (Cases 1-4). (c) Immunofluorescence staining with anti-p53β antibody TLQ40 in H358 cells (p53-null) with or without constitutive p53β expression. Scale bars = 10 μm. (d) Immunofluorescence staining with anti-Δ133p53 antibody MAP4 in H358 cells with (+Dox) and without (-Dox) induction of anti-Δ133p53 expression. Scale bars = 10 μm (e) p53β and Δ133p53 expression in GFAP-positive astrocytes (arrows). No expression of p53β and Δ133p53 was observed in GFAP-negative cells (arrow heads). AD tissue was case 1. Scale bars = 10 μm. (f) SA-beta-Gal staining in primary human astrocytes at passage number 5 and 10. Scale bar = 20 μm. (g) Western blot analysis of p53 isoform signature in early-passage (P5) and aged (P13) primary human astrocytes. Densitometric values are normalized to β-actin.

5.3 Autophagic degradation of Δ133p53 and SRSF3-mediated regulation of p53β in astrocytes

To investigate whether increased p53β protein and decreased Δ133p53 protein in aged astrocytes and in neurodegenerative diseases are due to regulation at the mRNA level, we performed qRT-PCR using RNA samples extracted from human brain tissues and cultured astrocytes (**Figure 5.3A-C**). Δ133p53 mRNA was expressed at similar levels between AD, ALS and ND age-matched tissues (**Figure 5.3A**), as well as between early-passage and aged astrocytes (**Figure 5.3C**). p53β, by contrast, significantly increases at the mRNA level both in neurodegenerative diseases (AD and ALS) (**Figure 5.3B**) and in aged astrocytes *in vitro* (**Figure 5.3C**). These data indicate that Δ133p53 expression is mainly regulated at the protein level, while p53β is regulated at the mRNA level in neurodegenerative diseases and during *in vitro* aging of human primary astrocytes, as we previously reported in human fibroblasts and $CD8^+$ T-lymphocytes (Fujita et al., 2009a; Mondal et al., 2013). Increased Δ133p53 mRNA in non-disease pediatric tissues compared to non-disease aged brain tissues (**Figure 5.3A**), however, may also suggest a transcriptional control of Δ133p53 during brain aging.

We tested whether Δ133p53 is degraded via selective autophagy in astrocytes, similar to senescent fibroblasts and $CD8^+$T-lymphocytes (Horikawa et al., 2014; Mondal et al., 2013). Senescent astrocytes (at passage number 15) with low levels of Δ133p53 were treated with a pharmacological inhibitor of autophagy, bafilomycin A1 (Mizushima et al., 2010), whose action was confirmed by increased LC3-II (Tanida et

al., 2008)(**Figure 5.3D**). Treatment with bafilomycin A1 resulted in the upregulation of Δ133p53 in senescent astrocytes (**Figure 5.3D**), suggesting that autophagic degradation of Δ133p53 contributes to its downregulation in these cells. The stabilization of Δ133p53 by bafilomycin A1 was also observed in an immortalized human astrocyte cell line (**Figure 5.3E**). Knockdown of p62/SQSTM1, which is a ubiquitin binding adaptor specifically functioning in the selective autophagy pathway (Johansen and Lamark, 2011), also stabilized Δ133p53 (**Figure 5.3F**), further supporting the degradation of Δ133p53 via selective autophagy. Since a splicing factor SRSF3 inhibits the alternative mRNA splicing generating p53β in human fibroblasts and $CD8^+$ T-lymphocytes (Mondal et al., 2013; Tang et al., 2013), we examined whether SRSF3 regulates p53β in astrocytes as well. Knockdown of SRSF3 through small interfering RNA (siRNA) in immortalized astrocytes resulted in a significant increase in p53β mRNA (**Figure 5.3G**), consistent with its negative regulation of p53β at the transcript level. These results indicate that the regulatory mechanisms for p53 isoform expression (autophagic degradation of Δ133p53 and SRSF3-mediated regulation of p53β splicing) are conserved across different cell types, including human fibroblasts, $CD8^+$ T-lymphocytes and astrocytes.

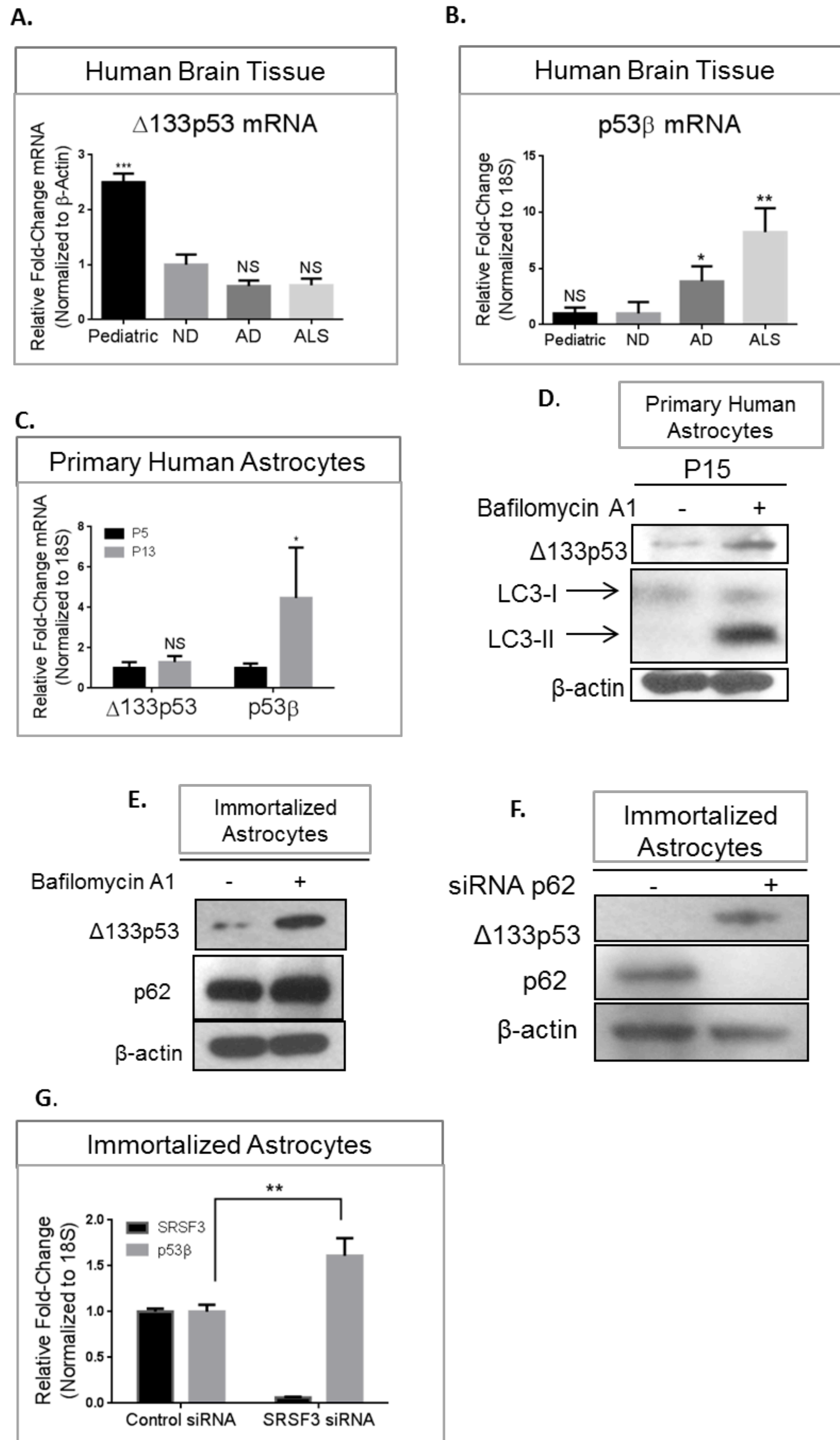

A.
Human Brain Tissue
Δ133p53 mRNA
Relative Fold-Change mRNA (Normalized to β-Actin)
***
NS
NS
Pediatric
ND
AD
ALS
B.
Human Brain Tissue
p53β mRNA
Relative Fold-Change (Normalized to 18S)
NS
*
**
Pediatric
ND
AD
ALS
C.
Primary Human Astrocytes
Relative Fold-Change mRNA (Normalized to 18S)
P5
P13
NS
*
Δ133p53
p53β
D.
Primary Human Astrocytes
P15
Bafilomycin A1
-
+
Δ133p53
LC3-I
LC3-II
β-actin
E.
Immortalized Astrocytes
Bafilomycin A1
-
+
Δ133p53
p62
β-actin
F.
Immortalized Astrocytes
siRNA p62
-
+
Δ133p53
p62
β-actin
G.
Immortalized Astrocytes
Relative Fold-Change (Normalized to 18S)
SRSF3
p53β
**
Control siRNA
SRSF3 siRNA

Figure 5.3: Autophagic degradation of Δ133p53 and SRSF3-mediated regulation of p53β. qRT-PCR analysis of (a) Δ133p53 and (b) p53β in pediatric (Cases 1-4), Alzheimer's disease (AD) (Cases 1-4), amyotrophic lateral sclerosis (ALS) (Cases 1-3), and non-disease (ND) control tissues (Cases 1-4). (c) qRT-PCR analysis of Δ133p53 and p53β in early-passage (P5) and aged (P13) primary astrocytes. (d) Δ133p53 expression is induced by bafilomycin A1 treatment (100 nM for 4 h) in senescent human astrocytes (P15). (e) Δ133p53 protein is stabilized by bafilomycin A1 treatment in immortalized human astrocytes. (f) Knockdown of p62/SQSTM1 stabilizes Δ133p53 in immortalized astrocytes. (g) SRSF3 knockdown induces p53β mRNA. Immortalized astrocytes were transfected with SRSF3 siRNA or control siRNA and examined by qRT-PCR for SRSF3 and p53β expression.

5.4 Loss of Δ133p53 induces senescent phenotype in astrocytes

Given a p53 isoform expression signature in neurodegeneration and senescent astrocytes *in vitro* (decreased Δ133p53 and increased p53β), we attempted to reproduce the senescence-associated expression signature in early-passage primary or immortalized human astrocytes (Ferenczy et al., 2013; Major et al., 1985) to examine whether it induces these cells to senesce. Immortalized astrocytes were transfected with siRNA specifically targeting Δ133p53 (Bernard et al., 2013) or scrambled siRNA control, followed by western blot analysis and immunofluorescence staining confirming Δ133p53 knockdown (**Figure 5.4A-B, G**). Three days following Δ133p53 knockdown, cells became growth-arrested (**Figure 5.4C, top panel**) with vacuolization (**Figure 5.4C, bottom panel**) and increased SA-β-Gal staining (**Figure 5.4D-E**), which are characteristics of cellular senescence. Increased mRNA expression of pro-inflammatory cytokines IL-6 and IL-1β, as well as $p21^{WAF1}$, was also observed in these senescent cells (**Figure 5.4F**), suggesting that loss of Δ133p53 induces SASP. Knockdown of Δ133p53 in early-passage primary astrocytes (confirmed by immunofluorescence; **Figure 5.4G**) also resulted in increased SA-β-Gal staining (**Figure 5.4H-I**) and the induction of SASP cytokines (**Figure 5.4J**).

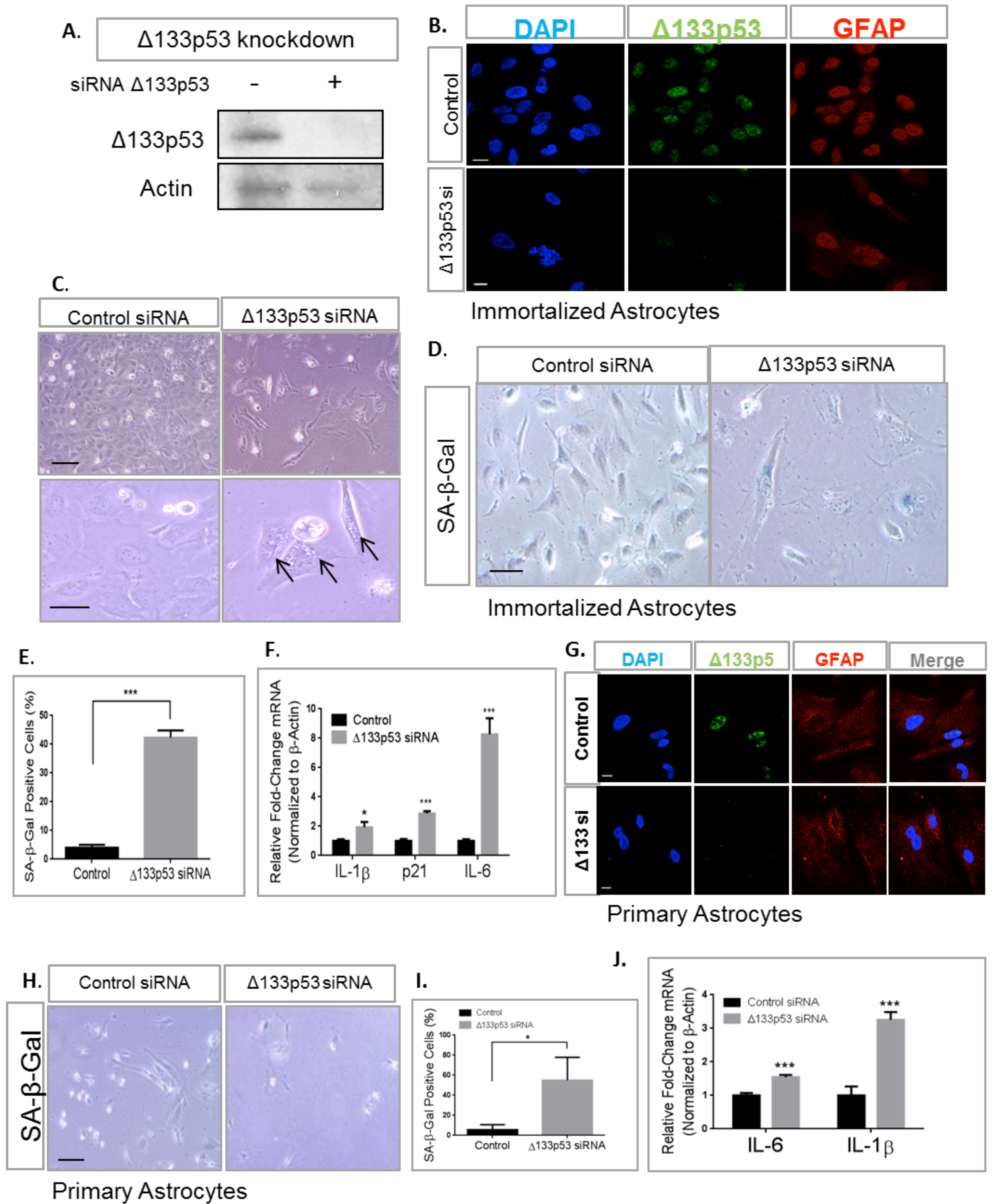

A.
Δ133p53 knockdown
siRNA Δ133p53 - +
Δ133p53
Actin
B.
DAPI
Δ133p53
GFAP
Control
Δ133p53 si
Immortalized Astrocytes
C.
Control siRNA
Δ133p53 siRNA
D.
Control siRNA
Δ133p53 siRNA
SA-β-Gal
Immortalized Astrocytes
E.
SA-β-Gal Positive Cells (%)
Control
Δ133p53 siRNA
F.
Relative Fold-Change mRNA (Normalized to β-Actin)
Control
Δ133p53 siRNA
IL-1β
p21
IL-6
G.
DAPI
Δ133p5
GFAP
Merge
Control
Δ133 si
Primary Astrocytes
H.
Control siRNA
Δ133p53 siRNA
SA-β-Gal
Primary Astrocytes
I.
SA-β-Gal Positive Cells (%)
Control
Δ133p53 siRNA
J.
Relative Fold-Change mRNA (Normalized to β-Actin)
Control siRNA
Δ133p53 siRNA
IL-6
IL-1β

Figure 5.4: Knockdown of Δ133p53 induces senescent phenotype in astrocytes. Immortalized astrocytes were transfected with Δ133p53 siRNA or control siRNA and analyzed after 3 days as below. (a-b) Confirmation of Δ133p53 knockdown via (a) Western blot and (b) immunofluorescence. (c) Phase-contrast images of growth arrest phenotype (upper panel, 20X magnification) and vacuolization (lower panel, 40X magnification) induced by Δ133p53 knockdown. Scale bars = 20 μm. (d-e) An increase in SA-β-Gal staining. (d) Representative images, Scale bar = 20 μm and (e) quantitative summary from triplicated experiments. (f) Increase in IL-6, IL-1β and $p21^{WAF1}$ mRNA expression by Δ133p53 knockdown. Primary human astrocytes at early-passage (P5) were transfected with Δ133p53 siRNA. (g) Confirmation of Δ133p53 knockdown via immunofluorescence. Scale bars = 10 μm. (h-i) Loss of Δ133p53 induces an increase in SA-β-Gal. Scale bars = 20 μm. (j) an increase in IL-6 and IL-1β mRNA expression.

5.5 Increased p53β induces senescent phenotype in astrocytes

To examine whether overexpression of p53β directly induces senescence, a lentiviral vector driving p53β expression or a vector control was transduced to immortalized astrocytes and its overexpression was confirmed by western blot analysis (**Figure 5.5A**). Similar to Δ133p53 knockdown, p53β overexpression inhibited cell proliferation (**Figure 5.5B**), increased SA-β-Gal-positive cells (**Figure 5.5C-D**), and induced pro-inflammatory SASP cytokines IL-6, IL-8, IL-1 β and $p21^{WAF1}$ (**Figure 5.5E**). Early-passage primary astrocytes with p53β overexpression (**Figure 5.5F**) were also growth-inhibited (**Figure 5.5G**), had increased SA-β-Gal staining (**Figure 5.5H-I**), and induction of SASP (**Figure 5.5J**). These results indicate that Δ133p53 downregulation and p53β upregulation play causative roles in inducing cellular senescence and SASP in astrocytes.

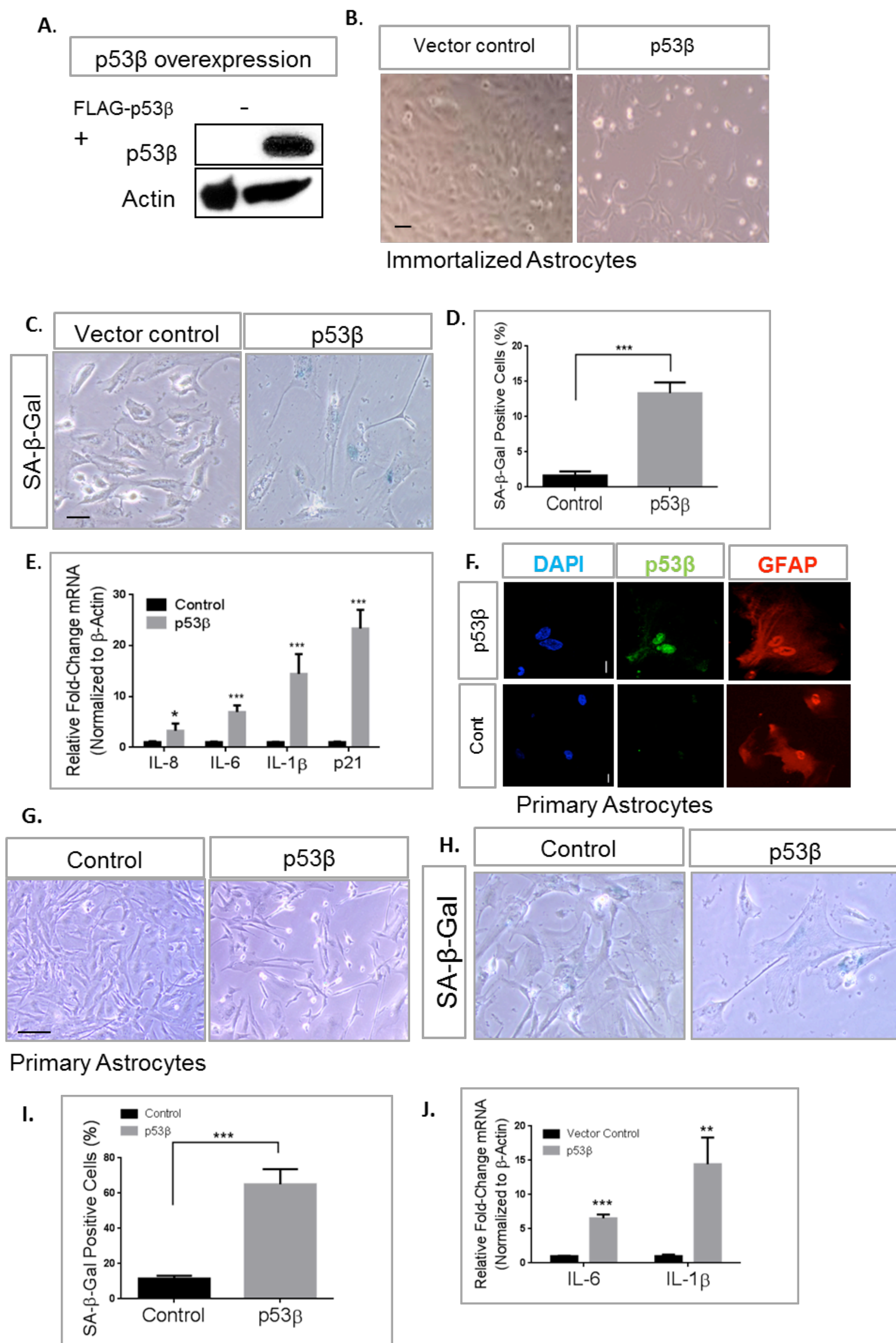

A.
p53β overexpression
FLAG-p53β
-
+
p53β
Actin
B.
Vector control
p53β
Immortalized Astrocytes
C.
Vector control
p53β
SA-β-Gal
D.
SA-β-Gal Positive Cells (%)
***
Control
p53β
E.
Relative Fold-Change mRNA (Normalized to β-Actin)
Control
p53β
IL-8
IL-6
IL-1β
p21
F.
DAPI
p53β
GFAP
p53β
Cont
Primary Astrocytes
G.
Control
p53β
Primary Astrocytes
H.
Control
p53β
SA-β-Gal
I.
Control
p53β
SA-β-Gal Positive Cells (%)
***
J.
Relative Fold-Change mRNA (Normalized to β-Actin)
Vector Control
p53β
IL-6
IL-1β

Figure 5.5: Overexpression of p53β induces senescent phenotype in astrocytes. Immortalized astrocytes were transduced with p53β lentiviral vector or control vector and analyzed after 3 days as below. (a) Confirmation of p53β overexpression via Western blot. (b) Phase-contrast images of growth arrest phenotype. Scale bar = 20 μm. (c-d) Representative images of increase in SA-β-Gal staining by p53β overexpression. (d) Quantitative summary from triplicated experiments. (e) Elevated mRNA expression of IL-8, IL-6, IL-1β and p21WAF1 by p53β overexpression. Primary human astrocytes at early passage (P5) were transduced with a p53β-overexpressing lentiviral vector or control. (f) Confirmation of p53β overexpression via immunofluorescence. Scale bars = 10 μm. (g) Phase-contrast images of growth arrest phenotype. Scale bars = 20 μm. (h-i) increased SA-β-Gal staining in p53β-overexpressing cells. (j) Elevated mRNA expression of IL-6 and IL-1β.

Summary

Here we show that cellular senesce exists in astrocytes in neurodegenerative diseases, including AD and ALS (**Figure 5.1**). Given that p53 isoforms Δ133p53 and p53β are endogenous regulators of senescence we investigated whether they modulate SASP in the CNS. We found that astrocytes are the main cell type that expresses Δ133p53 and p53β in neurodegeneration (**Figure 5.2**). Additionally, through manipulation of the isoforms in primary and immortalized astrocytes *in vitro,* we show that loss of Δ133p53 or gain of p53β directly induces SASP (**Figures 5.4 and 5.5**). Importantly, serial passed primary astrocytes and neurodegenerative disease brain tissue both demonstrate a signature p53 isoform signature of elevated p53β and reduced Δ133p53 (**Figure 5.2**), consistent with our findings in aged fibroblasts and T-cells (Fujita et al., 2009; Horikawa et al., 2014; Mondal et al., 2013; Tang et al., 2013), suggesting the presence of a common p53 isoform-mediated aging mechanism.

Chapter 6

Manipulation of Δ133p53 as a Therapeutic Strategy for Neurodegeneration

Introduction

THERAPIES targeting astrocytes have emerged as an important strategy to delay or inhibit the progression of neurodegeneration (Colin et al., 2009; Furman et al., 2012; Pehar et al., 2014; Vargas et al., 2008; Verkhratsky et al., 2012). Currently the only FDA-approved drug for ALS providing modest benefit is Riluzole, which targets astrocyte glutamate transporter, EAAT2 (Miller et al., 2012) and induces release of neurotrophic factors (Mizuta et al., 2001). The importance of astrocytes is also highlighted by the features of a rare astrocyte disease, Alexander disease, involving a mutation in the GFAP resulting in neuronal degeneration, astrocyte dysfunction, and

leukodystrophy (Brenner et al., 2001). Astrocytes exert both neuroprotective and neurotoxic effects in the brain in a context-dependent manner. Thus, therapies aimed at enhancing their protective function and minimizing their toxic functions have emerged as a promising strategy (Chen et al., 2006). In terms of their protective roles, astrocytes produce a variety of neurotrophic factors which enhance neuron survival in mouse models of MS (Flügel et al., 2001; Lessmann et al., 2003), AD (Nagahara et al., 2009; Tuszynski, 2000), and PD (Hyman et al., 1991; Lingor et al., 2000) and ALS (Das and Svendsen, 2014). For instance, astrocytes are the main source of nerve growth factor (NGF), glial-derived neurotrophic factor (GDNF), and insulin-like growth factor (IGF) (Eddleston and Mucke, 1993). These factors are deficient in PD (Nagatsu et al., 2000) and AD (Tuszynski, 2007) patients and also decrease to a lesser extent with general brain aging (Erickson et al., 2010).

On the other hand, astrocytes are also capable of eliciting neurotoxic functions. In co-culture experiments, astrocytes derived from patients with neurodegenerative diseases, such as ALS and HD, as well as from neurodegeneration mouse models, induce neuronal death in a non-cell autonomous manner (Bradford et al., 2009; Di Giorgio et al., 2007; Ilieva et al., 2009; Marchetto et al., 2008). Additionally, during neurodegeneration and also general aging, astrocytes lose many of their essential functions. In addition to decreased neurotrophic and growth factor support to neurons (Das and Svendsen, 2014; Genis et al., 2014), astrocyte have decreased glutamate uptake upon conditions of oxidative stress (Bal-Price and Brown, 2001; Gottfried et al., 2002; Papadopoulos et al., 1997; Pertusa et al., 2007) and increased pro-inflammatory

cytokine release (Jiang and Cadenas, 2014). One process that is central to all of these age- and neurodegenerative-induced changes is the induction of cellular senescence (Bitto et al., 2010; García-Matas et al., 2008; Morley et al., 2012). Thus, in this chapter we investigated whether p53-mediated isoforms regulate SASP in neurodegeneration.

Results

6.1 Increased neuronal death upon co-culture with Δ133p53-knocked-down or p53β-overexpressing astrocytes

To determine how the p53 isoform-mediated generation of senescent astrocytes with SASP contributes to neurodegeneration, we utilized a neuron-astrocyte co-culture system, in which human primary astrocytes were co-plated with induced pluripotent stem cell (iPSC)-derived mature neurons (Brennand et al., 2011; Haidet-phillips et al., 2012; Muratore et al., 2014), followed by assays for neurotoxicity (**Figure 6.1.1A-B**). Mature neurons were derived from iPSC (**Figure 6.1.1C**) and confirmation of neuronal phenotypes was achieved through expression of sequential markers of differentiation: iPSCs expressed TRA-1-81 demonstrating pluripotency (**Figure 6.1.1D**); neural stem cells (NSC) expressed Nestin and Sox1 (**Figure 6.1.1E**); and neurons expressed neuronal nuclei marker (NeuN) (**Figure 6.1.1F**). Motor neurons, a mature neuronal subtype that is primarily lost in ALS, were also derived from iPSC (Grunseich et al., 2014) and their differentiation was confirmed by expression of non-phosphorylated neurofilament marker, SMI-32 (**Figure 6.1.1G**).

When early-passage astrocytes with Δ133p53 siRNA or control siRNA (same cells as used in **Figure 5.4**) were co-cultured with motor neurons for 48h, Δ133p53-knocked-down astrocytes induced ~15-fold higher number of neurons positive for cleaved caspase 3, a final effector of neuronal apoptosis (Hartmann et al., 2000; Zhao et al., 2003), than control astrocytes (**Figure 6.1.2A-B**). Similarly, p53β-overexpressing

astrocytes (same cells as used in **Figure 5.5**) also resulted in ~20-fold increase in cleaved caspase 3-positive motor neurons (**Figure 6.1.2C-D**). To examine the effects of astrocytes on neurons in general and to obtain another quantitative measure, the co-culture experiment was performed using less specialized neurons (generated as in **Figure 6.1.1C**) and the number of surviving neurons (NeuN-positive) was counted after 48 hours co-culture with astrocytes. A significant decrease in the number of surviving neurons was observed following co-culture with Δ133p53-knocked-down astrocytes (**Figure 6.1.2E-F**) or p53β-overexpressing astrocytes (**Figure 6.1.2G-H**). An increase in neuronal apoptosis by p53β-overexpressing astrocytes was also confirmed in this experimental setting (**Figure 6.1.2I-J**). These findings indicate that the neurodegeneration-associated p53 isoform expression signature confers astrocytes with neurotoxic activity.

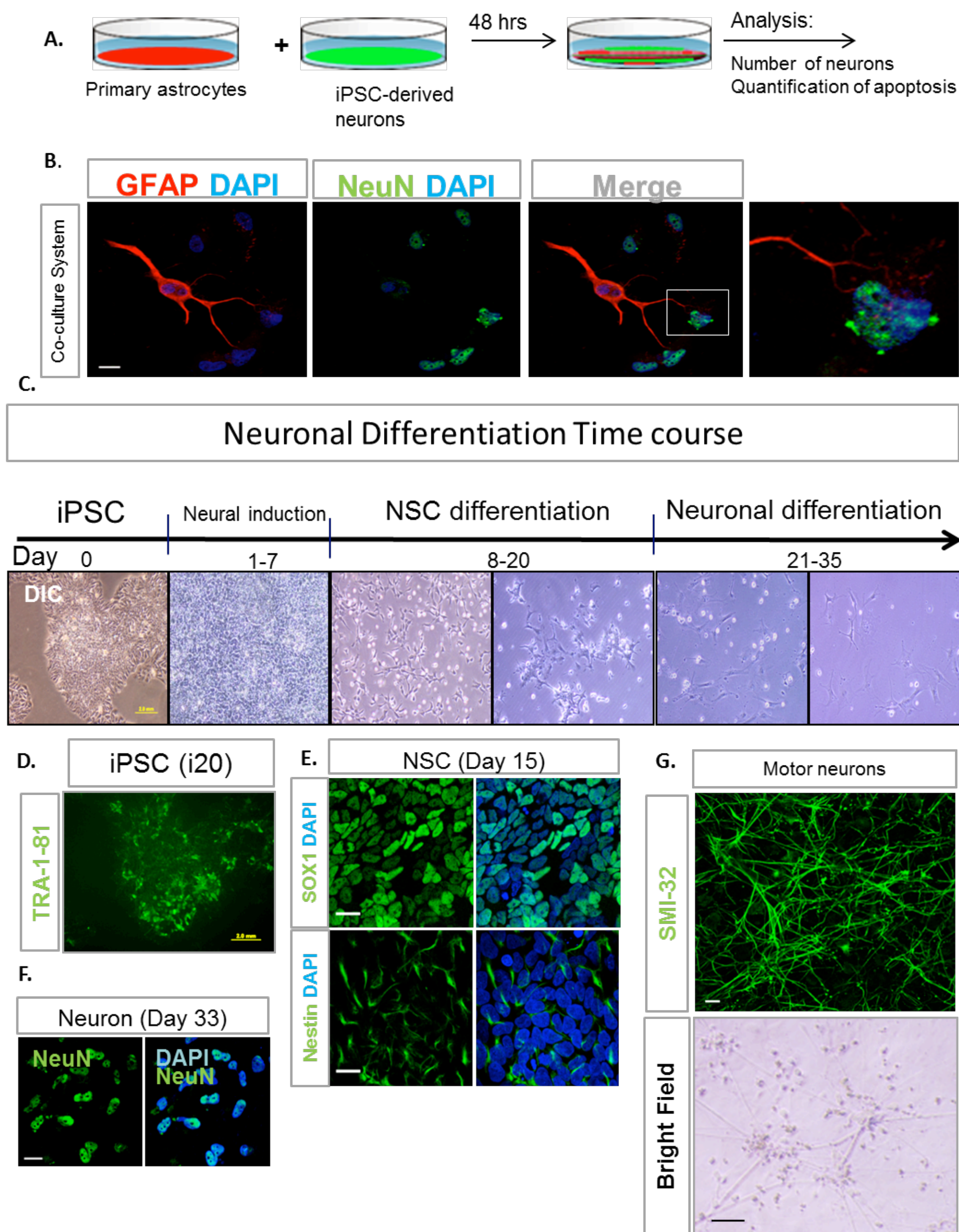
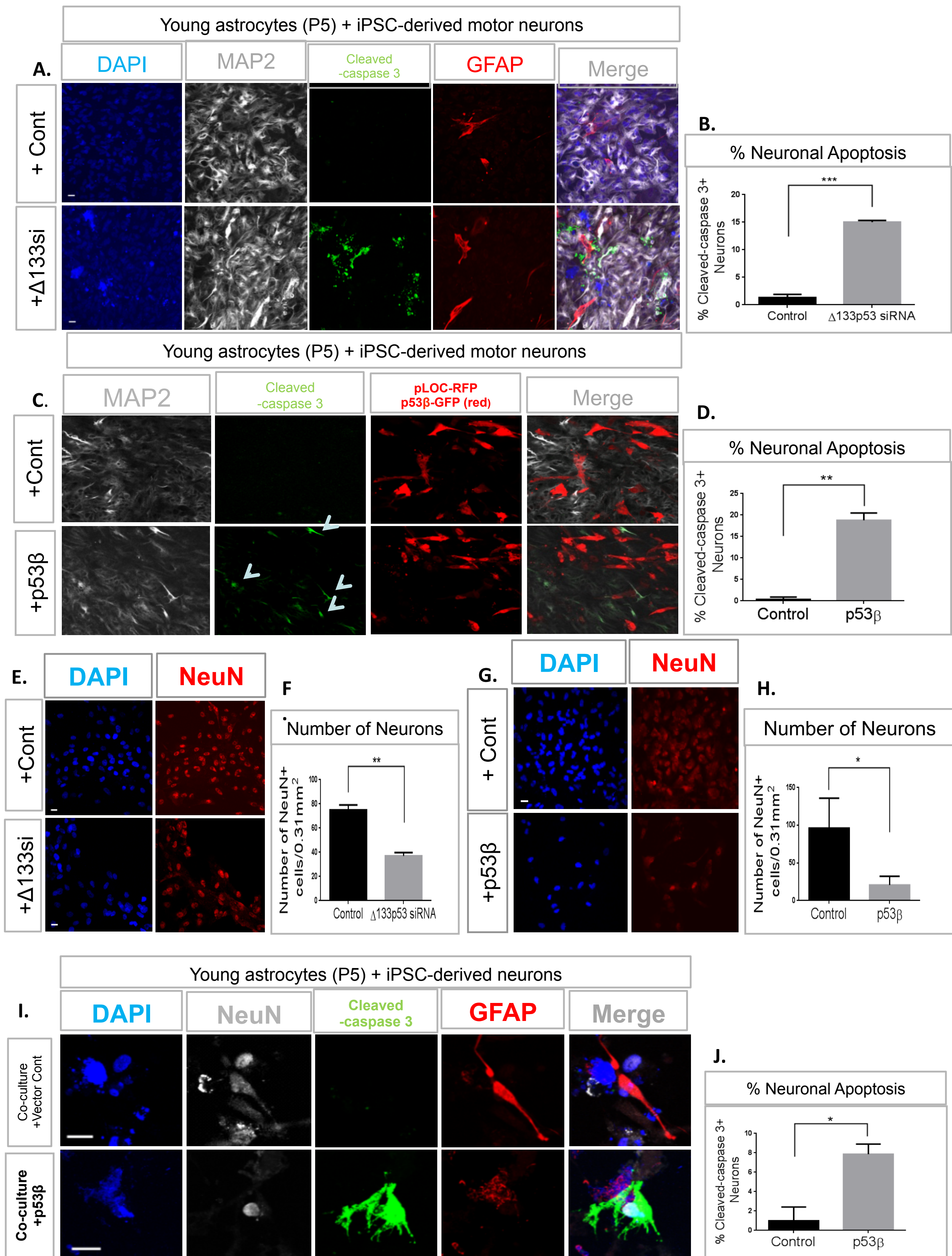


Figure 6.1.1 Generation of mature neurons from induced pluripotent stem cells (iPSC) and increased neurotoxicity by p53β overexpression in astrocytes. (a-b) Experimental protocol for co-culture system in which primary human astrocytes (red) are co-cultured with iPSC-derived neurons (green) for 48 hours. (c-f) Neuronal differentiation time course. Sequential differentiation of iPSC was confirmed though the expression of (d) TRA-1-81, (e) Sox1 and Nestin, and (f) NeuN. Scale bars = 10. (g) Motor neurons (Grunseich et al., 2014) were characterized by the expression of SMI-32. Scale bars =10 μm.



Young astrocytes (P5) + iPSC-derived motor neurons
A.
DAPI
MAP2
Cleaved -caspase 3
GFAP
Merge
+ Cont
+Δ133si
B.
% Neuronal Apoptosis
% Cleaved-caspase 3+ Neurons
Control
Δ133p53 siRNA
C.
MAP2
Cleaved -caspase 3
pLOC-RFP p53β-GFP (red)
Merge
+Cont
+p53β
D.
% Neuronal Apoptosis
Control
p53β
E.
DAPI
NeuN
F
Number of Neurons
Number of NeuN+ cells/0.31mm2
Control
Δ133p53 siRNA
G.
H.
Number of Neurons
Young astrocytes (P5) + iPSC-derived neurons
I.
Co-culture +Vector Cont
Co-culture +p53β
J.
% Neuronal Apoptosis

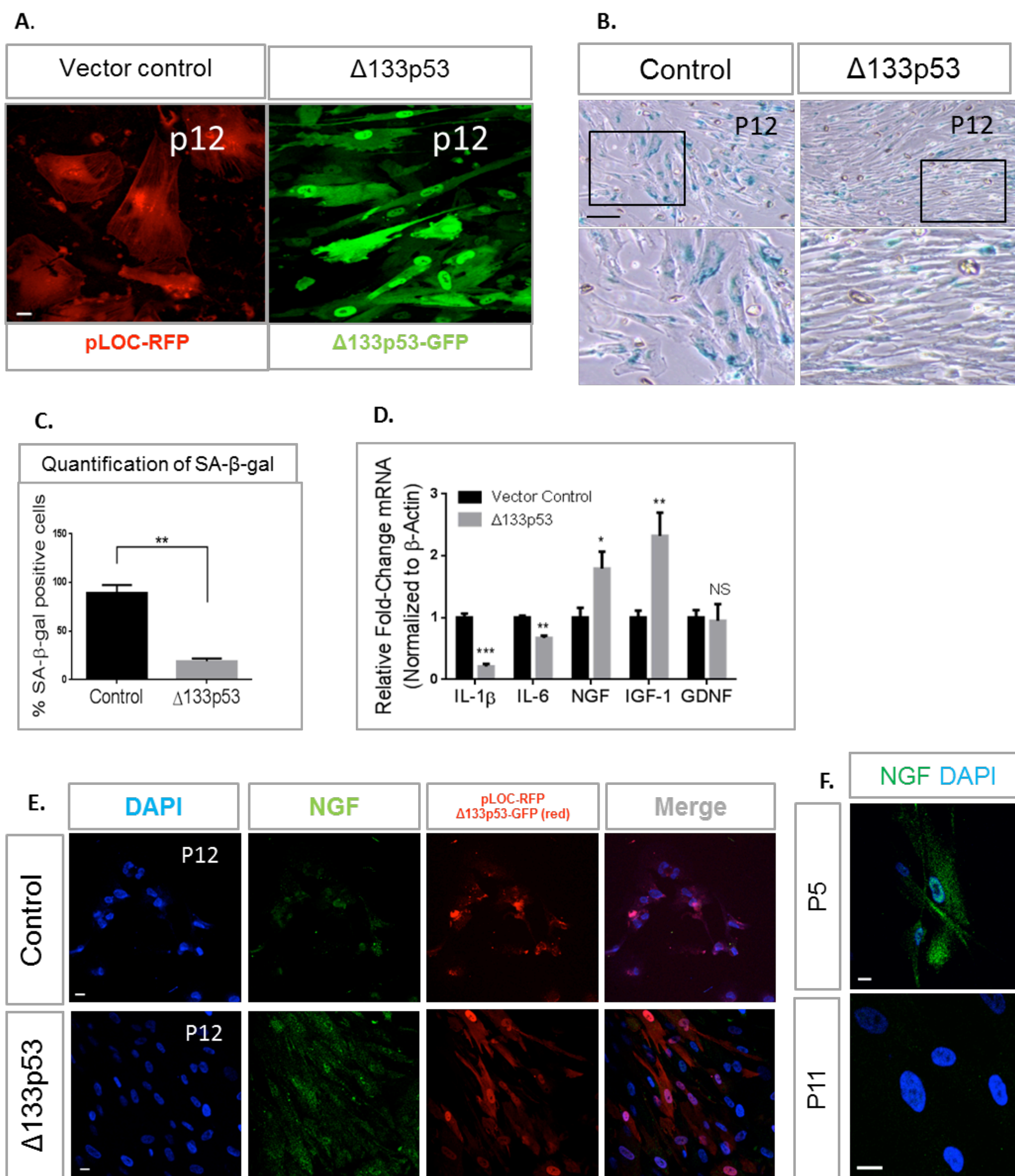
Figure 6.1.2.
Increased neuronal death upon co-culture with Δ133p53-knocked-down or p53β-overexpressing astrocytes. Early-passage primary astrocytes (P5) with Δ133p53 siRNA and control siRNA (generated as in Fig 5.4) (a, b, e and f) and those with p53β -overexpression or control vector (generated as in Fig 5.5) (c, d, g and h) were used in co-culture for 48 hours with motor neurons (Grunseich et al., 2014) (a-d, i-j) or less specialized neurons (generated as in Fig 6.1.1C-F) (e-h). (a) Immunofluorescence staining of cleaved-caspase 3, MAP2, and GFAP. Scale bars = 10 μm. (c) Fluorescent signals derived from the lentiviral vectors mark astrocytes. GFP from the Δ133p53 vector was pseudocolored red. Scale bars = 10 μm (b and d) Quantification of neuronal apoptosis. Cleaved caspase-3-positive neurons per total number of MAP2-positive neurons were counted in triplicate experiments in 5 microscopic fields (40X magnification). (e and g) Immunofluorescence staining of NeuN. (f and h) Quantification of number of neurons. Total number of NeuN-positive neurons were counted in at least 5 microscopic fields (40X magnification) in triplicate experiments. Scale bars = 10 μm. (i) Co-culture of iPSC-derived neurons and astrocytes. For quadruple-immunofluorescence in this experiment, a non-fluorescence-carrying retroviral vector was used for p53β overexpression in astrocytes. Antibodies were used for astrocyte specific marker, GFAP, neuron specific marker, NeuN, and apoptotic marker, cleaved-caspase 3. Scale bars = 10 μm. (j) Quantification of neuronal apoptosis was achieved by counting the number of cleaved caspase-3-positive neurons per total number of NeuN-positive neurons in triplicate experiments in 5 microscopic fields (40X magnification).

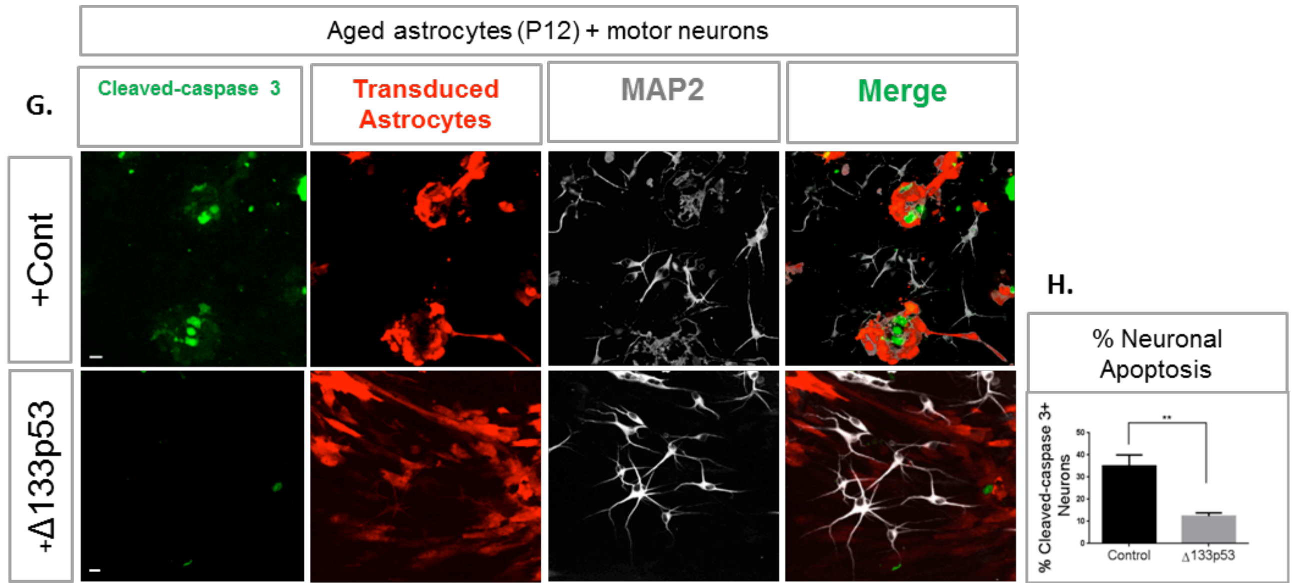
6.2 Δ133p53 prevents astrocytes from senescence and enhances their neuroprotective function

The induction of astrocyte senescence and neurotoxicity by manipulated expression of the p53 isoforms as described above prompted us to hypothesize that their manipulated expression (which in this case recapitulates the expression profile in early-passage astrocytes and non-disease brain tissues) may also lead to senescence inhibition and neuroprotective function in astrocytes. To test this hypothesis, Δ133p53 expression was reconstituted via lentiviral vector transduction in primary astrocytes approaching full senescence (at passage number 12) (**Figure 6.2A**). Reconstituted expression of Δ133p53 restored cell proliferation (**Figure 6.2B**), decreased SA-β-Gal positive cells (**Figure 6.2B-C**), and reduced the levels of pro-inflammatory SASP cytokines IL-6 and IL-1β (**Figure 6.2D**). Significantly, two neurotrophic growth factors, NGF and IGF-1, out of the three examined were 2- to 3-fold upregulated by Δ133p53 overexpression (**Figure 6.2D**). Immunofluorescence staining of NGF confirmed its increased expression in Δ133p53-reconstituted astrocytes (**Figure 6.2E**), which was comparable to that in early-passage astrocytes (**Figure 6.2F**).

We then used these Δ133p53-reconstituted astrocytes in the co-culture experiment (as performed in **Figure 6.1.2**). Vector control-transduced astrocytes (derived from passage number 12) resulted in a much larger number of motor neurons positive for cleaved caspase 3 (~35%; **Figure 6.2G-H**) than those derived from passage number 5 (0-2%; **Figure 6.1.2A-D**), indicating a senescence-associated progression of neurotoxicity in astrocytes. The reconstitution of Δ133p53 in these near-senescent astrocytes significantly reduced the number of cleaved caspase 3-

positive motor neurons (~12%; **Figure 6.1.2E-H**), suggesting that Δ133p53 in astrocytes functions to suppress neuronal apoptosis. Counting of NeuN-positive neurons following co-culture (**Figure 6.2I-J**) also showed that aged astrocytes *in vitro* were less neuroprotective than early-passage astrocytes (compare controls in **Figure 6.2J** versus **6.1.2F and 6.1.2H**), and that reconstituted expression of Δ133p53 restored the number of surviving neurons back to the level exerted by early-passage astrocytes (Δ133p53 in **Figure 6.2J**; compare with controls in **Figure 6.1.2F** and **6.1.2H**). These results indicate that Δ133p53 in astrocytes prevents cellular senescence, inhibits SASP, induces neurotrophic factors, and enhances their neuroprotective activity.


A.
Vector control
Δ133p53
p12
p12
pLOC-RFP
Δ133p53-GFP
B.
Control
Δ133p53
P12
P12
C.
Quantification of SA-β-gal
% SA-β-gal positive cells
150
100
50
0
**
Control
Δ133p53
D.
Relative Fold-Change mRNA
(Normalized to β-Actin)
Vector Control
Δ133p53
3
2
1
0
***
**
*
**
NS
IL-1β
IL-6
NGF
IGF-1
GDNF
E.
DAPI
NGF
pLOC-RFP
Δ133p53-GFP (red)
Merge
Control
Δ133p53
P12
P12
F.
NGF DAPI
P5
P11


Aged astrocytes (P12) + motor neurons
G.
Cleaved-caspase 3
Transduced Astrocytes
MAP2
Merge
+Cont
+Δ133p53
H.
% Neuronal Apoptosis
% Cleaved-caspase 3+ Neurons
Control
Δ133p53

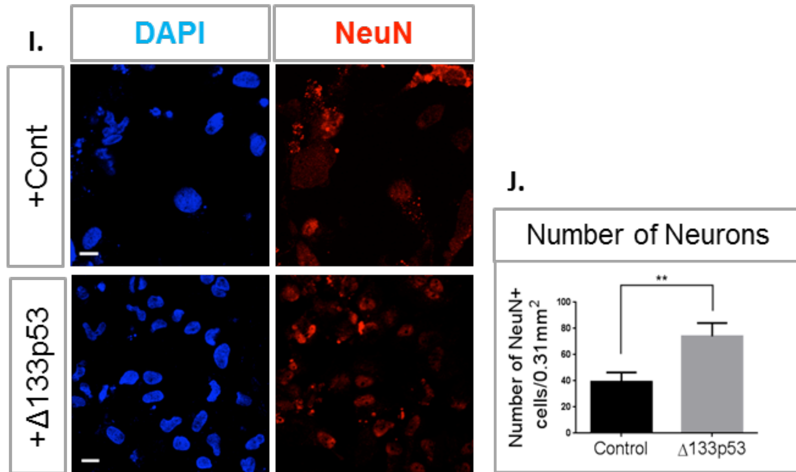

I.
DAPI
NeuN
+Cont
+Δ133p53
J.
Number of Neurons
Number of NeuN+ cells/0.31mm2
Control
Δ133p53

Figure 6.2: Δ133p53 prevents astrocyte SASP and enhances neuroprotection. Aged primary astrocytes (P12) were transduced with a lentiviral vector driving Δ133p53 or its control vector. (a) Confirmation of lentiviral transduction by RFP (vector control) or GFP (Δ133p53). Scale bars = 10 μm. (b-c) Decreased SA-β-Gal staining by reconstituted expression of Δ133p53. Representative images of SA-β-Gal staining (b, top panels, scale bars = 20 μm), enlarged images of the insets (b, bottom panels). (c) Quantitative summary from triplicated experiments. (d) Decreased expression of IL-1β and IL-6 and increased expression of NGF and IGF-1. qRT-PCR analysis was performed in triplicate. (e) Immunofluorescence staining showing increased NGF protein upon Δ133p53 reconstitution in aged (P12) astrocytes. Transduced astrocytes were marked by RFP (control) or GFP (Δ133p53); pseudocolored to red. Scale bars = 10 μm. (f) Immunofluorescence staining of NGF in early-passage (P5) and aged (P11) astrocytes. Merged images with DAPI shown. Scale bars = 10 μm. (g-j) Δ133p53 reconstituted astrocytes and control astrocytes were used in co-culture with motor neurons (g-h) or less specialized neurons (i-j), as performed in Figure 6. (g-j) Decreased percent cleaved caspse-3 motor neurons upon co-culture with Δ133p53-overexpressing aged (P12) astrocytes. (g) Immunofluorescence staining of cleaved-caspase 3 and MAP2. Scale bars = 10 μm. (h) Quantification of neuronal apoptosis was achieved by counting the number of cleaved caspase-3-positive neurons per total number of MAP2-positive neurons in triplicate experiments in 5 microscopic fields (40X magnification). (i) Immunofluorescence staining of NeuN. (j) Quantification of number of neurons. Total number of NeuN-positive neurons were counted in at least 5 microscopic fields (40X magnification) in triplicate experiments. Scale bars = 10 μm.

Summary

This study shows that p53 isoforms are endogenous regulators of cellular senescence in the CNS. A novel p53 isoform signature in neurodegenerative disease and aged astrocytes *in vitro* is identified (**Figure 5.2**). Importantly, we report for the first time that astrocyte senescence can be reversed though manipulation of p53 isoforms (**Figure 6.2**) and could lead to a viable therapeutic approach for neurodegenerative disease. Specifically, we show that p53 isoforms regulate astrocyte SASP *in vitro* (**Figure 5.4-5.5**) and in neurodegeneration (**Figure 5.2**). Importantly, we demonstrate a novel mechanism of neuroprotection though reconstitution of the Δ133p53 isoform, which increases levels of key neurotrophic factors, IGF-1 and NGF, while decreasing neurotoxic factors, IL-6 and IL-1β (**Figure 6.2**).

Chapter 7

Discussion and Concluding Remarks

ASTROCYTIC loss and dysfunction are emerging as crucial components of neurodegenerative disease pathogenesis. As the most abundant cell type in the brain, astrocytes play multiple key roles in providing structural, functional, and metabolic support to neurons (Barres, 2008). Accordingly, dysfunction of these astrocyte functions can profoundly influence neuronal survival. Specifically, senescence and apoptosis are two pathways that can disrupt astrocyte function in neurodegeneration (Bhat et al., 2012; Takuma et al., 2004). Senescent astrocytes increase the release of neurotoxic pro-inflammatory cytokines, such as IL-6 and IL-1β, and a decrease the release of neurotrophic factors, such as nerve growth factor NGF and IGF-1 (Farina et al., 2007). Additionally, astrocyte apoptosis is a key feature of neurodegeneration leading to loss of these supportive, metabolic, and inflammatory regulators in the CNS (Takuma et al.,

2004). Furthermore, astrocyte apoptosis can lead to the death of surrounding cells propagating neurodegeneration by disrupting the gap channels that connect astrocytes and neurons and mediate exchange of key factors for neuronal survival including glutamate (Lin et al., 1998). The importance of astrocytes in neurotoxicity is highlighted by the features of Alexander disease, a rare astrocyte disease involving a mutation in GFAP, results in progressive neuronal death (Brenner et al., 2001). Additionally, Riluzole, the only FDA (Food and Drug Administration)-approved drug for ALS, targets a glutamate transporter, EAAT2, in astrocytes (Miller et al., 2007). Thus, development of therapies targeting astrocytes is a subject of intense research for neurodegenerative diseases. (Furman et al., 2012; Graber et al., 2011; Verkhratsky et al., 2012).

The p53 signaling pathway is emerging as a major therapeutic target for neurodegeneration given its role regulating both apoptosis and senescence programs. There are several reports highlighting the role of p53 in astrocytes. p53 mediates oxidative stress-induced apoptosis in astrocytes, via transcription-independent signaling to the mitochondria (Intrinsic signaling pathway, Introduction **Figure 1.7**) (Bonini et al., 2004). However, loss of p53 increased astrocyte proliferation (Bogler et al., 1995) and is the earliest detected genetic event in the development of astrocytomas (Sidransky et al., 1992). Thus, striking the appropriate balance in the p53 network is essential. This work investigates p53-mediated mechanisms of apoptosis and senescence regulation in astrocytes via ASPP2 and p53 isoforms, respectively. Key findings are detailed below with an examination of study limitations and future directions.

7.1 ASPP2-mediated apoptosis in neurodegeneration

ASPP2, an activator of p53 and regulator of cell polarity, is identified as a novel regulator of astrocyte activation and apoptosis in neurodegenerative diseases (**Figure 7.1**). Its role in neurodegeneration provides insight to how inflammation at the brain barriers is sensed by cell death machinery to tip the balance between cell survival and death. ASPP2's function as a "gatekeeper" could be conferred through its ability to regulate cell polarity and RAS signaling. This property of ASPP2 is mediated through its N-terminal 360 amino acids. In polarized epithelial cells, including choroid plexus epithelial cells, ASPP2 is mainly located at apical/tight junctions in vitro and in vivo. (Sottocornola et al., 2010). Upon RAS activation, ASPP2 is translocated into the cytoplasm and enhances the apoptotic function of p53 (Wang et al., 2013). The ability of ASPP2 to stimulate the apoptotic function of p53 and p73 is mediated by its C-terminus, which is localized in the nucleus (Trigiante and Lu, 2006).

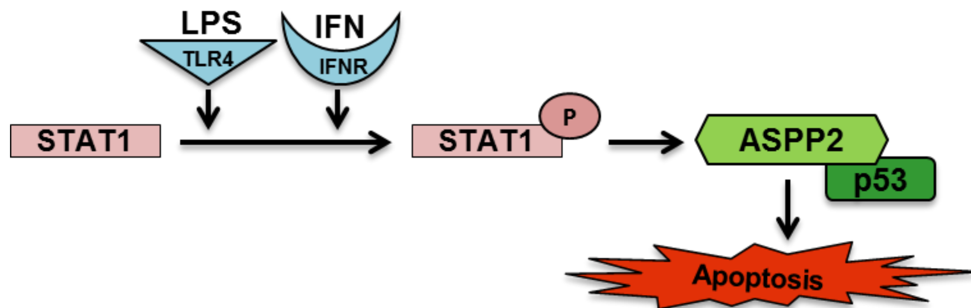


Figure. 7.1.
Proposed mechanism of ASPP2/STAT1-induced apoptosis in response to inflammatory stimuli.
In this model, STAT1 is activated via LPS or IFN binding their receptors on the cell membrane, STAT1 is phosphorylated and binds to the ASPP2 promoter/enhancer. ASPP2 translocates to the nucleus where it binds p53 and activates apoptosis. Adapted from (Turnquist et al., 2014).

STAT1 has been shown to potentiate p53- and p73-induced apoptosis by selectively enhancing its transcriptional activity on pro-apoptotic genes such as Bax (Soond et al., 2007)and Noxa (Townsend et al., 2004). However, it remains unclear how STAT1 selectively enhances the apoptotic function of p53 and family members. The identification of ASPP2 as a transcriptional target of STAT1 and its induction in astrocytes in neuroinflammatory disease provides a molecular explanation by which STAT1 signaling could be guided towards a pro-apoptotic path. Since ASPP2 is a common activator of the p53 family and able to enhance their apoptotic functions, the status of ASPP2 may play a key role in dictating the cell's response to inflammatory stimuli. Consistent with this, reduced ASPP2 expression dampened IFN-induced apoptosis in RAW264.7 cells and ASPP2-deficient mice displayed less LPS-induced apoptosis in the hippocampus. Since ASPP2 is a haploinsufficient tumor suppressor, cell polarity regulator, and activator of p53, the identified LPS/STAT1/ASPP2 pathway provides a novel link between inflammation, cell polarity and tumor suppression.

Additionally, the identified LPS/STAT1/ASPP2 pathway provides an example of so-called SMaRT signaling (**Figure 7.2**) (Lu et al., in preparation). SMaRT signaling is distinct from cascade and direct signaling because a single molecule plays 3 roles as a Sensor, Messenger and Regulator of Transcription. With regard to ASPP2's role in neuroinflammation, we show that:

1) It senses external signals at the BCSFB under basal condition where it co-localizes with tight junction proteins, ZO-1 and Par-3.
2) It acts as a messenger to integrate the signals to the nucleus by translocating from the BCSFB to the nucleus upon LPS or IFN stimulation.
3) It binds to and regulates transcription factors, such as p53.

Importantly, a SMaRT factor does not bind DNA and it is not a transcription factor itself. Additional examples of SMaRT factors include β-catenin and yes-associated protein (YAP). The concept of SMaRT signaling helps explain how a diversity of cellular signals that often come into contact with external cellular membranes can exert transcriptional affects.

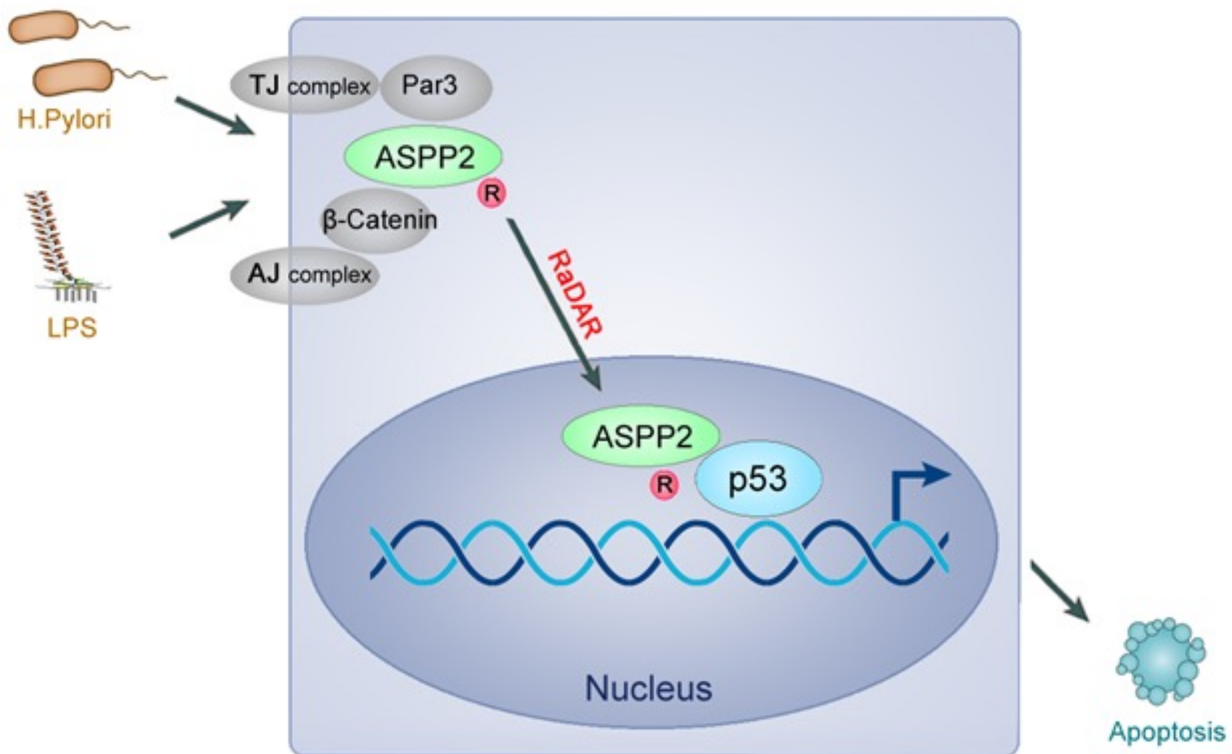


Figure 7.2
ASPP2 as a SMaRT factor.
First, ASPP2 senses external signals, such as tumorigenic pathogen helicobacter pylori (H. Pylori) and lipopolysaccharide (LPS) from gram-negative bacteria. Second, ASPP2 relays information from the cell-cell junction to the nucleus as a messanger. Finally, ASPP2 triggers cellular apoptosis in the nucleus though binding p53. ASPP2 enters the nucleus via a RaDAR import pathway, a non nuclear localization signal (NLS) pathway (Lu et al., 2014) Adapted from (Lu et al, in preparation).

Our study of the role of ASPP2 in neurodegeneration provides several lines of evidence that its induction occurs in humans (**Figures 3.2; 4.4**) as well as animal models of disease (**Figure 4.4**). Support for its role in cell death comes from data that ASPP2-deficient mice have reduced apoptosis upon LPS injection and that loss of ASPP2 abrogates apoptosis after treatment with LPS *in vitro* (**Figure 4.2**). However, further work is needed to directly relate this data to the pathogenesis of human disease in several ways. First, we demonstrate that ASPP2 is induced in astrocytes as well as choroid plexus epithelial cells in neurodegenerative/neuroinflammatory mouse models and in human disease, however more is to be explored in terms of astrocyte-neuron interactions. While loss of brain barrier integrity and astrocyte death are increasingly recognized as drivers of neurodegeneration, we have not yet examined how neurons are ultimately affected. ASPP2 likely plays a role in neurons as well, as evidenced by expanded neural progenitor cells in the ASPP2 Δexon3 mice (Sottocornola et al., 2010). Our study primarily focused on the initiation of neurodegeneration via loss of the brain barriers and neuroinflammatory sequelae, which are key aspects of the early events in neurodegeneration. However, future studies could utilize various co-culture systems – astrocyte and neuron, polarized epithelial cell and neuron – to investigate how ASPP2-depletion in non-neural cells may contribute to non-cell autonomous neuronal death. These types of studies were undertaken in Chapter 6 to investigate mechanisms of astrocyte-mediated neurotoxicity and could also be useful for studies in Chapter 4.

Additionally, it would be useful to examine how ASPP2-deficiency could be employed for therapeutic or diagnostic purposes. For instance, is ASPP2 deficiency

neuroprotective in humans? Previous studies have shown that the contact residues between ASPP2 and p53 are absent in human cancers (Gorina and Pavletich, 1996). Is the inverse true for neurodegeneration? Are ASPP2-p53 interactions enriched in neurodegeneration? If so, therapeutics would have to strike a balance between lowering levels of ASPP2 enough to promote neuronal survival, but not enough to drive tumorigenesis. Given the molecular links between cancer and neurodegeneration (see Introduction 1.1 and Introduction Part II), striking an equilibrium between these two opposing phenotypes has been a challenge for other therapeutics. For instance, Semagacestat, a compound that inhibits γ-secreatse, the enzyme that generates Aβ, was found to result in higher cancer incidence in a phase III trial (Doody et al., 2013). One proposed explanation is that p53 imbalance can lead to either cancer or neurodegeneration and p53 is known to regulate and be regulated by γ-secreatse (Checler et al., 2010). Inhibition of γ-secreatse might therefore increase the proliferative capacity of p53 in this context.

The intimate link between cancer and neurodegeneration has given them the name "face of two evils" (Curran and Christen, 2011), highlighting a major challenge in targeting p53-mediated pathways. One strategy would be to specifically target ASPP2 depletion in the brain. Recently exosomes have been investigated as a possible means of delivering siRNA to the brain (Alvarez-Erviti et al., 2011; El-Andaloussi et al., 2012). Another approach to further investigating how ASPP2 depletion may play a role in disease would be to cross ASPP2 deficient mice with various animal models of neuroinflammatory or neurodegenerative diseases and determine if there is any

phenotypic rescue or enhanced neuronal survival. The TMEV multiple sclerosis model may be ideal as we have already demonstrated that ASPP2 plays a role in this model (**Figure 4.4J**) and MS in humans through *in silico* analysis (**Figure 3.2D**). Another means to investigate this would be to delete ASPP2 from patient-derived iPSCs and differentiate them into astrocytes to determine whether they rescue any disease phenotypes or neuronal survival in co-culture studies.

7.2 Δ133p53 as a therapeutic strategy for neurodegeneration and other aging diseases

This study shows that p53 isoforms are endogenous regulators of cellular senescence in the brain. The senescence-associated p53 isoform signature (upregulation of p53β and downregulation of Δ133p53) is identified in neurodegenerative diseases and aged astrocytes *in vitro* (**Figure 5.2**), and is shown to induce astrocyte SASP (**Figures 5.4 and 5.5**) and neurotoxic effect (**Figure 5.6**). Reconstitution of Δ133p53 expression can rescue astrocyte senescence and enhance neuroprotection though increased expression of neurotrophic factors (**Figure 6.2**).

The mechanisms of p53 isoform regulation in astrocytes (autophagic degradation of Δ133p53 and SRSF3-mediated splicing regulation of p53β) (**Figure 5.3**) are consistent with other previously examined human cell types including aged fibroblasts and senescent $CD8^+$ T-lymphocytes (Fujita et al., 2009; Horikawa et al., 2014; Mondal et al., 2013; Tang et al., 2013), possibly suggesting a general aging program that is found in a variety of age-related diseases, including Alzheimer's disease and amyotrophic lateral

sclerosis. As observed in age-related disorders in general (Rubinsztein et al., 2011), neurodegeneration is associated with impaired activity of bulk autophagy (Nixon, 2013), which would stabilize autophagy substrates. However, Δ133p53, which is downregulated rather than stabilized in neurodegenerative diseases (**Figure 5.2**), does not seem to be degraded via bulk autophagy, highlighting the importance of a specific degradation of Δ133p53 via selective autophagy that is dependent on p62/SQSTM1 (Horikawa et al., 2014; Johansen and Lamark, 2011). Future studies will investigate whether selective autophagy and SRSF3-mediated alternative splicing can be manipulated to control Δ133p53 and p53β levels in neurodegenerative diseases.

Astrocytes exert both neuroprotective and neurodegenerative effects in a context-dependent manner, which are associated with either repression or induction of SASP, respectively (Morley et al., 2012; Pertusa et al., 2007). Our data suggest that the astrocyte-mediated effects of the p53 isoforms on neurons are exerted through their regulatory roles for SASP (**Figure 5.4F, 5.5E, 6.2D-F**). One main feature of astrocyte SASP driving neuronal loss is the release of pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β (Jiang and Cadenas, 2014; Mrak and Griffin, 2005). IL-6 and IL-1β were increased in the cerebral spinal fluid of Alzheimer's and Parkinson's disease patients (Blum-Degen, 1995; Jia et al., 2005). IL-6 has been revealed to cause neurodegeneration in the GFAP-IL-6 mouse model (Campbell et al., 1993). Consistent with the neurodegenerative role of these SASP cytokines, the induction of SASP in astrocytes either by Δ133p53 knockdown (**Figure 5.4F**) or p53β overexpression (**Figure 5.5E**) leads to increased neuronal apoptosis (**Figure 6.1.2**), while the repression of astrocyte

SASP by Δ133p53 restoration (**Figure 6.2D**) leads to decreased neuronal apoptosis (**Figure 6.2G-J**). Although SASP may in particular contexts play a beneficial role in tissue homeostasis and regeneration (Demaria et al., 2014; Serrano, 2014), this study does not support its beneficial role in astrocytes against neurodegeneration. Our data also suggest that the upregulation of neurotrophic growth factors such as NGF and IGF-1 in astrocytes (**Figure 6.2D-F**) mediates Δ133p53-induced neuroprotection (**Figure 6.2G-J**). NGF and IGF-1 are known to promote neuronal survival (Hefti, 1986; Wine et al., 2009) and are decreased in Alzheimer's and Parkinson's disease brain tissues (Nagatsu et al., 2000; Tuszynski, 2007). Furthermore, astrocytes utilize IGF-1 to protect neurons from oxidative stress (Genis et al., 2014).

Full-length p53 is known to transactivate pro-inflammatory cytokine genes such as IL-6 and IL-8 (Lowe et al., 2014). It is thus likely that the effect of p53β on SASP and neurodegeneration is through its cooperative activity with full-length p53 (Fujita 2009) (**Figure 7.3**). Since full-length p53 functions to inhibit the IGF-1 signaling pathway (Levine et al., 2006), the dominant-negative inhibition by Δ133p53 of full-length p53 activity (Fujita et al., 2009) may increase IGF-1 signaling towards neuroprotection. Another possibility is that Δ133p53 may directly upregulate the neurotrophic factors NGF and IGF-1 through its gain-of-function activity, which was recently reported to activate a set of genes for DNA-damage repair (Gong et al., 2015). Although *in silico* analysis of the NGF and IGF-1 genes did not identify a perfect match to the predicted Δ133p53-binding element (Gong et al., 2015), further studies will elucidate the

regulation of these neurotrophic factors by Δ133p53 in dominant-negative and gain-of-function manners (**Figure 7.3**).

A method to shift astrocytes from pro-inflammatory, neurodegenerative state to anti-inflammatory, neuroprotective state may be a promising therapeutic approach for inhibiting or delaying the progression of neurodegenerative diseases. In our proposed model (**Figure 7.3**), enhancement of Δ133p53 activity and/or inhibition of p53β activity could lead to such phenotypic shift of astrocytes, the former of which was demonstrated by lentiviral expression of Δ133p53 in this study (**Figure 6.2**) and the latter of which awaits establishment of effective knockdown of p53β in senescent astrocytes. Because increased neurotoxicity exerted by *in vitro* aged astrocytes (controls in **Figure 6.2H versus Figure 6.1.2B and D**), which was reverted by reconstituted expression of Δ133p53 (Δ133p53 in **Figure 6.2G**), is also characteristic of astrocytes derived from patients with neurodegenerative diseases (Bradford et al., 2009; Ilieva et al., 2009), our findings have implications in developing therapeutic invention for these diseases. This study provides a rationale for exploration of small molecules that can modulate the expression level or activity of the p53 isoforms to delay or inhibit the progression of neurodegeneration. Given that the senescence-associated p53 isoform expression signature and its regulatory mechanisms are conserved across different cell types, identification of such small molecules may lead to treatment of other age-related human diseases as well.

One important consideration when investigating the role of Δ133p53 in the rescue of neurodegeneration is to differentiate various potential gain-of-function roles

of Δ133p53. Δ133p53 was recently identified as a transcription factor capable of targeting DNA-damage repair genes, including Rad51 (Gong et al., 2015). In our study, we find specific neurotrophic factors are enhanced by the expression of Δ133p53 but have not identified the mechanism behind this upregulation. There are several possibilities including direct and indirect targeting. We performed *in silico* analysis of the NGF and IGF-1 sequences and found no predicted Δ133p53 binding sequences. However, interestingly their receptors and family members had about a dozen predicted binding sites, indicating that Δ133p53 may have an indirect influence on NGF and IGF-1 pathways. However, further studies are required to understand how Δ133p53 may target these pathways. One method of accomplishing this, which is currently underway, is microarray analysis comparing Δ133p53 overexpressing and control astrocytes for possible gene targets. Confirmation of any hits would require validation through chromatin-immunoprecipitation and luciferase assays using NGF and IGF-1 promoter/enhancer regions.

Another possibility is that Δ133p53 could modulate NGF and IGF-1 through full length-p53. Full-length p53 is known to transactivate pro-inflammatory cytokine genes such as IL-6 and IL-8 (Lowe et al., 2014), suggesting that the effect of p53β on SASP and neurodegeneration could be through its cooperative activity with full-length p53. Also, since full-length p53 functions to inhibit the IGF-1 signaling pathway (Levine et al., 2006), the dominant-negative inhibition by Δ133p53 of full-length p53 activity (Fujita et al., 2009) may increase IGF-1 signaling towards neuroprotection. To test this hypothesis, knock down of full-length p53 along with p53β could be performed to examine if SASP is

abrogated. Similarly, knockdown of full-length p53 along with overexpression of Δ133p53 would help to determine whether IGF-1 upregulation is mediated by full-length p53.

Other remaining questions are whether in fact fully senescent cells can indeed be rescued by Δ133p53 overexpression. It is possible that senescence rescue only occurs in nearly-senescent cells but not in fully senescent cells and that the expansion of these nearly-senescent cells becomes dominant in a heterogeneous population. While only partial rescue of senescence in nearly-senescent cells could lead to therapeutic benefit *in vivo*, nevertheless it will be important conceptually to understand the significance of Δ133p53 overexpression. There are several experiments that could be performed to address this question. The first is live imaging of a population of senescent (P15) and nearly-senescent (P10-P14) astrocytes that have been transduced with a Δ133p53 lentiviral vector tagged with GFP. This would enable single-cell tracking to determine which cells in the population are expanding. Co-staining with proliferation marker, Ki67, could differentiate the nearly-senescent versus fully-senescent cells. Another method would be to use a p16-MR transgene as developed by Campisi and colleagues (Demaria et al., 2014), which allows senescent cells to be tracked and could differentiate nearly-senescent from fully-senescent cells.

In terms of the neuroprotective roles of Δ133p53, this study would be enhanced by animal model data demonstrating that features of neurodegeneration, including neuronal loss could be rescued. These experiments could be performed in a co-culture system of neurons and astrocytes from disease model animals or via transplantation of

Δ133p53 overexpressing astrocytes *in vivo* and monitoring disease progression *in vivo*. A third method would be to test small molecules that enhance the expression of Δ133p53 as a potential therapeutic. The main challenge to this experiment is that only primates (including humans, crab-eating macaque, green monkey, Japanese macaque, and rhesus macaque) have the equivalent of Δ133p53. Nonhuman primate models of AD have been developed and present a unique opportunity to examine complex brain aging in an AD-free context, as nonhuman primates do not develop neurodegeneration (Jucker, 2010). It is interesting whether this primate-specificity of Δ133p53 is related to primate-specific accumulation of β-amyloid (Heuer et al., 2012; Toledano et al.) and other physiological or pathological processes specific or preferential to humans and other primates.

Alternatively, a chimeric mouse model could be employed. In this experiment, human astrocytes with or without overexpression of Δ133p53 would be transplanted to neurodegenerative disease mice. Su-Chun Zhang and colleagues performed such an experiment in NOD/ severe combined immunedeficient (SCID) mice whereby neural progenitors suspended in cerebral spinal fluid were transplanted to the cervical spinal cord (Chen et al., 2015). Human astrocytes in this model integrated into mouse neural networks and extended end feet around blood vessels. This same model was then used with human astrocytes derived from iPSC from ALS patients to demonstrate that astrocyte dysfunction plays a prominent role in motor deficits. This study demonstrates proof-of-principle that the chimeric animal model is feasible and would be a viable

approach to further investigate potential enhancement of neuronal survival of Δ133p53 in astrocytes.

Finally, given that the p53 isoform signature and mechanism of regulation is similar across a variety of diseases and cell types (Fujita et al., 2009; Horikawa et al., 2014; Mondal et al., 2013; Tang et al., 2013), it would be interesting to test the hypothesis that Δ133p53 induction could be a useful disease modifying therapeutic strategy for a number of diseases. Currently, we are developing a small molecule screen for this purpose and plan to test their efficacy in additional aging-diseases including early aging diseases: Werner's and Progeria syndromes, in which loss of senescent cells enhances survival in progeriod mouse model (Naylor et al., 2013). Other useful diseases to test Δ133p53 enhancing therapy would be early-onset AD as found in Down's syndrome patients.

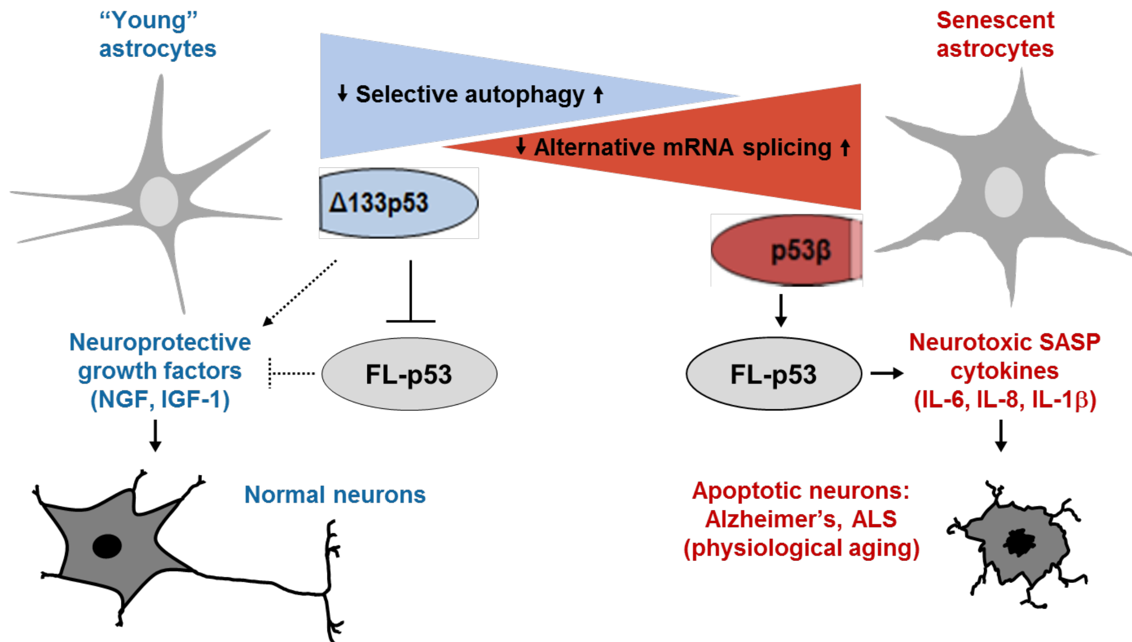


Figure 7.3.
Proposed model of p53 isoform regulation of neurodegeneration and neuroprotection. Δ133p53 plays a dominant-negative role towards full-length p53 and may also directly activate neurotrophic factors (Gong et al., 2015) (dashed arrow). p53β is a co-activator of full-length p53, drives a senescence-associated secretory phenotype in astrocytes and neurodegeneration. (Turnquist et al., in preparation).

Conclusion

The results presented in this thesis reinforce the notion that p53 signaling may be a fruitful target for neurodegenerative disease therapies. Here we examine regulators of p53-mediated apoptosis and senescence mechanisms in astrocytes through ASPP2 and p53 isoforms, respectively. Chapters 3 suggest that the p53 target and apoptosis regulator, ASPP2, is an early-activated gene in the STAT1 inflammatory cascade in the neuroinflammatory events in neurodegenerative diseases, such as MS and encephalitis. In Chapter 4 our results indicate that ASPP2-depletion promotes neuronal survival as ASPP2 communicates inflammation and cell polarity loss at the BCSFB to cell death machinery in the nucleus and ultimately results in astrocyte apoptosis. We then examined how p53 isoforms regulate astrocyte senescence in neurodegenerative diseases, such as ALS and AD. Chapter 5 shows that Δ133p53 and p53β display a signature expression in ALS and AD that is consistent with other aging diseases. Furthermore, we demonstrate that these isoforms function to control astrocyte SASP. Finally, Chapter 6 reveals that SASP modulation through the isoforms directly contributes to non-cell autonomous neurotoxicity that can be restored upon Δ133p53 reconstitution. Further work will be required to determine how these mechanisms may be channeled into disease-modifying therapies. A critical consideration for p53-modifying therapies is that they strike the right balance between the so-called "two faces of evil" (Curran and Christen, 2011): cancer and neurodegeneration.

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A

Appendix A: Figure permissions

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Table 1.1 Biological links between cancer and neurodegeneration.
Reference (Driver, 2012)

Driver, J. a. (2012). Understanding the link between cancer and neurodegeneration. *Journal of Geriatric Oncology*, *3*(1), 58–67.

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Figure 1.1 Is there a common aging process?
Reference (Campisi et al., 2011).

Campisi, J., Andersen, J.K., Kapahi, P., and Melov, S. (2011). Cellular senescence: a link between cancer and age-related degenerative disease? Semin. Cancer Biol.

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Figure 1.2. Origin of CNS glial cells.

Reference (Ransohoff and Cardona, 2010).

Ransohoff, Richard M, and Astrid E Cardona. 2010. "The Myeloid Cells of the Central Nervous System Parenchyma." *Nature* 468 (7321).

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Figure 1.3. Toll-like receptors and ligands

Reference (Medzhitov, 2001)

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nature Reviews. Immunology*, *1*(2), 135–45.

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Figure 1.4. Canonical IFN Type 1 signaling pathway

Reference (Ivashkiv and Donlin, 2014)

Ivashkiv, L. B., & Donlin, L. T. (2014). Regulation of type I interferon responses. *Nature Reviews. Immunology*, *14*(1), 36–49.

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Figure 1.5. Three distinct inducers of cellular senescence.
Reference**:** (Naylor et al., 2013)

Naylor, R. M., Baker, D. J., & van Deursen, J. M. (2013). Senescent cells: a novel therapeutic target for aging and age-related diseases. *Clinical Pharmacology and Therapeutics*, *93*(1), 105–16.

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Figure 1.6. Cellular senescence in neurodegeneration and physiological brain aging
Reference: (Tan et al., 2014).

Tan, F. C. C., Hutchison, E. R., Eitan, E., & Mattson, M. P. (2014). Are there roles for brain cell senescence in aging and neurodegenerative disorders? *Biogerontology*, *15*(6), 643–660.

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Figure 1.7. The role of caspase-3 in apoptosis.

Reference (Salvesen and Duckett, 2002)

Salvesen, G. S., & Duckett, C. S. (2002). IAP proteins: blocking the road to death's door. *Nature Reviews. Molecular Cell Biology*, *3*(6), 401–10.

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Figure 1.8. Microautophagy

Reference: (Knaevelsrud and Simonsen, 2010)

Knaevelsrud, H., & Simonsen, A. (2010). Fighting disease by selective autophagy of aggregate-prone proteins. *FEBS Letters*, *584*(12), 2635–45.

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Figure 1.9 Roles of p53 activation

Reference (Olivares-Illana and Fåhraeus, 2010)

Olivares-Illana, V., & Fåhraeus, R. (2010). P53 Isoforms Gain Functions. *Oncogene*, *29*(May), 5113–5119.

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Figure 1.10 **Diagram of 12 known p53 isoforms**

Reference: (Marcel et al., 2011)

Marcel, V., Dichtel-Danjoy, M.-L., Sagne, C., Hafsi, H., Ma, D., Ortiz-Cuaran, S., … Bourdon, J.-C. (2011). Biological functions of p53 isoforms through evolution: lessons from animal and cellular models. *Cell Death and Differentiation*, *18*(12), 1815–1824.

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Figure 1.12 Phenotype of ASPP2-deficient mice.

Reference (Vives et al., 2006)

Vives, V., Su, J., Zhong, S., Ratnayaka, I., Slee, E., Goldin, R., & Lu, X. (2006). ASPP2 is a haploinsufficient tumor suppressor that cooperates with p53 to suppress tumor growth. *Genes Dev*, *20*(10), 1262–1267.

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Figure 1.13 Histological abnormalities in the brain of ASPP2-deficient mice

Figure 1.14 The role of ASPP2 in cell polarity

Reference (Sottocornola et al., 2010)

Sottocornola, Roberta, Christophe Royer, Virginie Vives, Luca Tordella, Shan Zhong, Yihua Wang, Indrika Ratnayaka, et al. 2010. "ASPP2 Binds Par-3 and Controls the Polarity and Proliferation of Neural Progenitors during CNS Development." *Dev.*

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B

Appendix B: Published works

The papers published in collaboration with my colleagues have been included in Appendix B. My contribution to each publication is detailed below:

- The work presented in Chapters 3 and 4 was published in:

  Turnquist C, Wang Y, Severson DT, Zhong S, Sun B, Constantinescu SN, Angsorge O, Stolp HB, Molńar Z, Szele FG, Lu X (2014). STAT1-induced ASPP2 transcription identified a link between neuroinflammation, cell polarity and tumor suppression. Proc Natl Acad Sci USA 111(27): 9834-9839.

- My contribution to elucidating how maternal inflammation affects neocortical development was published in Stolp et al., 2011. Brain tissues used in this study were used as a model system for studying neuroinflammation *in vivo* included in Chapter 4.

  Stolp HB, **Turnquist C**, Dziegielewska KM, Saunders NR, Anthony DC, Molnar Z (2011). Reduced ventricular proliferation in the foetal cortex following maternal inflammation in mouse. Brain 134(11): 3236-3248.

- The results presented in Chapters 5 and 6 have been recently submitted for publication. The abstract is included in the appendix.

  Turnquist C, Horikawa I, Foran E Major EO, Vojtesek B, Lane DP, Lu X. Harris BT, Curtis C. Harris. p53 isoforms regulate astrocyte-mediated neuroprotection and neurodegeneration. (In preparation).

- The concept of SMaRT signaling is introduced in a review article currently in preparation. The LPS-ASPP2-STAT1 pathway discussed in Chapter 7 is a key example of SMaRT signaling.

  Lu M, et al. SMART signaling in infection, cancer and cell fate determination (In preparation).

p53 isoforms regulate astrocyte-mediated neuroprotection and neurodegeneration

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Astrocytes exert both neuroprotective and neurodegenerative roles and are targets for therapies in neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's disease. Their neurodegenerative effect is mediated via senescence-associated secretory phenotype (SASP), which increases with aging and neurodegenerative diseases. We report that astrocytes express p53 isoforms, Δ133p53 and p53β, and that these isoforms regulate SASP in astrocytes and their protective and toxic effects on neurons. Amyotrophic lateral sclerosis and Alzheimer's disease brains, as well as senescent astrocytes after *in vitro* passaging, show decreased Δ133p53 and increased p53β, which are attributed to autophagic degradation and SRSF3-mediated alternative splicing, respectively, conserved across different cell types. Early-passage astrocytes with Δ133p53 knockdown or p53β overexpression are induced to show SASP and to exert neurotoxicity in co-culture with neurons. Restored expression of Δ133p53 in near-senescent astrocytes results in repressed SASP, elevated neurotrophic factors and enhanced neuroprotection, suggesting a novel therapeutic approach for neurodegenerative diseases.

STAT1-induced ASPP2 transcription identifies a link between neuroinflammation, cell polarity, and tumor suppression

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Inflammation and loss of cell polarity play pivotal roles in neurodegeneration and cancer. A central question in both diseases is how the loss of cell polarity is sensed by cell death machinery. Here, we identify apoptosis-stimulating protein of p53 with signature sequences of ankyrin repeat-, SH3 domain-, and proline-rich region-containing protein 2 (ASPP2), a haploinsufficient tumor suppressor, activator of p53, and regulator of cell polarity, as a transcriptional target of signal transducer and activator of transcription 1 (STAT1). LPS induces ASPP2 expression in murine macrophage and microglial cell lines, a human monocyte cell line, and primary human astrocytes in vitro. LPS and IFNs induce ASPP2 transcription through an NF-κB RELA/p65-independent but STAT1-dependent pathway. In an LPS-induced maternal inflammation mouse model, LPS induces nuclear ASPP2 in vivo at the blood–cerebral spinal fluid barrier (the brain's barrier to inflammation), and ASPP2 mediates LPS-induced apoptosis. Consistent with the role of ASPP2 as a gatekeeper to inflammation, ASPP2-deficient brains possess enhanced neuroinflammation. Elevated ASPP2 expression is also observed in mouse models and human neuroinflammatory disease tissue, where ASPP2 was detected in GFAP-expressing reactive astrocytes that coexpress STAT1. Because the ability of ASPP2 to maintain cellular polarity is vital to CNS development, our findings suggest that the identified STAT1/ASPP2 pathway may connect tumor suppression and cell polarity to neuroinflammation.

TP53BP2 | TLR4 | multiple sclerosis

Neurodegenerative disease and cancer are two of the most common aging diseases and major medical challenges of the 21st century. Whereas neurodegeneration is characterized by accelerated cell death and a lack of self-renewal, cancers have an opposing phenotype, with excessive cell growth and dedifferentiation. Support for a molecular link between neurodegenerative disease and cancer is emerging and more intimate than previously understood. Recent studies indicate that patients with a history of cancer display a lower probability of developing Alzheimer's disease (AD), whereas patients with AD have lower rates of cancer development (1). Compounds that inhibit γ-secretase, the enzyme that generates β-amyloid, have been used as potential therapeutics to treat AD. However, such treatment resulted in higher cancer incidence in a phase III clinical trial of Semagacestat (2). These observations suggest the existence of common molecules controlling cell death and self-renewal programs, which may be deregulated in both cancer and neurodegeneration. One such example is the tumor suppressor p53, the most commonly mutated gene in human cancer.

Several studies have attributed p53-mediated apoptosis to a number of acute and chronic neurodegenerative disorders, including excitotoxicity, AD, Parkinson disease, multiple sclerosis, and Huntington disease (3). Accumulating evidence supports the role of reactive oxygen species in prompting DNA damage in neurodegenerative disease (4), leading to p53-dependent apoptosis. Consistent with a proapoptotic role of p53 in CNS cells, inhibition of p53 by the chemical inhibitor pifithrin-α (5) or deletion of the p53 gene in a mouse model of multiple sclerosis (6) enhanced cell survival. These findings indicate that tumor suppressor pathways involving p53 may be deregulated in neurodegenerative disorders.

Inflammation and cell polarity disruption represent another link between neurodegeneration and cancer. In epithelial cancers, loss of cell polarity is a hallmark of cancer malignancy (7) and often associates with tumor-infiltrating lymphocytes and inflammation (8). Likewise, loss of brain barrier function prompted by neuroinflammation is linked to neurodegenerative disease onset and progression. The blood–brain barrier and blood–cerebral spinal fluid barrier (BCSFB) are the brain's main barriers to infection (9). Previous studies have shown that a loss of cell polarity at these barriers prompts inflammatory changes, including the intrusion of immune cells and activation of microglia and astrocytes, which contribute to neurodegeneration (10). In the CNS, microglia, astrocytes, and macrophages participate in toll-like receptor (TLR) signaling. Mammalian TLRs are type I transmembrane receptors that recognize microbial pathogen-associated molecular patterns. TLR signaling culminates in the activation of

Significance

Two of the most debilitating and scientifically challenging diseases of the 21st century are cancer and neurodegeneration. Although cancer results from excessive cell growth, neurodegeneration is a consequence of excessive cell loss. Dysfunction of the same key regulators, including oncogenes and tumor suppressors, may cause both diseases. We report that LPS and IFN induce apoptosis-stimulating protein of p53 with signature sequences of ankyrin repeat-, SH3 domain-, and proline-rich region-containing protein 2 (ASPP2) transcription through a signal transducer and activator of transcription 1 (STAT1) -dependent but NF-κB RELA/p65-independent pathway and that ASPP2 mediates LPS-induced apoptosis. Thus, the identified STAT1/ASPP2 pathway reveals an important function of ASPP2 in the cellular response to inflammation and infection and connects neuroinflammation to cell polarity and tumor suppression.

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transcription factors, such as NF-κB, signal transducer and activator of transcription 1 (STAT1), and AP-1 (11). Increasing evidence implicates the LPS receptor TLR4 in a number of neurodegenerative diseases and CNS injury (12). In mouse models, systemic injection of LPS leads to progressive neurodegeneration (13). Additionally, the role of viral infection and excessive IFN production in neurodegeneration is underscored by animal models of multiple sclerosis (14, 15) as well as IFN transgenic mouse models. For instance, transgenic mice producing IFN-α1 in GFAP-expressing astrocytes develop progressive neurodegeneration (16). Consistent with the link between neuroinflammation and p53-mediated apoptosis, previous studies indicate that p53 activity is regulated by TLR and IFN signaling. In the murine macrophage cell line RAW264.7, LPS and IFN-γ induce NO synthase and p53-mediated cell death (17). Moreover, a genome-wide in silico search identified most human TLR genes as potential p53 targets (18), suggesting an autoregulation loop between infection and p53 activity. Hence, the apoptotic function of p53 in response to infection plays an important role in controlling the inflammatory response. Given the emerging link between p53-induced apoptosis and inflammation, a better understanding of how cells relay changes in barrier function and cell polarity to cell death signals is critical. We, therefore, hypothesized that p53 regulators and gatekeepers of cell polarity may fulfill these requirements by acting as (*i*) a sensor that surveys the integrity of cell polarity, (*ii*) a messenger that communicates changes in cell polarity to cell death machinery, and (*iii*) a regulator of transcription. We refer to factors that fulfill these three roles as SMRT factors. One p53 regulator that may act as an SMRT factor is apoptosis-stimulating protein of p53 with signature sequences of ankyrin repeat-, SH3 domain-, and proline-rich region-containing protein 2 (ASPP2), a haploinsufficient tumor suppressor, activator of p53, and apical polarity regulator.

ASPP2 belongs to the ASPP family that comprises three members: ASPP1, ASPP2, and iASPP. Although ASPP1 and ASPP2 stimulate the apoptotic function of p53 by promoting the transcription of its proapoptotic target genes, iASPP prevents p53-mediated apoptosis (19). ASPP2 cooperates with p53 to suppress tumor growth in vivo (20). ASPP2-deficient mice lacking exon 3 (ASPP2 Δ3/Δ3) display a loss of neuroepithelial cell polarity and an expansion of CNS neural progenitors (21). ASPP2 Δ3/Δ3 mice die of hydrocephalus and display a loss of tight junctions (TJs) between choroid plexus (CP) epithelial cells, which form the BCSFB. This function of ASPP2 is mediated by its ability to bind Par-3 and maintain the integrity of apical cell polarity and TJs. The importance of ASPP2 in maintaining epithelial polarity is supported by the fact that ASPP2 is a target of CagA, a toxin and oncoprotein of a gastric cancer-associating bacterium *Helicobacter pylori* (22). Prompted by the emerging roles of cell polarity, inflammation, and p53 in cancer and neurodegeneration, in this study, we tested whether inflammatory stimuli regulate ASPP2 expression.

Results

LPS Induces ASPP2 in Macrophages, Microglia, and Astrocytes. Recent reports support the role of TLR4 in a number of cerebral inflammatory disorders (12, 23). Because *Helicobacter pylori* infection induces ASPP2 in gastric cancer cells (22), we used the TLR4 ligand LPS to examine whether ASPP2 is responsive to inflammatory signaling. RAW264.7 (mouse macrophage), BV-2 (mouse microglial), and THP-1 (human monocyte) cell lines and primary human astrocytes were treated with 1 μg/mL LPS over the indicated time points. As a positive control for LPS treatment, we first examined the expression level and/or phosphorylation status of major inflammatory signaling transcription factors p65 and STAT1. All primary and secondary antibodies used are listed in Tables S1 and S2. A small increase in phospho-STAT1 and phospho-p65 was detectable by 1 h, and a clear increase was detectable by 2 h (Fig. 1*A*). Phospho-STAT1 and phospho-p65 decreased 6 h after LPS treatment. Expression of ASPP2 increased in all cell lines examined on LPS treatment (Fig. 1 *A* and *B* and Fig. S1 *A–C*), with maximum induction of ASPP2 apparent at 3 h in RAW264.7 cells. Similar to phospho-STAT1 and phospho-p65, elevated ASPP2 levels began to decrease 6 h after LPS treatment (Fig. 1*A*).

To determine whether LPS regulates ASPP2 at the protein or mRNA level, we examined the effect of LPS treatment on ASPP2 mRNA levels using quantitative RT-PCR (qRT-PCR). Primers used for qRT-PCR are listed in Table S3. Similar to ASPP2 protein level, up-regulation of ASPP2 mRNA was detected in a time-dependent manner after LPS treatment in RAW264.7 cells, with 1 h being the earliest time point of induction (Fig. 1*A*). Importantly, LPS did not induce iASPP mRNA expression, suggesting that LPS specifically induces ASPP2 expression (Fig. S1*D*). To determine whether LPS treatment affects ASPP2 protein stability, cycloheximide was used to block protein synthesis. ASPP2 expression levels were measured in RAW264.7 cells over a cycloheximide incubation time course alone or combined with LPS. The presence of LPS had a minimal impact on the kinetics of ASPP2 expression (Fig. S1*E*). LPS treatment is known to induce p65 nuclear localization (24). In RAW264.7 cells, ASPP2 expression patterns were similar to those of p65 (Fig. 1*B*). Nuclear and cytoplasmic fractionation indicated that ASPP2 was mainly induced in the nuclear fraction in RAW264.7 cells after 2 h (Fig. 1*C*). In primary human astrocytes, LPS-induced ASPP2 was mainly cytoplasmic, with a pattern overlapping that of the intermediate filament GFAP (Fig. S1*C*). The underlying molecular mechanism for this difference in expression pattern in astrocytes is unknown.

Genome-wide analysis of LPS-regulated genes has been studied extensively. Because LPS induces ASPP2 mRNA, bioinformatics analysis of ASPP2 mRNA expression in response to inflammatory stimuli was performed using publicly available gene array data in NextBio database (25). In agreement with our findings, LPS-induced ASPP2 mRNA expression was an early response in mouse macrophages (Fig. S1*F*) and human myeloid lineage cells (Fig. S1*G*). Additionally, increased ASPP2 mRNA was detected in rheumatoid arthritis or tuberculosis-infected latent, meningeal, or pulmonary human macrophages compared with control macrophages (Fig. S1*H*). Increased ASPP2 mRNA expression was also found in brain tissue from patients with neurodegenerative disorders, including multiple sclerosis, Parkinson disease, and Huntington disease, compared with control tissue (Fig. S1*I*). These data support the conclusion that inflammatory stimuli or disease states induce ASPP2 expression in both mouse and human.

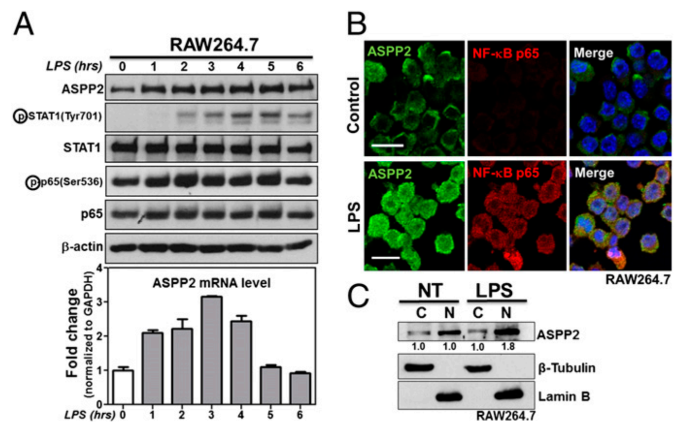

Fig. 1. ASPP2 is induced by LPS. (*A*) LPS time course showing increased ASPP2 expression at protein and mRNA levels in RAW264.7. Expression levels of signaling pathways downstream of LPS were examined, including STAT1 and p65. (*B*) IF staining of ASPP2 and p65 after 2 h of LPS treatment in RAW264.7. (Scale bars: 10 μm.) (*C*) Nuclear (N) and cytoplasmic (C) fractionations of ASPP2 on LPS treatment in RAW264.7. Quantification was performed using densitometry analysis. NT, no treatment.

ASPP2 Is a Bona Fide Transcriptional Target of STAT1. LPS binds TLR4 and its coreceptor MD-2 to initiate the TLR4 signaling cascade (26, 27). We, thus, introduced TLR4 or MD-2 siRNA to RAW264.7 cells in the presence or absence of LPS. As expected, TLR4 siRNA almost prevented LPS from inducing ASPP2 in RAW264.7 cells (Fig. 2*A*). MD-2 siRNA also dampened LPS-induced ASPP2 in RAW264.7 cells (Fig. S2*A*). These data suggest that intact TLR4 signaling is required for LPS to induce ASPP2.

The downstream effectors of LPS/TLR4 are canonical MYD88-p65–dependent and noncanonical MYD88-p65–independent pathways. As a result, LPS/TLR4 activates a number of downstream transcription factors, including p65, STAT1, IRF-3, and AP-1. Analysis of the ASPP2 promoter region in conjunction with ENCODE transcription factor binding data (28) suggested that, in addition to the previously identified E2F site (29), both human and mouse ASPP2 promoters contain potential p65 and STAT1 binding sites but do not contain IRF-3 and AP-1 sites (Fig. S2*B*). In RAW264.7 and THP-1 cells, p65 and STAT1 siRNA reduced STAT1 and p65 expression with similar efficiency. Interestingly, only STAT1 siRNA but not p65 siRNA diminished ASPP2 induction after 3 h of LPS treatment in RAW264.7 cells (Fig. 2*B* and Fig. S2*C*) or after 10 h of LPS treatment in THP-1 cells (Fig. S2*D*). In THP-1 cells, ASPP2 induction after 2 h of LPS treatment was unaffected by p65 or STAT1 depletion, indicating the involvement of other unknown factors at this time point in this cell line. Because IFN is upstream of the JAK/STAT1 pathway, ASPP2 induction was examined with IFN treatment in RAW264.7 and THP-1 cells. IFN-β was able to induce ASPP2 expression in RAW264.7 cells (Fig. 2*C*), whereas only IFN-γ, not IFN-β, induced ASPP2 in THP-1 cells (Fig. S2 *E* and *F*).

To determine the STAT1 binding site in the ASPP2 promoter/enhancer, we first performed a ChIP assay. Primers used for the ChIP assay are listed in Table S4. An anti-STAT1 antibody was used to precipitate formaldehyde cross-linked STAT1–DNA complexes in RAW264.7 and THP-1 cells treated with or without LPS. The presence of ASPP2 promoter/enhancer DNA sequences was verified by PCR using primers surrounding distinct but overlapping regions of the mouse and human ASPP2 promoter/enhancer. In RAW264.7 cells, three LPS-responsive sites were identified by the ChIP assay: −1,058 to −799, −844 to −577, and −597 to −331 (Fig. S2*G*). To further locate the responsive sequence, additional primers were designed within the responsive region, and sequence −645 to −507 was identified as the maximally LPS-responsive ASPP2 promoter/enhancer region containing the putative STAT1 binding site (Fig. 2*D*). Three mouse ASPP2 promoter/enhancer fragments were cloned into a pGL4.23 (luc2/minP) luciferase reporter plasmid. The ASPP2 (−645 to −507) -Luc construct contains the putative STAT1 binding site, whereas ASPP2 (−765 to −608) -Luc does not. ASPP2 Δ(−590 to −582) -Luc contains a deletion of the STAT1 binding sequence that is present in ASPP2 (−645 to −507) -Luc. The responsiveness of these three promoter/enhancer fragments was tested in 293T cells, which enable high transfection efficiency and also lack TLR4 (26, 27). LPS treatment together with transfected TLR4 resulted in a time-dependent increase in ASPP2 (−645 to −507) -Luc activity. Plasmids used are

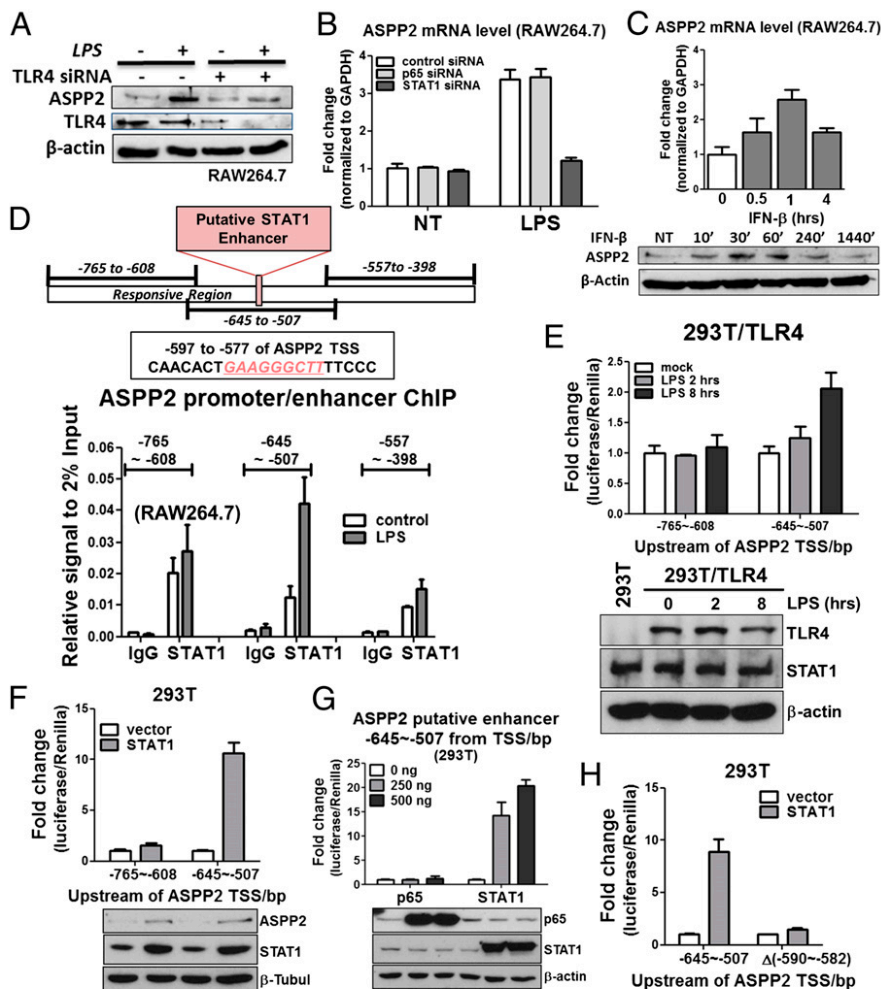

Fig. 2. ASPP2 is a target of STAT1. (*A*) TLR4 siRNA reduces ASPP2 induction after LPS treatment in RAW264.7 cells. (*B*) STAT1 siRNA but not p65 siRNA reduces ASPP2 induction after LPS treatment in RAW264.7 cells. (*C*) IFN-β time course showing increased ASPP2 expression at protein and mRNA levels in RAW264.7. (*D*) Illustration of the LPS-responsive region of the ASPP2 enhancer region. Putative STAT1 binding site is within the −645 to −507 region. Results of STAT1 ChIP in RAW264.7. The regions corresponding to each of the primer sets are shown. Results are the average of duplicate treatments, and error bars show the range of the duplicates. (*E*) ASPP2 (−645 to −507) -Luc shows increased activity after LPS treatment for 8 h, whereas ASPP2 (−765 to −608) -Luc shows no response. (*F*) Only ASPP2 (−645 to −507) -Luc is activated after STAT1 exogenous expression. ASPP2 (−765 to −608) -Luc remains unresponsive. (*G*) STAT1 but not p65 is able to induce ASPP2 (−645 to −507) -Luc activity. (*H*) After deletion of the STAT1 binding sequence located at −590 to −582, ASPP2 (−645 to −507) -Luc activity after STAT1 exogenous expression is abolished. NT, no treatment.

listed in Table S5. Under the same conditions, ASPP2 (−765 to −608) -Luc activity was unchanged (Fig. 2*E*).

Consistent with the notion that STAT1 is a downstream effector of TLR4, STAT1 overexpression was sufficient to induce endogenous ASPP2 expression in TLR4-deficient 293T cells. Although ASPP2 luciferase activity was induced 9- to 10-fold in the ASPP2 (−645 to −507) -Luc, the ASPP2 (−765 to −608) -Luc showed no change in activity (Fig. 2*F*). To further confirm that ASPP2 promoter/enhancer activity is responsive to STAT1 and not p65, ASPP2 (−645 to −507) -Luc was transfected with a gradient of p65 or STAT1-expressing plasmids. STAT1, but not p65, induced ASPP2 (−645 to −507) -Luc reporter activity (Fig. 2*G*). Importantly, exogenous STAT1 expression induced ASPP2 (−645 to −507) -Luc activity but failed to stimulate the transcriptional activity of ASPP2 Δ(−590 to ∼−582) -Luc, in which the binding site was excised (Fig. 2*H*). A similar approach was used to identify the STAT1 binding site in the human ASPP2 promoter. ChIP assays were performed in human THP-1 cells, which showed two LPS-responsive sites (Fig. S2*H*). These sites were confirmed using luciferase assays. STAT1 overexpression resulted in increased ASPP2 promoter/enhancer activity in the responsive human fragments (Fig. S2*I*). These data showed that ASPP2 is a bona fide transcriptional target of STAT1.

LPS Induces Nuclear ASPP2 Expression in a Mouse Model of Maternal Inflammation and Mediates Apoptosis. To see whether LPS-induced ASPP2 induction could be observed in vivo and understand the role of LPS-induced ASPP2 transcription in the context of cerebral inflammatory disease, ASPP2 expression was examined in an LPS-induced model of maternal inflammation. In this model, LPS was injected i.p. into pregnant mice carrying embryos at embryonic day (E) 13.5 (30). Pup brains were then examined at postnatal day (P) 8. Under basal conditions, ASPP2 is expressed in the TJs of CP epithelial cells, where it binds Par-3 to maintain cell polarity (21). ASPP2 was expressed at the junctions of CP epithelial cells in animals receiving saline injections as a control. In the LPS injection group, however, ASPP2 expression was up-regulated and accumulated in the nucleus rather than at the TJs (Fig. 3*A* and Fig. S3*A*). Nuclear p53 was also observed in the nucleus of CP epithelial cells on LPS treatment (Fig. 3*B* and Fig. S3*B*).

Previous studies have shown that STAT1 cooperates with p53 to induce apoptosis by selectively enhancing the transcriptional activity of p53 on p53 target gene promoters bearing an ASPP2 signature, such as Bax (31) and Noxa (32). Because LPS-induced STAT1 activity induces ASPP2 expression, we tested whether LPS-induced nuclear ASPP2 and p53 may play a proapoptotic role in response to inflammatory stimuli. To test if ASPP2 plays a proapoptotic role, ASPP2 siRNA was used in RAW264.7 cells, which express WT p53. On treatment with LPS, the expression level of cleaved polyADP ribose polymerase (PARP) diminished in the ASPP2-depleted cells but not the control cells (Fig. S3*C*). To further examine the role of ASPP2 in apoptosis, ASPP2-deficient ASPP2 Δ3/Δ3 and WT mice were injected with LPS or saline i.p. Previous studies have shown that systemic administration of LPS can induce apoptosis in the brain, particularly in the hippocampus (33). Levels of cleaved caspase-3 were compared in hippocampal brain sections from ASPP2 Δ3/Δ3 and WT mice compared with saline-injected controls. Cleaved caspase-3–positive cells were quantified and found to be significantly less prevalent in ASPP2 Δ3/Δ3 vs. WT mice injected with LPS, supporting a proapoptotic role of ASPP2. Few cleaved caspase-3–positive cells were found in mice receiving saline injections (Fig. 3*C*). These data support the role of ASPP2 in mediating LPS-induced apoptosis in vitro and in vivo.

ASPP2 Deficiency Enhances Neuroinflammation in Vivo. Because ASPP2 was previously shown to maintain the TJs between CP epithelial cells, the brain's main barrier to inflammation, we examined whether ASPP2 Δ3/Δ3 mice display neuroinflammation. Immunohistochemistry (IHC) staining showed that ASPP2 Δ3/Δ3 mice possess IBA1-positive microglia and GFAP-positive astrocytes throughout the parenchyma at E15.5 (Fig. 4*A*) and P20 (Fig. S4*A*). Using qRT-PCR with cortical tissue from ASPP2 Δ3/Δ3 and WT mice, we observed that ASPP2 Δ3/Δ3 mice displayed a significant increase in several proinflammatory cytokines, including TNF-α at E15.5 (Fig. S4*B*). The extent of neuroinflammation increased by P20 as the production of TNF-α and IL-1β in cortical brain tissues increased dramatically compared with age-matched WT mice (Fig. 4*B*). Thus, the localization of ASPP2 at the TJs of the BCSFB under basal conditions and the neuroinflammatory phenotype of ASPP2 Δ3/Δ3 mice suggest that ASPP2 may act as a barrier to inflammation.

Fig. 3. LPS induces nuclear ASPP2 expression in a model of maternal inflammation, and ASPP2 mediates apoptosis. (*A*) On LPS injection, ASPP2 is disrupted from the TJs and relocalized to the nucleus of CP epithelial cells. (Scale bar: 25 μm.) (*B*) After LPS injection, p53 appears in the nucleus of CP epithelial cells. (Scale bar: 25 μm.) (*C*) IF staining of cleaved caspse-3 in LPS-injected and control saline-injected WT and ASPP2 Δ3/Δ3 mice. Arrows indicate cleaved caspase-3–positive cells. (Scale bars: 25 μm.) Quantification of the number of cleaved caspase-3–positive cells in the hippocampus after LPS or saline injection (n = 4). **$P < 0.01$.

ASPP2 Is Highly Expressed in Reactive Astrocytes in Mouse Neuroinflammation Models and Human Neuroinflammatory Disease. To test whether ASPP2 is involved in neuroinflammatory and neurodegenerative disorders involving STAT1 signaling, we used mouse models in which IFN activation is implicated in the disease pathology, including multiple sclerosis and stroke (34, 35). Tissue sections were obtained from an animal model of experimental stroke, middle cerebral artery occlusion, and the Theiler's murine encephalomyelitis virus infection model of multiple sclerosis. ASPP2 IHC revealed marked up-regulation in cells with astrocytic morphology found in the dentate gyrus of the hippocampus ipsilateral to the cerebral infarct in the middle cerebral artery occlusion model (Fig. S5*A*) and the cerebral cortex of the Theiler's murine encephalomyelitis virus model (Fig. 5*A*). ASPP2 was not as highly expressed on the hemisphere contralateral to the cerebral infarct, indicating that ASPP2 up-regulation was specific to areas of the brain in which major damage was present.

We next examined human tissue samples obtained from a variety of disorders associated with neuroinflammation and neurodegeneration. IHC staining showed low to no expression of ASPP2 in noninflamed tissue. However, high expression of ASPP2 was observed in cerebral infarct (Fig. S5*B*) and subacute/chronic encephalitis (Fig. S5*C*). Morphological analysis suggested that reactive astrocytes were the dominant ASPP2-expressing cell type. To confirm that ASPP2 is expressed in astrocytes, we performed double immunofluorescence (IF) staining with anti-ASPP2 and anti-GFAP antibodies on biopsy tissue, which enabled ASPP2 antigen preservation. ASPP2 was found to be highly expressed in the cytoplasm of reactive GFAP-positive

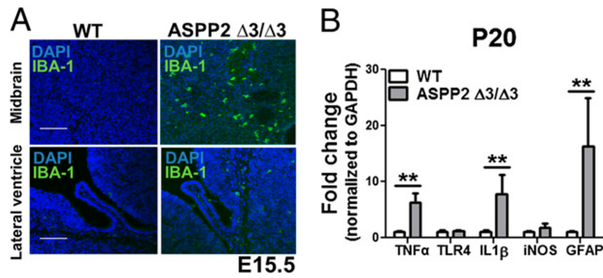

Fig. 4. ASPP2-deficient mice possess neuroinflammation. (*A*) Increased IBA1-positive microglia in ASPP2 Δ3/Δ3 mice at E15.5. (Scale bars: 100 μm.) (*B*) Increased proinflammatory cytokines in cortical brain tissue of ASPP2 Δ3/Δ3 mice at P20. **$P < 0.01$.

astrocytes, particularly those with gemistocytic morphology (Fig. 5*B* and Fig. S5*D*). The detected increase in ASPP2 expression is specific, because iASPP was not highly expressed in either encephalitis or control tissue (Fig. S5*E*). The number of ASPP2-positive cells was quantified per cell subtype and revealed that ~83% of GFAP-positive cells had high ASPP2 expression (Fig. S5*F*). Because encephalitis is known to arise from viral infection and because IFN secretion in encephalitis is linked to neuronal dysfunction (36), we carried out triple IF staining to examine whether STAT1-mediated ASPP2 induction was present in astrocytes in human tissue samples. In agreement with this hypothesis, ASPP2 was found to be up-regulated in STAT1- and GFAP-expressing reactive astrocytes (Fig. 5*C*). The role of ASPP2 induction in astrocytes was tested by treating primary human astrocytes with IFN-β for 24 h, after which time they began to undergo apoptosis. Increased ASPP2 expression was found in cells that also express Annexin V and cleaved caspase-3 (Fig. S5 *G* and *H*). These findings are consistent with the conclusions that ASPP2 is a transcriptional target of STAT1 and that it plays a proapoptotic role in response to inflammatory stimuli such as LPS and IFN.

Discussion

We identify ASPP2, a known tumor suppressor, activator of p53, and regulator of cell polarity, as a bona fide transcriptional target of STAT1 and regulator of neuroinflammation. Its dynamic cellular localization and diverse functions place ASPP2 in an ideal position to act as an SMRT factor that can sense inflammatory stimuli at the apical cell membrane, act as a messenger to transcriptional machinery in the nucleus, and determine cell fate as a regulator of transcription. When ASPP2 binds Par-3 through its N terminus, it maintains the integrity of the apical polarity complex and the TJs of the BCSFB. In this way, ASPP2 may act as a defender against infection and inflammation. The identification of ASPP2 as a novel transcriptional target of STAT1 and the finding that LPS and IFNs can induce ASPP2 expression suggest that it may also act as a sensor of infection. ASPP2 may also promote phosphorylation of STAT1 through the potentiating RAS-MAPK pathway (37), which forms an autoregulation loop (Fig. 5*D*). The kinetics of ASPP2 induction by LPS suggest that ASPP2 is likely to be involved in the early phase of infection through its ability to induce apoptosis, a type of cell death that halts additional inflammation, in contrast to necroptosis, where a failure to eliminate damage may further propagate inflammation (38). Consistent with this hypothesis, ASPP2-deficient mice possess enhanced neuroinflammation and a reduced apoptotic response after LPS injection.

The function of ASPP2 as a gatekeeper could be caused by its ability to regulate cell polarity and RAS signaling through its N terminus (37). In polarized epithelial cells, including CP epithelial cells, ASPP2 is located at TJs (21). On RAS activation, ASPP2 is translocated to the cytoplasm and enhances the apoptotic function of p53 (37). The ability of ASPP2 to stimulate the apoptotic function of p53 and p73 is mediated by its C terminus, which is localized in the nucleus (19). The up-regulation of ASPP2 in the LPS-induced maternal inflammation model shows that inflammatory stimuli could induce ASPP2 in vivo. When the TJs of the BCSFB are disrupted, ASPP2 is displaced from the cell junctions and relocalizes to the nucleus. Like many other ankyrin repeat-containing proteins without an identifiable nuclear localization signal, ASPP2 may enter the nucleus through a newly identified RanGDP/Ankyrin Repeats binding nuclear import pathway (39).

Bacterial and viral infections induce inflammatory cellular responses through TLRs. LPS and IFN are often used as inflammatory stimuli to mimic infections induced by Gram-negative bacteria and viral RNA, respectively. LPS and IFN were previously found to have cell- and context-dependent pro- or antiapoptotic functions. LPS is able to induce MYD88-mediated antiapoptotic pathways through p65 (40), and LPS/TLR4 can also induce STAT activation through an MYD88-independent and IFN-dependent pathway. Interestingly, STAT1 has been shown to potentiate p53- and p73-induced apoptosis by selectively enhancing its transcriptional activity on proapoptotic genes, such as Bax (31) and Noxa (32). However, it remains unclear how STAT1 selectively enhances the apoptotic function of p53 family members. The identification of ASPP2 as a transcriptional target of STAT1 explains how STAT1 signaling could be guided to a proapoptotic path. Because ASPP2 is a common activator of

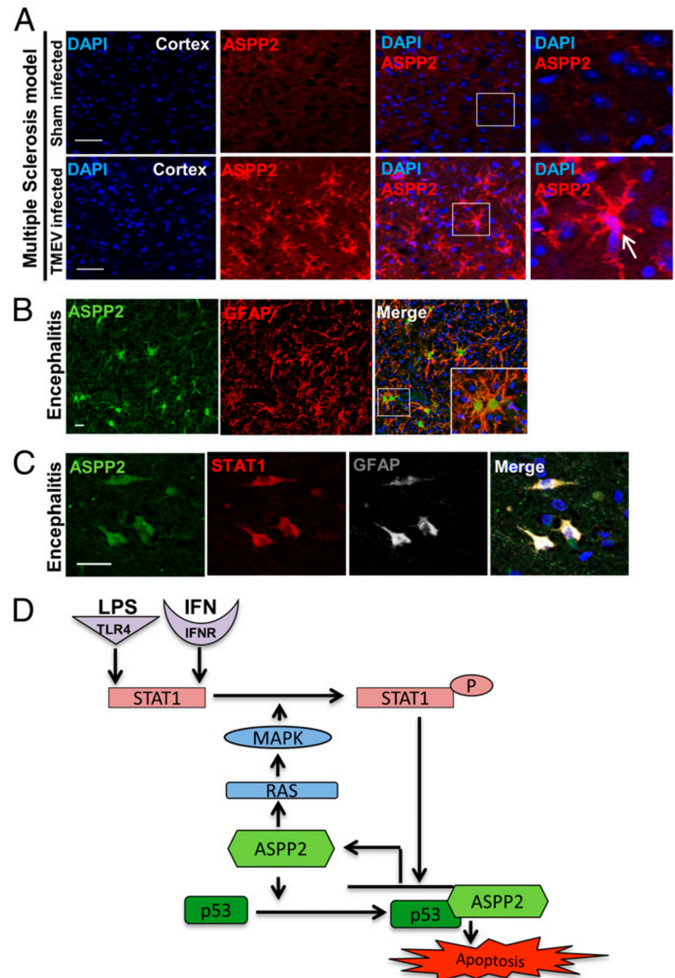

Fig. 5. ASPP2 is up-regulated in mouse models and human neuroinflammatory disorders. ASPP2 induction in (*A*) an animal model of multiple sclerosis and (*B*) human encephalitis. (*C*) ASPP2 is up-regulated in GFAP-positive reactive astrocytes. STAT1 (red) and ASPP2 (green) coexpression in GFAP-positive reactive astrocytes (white). (Scale bars: 25 μm.) (*D*) Proposed mechanism of ASPP2/STAT1-induced apoptosis in response to inflammatory stimuli.

the p53 family, the status of ASPP2 may play a key role in dictating the response of the cell to inflammatory stimuli. Consistent with the role of ASPP2 in apoptosis, reduced ASPP2 expression dampened LPS-induced apoptosis in RAW264.7 cells, and ASPP2-deficient mice displayed less LPS-induced apoptosis in the hippocampus.

Interestingly, we also observed a biphasic induction of ASPP2 in human THP-1 cells by LPS. The underlying mechanism for the biphasic induction is currently unknown. Also, in THP-1 cells, only IFN-γ induced ASPP2 expression and not IFN-β. The failure of IFN-β to induce ASPP2 is not because of a lack of activity in THP-1 cells, because both IFN-γ and IFN-β induce phopspho-STAT1 expression in THP-1 cells with similar kinetics. The induction of ASPP2 by IFN-γ alone may indicate that, in THP-1 cells, IFN-γ specifically induces the formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind interferon-gamma activated site (GAS) elements (41) that are present in the promoter/enhancer of ASPP2, thereby initiating the transcription of ASPP2. Also, the identified STAT1 binding sites in the mouse (-590*GAAGGGCTT*-582) and human (-1090*GAAAGAATT*-1081) ASPP2 promoter/enhancer are GAS elements (Fig. S2*B*). In RAW264.7 cells and human astrocytes, IFN-β also induces the formation of STAT1–STAT1 homodimers (41) and binds GAS elements in the ASPP2 promoter/enhancer. However, in THP-1 cells, IFN-β may fail to do so. Furthermore, our data indicate that STAT1 induces transcription of ASPP2 in its activated tyrosine phosphorylated form on activation by IFNs or overexpression.

The identification of ASPP2 as a transcriptional target of STAT1 in response to LPS and IFN signaling reveals an important function of ASPP2 in the response of the cell to infection and inflammation. Increased ASPP2 expression in mouse neuroinflammation models and human neuroinflammatory disorders as well as the finding that ASPP2 Δ3/Δ3 mice have reduced apoptosis in response to systemic LPS injection also support the potential importance of ASPP2 in sensing, integrating, and dictating the cellular response to inflammatory stimuli. Because ASPP2 is a haploinsufficient tumor suppressor, cell polarity regulator, and activator of p53, the identified STAT1/ASPP2 pathway provides an important link between infection, inflammation, cell polarity, and tumor suppression.

Materials and Methods

ASPP2 Δexon3 C57BL/6Jx129SvJ mice were backcrossed in a BALB/c background for nine generations. All animal procedures were approved by the University of Oxford's ethical review committee and licensed by the UK Home Office (license number PPL 30/2862). *SI Materials and Methods* provides complete experimental methods. It includes reagents and details of IHC, IF, immunoblotting, RNA extraction, cDNA preparation, qRT-PCR, cell culture, ChIP assay, luciferase assay, and statistical analysis.

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