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Role of the ASPP Family in the Regulation of p53-Mediated Apoptotic Death of Retinal Ganglion Cells after Optic Nerve Injury

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« Role of the ASPP Family in the Regulation of p53-Mediated Apoptotic Death of Retinal Ganglion Cells after Optic Nerve Injury»

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RÉSUMÉ

Le glaucome est la première cause de cécité irréversible à travers le monde. À présent il n'existe aucun remède au glaucome, et les thérapies adoptées sont souvent inadéquates. La perte de vision causée par le glaucome est due à la mort sélective des cellules rétiniennes ganglionnaires, les neurones qui envoient de l'information visuelle de la rétine au cerveau. Le mécanisme principal menant au dommage des cellules rétiniennes ganglionnaires lors du glaucome n'est pas bien compris, mais quelques responsables putatifs ont été proposés tels que l'excitotoxicité, le manque de neurotrophines, la compression mécanique, l'ischémie, les astrocytes réactifs et le stress oxidatif, parmis d'autres. Indépendamment de la cause, il est bien établi que la perte des cellules rétiniennes ganglionnaires lors du glaucome est causée par la mort cellulaire programmée apoptotique. Cependant, les mécanismes moléculaires précis qui déclenchent l'apoptose dans les cellules rétiniennes ganglionnaires adultes sont mal définis. Pour aborder ce point, j'ai avancé l'**hypothèse centrale** que l'identification de voies de signalisations moléculaires impliquées dans la mort apoptotique des cellules rétiniennes ganglionnaires offrirait des avenues thérapeutiques pour ralentir ou même prévenir la mort de celles-ci lors de neuropathies oculaires telles que le glaucome.

Dans la première partie de ma thèse, j'ai caractérisé le rôle de la famille de protéines stimulatrices d'apoptose de p53 (ASPP), protéines régulatrices de la famille p53, dans la mort apoptotique des cellules rétiniennes ganglionnaires. p53 est un facteur de transcription nucléaire impliqué dans des fonctions cellulaires variant de la transcription à l'apoptose. Les membres de la famille ASPP, soit ASPP1, ASPP2 et iASPP, sont des protéines de liaison de p53 qui régulent l'apoptose. Pourtant, le rôle de la famille des ASPP dans la mort des cellules rétiniennes ganglionnaires est inconnu. ASPP1 et ASPP2 étant pro-apoptotiques, l'hypothèse

de cette première étude est que la baisse ciblée de ASPP1 et ASPP2 promouvrait la survie des cellules rétiniennes ganglionnaires après une blessure du nerf optique. Nous avons utilisé un modèle expérimental bien caractérisé de mort apoptotique neuronale induite par axotomie du nerf optique chez le rat de type Sprague Dawley. Les résultats de cette étude (Wilson et al. *Journal of Neuroscience*, 2013) ont démontré que p53 est impliqué dans la mort apoptotique des cellules rétiniennes ganglionnaires, et qu'une baisse ciblée de ASPP1 et ASPP2 par acide ribonucléique d'interference promeut la survie des cellules rétiniennes ganglionnaires.

Dans la deuxième partie de ma thèse, j'ai caractérisé le rôle d'iASPP, le membre anti-apoptotique de la famille des ASPP, dans la mort apoptotique des cellules rétiniennes ganglionnaires. L'hypothèse de cette seconde étude est que la surexpression d'iASPP promouvrait la survie des cellules rétiniennes ganglionnaires après axotomie. Mes résultats (Wilson et al. *PLoS ONE*, 2014) démontrent que le knockdown ciblé de iASPP exacerbe la mort apoptotique des cellules rétiniennes ganglionnaires, et que la surexpression de iASPP par virus adéno-associé promeut la survie des cellules rétiniennes ganglionnaires.

En conclusion, les résultats présentés dans cette thèse contribuent à une meilleure compréhension des mécanismes régulateurs sous-jacents la perte de cellules rétiniennes ganglionnaires par apoptose et pourraient fournir des pistes pour la conception de nouvelles stratégies neuroprotectrices pour le traitement de maladies neurodégénératives telles que le glaucome.

Mots clés : cellule ganglionnaire de la rétine, mort neuronale, protéine stimulatrice d'apoptose de p53, axotomie du nerf optique

SUMMARY

Glaucoma is the leading cause of irreversible blindness worldwide. At present, there is no cure for glaucoma, and current therapies are often inadequate. Loss of vision in glaucoma results from the death of retinal ganglion cells, the neurons that send visual information from the retina to the brain. The principal mechanism leading to retinal ganglion cell damage during glaucoma is not well understood, however, putative culprits have been proposed including excitotoxicity, neurotrophin deprivation, mechanical compression, ischemia, reactive astrocytes and oxidative stress. It is well established that retinal ganglion cell loss during glaucoma is caused by apoptotic programmed cell death, however, the precise mechanisms that lead to apoptotic death of adult retinal ganglion cells are poorly defined. To address this point, I put forth the **central hypothesis** that the identification of signaling pathways involved in apoptotic retinal ganglion cell death would offer therapeutic avenues to slow or prevent retinal ganglion cell death during ocular neuropathies such as glaucoma.

In the first part of my thesis, I characterised the role of Apoptosis Stimulating Protein of p53 family (ASPP) proteins, which are regulators of p53, in the apoptotic death of retinal ganglion cells. p53 is a nuclear transcription factor implicated in cellular functions ranging from transcription to apoptosis. ASPP family members ASPP1, ASPP2 and iASPP are p53 binding proteins that belong to a family of protein regulators of p53-dependent apoptotic death. However, the role of ASPP family members in retinal ganglion cell death is unknown. As ASPP1 and ASPP2 are pro-apoptotic, the hypothesis of our first study was that the knockdown of ASPP1 and ASPP2 gene expression would lead to retinal ganglion cell survival after an optic nerve lesion. We used a well-characterized experimental model of neuronal apoptosis induced by optic nerve axotomy in Sprague Dawley rats. The results of this study

(Wilson et al. *Journal of Neuroscience*, 2013) demonstrated that p53 is implicated in retinal ganglion cell death, and that targeted knockdown of ASPP1 and ASPP2 by short interference ribonucleic acid promotes retinal ganglion cell survival. The knockdown of ASPP2 correlates with a reduction in the levels of pro-apoptotic p53 regulated targets PUMA and Fas/CD95.

In the second part of my thesis, I characterized the role of the anti-apoptotic member of the ASPP family, iASPP, in the apoptotic death of retinal ganglion cells. The hypothesis of this second study is that the overexpression of iASPP would promote retinal ganglion cell survival after axotomy. The data (Wilson et al. *PLoS ONE*, 2014) demonstrate that the targeted knockdown of iASPP by short interference ribonucleic acid exacerbates retinal ganglion cell death, and that the overexpression of iASPP by adeno-associated virus promotes retinal ganglion cell survival. The overexpression of iASPP correlates with a reduction in protein levels of PUMA and Fas/CD95.

In conclusion, the findings presented in this thesis contribute to a better understanding of the pathological mechanisms underlying retinal ganglion cell loss by apoptosis and might provide insights into the design of novel neuroprotective treatments for neurodegenerative diseases such as glaucoma.

Key words : retinal ganglion cell, neuronal death, apoptosis-stimulating protein of p53, axotomy

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

LIST OF ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
ADAM	a disintegrin and metalloproteinase
ADAM17	ADAM metallopeptidase domain 17
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor 1
ASK1	apoptosis signal regulating kinase 1
ASPP	apoptosis stimulating protein of p53 (or ankyrin-repeat, SH3 and Proline- rich domain containing protein)
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BDNF	brain-derived neurotrophic factor
Bid	BH3 interacting domain death agonist
BIRC2	baculoviral IAP repeat-containing protein 4 (also called cIAP1)
BIRC4	baculoviral IAP repeat-containing protein 4
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CBA	chicken beta actin
CBP	CREB-binding protein
cIAP-1	cellular inhibitor of apoptosis protein-1

CMV	cytomegalovirus
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cAMP response element-binding protein
DBD	DNA binding domain
DIABLO	direct inhibitor of apoptosis (IAP)-binding protein with low pI
DNA	deoxyribonucleic acid
EndoG	endonuclease G
ERK 1/2	extracellular signal-regulated kinases 1/2
FG	fluorogold
GLAST	glutamate aspartate transporter
GMP	good manufacturing practice
HIV	human immunodeficiency virus
HTRA2	high-temperature-requirement protein A2
IAP	inhibitor of apoptosis
ICE	interleukin-1β-converting enzymes
ITR	inverted terminal repeats
JNK	c-Jun N-terminal kinase
Kb	kilobase
kDa	kilodalton
LTR	long terminal repeat
LV	lentivirus
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MDM2	mouse double minute 2

MDMX	mouse double minute X (also called MDM4)
miRNA	microRNA
mRNA	messenger RNA
Noxa	latin for *damage*
OD	oligomerization domain
ONH	optic nerve head
OPA-1	optic atrophy 1
PACG	primary angle-closure glaucoma
PBS	phosphate-buffered saline
PI3K	phosphatidylinositide 3-kinase
PKC	protein kinase C
PNS	peripheral nervous system
POAG	primary open-angle glaucoma
PTPC	permeability transition pore complex
PUMA	p53 upregulated modulator of apoptosis
qPCR	quantitative polymerase chain reaction
RBPMS	RNA-binding protein with multiple splicing
RGC	retinal ganglion cell
RING	really interesting new gene
RISC	RNA-induced silencing complex
RPE	retinal pigment epithelium
ROS	reactive oxygen species
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction

SAM	sterile alpha motif
SAPK	stress activated protein kinase
shRNA	short hairpin RNA
siRNA	short interfering RNA
SMAC	second mitochondria-derived activator of caspases
syn1	synapsin 1
TA	transactivation domain
TACE	TNFα-converting enzyme
TM	trabecular meshwork
TNFα	tumour necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRPV1	transient receptor potential vanilloid type 1
Trk	tropomyocin receptor kinase
µm	micrometer
XIAP	X-linked inhibitor of apoptosis protein
YY1	ying yang 1

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The eye owes its existence to the light. Out of indifferent animal organs the light produces an organ to correspond to itself...

J W von Goethe

To my mother, for her joy and understanding

To my father, for his boundless curiosity

To my sister Alexandra, for her encouragement

To my husband Marc, for his endless support

CHAPTER 1

I. GENERAL INTRODUCTION

I.1. THE RETINA AS AN EXPERIMENTAL MODEL SYSTEM

When the Roman physician Galen of Pergamon observed the superficial blood vessels spanning across the retina, he concluded that the purpose of the retina was to feed the eye, which he described in his manuscript *De Usu Partium Corporis Humani* (On the Usefulness of the Parts of the Body) in the second century A.D. That beneath these blood vessels lay nervous tissue only became apparent when Johannes Kepler‟s optical description of the eye identified the retina as the sensitive receptor of the eye (Kepler, 1604). William Bowman, a British surgeon, furthered this realisation by stating that "the eyeball [...] consists primarily and essentially of a sheet of nervous matter visually endowed", the retina (Bowman, 1849). Furthermore, the exquisite drawings of the Spanish histologist Santiago Ramón y Cajal laid the groundwork for the understanding of how the retina functions (Ramón y Cajal, 1899).

Cajal teased out the inter-relationships between neurons and traced the interweavings of neural communities. The quality of Cajal‟s observation revealed that the nervous system was made up of many independent, but interlinked, cells. Cajal not only identified a diversity of neurons, but also hypothesized that neurons receive electrical impulses through incoming dendrites, and conduct signals through outgoing axons (Ramón y Cajal, 1899). His findings revealed that the nervous system was to be understood not just through the behaviours of individual neurons, but through their connections with other cells.

I.1.1. Retinal cytoarchitecture

The retina is comprised of many cell types: photoreceptors, bipolar cells, horizontal cells, amacrine cells, retinal ganglion cells (RGC) and Müller glia (Figure 1). There are two types of photoreceptors in the retina: rods, which function as low light photoreceptors, and

cones, which are responsible for colour vision. Photoreceptors are light-sensitive cells that have an outer segment, composed of membranous disks that contain photopigment and lies adjacent to the pigment epithelial layer, and an inner segment that contains the cell nucleus and gives rise to synaptic terminals that contact bipolar or horizontal cells. Absorption of light by the photopigment (rhodopsin or cone opsins) in the outer segment of the photoreceptors initiates a cascade of events that changes the membrane potential of the photoreceptor, and therefore the amount of neurotransmitter released by the photoreceptor synapses onto the cells they contact. The synapses between photoreceptor terminals and bipolar or horizontal cells occur in the outer plexiform layer. The cell bodies of photoreceptors make up the outer nuclear layer, whereas the cell bodies of bipolar cells lie in the inner nuclear layer. The short axonal processes of bipolar cells make synaptic contacts on the dendritic processes of RGCs in the inner plexiform layer. The much larger axons of the RGCs form the optic nerve and carry retinal nerve impulses to the brain.

Horizontal and amacrine cells have their cell bodies in the inner nuclear layer and are primarily responsible for lateral interactions within the retina. The processes of amacrine cells, which extend laterally in the inner plexiform layer, are postsynaptic to bipolar cell terminals and presynaptic to the dendrites of ganglion cells. Müller glia, which represent the principal glial cells of the retina, span radially across the entire retina, with their endfeet enveloping RGCs and displaced amacrine cells in the ganglion cell layer. Their cell bodies are found at the level of the inner nuclear layer.

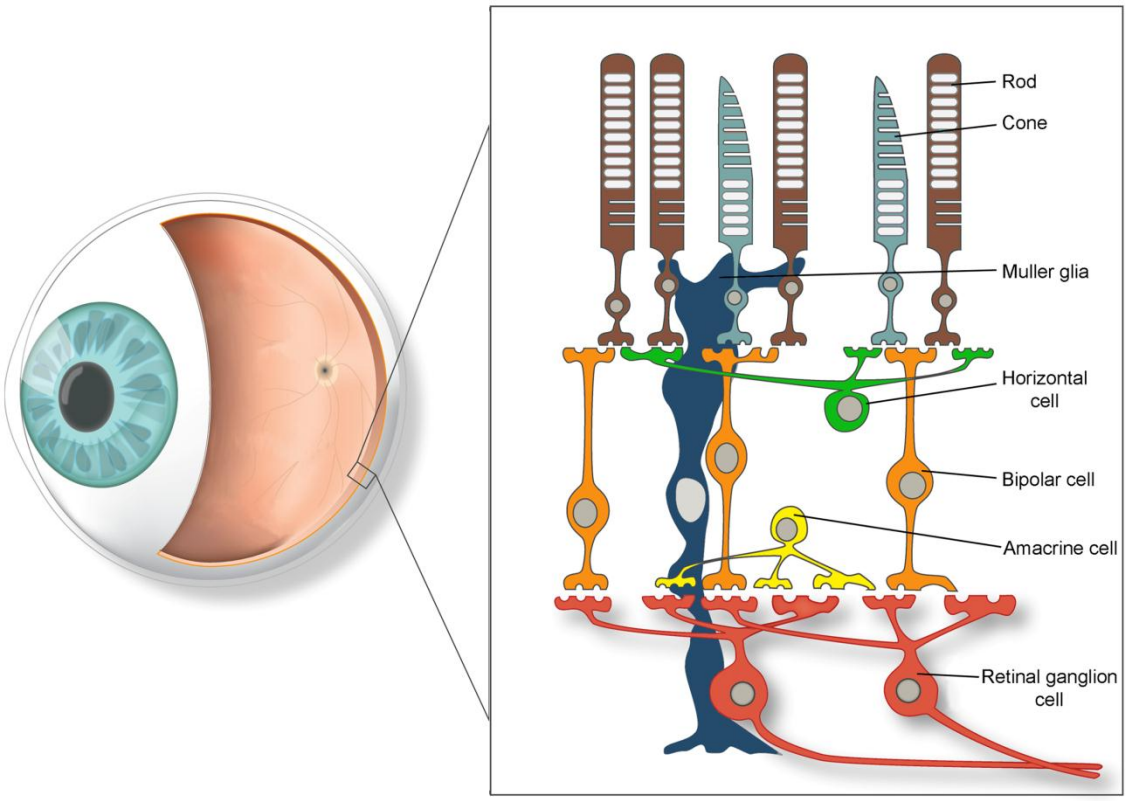

Figure 1. A schematic diagram of the retina demonstrating the principal cell types involved in retinal signaling.

The main retinal cell types are depicted as follows, with their respective attributed colour in parenthesis: rod photoreceptor (brown), cone photoreceptor (light grey), Müller glia (dark blue), horizontal cell (green), bipolar cell (orange), amacrine cell (yellow), retinal ganglion cell (red). Source: adapted from Wilson et al., Gene Therapy. 2012

I.1.2. The retina as a model system to study neurodegenerative mechanisms

RGCs are classified in 10 to 15 subtypes based on morphology or physiology in mammalian species (Masland, 2001a, b, Rockhill et al., 2002) but, despite their diversity, RGCs display the typical properties of CNS neurons, and comprise a cell body, dendrites and an axon. The RGC axons converge at the optic nerve head (ONH) to form the optic nerve which projects to four main targets: 1) the lateral geniculate nucleus of the thalamus, 2) the superior colliculus, 3) the pretectal nucleus, and 4) the suprachiasmatic nucleus. In rodents, a majority of axons project to the superior colliculus, whereas in humans the lateral geniculate nucleus is the predominant projection site (Linden and Perry, 1983, Purves, 2001). The optic nerve is the second of twelve paired cranial nerves but it is part of the CNS as it is derived from an evagination of the diencephalon during embryonic development and, as a consequence, the fibers are covered with myelin produced by oligodendrocytes, rather than Schwann cells found in the PNS. The accessibility of the retina renders it an ideal model in which to study neurodegenerative processes. One such disease is glaucoma, models of which will be discussed in the following section.

I.2. GLAUCOMA MODELS

I.2.1. Definition, risk factors and pathological features of glaucoma

Glaucoma refers to a group of chronic optic neuropathies characterized by progressive optic nerve damage and selective loss of RGCs, which functionally translates to progressive visual field defects leading to irreversible blindness. It is estimated that more than 60 million people suffer from glaucoma worldwide (Quigley and Broman, 2006) and according to the World Health Organization, glaucoma is the second leading cause of blindness after cataracts,

accounting for 12% of total cases of blindness globally (Foster and Resnikoff, 2005). Furthermore, glaucoma is the first irreversible cause of blindness worldwide, as vision loss from cataracts is reversible. Age and ethnic background are important risk factors for developing glaucoma. Individuals over the age of 40 are at a much higher risk of developing this ocular neuropathy (Coleman and Miglior, 2008). Individuals of African descent are at higher risk of developing glaucoma and have the highest rate of blindness due to this disease (Leske et al., 2007). Elevated intraocular pressure (IOP) is also an important risk factor, and the only modifiable one, for developing glaucoma. Ocular hypertension is characterized by an IOP in the human eye of over 21 mmHg, whereas a value of 15 mmHg is considered normal (Quigley et al., 1994). Although IOP is regarded as an important risk factor, it is not an accurate predictor of glaucoma since over 30% of glaucoma patients have an IOP in the normal range (Nemesure et al., 2007).

Glaucoma is characterized by damage to the neural components of the visual pathway including the retina, the optic tract, and the brain at the level of the lateral geniculate nucleus and the visual cortex. One of the determining features of glaucoma pathology is the selective loss of RGCs, which is characteristic of all glaucoma patients (Kendell et al., 1995, Quigley, 1999).

I.2.2. Implication of the aqueous humour in experimental models of glaucoma

As IOP is an important risk factor linked to glaucoma pathogenesis, many animal models have been developed based on inducible high IOP, which results in ONH damage and gradual RGC death. Incidentally, the production, circulation and drainage of the aqueous humor -the clear fluid which fills the anterior and posterior chambers of the eye- are

determining factors in the regulation of IOP levels. To understand how these animal models of glaucoma induce IOP increase, we will review the anatomical structures of the eye involved in the production and circulation of the aqueous humour.

The aqueous humour, produced by the ciliary epithelium in the ciliary body, provides nutrients for the lens and removes waste as it flows through the pupil into the anterior chamber of the eye (Krupin et al., 1986). The aqueous humor fills the anterior chamber and provides nutrients to the cornea as well. Within the anterior chamber, the cornea and iris join and it is here where the drainage of the aqueous humour takes place (Figure 2). The angle with which the cornea and iris join is of particular interest, as the two major categories of glaucoma are defined by the formation of an open or closed angle between the cornea and the iris: primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG).

Aqueous fluid flows toward the angle where it enters the trabecular meshwork (TM) (Tamm, 2009), which filters and directs the aqueous fluid into the Schlemm‟s canal (Johnstone, 2004). Several models of inducible and spontaneous glaucoma rely on a blockade of the aqueous humour drainage and will be discussed in subsequent sections.

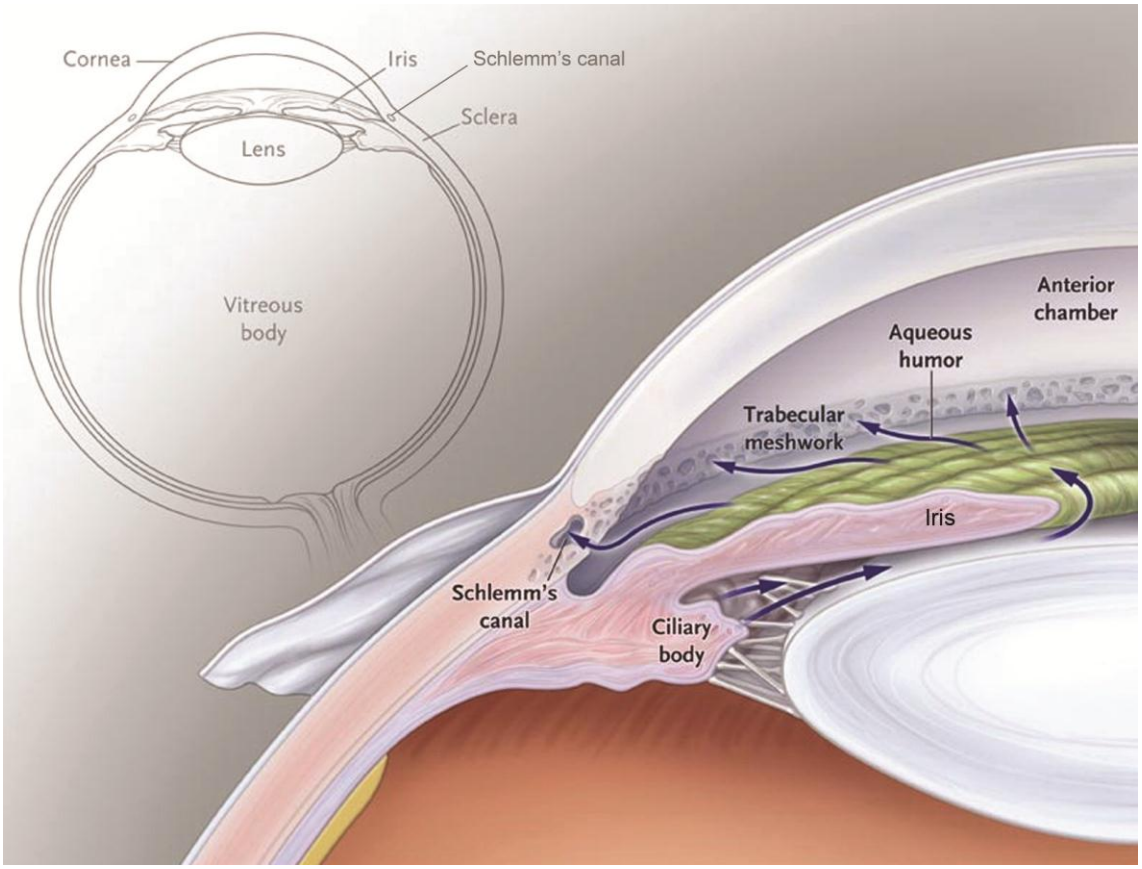

Figure 2. Schematic presentation of the aqueous humour circulation within the eye. Aqueous humour is produced by the ciliary body and enters the anterior chamber through the pupil. The trabecular meshwork (TM) is located in the angle between cornea and iris and provides an outlet for aqueous drainage by directing it into the Schlemm's canal. Reproduced with permission from (Kwon et al., 2009), Copyright Massachusetts Medical Society.

I.2.3. Experimental models of glaucoma

I.2.3.1. Primary-angle closure glaucoma

Primary-angle closure glaucoma (PACG) is characterized by the blockade of the aqueous humour drainage and/or its circulation. This results in increased IOP and consequent damage to the retina and optic nerve. Several breeds of dogs are prone to developing spontaneous glaucoma (Reinstein et al., 2009) however the high cost of purchasing and housing dogs, and complications of handling dogs in large experimental groups have resulted in the limited use of this model. A model of laser photocoagulation to induce the closure of the anterior chamber angle has been adapted for mice (Aihara et al., 2003). This model employs a diode laser which creates burn spots causing the iris root to attach to the peripheral cornea, which consequently obstructs the aqueous outflow and results in an elevation of IOP (Aihara et al., 2003). The IOP elevation in this model is accompanied by significant loss of RGC axons (Mabuchi et al., 2003). However, the small size of mice eyes demands a high level of dexterity by the experimenter, as excessive or misplaced laser burns could result in an inflammatory response and retinal damage.

I.2.3.2. Open-angle glaucoma

Primary open angle glaucoma (POAG) is the most common form of glaucoma worldwide. POAG is characterized by changes in the optic nerve head (also called optic disk), damage to RGC axons in the optic nerve, and loss of RGCs in the retina (Quigley, 2005). POAG is not necessarily associated with IOP elevation, however high IOP is a major risk factor for developing POAG (Leske et al., 2003).

In primate models of POAG, Rhesus or Cynomolgus monkeys are subjected to laser photocoagulation whereby burn spots are created on the circumference of the TM, resulting in moderate IOP increase (Wang et al., 1998). Loss of RGCs and visual deficits are well documented in this model (Hood et al., 1999, Morgan et al., 2000, Yücel et al., 2000, Hare et al., 2001, Yücel et al., 2003). However, the high cost of monkeys, their limited availability and difficulty to work with are drawbacks of this model.

The laser photocoagulation technique has also been used in rodent models of POAG (Ueda et al., 1998, Levkovitch Verbin et al., 2002, Gross et al., 2003, Ji et al., 2005). Another rodent model of POAG is the Morrison rat model, in which a hypertonic saline solution is injected into an episcleral vein of Brown Norway rat eyes. Hypertonic saline disrupts the structure of the TM and gradually reduces the aqueous outflow, resulting in IOP elevation (Morrison et al., 1997). Another method of reducing the aqueous outflow is by cauterizing the episcleral veins (Shareef et al., 1995). In this model, two to three veins are isolated and blocked using an ophthalmic cautery instrument. Obstruction of the TM and elevation of IOP can also be achieved by injection of sterile latex microspheres (Weber and Zelenak, 2001, Urcola et al., 2006) or microbeads into the anterior chamber of rodent eyes (Sappington et al., 2010, Chen et al., 2011). Finally, a hereditary mutation in the DBA/2J mice leads to iris pigment dispersion and adhesion of the iris to the cornea, which results in significant elevation of IOP by 6 months of age (John et al., 1998).

1.2.4 Glaucoma model caveats

The aforementioned glaucoma models each have their advantages and disadvantages. Chronic animal models of glaucoma rely on OHT induction to ultimately cause RGC

neurodegeneration, and yet not all glaucoma patients exhibit an increase in IOP. Furthermore, signaling pathways activated in chronic experimental models of glaucoma, much like the disease, are complex and variable. The complexity and variability in these models are not ideal for the detection of precise molecular mechanisms leading to apoptotic RGC death, a crucial aspect in the pathophysiology of all forms of glaucoma. The identification of key molecular mechanisms involved in neuronal death in vivo often requires a model that allows reliable, reproducible and predictable time-course of RGC death.

I.3. OPTIC NERVE AXOTOMY MODEL

Following traumatic injury to the mammalian CNS, neurons die by apoptosis, necrosis, or autophagy. Neurons that have their axons sheared open will undergo fundamental cellular and biochemical changes. Notably, the physical separation of the cell body from its target effectively prevents retrograde transport of neurotrophic factors thought to be required for survival (Oppenheim, 1991). Furthermore, calcium influx destabilizes the cytoskeleton and contributes to the activation of apoptotic pathways and the ensuing cellular degradation (Trump and Berezesky, 1995). Neurons that survive are severely impaired and rendered inactive, at least transiently, by the dramatic variations in their ionic and metabolic environment. In brief, adult mammalian CNS neurons are unable to regenerate an axon and their soma dies or atrophies after lesion (Ramon y Cajal, 1928).

I.3.1. Acute optic nerve injury and RGC death

The optic nerve transmits visual input from the retina to the visual cortex where image processing occurs. Damage to the optic nerve will affect this transmission of visual information, thus compromising vision. Optic nerve afflictions include: 1) glaucoma, in which

the optic nerve at the optic nerve head are damaged; 2) optic neuritis, an inflammation of the optic nerve which leads to the degradation of the myelin sheath enveloping the optic nerve; 3) cancerous tumours such as a pituitary tumour which compresses the nerve at the level of the optic chiasm, and 4) acute trauma such as an orbital fracture. Although a full transection (axotomy) is unlikely to occur and traumatic injury of the optic nerve is very rare (Steinsapir and Goldberg, 1994), the optic nerve axotomy produces a well characterized time course of RGC death, permitting the accurate evaluation of neuroprotective and regenerative strategies. In addition, RGCs die by apoptosis after optic nerve axotomy as they do in glaucoma (Garcia-Valenzuela et al., 1995) and other CNS diseases. However, because a larger number of RGCs die abruptly after axotomy, the molecular mechanisms that promote neuronal apoptosis might be more readily identified in this simpler model of RGC death. Furthermore, the initial wave of apoptotic RGC death is more reproducible in an axotomy model than in experimental rat glaucoma models, allowing for a spatiotemporal correlation between pro-apoptotic gene expression and RGC loss *in vivo*. Thus, strategies to promote cell survival in this system may be extrapolated to other neurodegenerative diseases affecting other neuronal populations. For these reasons, the optic nerve axotomy model was selected for the studies presented in this dissertation. Importantly, the axotomy model has proved extremely useful in the study and treatment of traumatic CNS injuries. Indeed, optic nerve axotomy has been extensively used to study the molecular mechanisms underlying retinal neuron death. For example, this model was used to detect the activation of apoptosis initiator caspase 9 (Kermer et al., 2000, Koeberle, 2009) and cleavage of effector caspase 3 in RGCs (Kermer et al., 1999, Hu et al., 2012) after optic nerve lesion, and subsequent TUNEL reactivity (Berkelaar et al., 1994, Quigley et al., 1995). This model has also been useful to evaluate the effect of neuroprotective strategies *in*

vivo; for example with the demonstration that caspase inhibitors reduced RGC loss following optic nerve axotomy (Kermer et al., 1998, Chaudhary et al., 1999, Ahmed et al., 2011, Monnier et al., 2011). Moreover, this was an effective model to investigate the signal transduction pathways involved in RGC survival, including TrkB signaling (Cheng et al., 2002b), the Erk1/2 pathway (Pernet et al., 2005, Almasieh et al., 2011), and the opposing effects of TrkA and p75 receptor signaling pathways (Lebrun-Julien et al., 2009).

I.3.2. Pattern of RGC cell death in the axotomy model

Transection of the adult rat optic nerve leads to a bi-phasic pattern of cell death. The first phase is prolonged, as within the first 4 to 5 days after lesion only a negligible number of RGCs die (Berkelaar et al., 1994, Peinado-Ramón et al., 1996). The second stage is rapid, however, and is characterized by massive cell death. Indeed, seven days after axotomy, only 50% of RGCs survive and less than 10% remain 14 days after injury (Berkelaar et al., 1994, Quigley et al., 1995). Interestingly, approximately 5% of RGCs remain up to 20 months after transection (Villegas-Perez et al., 1993), however, the molecular basis for this apparent resilience is currently unknown.

Following optic nerve axotomy, RGC death has been described as apoptotic (Berkelaar et al., 1994, Garcia-Valenzuela et al., 1994). Indeed, RGCs die by apoptosis in optic nerve acute lesion models, such as the optic nerve axotomy or crush models (Berkelaar et al., 1994), experimental glaucoma (Quigley et al., 1995), human glaucoma (Kerrigan LA, 1997), an observation that has been confirmed by *in vivo* real-time visualization in ocular hypertensive rat eyes (Cordeiro et al., 2004). Apoptosis is an energy consuming process that requires *de novo* protein synthesis. Apoptosis is a common mechanism of neuronal loss in the injured or

degenerating visual system. The hallmark structural features of apoptosis are cellular round-up, retraction of pseudopodia, reduction of cellular volume, nuclear fragmentation, modification of cytoplasmic organelles, plasma membrane blebbing, and engulfment by resident phagocytes (Kerr, 1972, Kerr et al., 1995). The apoptotic process can be triggered by various stimuli and involves the death receptor and/or mitochondrial apoptotic pathways.

Autophagy, traditionally defined as a non-apoptotic type of programmed cell death, involves lysosomal degradation of dysfunctional cellular components. Mutations in the Toll-Like Receptor 4 (TLR4) gene, a sensor for autophagy, have been associated with normal tension glaucoma in a Japanese cohort study (Shibuya et al., 2008). There is evidence that autophagy occurs in glaucoma, as LC3-B and Beclin-1 upregulation as well as an accumulation of autophagosomes were detected in RGCs after IOP elevation in a hypertensive rat glaucoma model (Park et al., 2012), and in a rhesus monkey hypertensive glaucoma model (Deng et al., 2013). In addition, an upregulation of genes linked to autophagy was detected in ocular hypertensive astrocyte samples (Tezel et al., 2012). Autophagy is also activated following optic nerve transection, and was compellingly revealed to have a neuroprotective role following axotomy (Kim et al., 2008, Rodriguez Muela and Boya, 2012).

Although apoptosis is the active process by which RGCs die after axonal damage, a small number of cells die by necrosis due to mechanical or inflammatory damage inflicted by the injury (Thanos et al., 1993, Bien et al., 1999). Necrosis typically occurs after toxic insult, hypoxia, energy depletion or other exogenous insults. It results in a swelling of the cell body and mitochondria, followed by perforation of the cell membrane resulting in the leakage of cellular contents and consequent inflammatory response. It is likely that there is a continuum

between axotomy-induced apoptotic and necrotic cell death, as both modes share common characteristics (Nicotera et al., 1999). While the orchestral role of p53 in apoptotic pathways is well established, recent findings have reported a novel role in necrosis, whereby p53 opens the mitochondrial permeability transition pore to trigger necrosis (Vaseva et al., 2012). An understanding of p53 function in RGC death is therefore warranted. We opted to focus on apoptotic cell death in our studies because of the central role apoptosis plays in RGC death during glaucoma. Furthermore, despite potential crosstalk amongst apoptotic, necrotic and autophagic pathways, there is no strong evidence of necrosis occurring in glaucoma (Osborne et al., 1999b), and the understanding of autophagic processes in glaucomatous neurodegeneration is incipient.

I.4. p53 AND ITS REGULATORS

I.4.1. The p53 family

The p53 transcription factor belongs to a family of proteins called the p53 family, comprised of three evolutionarily conserved transcription factors, p53, p63 and p73. The p53 family proteins are involved in many important cellular functions, including tumour suppression (p53 and p73), epithelial cell stratification (p63), and CNS development (p73). Furthermore, *p53*, *p63* and *p73* genes can independently mediate apoptosis (Sheikh and Fornace, 2000). All p53 family members are expressed during retinal development, but their levels are downregulated in the adult eye (Vuong et al., 2012). Upregulation of p63 and p73, but not p53, are observed in human retinoblastoma tumour samples, an eye cancer affecting the retina (Adithi et al., 2008).

All three members of the p53 family share significant homology at the genomic and protein levels. They share common structural motifs, including a transactivation domain (TA), a DNA-binding domain (DBD) and an oligomerization domain (OD). In addition, p63 and p73, but not p53, contain long C-termini containing a sterile alpha motif (SAM), which is a protein-protein interaction domain (Chi, 1999) (Figure 3). *p63* and *p73* genes have been found to encode several proteins whose structure and functions are similar yet not identical to those of *p53* (Kaghad et al., 1997, Yang et al., 1998). *p53*, *p63* and *p73* encode differentially spliced mRNAs, with most of splicing occurring at the 3‟ end for p63 and p73, creating proteins with varying C-termini lengths (Kaghad et al., 1997, De Laurenzi et al., 1998, De Laurenzi et al., 1999, Ueda et al., 1999). Not only do all three p53 family members express multiple splice variants, they also contain different internal promoters in addition to their proximal promoters, yielding truncated or full length variants, respectively. This allows for a multitude of transcript variants arising from *p53*, *p63* and *p73* gene transcription. Altogether, the *p53* gene can transcribe ten different variants: p53 (α, β, γ), Δ40p53 (α, β, γ), Δ133p53 (α, β, γ) and Δp53 (Courtois et al., 2004, Bourdon et al., 2005, Mills, 2005, Rohaly et al., 2005) (Figure 3). In the case of *p63* and *p73*, the promoter and differential splicing options yield α, β, γ, δ, ε, δ, ε, and φ isoforms for both full length and truncated forms (Figure 3). Furthermore, p73 has 3 additional truncated forms due to an additional amino-terminal splicing site (Moll and Slade, 2004). As the N terminus is crucial for the transactivation of target genes, full-length isoforms of p53, p63 and p73 can be functionally distinguished from the transactivation-compromised ΔN isoforms that show anti-apoptotic and dominant–negative properties.

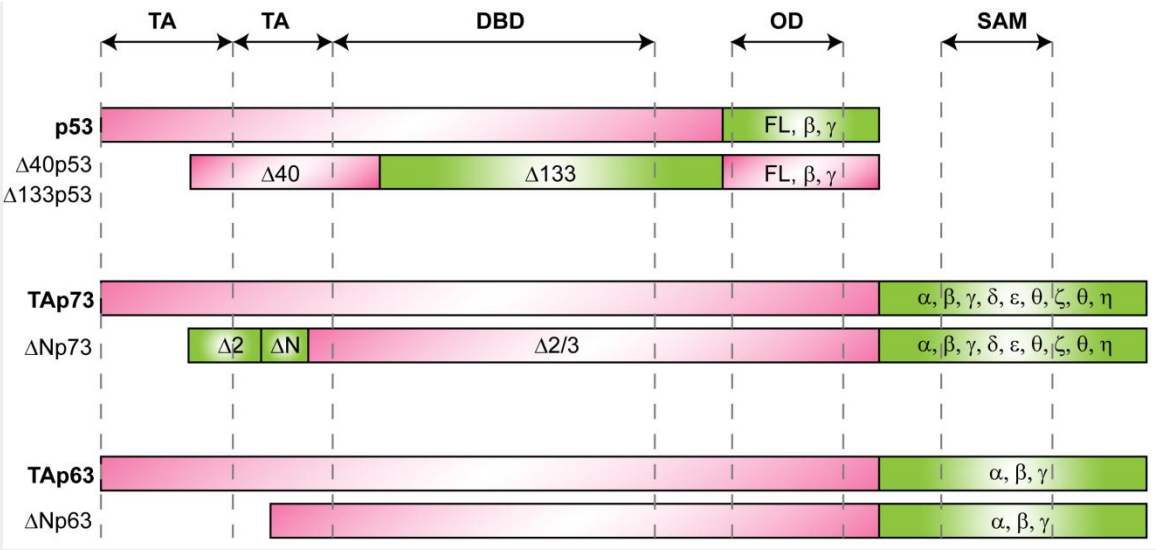

Figure 3: p53 family structural motifs. The overall protein architecture of the p53 family is highly conserved, and consists of a central sequence-specific DNA binding domain (DBD), an N-terminal transactivation domain (TA) and a C-terminal oligomerization domain (OD). Both p63 and p73 have a sterile alpha motif (SAM) domain implicated in protein–protein interactions, whereas p53 does not. The highest degree of homology is seen within the DBD, where >97% of all tumour-associated p53 mutations are located. All three genes express many differently spliced isoforms, and contain a second intronic promoter that generates N-terminally truncated proteins (Δ133p53, ΔNp63 and ΔNp73). Alternative splicing of C-terminal exons yields many different isoforms (α, β, γ, δ, ε, δ, ε, and ζ) with still incompletely understood DNA-binding properties, transcriptional activities and biological functions. Source of image adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Stiewe, 2007), copyright (2007).

I.4.2. The p53 transcription factor

I.4.2.1. p53 in ocular development

The role of p53 during ocular development has been assessed in p53 knockout mice of varying strains, which revealed striking differences. Indeed, in two strains, the C57BL x CBA and 129/Sv x C57BL/6 mice, there were no reported ocular developmental defects (Donehower et al., 1992, Jacks et al., 1994). However, abnormalities were detected in the p53 knockout mice with C57BL/6 and BALB/c OlaHsd backgrounds. Indeed, cataract formation was detected in p53-null BALB/c OlaHsd mice, as well as aberrant hyaloid vasculature, which is a transient embryonic vasculature that regresses with the formation of adult retinal vessels (Reichel et al., 1998). In addition to abnormal hyaloid structure, p53-null mice in a C57BL/6 background also exhibited retinal folding and underdeveloped (hypoplastic) optic nerves (Ikeda et al., 1999). It was hypothesized that the phenotype discrepancy between p53-null mice with 129/Sv or C57BL/6 backgrounds was caused by protective and compensatory alleles for p53 loss present in 129/Sv mice (Ikeda et al., 1999).

p53 transcript levels during murine retinal development peak at embryonic days E17-E18 and decrease gradually until postnatal day P15, where they remain low in the post-mitotic retina (Vuong et al., 2012). Likewise, p53 protein was readily detected at E18, after which time point protein levels began to decrease, and were undetectable by P7 (Vuong et al., 2012). This decrease in p53 levels in the murine retina coincides with the developmental time point that retinal cells exit the cell cycle, differentiate, and become postmitotic (Cepko et al., 1996). *p53* is not required for developmental programmed death of RGCs, as mice deficient for one

or both alleles of p53 do not exhibit developmental changes in RGC number in comparison to wild-type animals (Li et al., 2002).

I.4.2.2. p53 in post-mitotic neurons

The tumour suppressor and nuclear transcription factor p53 mediates the apoptosis of post-mitotic neurons exposed to a wide range of insults such as DNA damage, neurotrophic factor deprivation, oxidative stress, ischemia and excitotoxicity (Culmsee and Mattson, 2005). Stress signals lead to activation and accumulation of p53, which then promotes the transcription of pro-apoptotic genes (Michalak et al., 2005). Neuronal apoptosis induced by p53 has been well documented in models of neurodegenerative disorders including Alzheimer‟s disease, Parkinson‟s disease, Huntington‟s disease and amyotrophic lateral sclerosis (de la Monte et al., 1997, González de Aguilar et al., 2000, Martin, 2000, Duan et al., 2002, Tamagno et al., 2003, Ryan et al., 2006), suggesting a key role for this transcription factor in the regulation of neuronal viability after neural injury. Given its critical role in the control of cell death, several mechanisms exist to ensure a tight regulation of p53 activity. The level of p53 protein is normally kept low in most cell types, including neurons (Soussi, 2000), via rapid and continuous degradation following ubiquitination by the E3 ubiquitin ligases Mdm2 and MdmX (Wade et al., 2010). Additional control of p53 function is exerted via post-translational acetylation, phosphorylation or methylation; and by interactions with protein partners including the apoptosis-stimulating proteins of p53 (ASPP) family (Iwabuchi, 1994, Nagase et al., 1998, Boehme and Blattner, 2009).

p53 has been shown to exert an important pro-oxidant activity in the retina (Chatoo et al., 2009). Inactivation of the p53 gene, or reduced p53 expression, has been shown to protect

RGCs against retinal ischemia or excitotoxic death (Rosenbaum, 1998, Li et al., 2002). Pro-apoptotic genes induced by p53 including Gadd45a and Ei24 are upregulated in the retina after optic nerve transection and in experimental glaucoma models (Levkovitch-Verbin et al., 2006). Attempts to link single nucleotide polymorphisms in the p53 gene with glaucoma have been contradictory: while some studies found a correlation in Caucasian and Chinese populations (Ressiniotis et al., 2004, Daugherty et al., 2009) an association has not been found in other ethnic groups (Acharya et al., 2002, Dimasi et al., 2005, Mabuchi et al., 2009, Saglar et al., 2009, Silva et al., 2009). A recent study demonstrated that loss of WDR36, a gene of unknown function identified as causative for glaucoma (Monemi et al., 2005), leads to activation of the p53 stress-response pathway in zebrafish (Skarie and Link, 2008). These studies suggest that defects in p53 pathway genes may increase the risk of certain populations to develop glaucoma (Fan et al., 2010). Furthermore, p53 has been implicated in age-related macular degeneration (AMD): AMD is generally believed to start with retinal pigment epithelium (RPE) cell death, and p53 is upregulated in RPE cells in response to high-energy light exposure, inducing apoptosis (Westlund et al., 2009, Bhattacharya et al., 2012). In retinoblastoma, Mdm4 is overexpressed in response to loss of *RB1*, leading to degradation of p53 (Laurie et al., 2006, Wallace, 2006).

I.4.3. p53 regulation

There are over 100 known p53 binding proteins, whose interactions with p53 along with p53 post-translational modifications frequently dictate p53 function, for example whether it induces cell cycle arrest or apoptosis (Braithwaite et al., 2005). These regulating mechanisms range from inhibition to coactivation of p53. The identification of p53 in 1979 has led to nearly 70,000 peer-reviewed publications, and was named the „Molecule of the

year" in 1993 by the journal *Science* (Koshland, 1993). p53 is mutated in nearly half of all human cancers (Petitjean, 2007), a trait rarely found in p63 and p73 family members (Strano et al., 2007). Not only does p53 act as a tumour suppressor, it is activated in response to various stress signals. p53 activation occurs via multiple mechanisms including increased p53 protein concentration, often caused by decreased p53 degradation (Ashcroft et al., 2000), nuclear translocation (Liang and Clarke, 2001), and post-translational protein modifications including phosphorylation and acetylation (Jayaraman and Prives, 1999). Furthermore, p53 can also be activated by non-covalent modifications such as electrostatic interactions with other proteins (Benyamini and Friedler, 2011). p53 is modified by as many as 50 individual posttranslational modifications, which mediate precise protein-protein interactions, an array of modifications that is interdependent (Meek and Anderson, 2009).

The p53 interactome is continuously being updated, however relatively few of these p53 binding partners have been studied in the CNS. The following sections on p53-regulating proteins focus on the ASPP family, and additional p53 regulating proteins whose roles have been assessed in the retina.

I.4.3.1. The ASPP family

The ASPP proteins constitute a recently discovered family of proteins that bind and modulate p53-dependent apoptosis (Trigiante and Lu, 2006). Their name is an acryonym either based on the domain organization of the proteins (Ankyrin-repeat, SH3, and Proline-rich domain containing Protein) or their function (Apoptosis-Stimulating Protein of p53). The founding member of the family, ASPP2, was initially identified as 53BP2 (p53 binding protein 2) in a yeast two-hybrid screen, using the p53 DNA binding core domain as bait (Iwabuchi,

1994). ASPP1 was identified later in a homology search (Nagase et al., 1998). Functional studies revealed that p53-induced apoptosis was substantially enhanced in the presence of ASPP1 or ASPP2 (Lopez et al., 2000, Ao et al., 2001, Samuels-Lev, 2001) and that complexes with ASPP1 or ASPP2 increased the affinity of p53 for promoters of pro-apoptotic genes (Samuels-Lev, 2001, Bergamaschi et al., 2006). Furthermore ASPP1 and ASPP2 selectively enhance the apoptotic-promoting ability of p53 without affecting p53 cell cycle arrest functions (Bergamaschi et al., 2003). This preferential activation of apoptotic genes in the presence of ASPP1 and ASPP2 was also observed for p63 and p73 (Bergamaschi et al., 2004). The third member of the ASPP family, iASPP, was originally identified as an inhibitor of the nuclear factor kappa β (NFk β) (Yang, 1999). iASPP is the p53 inhibitor within the ASPP family, blocking apoptosis by repressing the transactivation potential of p53 (Bergamaschi et al., 2003, Bergamaschi et al., 2006) (Figure 4). Notably, the ability of iASPP to inhibit p53 mediated apoptosis is conserved from *C. elegans* to humans.

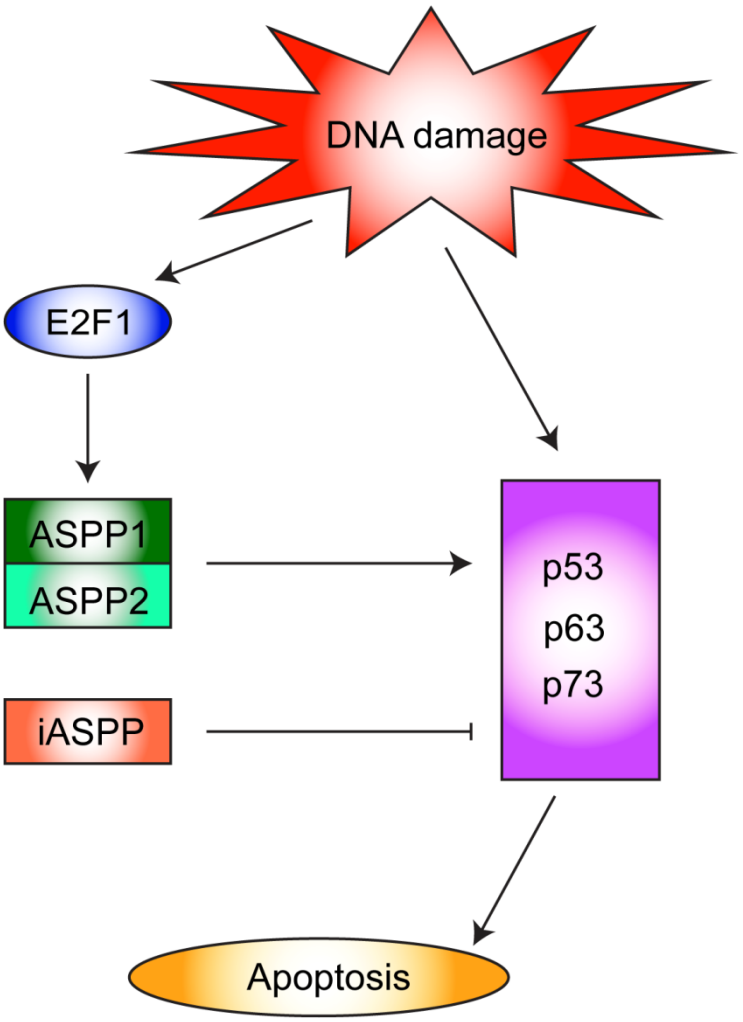

Figure 4. ASPP family apoptotic signaling pathway. The pro-apoptotic ASPP1 (ankyrin-repeats-, SH3-domain- and proline-rich-region-containing protein 1) and ASPP2 are induced by the E2F1 transcription factor and cooperate with the p53 transcription factor and its family members p63 and p73 in transactivating pro-apoptotic genes to promote apoptosis. The inhibitory family member iASPP functions as a transrepressor of the same genes that ASPP1 and ASPP2 transactivate. (Image generated by Ariel Wilson).

The regions of ASPP family members that interact with p53 have been mapped to their C-termini (Patel et al., 2008, Robinson, 2008). The N termini of ASPP1 and ASPP2 confers their apoptotic activity (Samuels Lev et al., 2001), and show no sequence similarity with the N terminal of iASPP (Bergamaschi et al., 2003). ASPP1/2 and iASPP bind to the core domain of p53 (Robinson, 2008), and iASPP also binds to the proline-rich region of p53 (Bergamaschi et al., 2006) (Figure 5).

Pro-apoptotic ASPP1 and ASPP2 are frequently downregulated in tumours, and anti-apoptotic iASPP is frequently upregulated (Bergamaschi et al., 2003, Jiang et al., 2011, Li et al., 2011, Li et al., 2012, Mak et al., 2013, Zhao et al., 2013). Post-translational modifications of ASPP family members in cancer cells have been discovered. Indeed, ASPP2 is phosphorylated by the Ras/MAPK pathway in an osteocarcinoma cell line (Godin Heymann et al., 2013), and Cyclin B1/CDK1 phosphorylates iASPP in melanoma cells (Lu et al., 2013). Other mechanisms of ASPP regulation have been uncovered, such as the study of *ASPP1* and *ASPP2* epigenetic regulation which revealed that in tumour cells expressing wild-type p53, *ASPP1* and *ASPP2* promoters are hypermethylated and subsequently downregulated in hepatocellular carcinoma tumours (Zhao et al., 2010) as well as in breast cancer and lung carcinoma tumour cell lines (Liu et al., 2005). Furthermore, microRNA downregulation of iASPP protein levels was detected in a cerebral ischemia mouse model (Liu et al., 2013).

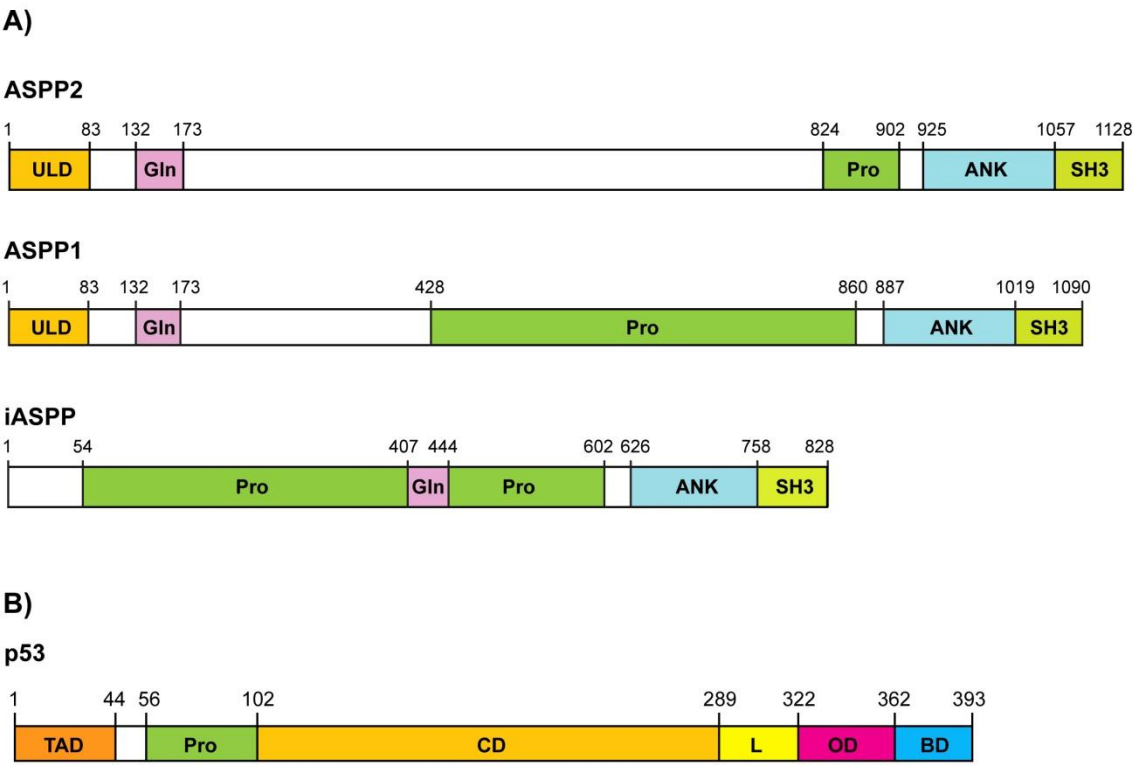

Figure 5. Amino acid sequences and domain organisation of ASPP and p53 proteins. A) Domain organization of ASPP2, ASPP1 and iASPP. Individual domains are as follows: ubiquitin-like (ULD), glutamine-rich (Gln), proline-rich (Pro), ankyrin repeats (ANK), and Src homology 3 (SH3). B) Domain organization of p53. Individual domains are the transcription activation (TAD), the proline-rich (Pro), the DNA binding or core (CD), the linker (L), the oligomerization or tetramerization (OD), and the basic (BD) domains. Source of image: adapted from Ahn et al., 2009, originally published in the Journal of Biological Chemistry © the American Society for Biochemistry and Molecular Biology.

I.4.3.2. MDM2/MDMX

The murine double minute 2 (MDM2) and murine double minute X (MDMX) (also known as MDM4) inhibit p53 activity by engaging its N-terminus transactivation domain (Momand et al., 1992, Kussie et al., 1996, Laurie et al., 2006). MDM2 and MDMX are RING (Really Interesting New Gene) domain proteins, and many proteins containing a RING domain have been shown to play a key role in the ubiquitination pathway (Joazeiro and Weissman, 2000). However, despite their similar structures, only MDM2 has intrinsic E3 ubiquitin ligase activity, conferring MDM2 the ability to target p53 for degradation by the proteasome (Honda et al., 1997). Although MDM2 alone can inhibit p53, its RING-dependent heterodimerization with MDMX has an important role in p53 inhibition. Indeed, MDM2 inhibits the transactivation ability of p53 (Momand et al., 1992), and MDMX stabilizes MDM2 by preventing it to self-ubiquitinate (Stad et al., 2001).

Within the retina, the role of MDM2 was assessed in RPE cells, which are essential for photoreceptor function by regulating cell homeostasis and serving as blood-retinal barrier (Strauss, 2005). Inhibition of MDM2 in primary RPE cultured cells resulted in an increase of pro-apoptotic targets, sensitizing RPEs to apoptosis (Bhattacharya et al., 2011). In aged RPEs, the MDM2/p53 pathway interaction is disrupted, leading to an age-dependent increase in apoptosis (Bhattacharya et al., 2012). Furthermore, MDM2 has been studied in the context of retinoblastoma. Mutations in the tumour suppressor *RB1* gene are pivotal in the development of this early childhood cancer of the retina. Indeed, loss of RB1 function in the developing retina leads to *MDM2* and *MDMX* amplification, contributing to p53 pathway inactivation (Laurie et al., 2006), which facilitates retinal cell transformation and tumorigenesis.

I.4.3.3. YY1

Yin yang 1 (YY1) is a transcription factor that belongs to the Polycomb family, a group of chromatin modulators that are critical to homeobox gene regulation during development. First identified in 1991 (Shi et al., 1991), YY1 is a highly conserved and multifunctional transcription factor. Its name, representing interconnected yet seemingly opposing forces, stems from the fact that YY1 can act as an activator or repressor of transcription (Shi et al., 1997). YY1 has been implicated in cell proliferation, differentiation and apoptosis (Gordon et al., 2006), and is crucial for embryonic development as YY1 deficiency results in peri-implantation lethality, i.e. lethality occurring in the period between blastocyst formation and uterine implantation (Donohoe et al., 1999).

YY1 negatively regulates p53 protein levels and activity (Sui et al., 2004). YY1 acts as an MDM2 cofactor, facilitating MDM2-p53 interaction, and YY1 downregulation results in p53 accumulation due to a decrease in p53 ubiquitination levels. In contrast, YY1 overexpression increases p53 ubiquitination and degradation (Sui et al., 2004). YY1 is ubiquitously expressed in the CNS during early embryonic development (Kwon and Chung, 2003), and is highly expressed in the adult neural retina (Bernard and Voisin, 2008). In mouse embryos and *Xenopus* oocytes, YY1 was shown to activate Otx2, a transcription factor that controls photoreceptor cell fate (Kwon and Chung, 2003, Nishida et al., 2003, Takasaki et al., 2007). In the adult retina, YY1 is mainly expressed by photoreceptors at the level of the inner segments and nuclei in the intact chicken retina, and weakly in the inner nuclear layer (Bernard and Voisin, 2008).

I.4.3.4. P300/CBP

p300 and CREB Binding Protein (CBP) are highly homologous nuclear proteins that play a key role in transcriptional regulation. The interaction of p53 with p300 and/or CBP (p300/CBP) regulates the ability of p53 to bind to its cognate DNA sequences and activate transcription. It is specifically the acetyltransferase activity of p300/CBP that has been implicated in the regulation of p53 function. Indeed, in response to DNA damage, transcription coactivators p300/CBP bind to and acetylate p53, which stimulates the DNA binding activity of p53 (Gu and Roeder, 1997, Prives and Hall, 1999, Grossman, 2001).

There is evidence that p300/CBP are involved in retinal function. For instance, mutations in CBP or p300 are responsible for a subset of Rubinstein-Taybi syndrome cases (Schepis et al., 2001, Zimmermann et al., 2007), a disease in which retinal dystrophy and glaucoma are common (van Genderen et al., 2000). The study of p300/CBP in animal models revealed that p300 and CBP knockout mice are embryonic lethal (Yao et al., 1998, Tanaka et al., 2000). The development of photoreceptor-specific p300/CBP conditional knockout mice was instrumental to demonstrate a key role for p300/CBP in photoreceptor gene expression (Hennig et al., 2013). Furthermore, p300 and CBP are expressed by adult RGCs, and adenoviral-mediated overexpression of p300 in RGCs has been shown to promote optic nerve regeneration (Gaub et al., 2011). Although p53 C-terminal acetylation by p300 is known to be involved in the fine-tuning of p53 stress responses (Krummel et al., 2005), these modifications do not appear to play a crucial role in RGC death. Indeed, in an acetylation-deficient missense mutant mouse model, the loss of p53 acetylation at its C terminus by CBP/p300 was not required for p53 transactivation (Krummel et al., 2005).

The ASPP family, MDM2/MDMX, YY1, and CBP/p300, along with additional p53 regulating proteins, are implicated in the apoptotic function of p53. The signaling cascades occurring during apoptosis in RGCs will be discussed in the subsequent section.

I.5. APOPTOTIC PATHWAYS ACTIVATED IN RGCs

The apoptotic machinery is present in all cells and is therefore considered to be an intrinsic suicide program. Neuronal survival relies on a detailed interaction between the cell and its environment. The neuron is thought to be continuously on the verge of apoptosis, requiring survival signals to prevent its death (Raff, 1992, Raff et al., 1993). Neurotrophins, cytokines, growth factors such as brain derive neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), and other peptide ligands stimulate transmembrane receptors, which activate intracellular pathways and ultimately inhibit this internal death program (Jacobson et al., 1997) (Figure 6). The apoptotic pathway can be induced either through a mitochondrial pathway (Green and Reed, 1998), or by stimulation of cell surface death receptors (Ashkenazi, 1998), which are referred to as the intrinsic and extrinsic apoptotic pathways, respectively.

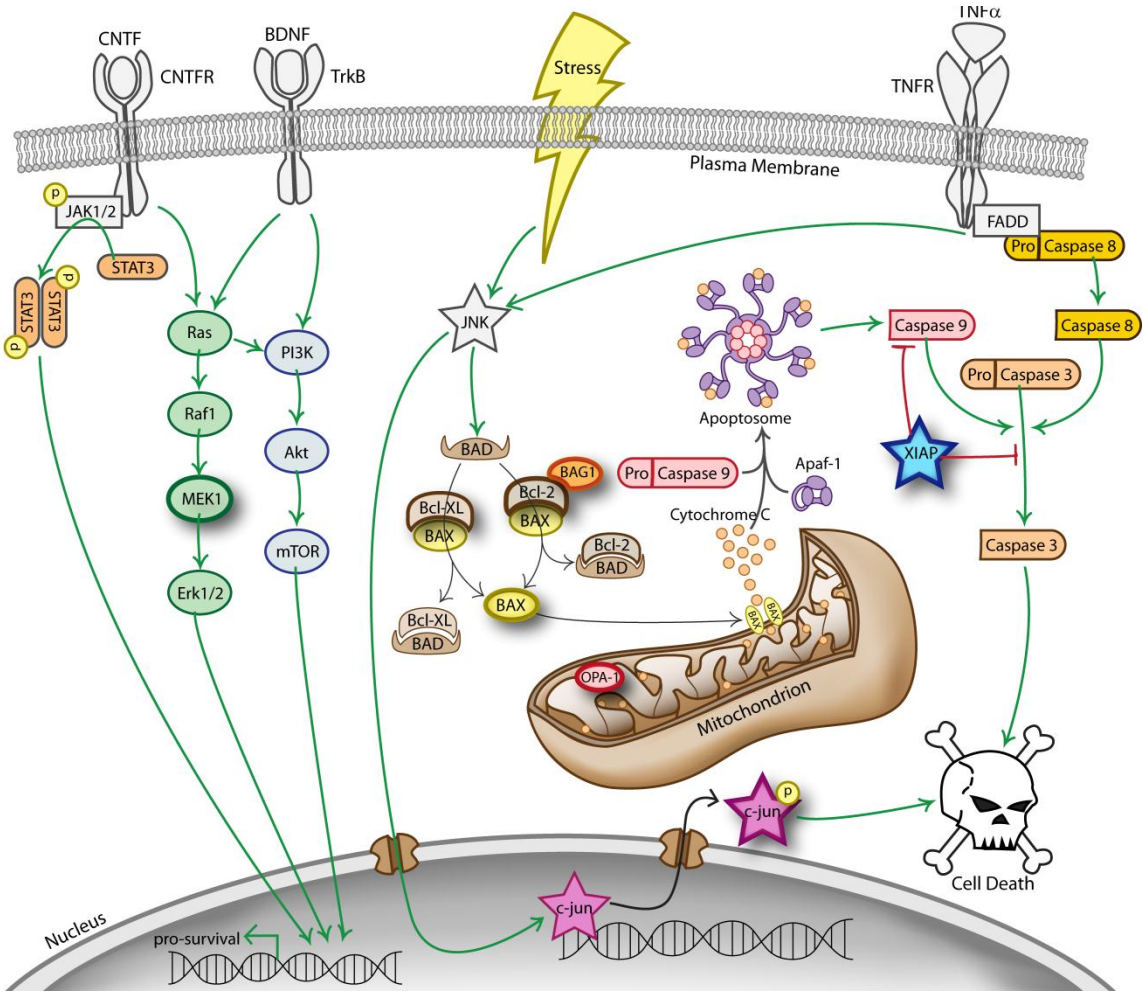
CNTF
CNTFR
BDNF
TrkB
Stress
TNFR
TNFα
Plasma Membrane
JAK1/2
STAT3
STAT3
STAT3
Ras
PI3K
Raf1
Akt
MEK1
mTOR
Erk1/2
JNK
FADD
Pro Caspase 8
Caspase 8
Caspase 9
Pro Caspase 3
Caspase 3
XIAP
Apoptosome
Apaf-1
Pro Caspase 9
Cytochrome C
BAD
BAG1
Bcl-2
Bcl-XL
BAX
Bcl-2
BAD
Bcl-XL
BAD
BAX
BAX
BAX
OPA-1
Mitochondrion
c-jun
P
c-jun
Cell Death
Nucleus
pro-survival

Figure 6. Extrinsic and intrinsic apoptotic pathways regulating retinal ganglion cell death. The apoptotic death of RGCs can be triggered by various stimuli and involves extrinsic and intrinsic pathways. Extrinsic signals include the death-receptor ligands TNF-α, Fas-L, and TRAIL, and their respective receptors, which induce RGC death. Lack of neurotrophic factors may result in deficits of pro-survival pathways including Erk1/2 and PI3K. The intrinsic pathway converges on the Bcl-2 family members: pro-apoptotic (Bax, Bad, Bid) or anti-apoptotic (Bcl-2, Bcl-XL), which regulate the mitochondrial outer membrane permeabilization. These proteins control the release of cytochrome c into the cytosol which can activate the caspases, executioners of apoptosis. Source: from Wilson et al., Gene Therapy. 2012

I.5.1 The intrinsic apoptotic pathway

I.5.1.1. Pro-apoptotic kinases

The mitogen-activated protein kinases (MAPKs) are a large family of protein Ser/Thr kinases that relay extracellular signals to the intracellular milieu and, as such, are central regulators of many cellular functions (Cargnello and Roux, 2011). The conventional MAPKs family comprises extracellular signal-regulated kinases 1 and 2 (Erk1/2), c-Jun N-terminal kinases (JNKs), p38 isoforms and Erk5. In contrast to the RGC survival response associated with activation of the Erk1/2 pathway, pro-apoptotic MAPK are typically activated by a variety of stress signals and have been proposed to contribute to RGC death. One such MAPKs subfamily are the JNKs, also known as stress-activated protein kinases (SAPKs), which play central roles in the regulation of signal transduction in the mammalian brain (Brecht et al., 2005). The elucidation of JNKs function *in vivo* has been challenging due to the presence of ten different JNK isoforms that result from alternative splicing (Kyriakis et al., 1994) and display differential specificity towards their target proteins (Gupta et al., 1996, Kallunki et al., 1996, Chang and Karin, 2001). C-jun, a transcription factor activated by JNK phosphorylation, mediates the transcription of pro-apoptotic genes (Curran and Franza, 1988) (Figure 6). The JNK/c-jun pathway is upregulated in RGCs after optic nerve axotomy or crush (Herdegen et al., 1993, Hull and Bahr, 1994, Robinson, 1994, Isenmann and Bähr, 1997, Kreutz et al., 1999, Takeda et al., 2000, Yang et al., 2007), during excitotoxic damage (Bessero et al., 2010), in rodent models of experimental glaucoma (Levkovitch-Verbin et al., 2005, Kwong and Caprioli, 2006, Yang et al., 2007), and in human glaucoma (Tezel et al., 2003). Of interest, long-term activation of c-Jun has been observed in optic nerve head astrocytes of monkeys subjected to experimental ocular hypertension (Hashimoto 2005). Short

interfering RNA (siRNA)-mediated knockdown of c-Jun resulted in RGC survival after optic nerve lesion (Lingor et al., 2005), and administration of a JNK inhibitor conferred moderate RGC protection in an acute ocular hypertension model (Sun et al., 2011). To assess the functional role of JNK activation on RGC survival, recent studies have examined the effect of genetic deletion of JNK genes in mouse models of optic nerve injury. Induction of ocular hypertension in JNK3 null mice did not show significant RGC neuroprotection (Quigley et al., 2011). In contrast, mice lacking both JNK isoforms (*Jnk2*$^{-/-}$*Jnk3*$^{-/-}$) displayed marked RGC survival after injury and this response involved c-jun and Bim activation (Harder et al., 2011). These results suggest that the different JNK isoforms might act to compensate for deficiencies in this pathway to ensure apoptosis of injured RGCs. Future work is required to establish whether combined deletion of JNK2 and JNK3 influence RGC fate in experimental glaucoma.

The p38 MAPKs, a subfamily comprising four isoforms (p38α, p38β, p38γ, p38δ), is a group of kinases that are highly responsive to stress stimuli (Cuadrado and Nebreda, 2010). p38α is expressed at higher levels than the other isoforms; therefore the majority of the published studies to date refer to p38α form. p38 is strongly activated by various environmental signals such as inflammatory cytokines, chemokines, oxidative stress and ischemia, and plays a role in apoptotic death signaling by affecting death receptor function and Bcl-2 family members (Cuenda and Rousseau, 2007). The active, phosphorylated form of p38 has been shown to increase in RGCs following optic nerve axotomy (Kikuchi et al., 2000), in a translimbal photocoagulation laser model of rat glaucoma (Levkovitch Verbin et al., 2007) and in human glaucomatous eyes (Tezel et al., 2003). A time-course analysis revealed a sustained increase in phosphorylated p38, which appeared rapidly after intraocular pressure

elevation (Levkovitch Verbin et al., 2007). Intravitreal injection of a p38 inhibitor at the time of axotomy increased the number of surviving RGCs (Kikuchi et al., 2000) supporting a potential functional role for p38 in the regulation of RGC death. However, functional studies to assess the role of p38 in RGC death in experimental glaucoma are currently lacking.

Both JNKs and p38 are direct protein targets of the apoptosis signal regulating kinase 1 (ASK1), a SAPK and mitogen-activated protein kinase kinase kinase (MAPKKK) family member that plays key roles in human neurodegenerative diseases (Hattori et al., 2009). ASK1 has been shown to activate JNK and p38 in response to diverse stress stimuli, particularly inflammatory cytokines and oxidative stress (Ichijo et al., 1997, Saitoh et al., 1998, Hatai et al., 2000, Nishitoh et al., 2002, Zhang et al., 2007, Min et al., 2008). In a recent study, Harada et al. demonstrated that ASK1 is primarily expressed by RGCs, and that a substantial number of these neurons are protected from ischemic injury in ASK1 null mice (Harada et al., 2010). Furthermore, both p38 MAPK and caspase-3 activation were suppressed in mice lacking ASK1, suggesting a role for these downstream signaling components. ASK1 deficiency was also shown to attenuate RGC death in mice lacking the glial glutamate/aspartate transporter (GLAST) (Harada et al., 2010), which display spontaneous RGC death and optic nerve degeneration without elevated intraocular pressure (Harada et al., 2007).

I.5.1.2. The Bcl-2 family

A major class of intracellular regulators of apoptosis is the Bcl-2 family, which comprises evolutionarily conserved proteins from worms to humans (Adams and Cory, 1998). *Bcl-2*, a gene activated by chromosomal translocation in human follicular lymphoma (Bakhshi et al., 1985, Tsujimoto et al., 1985, Cleary et al., 1986), was initially found to promote the

survival of hematopoietic cells in the absence of an essential cytokine (Vaux et al., 1988). A hydrophobic tail on Bcl-2 enables it to bind to the cytoplasmic face of the outer mitochondrial membrane, but Bcl-2 can also be present in the endoplasmic reticulum and nuclear envelope (Green and Reed, 1998, Zamzami et al., 1998). Bcl-2 can inhibit apoptosis by blocking the release of cytochrome C from the mitochondria and consequent activation of proteases involved in the dismantling of the cell (Zou et al., 1997, Green and Reed, 1998, Zamzami et al., 1998). Several Bcl-2 family members have been identified in mammals: some that inhibit apoptosis (e.g. Bcl-2, Bcl-XL) and others that promote programmed cell death (e.g. Bax, Bad and Bid) (Figure 6 and 7). Bcl-2-related proteins contain at least one of four Bcl-2 homology domains (BH1-4), used for protein-protein interactions among family members (Kelekar and Thompson, 1998). A sub-group of Bcl2-related proteins only carry a BH3 domain (e.g. Bim, Noxa and PUMA) and have primarily pro-apoptotic functions (Zong et al., 2001) (Figure 7). The pro- and anti-apoptotic members of the Bcl-2 family can interact to form heterodimers acting as self-regulators of each other‟s function (Oltval et al., 1993). Based on this, it was proposed that the relative concentration of these proteins acts as a rheostat for activation of apoptosis (Korsmeyer et al., 1993, Nickells, 2010).

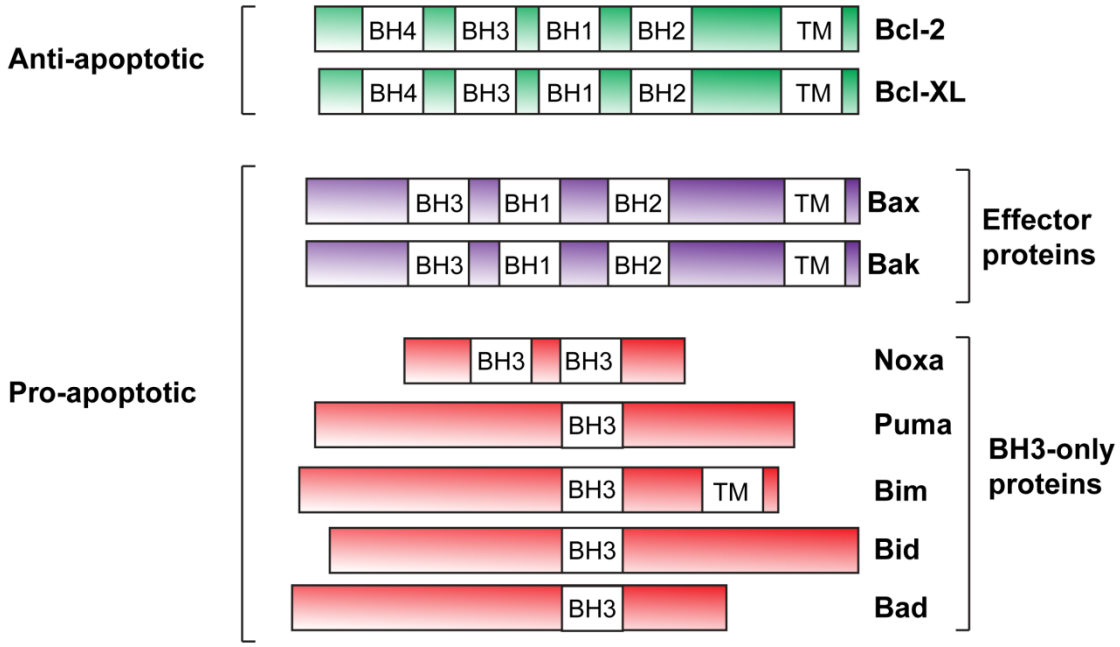

Figure 7. The Bcl-2 family of proteins. The Bcl-2 family of proteins is comprised of anti-apoptotic and pro-apoptotic members. The anti-apoptotic members include Bcl-2 and Bcl-XL, which contain four Bcl-2 homology (BH) domains: BH1, BH2, BH3 and BH4. They also have transmembrane (TM) domains. The pro-apoptotic proteins can be subdivided into two groups: effector and BH3-only members. The effectors proteins Bak and Bax also contain BH1-BH4 domains, while the BH3-only proteins solely have one BH domain, BH3, which is required for interactions with anti-apoptotic and effector proteins. The BH3-only proteins include Noxa, Puma, Bim, Bid, and Bad. Source of image adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Jesenberger and Jentsch, 2002), copyright (2002).

The function of the *Bcl-2* gene family on RGC survival in acute and chronic models of optic nerve lesion has been studied by several groups (Nickells et al., 2008). Among anti-apoptotic Bcl-2 family members, Bcl-XL appears to be the predominant anti-apoptotic protein in the rat retina (Levin et al., 1997). Both Bcl-2 and Bcl-XL retinal mRNA levels have been shown to decrease after optic nerve axotomy (Chaudhary et al., 1999). Consistent with this, AAV-mediated gene transfer of Bcl-XL promoted the survival of axotomized RGCs (Malik et al., 2005). Nevertheless, Bcl-2 also seems to play an important role based on initial reports showing that increased numbers of RGCs are found in transgenic mice overexpressing Bcl-2, both during developmental cell death and after optic nerve axotomy (Bonfanti et al., 1996, Cenni et al., 1996). More recently, gene transfer of BAG1, a Bcl-2-associated protein, protected RGCs in optic nerve cut or crush rat models (Planchamp et al., 2008).

Among pro-apoptotic family members, Bax has been shown to play a pivotal role in the regulation of RGC death. Deletion of the *Bax* gene results in RGC neuroprotection following developmental pruning, in optic nerve cut or crush models and in DBA/2J mice (Mosinger Ogilvie et al., 1998, Li et al., 2000, Libby, 2005). Of interest, deletion of the pro-apoptotic *Bax* gene was shown to be neuroprotective for RGC soma but failed to prevent axon degeneration in DBA/2J mice (Libby, 2005) suggesting a compartmentalized effect of *Bax* in neurodegeneration. Gene dosage experiments in mice carrying a single wild-type *Bax* allele showed that genetic background can influence neuronal death, including RGC loss in experimental glaucoma (Knudson et al., 1995, Deckwerth et al., 1998, Sun and Oppenheim, 2003, Libby, 2005). *Bax* heterozygote DBA/2J mice displayed complete protection of RGCs while Bax heterozygote 129B6 mice exhibited substantial RGC loss (Libby, 2005, Semaan et al., 2010). These distinct phenotypes have been recently attributed to differential *Bax*

transcriptional activity, which leads to higher levels of Bax mRNA in susceptible RGCs from the 129B6 genetic background (Nickells, 2010, Semaan et al., 2010). Bax knockdown by injection of siRNA into the optic nerve did not enhance the survival of axotomized RGCs (Lingor et al., 2005), which may reflect that reduction of Bax levels achieved with this siRNA strategy might not have been below a minimum threshold required to confer resistance against cell death.

BH3-only proteins, usually pro-apoptotic, have also been shown to be expressed by RGCs including Bad (Rickman et al., 1999, Huang et al., 2005c, Yang et al., 2008, Levkovitch-Verbin et al., 2010), Bim (Näpänkangas et al., 2003, McKernan and Cotter, 2007) and Bid (Huang et al., 2005a, Das et al., 2006). A recent study demonstrated that the Bad-interacting protein 14-3-3, a scaffold protein responsible for sequestering Bad in the cytoplasm, is phosphorylated in ocular hypertensive eyes resulting in Bad translocation to the mitochondria and RGC apoptosis (Yang et al., 2008). Although the functional role of BH3-only proteins in RGC death in glaucoma is still obscure, they have been proposed to serve as important molecular checkpoints in the apoptotic process, therefore future work is warranted to assess their contribution to neurodegeneration in this disease.

I.5.1.3. Mitochondrial dysfunction

The mitochondria has been aptly named the battleground of cell fate, as this organelle is the center stage for interactions between anti- and pro-apoptotic Bcl-2 family members (Kroemer, 2007). When the balance is tilted towards apoptosis, there is increased mitochondrial membrane permeability and release of a variety of mediators of cell death (Scaffidi, 1998). Mitochondrial membrane permeabilization is a point-of-no-return in the

apoptotic process, after which cell loss is irreversible, and it is a conserved feature of all cell types undergoing this death modality. In healthy cells, mitochondria typically exhibit a high transmembrane potential and the permeability transition pore complex (PTPC) mediates the exchange of metabolites. Pro-apoptotic signals have been proposed to destabilize the lipid organization in the mitochondrial outer membrane allowing the formation of pores and subsequent release of intermembrane space molecules. For example, in physiological conditions Bax is a cytosolic protein but, upon an apoptotic stimulus, it can insert into the outer mitochondrial membrane (Wolter et al., 1997) where it forms Bax homo- or hetero-oligomeric pores (in association with Bak or truncated Bid) that facilitate the release of death-promoting factors (Kuwana et al., 2002). Once in the cytoplasm, these proteins can activate caspase-dependent and caspase-independent pathways. The first mitochondrial protein shown to be released into the cytosol was cytochrome c, an essential protein component of the respiratory chain (Kluck et al., 1997, Yang et al., 1997). Once in the cytoplasm, cytochrome c binds to the apoptotic protease-activating factor-1 (Apaf-1) to form the apoptosome, a complex that recruits and activates procaspase 9 (Li et al., 1997) (Figure 6). Other well-characterized intermembrane space proteins that are released from mitochondria during apoptosis include the second mitochondria-derived activator of caspases (SMAC), also known as DIABLO (direct inhibitor of apoptosis (IAP)-binding protein with low pI), the apoptosis-inducing factor (AIF), endonuclease G (EndoG) and the high-temperature-requirement protein A2 (OMI/HTRA2) (Saelens et al., 2004). Optic nerve axotomy leads to early cytochrome c release in injured RGCs (Cheung et al., 2003) but, at present, the specific role of toxic mitochondrial proteins in RGC death in glaucoma remains ill defined.

RGCs have a high metabolic activity and energy demand, which is reflected by the numerous mitochondria in RGC soma and the intra-retinal portion of their axons (Hollander et al., 1995, Wang et al., 2003). Mitochondria are also present (albeit at lower densities) in the myelinated portion of RGC axons within the optic nerve (Bristow et al., 2002). It is possible that nuclear and mitochondrial DNA damage increases with age (Brasnjevic et al., 2008), especially considering the proximity of the mitochondrial DNA to the source of reactive oxygen species (ROS) within this organelle (de Souza-Pinto et al., 2008). Moreover, a study using mitochondria isolated from primary open angle glaucoma (POAG) patients showed numerous sequence alterations in the mitochondrial DNA, suggesting a pathogenic potential due to decreased respiratory activity (Abu Amero et al., 2006). The rate of ATP production by mitochondria decreases in the aging brain (Navarro and Boveris, 2007) and, given the age-related nature of glaucoma onset and progression, it is possible that a decline in mitochondrial function is involved. A recent study demonstrated that the ATP content in the mouse optic nerve dropped with age, and that the rate of ATP decline was amplified by increased intraocular pressure in DBA/2J mice leading to RGC axon dysfunction (Baltan et al., 2010). A cellular energy crisis due to reduced ATP availability could negatively impact RGC function because it disables Na^+/K^+ ion pumps, which in turn blocks normal transduction of action potentials along RGC axons (Gordon et al., 1990, Ames et al., 1992, Ames, 2000).

OPA-1, a protein embedded in the inner mitochondrial membrane, is mutated in the majority of patients with autosomal-dominant optic atrophy, a condition characterized by RGC degeneration and childhood blindness. OPA-1 is a GTPase dynamin-like protein that mediates mitochondrial fusion, thus loss of OPA-1 function leads to mitochondrial fragmentation, cytochrome C release, mitochondrial DNA damage and increased reactive oxygen species

(Cipolat et al., 2006). Of interest, overexpression of OPA-1 by AAV-mediated gene transfer increased RGC survival while decreasing the number of reactive astroglia and microglia in DBA/2J mice (Ju et al., 2010).

I.5.1.4. Caspases

Caspases are a family of aspartate-specific cysteine proteases, with homology to the interleukin-1β-Converting Enzymes (ICE), that play central roles in the execution of the apoptotic death program (Thornberry and Lazebnik, 1998). Caspases are expressed as pro-enzymes, which have little catalytic activity, and then undergo proteolytic cleavage and dimerization to become active (Gu et al., 1995). Caspases are categorized by their roles as either initiators of apoptosis (caspase-2, -8, -9, -10), executioners of apoptosis (caspase-3, -6, -7), or mediators of inflammation (caspase-1, -4, -5, -11, -12, -13, -14) (Fan et al., 2005). Initiator caspases (e.g. caspase 9) contain a long N-terminal region (prodomain) with caspase recruitment and death effector domains that are essential for their function, including cleavage and activation of executioner caspases (e.g. caspase 3). The initiator caspases are thought to undergo auto-activation and often require the assembly of multi-component complexes (Adams and Cory, 2002). Effector caspases contain short (20-30 residues) prodomain sequences (Riedl and Shi, 2004) and, once activated, are responsible for the proteolytic cleavage of many protein substrates resulting in cell death. The intrinsic and extrinsic apoptotic pathways involve the activation of distinct initiator caspases (Figure 6). In the intrinsic pathway, cytochrome C is released from the mitochondria and together with Apaf-1 and procaspase 9 forms the apoptosome, which facilitates caspase-9 activation and downstream cleavage of caspase-3 (Li et al., 1997). In the extrinsic pathway, an active death receptor recruits the intracellular adaptor protein Fas-associated death domain which in turn

recruits procaspase-8 to form a death-inducing signaling complex (Kischkel, 1995). Caspase-8 is cleaved and activated through autoproteolysis leading to subsequent activation of caspase-3 (Muzio et al., 1998).

The expression of both initiator and effector caspases has been investigated in RGCs following acute or chronic optic nerve injury. Active, cleaved caspases -3, -8 and -9 have been detected after optic nerve transection or crush (Chaudhary et al., 1999, Kermer et al., 1999, Kermer et al., 2000, Weishaupt et al., 2003, Cheung et al., 2004, Grosskreutz et al., 2005, Homma et al., 2007), ocular hypertension (Hänninen VA, 2002, McKinnon et al., 2002a) as well as ischemic injury (Vidal-Sanz et al., 2000, Harada et al., 2006). To assess the role of apoptosis on primary and secondary waves of RGC degeneration (Yoles and Schwartz, 1998), RGC death was monitored for 6 months after partial lesion of the optic nerve (Levkovitch-Verbin et al., 2003). Although caspase 3 was implicated in both waves of RGC apoptosis, it was active for a longer period of time and with greater intensity during the primary wave of RGC loss (Levkovitch Verbin et al., 2010). The inhibition of caspases to extend RGC survival after optic nerve injury is a strategy that has been tested with varying degrees of success. Intraocular injection of caspase-3 or capase-9 inhibitors promoted some RGC protection from axotomy-induced apoptosis (Kermer et al., 1998, Chaudhary et al., 1999, Kermer et al., 2000). siRNA-based gene knockdown of Apaf-1, a key mediator of caspase-induced cell death, was shown to enhance survival of axotomized RGCs (Lingor et al., 2005). A recent study demonstrated that caspase-2 is expressed and activated primarily in RGCs following optic nerve injury (Ahmed et al., 2011, Vigneswara et al., 2012). Moreover, inhibition of caspase-2 expression using a chemically modified siRNA delivered intravitreally led to robust RGC survival after optic nerve crush or cut (Ahmed et al., 2011), as did pharmacological inhibition

of caspase-2 with z-VDVAD (Vigneswara et al., 2012). Caspase-6 was also recently shown to be upregulated in injured RGCs, and selective inhibition of caspase-6 enhanced RGC survival and axonal regeneration (Monnier et al., 2011). During development, caspases play an important role in dendritic pruning (Kuo et al., 2006, Williams et al., 2006, Schoenmann et al., 2010), in addition to axonal pruning (Nikolaev et al., 2009, Simon et al., 2012). Although caspases are not implicated in Wallerian degeneration, i.e. the process of axonal degeneration after injury (Finn et al., 2000, Simon et al., 2012), the question of whether caspases are involved in dendritic remodelling occurring in the neurodegenerative context of glaucoma (Weber et al., 1998, Weber and Harman, 2005) has not yet been addressed. Although caspases do not appear to be involved in dendritic remodelling following axonal injury in drosophila (Tao and Rolls, 2011), their role in dendritic remodelling in a mammalian system remains unknown.

The inhibitor of apoptosis (IAP) family is composed of proteins with the ability to bind and inhibit caspases through their baculovirus IAP repeat (BIR) domains, thus preventing caspase-dependent cell death (Gyrd-Hansen and Meier, 2011). The cellular inhibitor of apoptosis-1 (cIAP1), a member of the IAP family, is downregulated during retinal development at the time of RGC maturation and is nearly absent in adult RGCs (Kisiswa et al., 2010). IAP-1, another family member, is upregulated in both the primary and secondary phases of RGC death after partial optic nerve lesion (Levkovitch-Verbin et al., 2011). An effective approach has been to target the Baculoviral IAP Repeat-Containing 4 (BIRC4), also known as X-linked inhibitor of apoptosis protein (XIAP), an IAP family member that can directly inhibit several death effectors including caspases 3, 7, and 9 (Deveraux et al., 1997). AAV-mediated gene transfer of BIRC4/XIAP successfully promoted RGC protection in

chronic and acute ocular hypertension models (McKinnon et al., 2002b, Renwick et al., 2005). Of interest, combined administration of adenoviral vectors encoding BIRC4/XIAP or glia-derived neurotrophic factor (GDNF) had a synergistic effect on the survival of axotomized RGCs which was greater than upregulation of each individual pathway (Straten et al., 2002). Therefore, the use of strategies that target multiple anti-apoptotic pathways appears to be a promising avenue to increase RGC survival after optic nerve damage.

I.5.1.5. Calcium-dependent mechanisms

Calcium (Ca^{2+}) is a vital intracellular messenger indispensable for a broad range of cellular functions. However, Ca^{2+} overload can compromise the integrity of organelles such as the mitochondria and the endoplasmic reticulum, and leads to activation of intracellular proteins that trigger neuronal death (Orrenius et al., 2003). Increased Ca^{2+} influx into RGC soma and axons has been proposed to contribute to neurodegeneration in glaucoma (Osborne et al., 2004, Whitmore et al., 2005, Crish and Calkins, 2011). Extracellular Ca^{2+} accumulation in RGC axons occurs through the reversal of the Na^{+}/Ca^{2+} exchanger and has been implicated in neuronal damage following ischemia (Stys et al., 1992). In addition, Ca^{2+} release from intra-axonal stores also plays a detrimental role on RGC axon function (Nikolaeva et al., 2005). Real-time imaging of Ca^{2+} dynamics in RGC-5 cells subjected to elevated hydrostatic pressure revealed a strong temporal correlation between calcium peak occurrence and morphological changes including neurite retraction and cell body shrinkage (Lee et al., 2010). When intracellular Ca^{2+} levels are elevated, either by influx from the extracellular space or by release from intracellular calcium stores, multiple Ca^{2+}-dependent pro-apoptotic enzymes are activated. Calpain is a Ca^{2+}-dependent, non-lysosomal cysteine protease that is expressed ubiquitously in mammalian cells. Calpain becomes active in rat RGCs after axotomy

(McKernan et al., 2007a), hypoxia (Tamada et al., 2005), and ocular hypertension (Huang et al., 2010). Calcineurin, a Ca^{2+}-dependent phosphatase, is cleaved and activated by calpain in experimental glaucoma (Huang et al., 2010). Constitutively active calcineurin has been detected in the retinas of ocular hypertensive rats and was associated with Bad dephosphorylation, cytochrome C release and RGC death (Huang et al., 2005b).

Several strategies aimed at the blockade of Ca^{2+} influx into RGCs or the inhibition of pro-apoptotic Ca^{2+}-dependent proteins in these neurons have been examined with relative success. Pharmacological agents that reduce intraocular pressure and increase vascular nutrition while exerting a dual function as blockers of Ca^{2+} and Na^{+} channels have been shown to confer RGC neuroprotection (Osborne et al., 2005). ß-adrenoceptor antagonists such as betaxolol, levobetaxalol, levobunolol and timolol, compounds that increase impaired blood flow and are known to have Na^{+}- and/or Ca^{2+}-blocking activities, promote RGC neuroprotection and increase retinal function in experimental animals with ischemia/reperfusion damage or high intraocular pressure (Gross et al., 1999b, Osborne et al., 1999a, Hirooka et al., 2000, Schuettauf et al., 2002, Wood et al., 2003, Osborne et al., 2004). Ca^{2+} channel antagonists can, in principle, block Ca^{2+} influx into RGCs and protect them from injury without the need to induce vasodilation. Other Ca^{2+} channel blockers including nifedipine, nimodipine, lomerizine, flunarizine, riluzole have also been shown to attenuate RGC death in animals models of ischemia and ocular hypertension (Araie and Mayama, 2011). The transient receptor potential vanilloid 1 (TRPV1) channel was recently implicated in Ca^{2+} influx and apoptosis of primary cultured RGCs exposed to increased hydrostatic pressure and blockers of TRPV1 resulted in neuroprotection (Sappington et al., 2009). The inhibition of calpain and calcineurin following increased Ca^{2+} influx into RGCs has been

investigated in models of optic nerve injury. For example, calpain inhibitors have been shown to attenuate RGC death after axotomy (McKernan et al., 2007b) and ocular hypertension/ischemia (Oka et al., 2006a, Oka et al., 2006b). Administration of FK506, a calcineurin inhibitor, was shown to block caspase-9 activation and protect RGCs following optic nerve crush (Freeman and Grosskreutz, 2000, Grosskreutz et al., 2005) and in experimental glaucoma (Huang et al., 2005c).

I.5.2. The extrinsic apoptosis pathway

Extrinsic apoptotic signals include an array of death-receptor ligands: TNF-α, FasL, and TNF-related apoptosis-inducing ligands (TRAIL) that bind to their respective receptors, TNF-R, Fas/CD95, and TRAIL-R, to induce cell death. Death receptor activation results in the recruitment of intracellular adaptor Fas-associated death domain (FADD), which typically recruits the initiator procaspase-8 leading to caspase-8 activation followed by executioner caspase-3 activation and cell death. These molecular pathways are being increasingly recognized as important signals in the regulation of RGC death in glaucoma.

I.5.2.1. Tumour Necrosis Factor alpha (TNFα)

TNFα is a pro-inflammatory cytokine that acts on two distinct receptors, TNFR1 and TNFR2. Initially discovered for its ability to cause hemorrhagic necrosis of experimental cancers (Carswell et al., 1975), TNFα has emerged as a critical element in immune homeostasis and mediator of apoptosis. Elevated levels of TNFα have been observed in the brain, cerebrospinal fluid, and serum of patients with Alzheimer‟s disease, Parkinson‟s disease, multiple sclerosis and HIV-dementia, and following traumatic brain injury or chemically-induced neurotoxicity (Sriram and O‟Callaghan, 2007). TNFα is produced as a 22-

kDa membrane-bound precursor (pro-TNFα) that is cleaved by a cell surface trans-membrane protease known as ADAM17 or TACE (for TNFα-converting enzyme) to release the soluble 17-kDa protein (Moss et al., 1997). Interestingly, both the trans-membrane and secreted forms of TNFα are biologically active and play distinct roles *in vivo* (Alexopoulou et al., 2006). The blockade of TNFα has been successfully introduced in the clinic for the treatment of rheumatoid arthritis, psoriasis and inflammatory bowel disease (Feldmann and Maini, 2001), however, side effects such as susceptibility to infection and auto-immune reactions have been reported (Slifman et al., 2003).

Intravitreal injection of TNFα leads to RGC loss and optic nerve degeneration (Nakazawa et al., 2006, Lebrun-Julien et al., 2010), and RGCs lacking TNFR1 are protected from mechanical damage and ocular hypertension (Tezel et al., 2004, Nakazawa et al., 2006). Notably, TNFα and TNFR1 are upregulated in experimental glaucoma (Nakazawa et al., 2006) and human donor eyes with glaucoma (Yan et al., 2000, Yuan and Neufeld, 2000, Tezel et al., 2001). Moreover, TNFα levels are increased in the aqueous humor of glaucoma patients (Sawada et al., 2010, Balaiya et al., 2011), and TNFα gene polymorphisms have been correlated with the most common form of glaucoma, Primary Open Angle Glaucoma (POAG) (Lin et al., 2003, Funayama et al., 2004, Fan et al., 2010, Bozkurt et al., 2011). TNFα gene expression is strongly induced by the transcriptional activity of NF-κB (Shakhov et al., 1990, Hiscott et al., 1993, Mori and Prager, 1996). A recent study demonstrated that excitotoxic damage leads to selective activation of NF-κB in Müller glia resulting in robust production of TNFα by these cells (Lebrun-Julien et al., 2009). Furthermore, inhibition of TNFα activity in the retina using pharmacological or genetic approaches resulted in marked RGC neuroprotection (Lebrun-Julien et al., 2009), thus Müller glia-derived TNFα is a potent

mediator of RGC death. In experimental glaucoma, upregulation of the truncated TrkC or $p75^{NTR}$ receptor activation in Müller glia has been correlated with increased TNFα production and RGC death (Bai et al., 2010a, Bai et al., 2010b).

Ligand binding to TNFR1 typically triggers apoptosis by induction of caspase-8 activation which in turn activates caspase-3 and promotes cell death (Boldin et al., 1996, Hsu et al., 1996). Previous studies have shown that TNFα released from microglia can kill primary cortical neurons via a caspase 8-dependent mechanism (Velier et al., 1999, Kaushal and Schlichter, 2008), however, caspase-8 inhibition had no effect on glia-derived TNFα-mediated apoptosis of RGCs *in vivo* (Lebrun-Julien et al., 2009). Indeed, studies using primary RGC cultures demonstrated that although TNFα leads to the loss of the mitochondrial membrane potential and subsequent release of cytochrome c and AIF, caspase inhibitors did not confer neuroprotection (Tezel and Yang, 2004). These findings suggest that caspase-independent events might contribute to TNFα-mediated RGC death. The study of alternative mechanisms by which TNFα might induce RGC death becomes a priority to understand how this cytokine contributes to neurodegeneration. Along these lines, TNFα has emerged as a crucial regulator of neuronal glutamate receptors in the CNS (Pickering et al., 2005, Beattie et al., 2010). For example, glial TNFα increases the surface expression of neuronal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) hence increasing synaptic efficacy (Beattie et al., 2002, Stellwagen and Malenka, 2006). Recently, TNFα was shown to mediate the insertion of Ca^{2+}-permeable AMPAR during excitotoxic injury (Lebrun-Julien et al., 2009) and in experimental glaucoma (Cueva Vargas et al., 2011) which correlated with devastating loss of RGCs. Consistent with this, Ca^{2+}-permeable AMPAR inhibitors protected

RGCs from excitotoxicity and ocular hypertension damage (Lebrun-Julien et al., 2009, Cueva Vargas et al., 2011). These findings highlight the possibility that TNFα mediates RGC death by modulating membrane insertion of Ca^{2+}-permeable channels and increasing neuronal susceptibility to damage.

I.5.2.2. Fas ligand and Fas/CD95

Fas ligand (FasL), a member of the TNF superfamily of cytokines, was initially identified as a mediator of T-cell apoptosis (Kägi et al., 1994, Ju et al., 1995). FasL is synthesized as a trans-membrane protein which is then cleaved by metalloproteinases to produce soluble FasL (Kayagaki et al., 1995, Tanaka et al., 1995, Mitsiades et al., 2001). Once processed, soluble FasL (sFasL) is not toxic and can compete with membrane-bound FasL (mFasL), while the mFasL form has pro-apoptotic activity (Suda et al., 1997, Schneider et al., 1998, Holler et al., 2003). The FasL receptor Fas/CD95 is a type I trans-membrane receptor which, like TNFR, belongs to the death receptor family. In addition to cell death, FasL-induced Fas/CD95 activation mediates a host of cellular responses including inflammation, cell growth and proliferation (Magnusson and Vaux, 1999). Both FasL and Fas/CD95 have been shown to be expressed in neurons and glial cells including oligodendrocytes, astrocytes, microglia and Schwann cells (Choi and Benveniste, 2004).

An early study demonstrated that the FasL-Fas/CD95 system participates in the maintenance of immune privilege in the eye (Griffith et al., 1995). For example, inflammatory cells that enter the eye in response to a viral infection are eliminated through FasL-mediated Fas/CD95 activation thus abrogating tissue damage. In contrast, mice that lacked functional FasL displayed massive infiltration of inflammatory cells into ocular tissues (Griffith et al.,

1995). The current information on the role of FasL and Fas/CD95 in RGC death after optic nerve injury and in glaucoma is rather limited; however, recent studies suggest a role for this pathway in RGC neurodegeneration. For example, increased expression of FasL in retinal microglia was reported in rats with chronic ocular hypertension (Ju et al., 2006) and in DBA/2J mice (Gregory et al., 2011), and loss of RGCs induced by autoimmunity to heat shock proteins was shown to be mediated through T-cell-derived FasL (Wax et al., 2008). A recent study reported opposing roles of sFasL versus mFasL in RGC death induced by exogenous administration of TNFα or in DBA/2J mice (Gregory et al., 2011). Using mice deficient in FasL or mice in which cleavage of membrane-bound FasL was impaired, this group demonstrated that while microglia-derived sFasL played a neuroprotective role, mFasL was neurotoxic and caused more extensive RGC death. The interesting contrast between sFasL neuroprotection and mFasL neurotoxicity suggests that the ratio of these two FasL forms, regulated at the level of FasL cleavage by matrix metalloproteinases, might play a key role in experimental glaucoma (Gregory et al., 2011). However, the precise molecular mechanisms through which sFasL protects and mFasL kills RGCs in glaucoma remain unknown.

I.6. TOOLS FOR GENE DELIVERY TO INJURED RGCS

Gene therapy for blinding diseases is based on the principle that the genetic material of a retinal cell can be manipulated by the insertion, removal or modification of a gene with the objective of restoring vision. When a specific genetic mutation is linked to disease onset or progression, the strategy is to deliver a healthy copy of the gene to the affected cell to restore functional deficits and promote neuroprotection. This approach has been particularly successful for inherited retinal degenerations of the outer retina, characterized by photoreceptor loss, for which the genetic basis of disease has been well established. For

example, gene therapy for Leber‟s congenital amaurosis, a form of inherited retinal degeneration caused by deficiency of retinal pigment epithelium-specific-65-kDa protein (RPE65), demonstrated promising results in the laboratory and is now being tested in clinical trials (Buch et al., 2008, MacLaren, 2009, Cideciyan, 2010). The elucidation of the genetic defects underlying glaucoma and other age-related optic neuropathies has been substantially more difficult because the prevalent adult-onset of disease limits the number of individuals available for linkage analysis. At least 29 genetic loci for various forms of glaucoma and 12 causative genes have been identified by linkage studies (Fan and Wiggs, 2010). However, further work is required to clarify the relationship between gene defects and RGC pathophysiology in glaucoma before gene replacement therapies for these neurons can be developed. In addition, multiple genes, individual risk factors and environmental factors are likely to contribute to glaucoma onset. For these reasons, most gene therapy strategies for RGC neuroprotection to date are based on enhancing neuronal survival rather than correcting the primary genetic defect or injury source. Since RGC death in human glaucoma typically occurs over several decades, strategies that do not address the initial cause but can delay RGC death and support functional vision throughout life are likely to be beneficial. Given the wide range of tools currently available for retinal gene transfer, we will focus here only on those that have had a significant impact on studies of RGC neuroprotection and discuss the advantages and disadvantages of each system.

I.6.1. Virus vector-based approaches to target RGCs or surrounding cells

Viruses have the evolutionary advantage of using molecular mechanisms to effectively transfer their genomes into infected cells. Based on this, recombinant virus-based vectors have been developed to infect post-mitotic cells in the retina as a means to promote transgene

expression. Improvements in the design of viral vectors and the methods to increase viral titers and purity suitable for *in vivo* delivery have contributed to their popularity. The ideal viral vector is expected to have the following characteristics: i) it can transduce large numbers of the target cell, ii) it mediates gene expression specifically in the infected cells, iii) it allows stable expression of the delivered gene product at physiologically adequate levels, iv) it is safe, entailing no toxic or immunological responses in the target tissue, v) it has no limitation in the size of DNA that it can accommodate, and vi) it is easy to produce in high purity, high titer stocks that can be scaled up for human use. Although at present there is not a single vector system that meets all these criteria, the next sections describe a number of useful viral vectors that are currently used in gene transfer therapies for RGC neuroprotection.

I.6.1.1. Adeno-associated virus (AAV)

Among all currently available viral vector systems, the adeno-associated virus (AAV) vector has emerged as a favored tool for targeting adult RGCs. AAV is a member of the *parvoviridae* family that requires a helper virus for replication, hence its initial identification as a contaminant of adenovirus preparations. The wild-type AAV houses a single-stranded genome of 4.7 kilo base-pairs (kb) containing two genes, *rep* and *cap*, that encode proteins involved in replication and encapsidation respectively. The AAV genome is flanked by two identical 145-bp inverted terminal repeats (ITRs), which are essential for packaging, replication and integration. Recombinant AAV vectors were originally derived from human parvovirus AAV by substituting all viral sequences, with the exception of ITRs, for a transgene of interest. Packaging of functional AAV particles requires the presence of the *rep* and *cap* gene products typically provided in *trans*.

The ability of AAV to infect distinct retinal cell-types depends on the virus serotype, the route of vector administration and the age of the host animal. Early studies in adult rat and mouse retinas identified AAV as an effective tool to transduce a large number of RGCs following intravitreal injection of the vector (Cheng et al., 2002b, Harvey et al., 2002), whereas subretinal AAV administration led to infection of photoreceptors and RPE (Ali et al., 1996, Bennett et al., 1999). A variety of different AAV serotypes have been tested for retinal gene transfer, but the general consensus is that AAV serotype 2 displays the highest transduction of RGCs after intravitreal injection (Hellstrom et al., 2009). Although RGC-specific promoters have not yet been used to drive transgene expression following AAV transduction, constitutive viral promoters such as the cytomegalovirus (CMV) promoter with a chicken β-actin (CBA) enhancer have been shown to be effective. The combination of a CBA-containing promoter with the woodchuck hepatitis posttranscriptional regulatory element allowed transduction of ~85% of rat RGCs within 2 weeks of intravitreal virus injection (Martin et al., 2002). Tetracycline-regulatable promoter systems are currently being investigated as a means to regulate AAV-mediated transgene expression (Stieger et al., 2009).

Other advantages of the AAV vector system for *in vivo* gene delivery include that it is not pathogenic and has not been implicated in the etiology of any known human disease, it mediates long-term transgene expression that can last for several years in the retina (Stieger et al., 2008), and it has low immunogenicity. In the absence of helper virus, wild-type AAV can integrate at a specific site on the q arm of chromosome 19 to establish latent infection. However, the lack of *rep* proteins has been shown to compromise integration specificity leading to random insertion of recombinant AAV. Although viral integration into the genome may contribute to the stability of AAV-mediated transgene expression, a careful evaluation of

the risks associated with insertional mutagenesis is required before implementing AAV-based therapies. A disadvantage of AAV has been the size constraint for packaging genes larger than 4.7-kb. Although methods have been developed to increase the size of delivered transgenes by trans-splicing two independent vectors co-administered to the same tissue (Lai et al., 2005), this remains a limitation of the AAV system. The laborious work needed to produce AAV vectors has often been regarded as a disadvantage. However, recent improvements in the protocols have facilitated the preparation of high-titer and pure AAV stocks following good manufacturing practice (GMP), which is a requirement for use in humans.

I.6.1.2. Adenovirus

Adenovirus (Ad) contains a linear double-stranded DNA genome of approximately 36-kb encapsidated in an icosahedral protein shell. Immediate early genes (E1, E2, E3 and E4) orchestrate viral gene transcription and suppression of the host immune response, while late genes are necessary for viral assembly. Ad vectors were initially generated with deletions of the early region 1 (ΔE1), that contains genes required for virus replication, rendering vectors replication defective and more suitable for gene transfer into mammalian cells. A major disadvantage of these early Ad vectors is the strong cytotoxic and immune response elicited upon infection of the host cells. Recent versions of Ad vectors have been produced in which the entire viral genome, except for the terminal repeat regions required for viral assembly, has been replaced by exogenous gene sequences. These so-called "gutless" vectors, also referred to as Helper-dependent adenovirus (Hd-Ad), exhibit considerably reduced immune response but can only be produced in the presence of a helper virus that provides all the proteins required for viral replication (Kumar-Singh, 2008). These new vectors are less immunogenic and mediate long-term transgene expression following subretinal or intravitreal injection

(Takahashi et al., 2003, Oshima et al., 2004a, Oshima et al., 2004b, Lamartina et al., 2007). For example, intravitreal administration of helper-dependent Ad promoted transgene expression for up to 1 year in transduced Müller glia.

A cardinal feature of Ad vectors in the adult retina is that it efficiently infects non-neuronal cell-types - epithelial cells or glia - whereas it has a poor ability to infect neurons. Intravitreal injection of Ad results in preferential transduction of Müller cells, the predominant glial cell in the retina, and one of the primary targets for Ad infection *in vivo* (Di Polo et al., 1998). This approach has proved to be useful for delivery of genes encoding diffusible factors to promote neuroprotection of injured RGCs (Di Polo et al., 1998, Isenmann et al., 1998). Ad vectors have also been shown to effectively transduce the RPE following subretinal injections (Bennett et al., 1994, Li et al., 1994). Under some experimental conditions, limited transduction of RGCs by Ad has been observed. For example, introduction of Ad to the superior colliculus or to the transected optic nerve stump results in retrograde transport of viral particles and subsequent gene expression in some RGCs (Kugler et al., 2000).

I.6.1.3. Lentivirus

Lentivirus (LV), a genus of retroviruses, consists of two identical single-stranded RNA molecules and enzymes required for replication within a viral protein core. Following virus internalization, the viral RNA is reverse transcribed into double-stranded DNA and transported to the cell nucleus. Viral DNA is then permanently integrated into the host genome to become a provirus. The retrovirus genome contains *gag*, *pol* and *env* genes flanked by long-terminal repeats (LTRs), genes that encode proteins essential for replication, encapsidation, internalization and reverse transcription. Replication-deficient recombinant retroviral vectors

have been generated by substituting all viral genes for a transgene of interest with the exception of the *cis*-acting sequences required for vector propagation. Most retroviral vectors can only transfer genes into cells that are actively proliferating, thus their use in neuroprotective strategies which typically involve gene transfer into fully differentiated cells is rather limited. An exception to the rule is LV, such as the human immunodeficiency virus (HIV), which can efficiently infect non-mitotic cells. This ability relies on nuclear localization signals in the pre-integration complex that allow entry into the nucleus without the need for nuclear membrane fragmentation (Roe et al., 1993). Advantages of the LV system are its relatively large cloning capacity, close to 10 kb, its ability to mediate high levels of transgene expression *in vivo* and the low immunogenicity elicited in the target tissues. The main concern with LV vector systems is the risk of generating replication competent recombinant virus during the production of viral stocks. Because HIV is a human pathogen, considerable work has been done to increase biosafety of LV production systems. Other concerns include low vector titers and the risks associated with insertional mutagenesis as the vector integrates into the host genome. The LV tropism in the retina was first characterized by subretinal injection which demonstrated LV-mediated gene expression in photoreceptors and RPE cells as well as some bipolar and Müller cells (Miyoshi et al., 1997). Although some studies have observed limited transduction of the inner retina when LV vectors are delivered intravitreally (Harvey et al., 2002), others have reported transduction of RGCs without apparent cytotoxicity (Cheng et al., 2002a).

I.6.2. DNA- and RNA-based technologies to modify RGC gene expression

Non-viral gene transfer strategies have the advantage of circumventing the safety concerns that stem from the potential immunogenic response and risk of chromosomal

integration associated with viral vectors. The downside of non-viral approaches is that they yield low DNA transduction rates resulting in limited and often short-lived transgene expression *in vivo*. These gene transfer strategies might be useful when transgene expression is required only during a critical time window to boost RGC survival. Sustained transgene expression using non-viral vectors might be attainable through multiple intraocular injections, topical (corneal) applications or intra-nasal delivery.

I.6.2.1. Naked DNA

DNA plasmids or oligonucleotides are easy to prepare and can be readily injected into the eye, but they are not easily taken up by cells. Antisense oligonucleotides against pro-apoptotic molecules have been delivered to RGCs by intravitreal injection or by retrograde transport via injection in the superior colliculus, but these approaches resulted in only modest protection of axotomized RGCs due to limited transfection efficiency (Isenmann S, 1999, Thaler S, 2006). In general, the ability of DNA molecules to transduce mammalian cells can be enhanced by chemical methods (e.g. liposomes, polymers); or by physical methods such as ballistic delivery (gene gun), micro-injection, electroporation, iontophoresis, laser or ultrasound. Liposomes are artificially prepared vesicular systems generated by the self-assembly of lipid bilayers. Cationic lipids, such as cholesterol, are often used in liposome preparations because they effectively bind DNA to form stable lipoplexes useful for gene delivery. Although liposomes have been shown to facilitate intravitreal or topical delivery of DNA to the inner retina, the usefulness of this approach in RGC neuroprotection remains largely unexplored. Among the physical methods to deliver DNA plasmids to the RGCs, electroporation has received special attention because it is safe while providing effective gene transfer to these neurons *in vivo*. The underlying principle of gene transfer by electroporation

is that electrical pulses increase membrane permeability and induce electrophoretic influx of DNA into the target cells. Electric field strength, pulse duration and stimulation pattern can be controlled to achieve maximum transduction efficiency of RGCs. Transgene expression using electroporation can be detected as early as 2-3 days after gene delivery *in vivo*, which can last up to 3 weeks. This approach has been successfully used to deliver neurotrophic factor and thioredoxin genes to promote RGC survival in different models of optic nerve injury (Mo X, 2002, Ishikawa et al., 2004, Caprioli et al., 2009).

I.6.2.2. Small Interference RNA (siRNA)

RNA interference (RNAi) is a conserved cellular mechanism by which gene expression is tightly regulated in living cells. Two types of small RNA molecules are crucial in the RNA interference process: miRNA and siRNA. The endogenous RNA interference pathway is initiated in the nucleus with the expression of primary miRNAs that are cleaved into precursor miRNAs and exported to the cytoplasm. Once in the cytosol, miRNAs are further processed by Dicer nuclease and incorporated into the RNA-induced silencing complex (RISC). This complex is directed to a target mRNA sequence leading to its cleavage and degradation, ultimately resulting in gene silencing. A similar mechanism mediates the processing of siRNA but, in contrast to miRNA, siRNA perfectly matches the target mRNA sequence hence its robust ability to suppress gene expression. In general, the siRNA technology is based on sequence-specific silencing of gene expression using short double-stranded RNAs of approximately 20-25 nucleotides in length. A limitation of siRNA-based strategies is the potential for non-specific effects that occur when the siRNA activates innate immune receptors, a problem that can overcome by optimizing the sequence design (Samuel-Abraham and Leonard, 2010). siRNA has been successfully delivered to RGCs via injection

into the superior colliculus (Lingor et al., 2005, Koeberle et al., 2009), however, the highly invasive nature of this approach limits its clinical application. Alternatively, siRNA can be injected intravitreally leading to effective delivery to RGCs soon after administration. Although other retinal cells are likely to uptake siRNA delivered to the vitreous chamber, this strategy might be suitable for silencing genes that are specific or highly enriched in RGCs. Intravitreal administration of siRNA against vascular endothelial growth factor-1 in patients with age-related macular degeneration was well-tolerated and led to improvements in visual acuity (Kaiser et al., 2010). Another approach is to use viral vectors, such as AAV, to express functional siRNA from short hairpin RNA (shRNA) or miRNA-based shRNA (Gorbatyuk et al., 2007, Georgiadis et al., 2010) to increase cell-specificity and reduce off-target effects.

I.7. OBJECTIVES OF THE THESIS, HYPOTHESES AND EXPERIMENTAL APPROACHES

Problem: The principal mechanisms leading to RGC damage in glaucoma are not well understood, however it is well established that RGC loss is caused by apoptotic cell death. Elucidating the signaling pathways involved in RGC apoptosis is necessary to develop novel therapeutic approaches to slow or halt the progression of glaucoma and other optic neuropathies affecting RGC viability.

Hypothesis: In this thesis, we explore the role of ASPP family members in the apoptotic death of RGCs. We put forth the hypothesis that the ASPP family is implicated in RGC apoptosis. As ASPP proteins are regulators of p53, we first assessed the role of p53 in axotomy-induced RGC apoptosis. We then selected two different genetic approaches to address the roles of ASPP proteins in RGC survival after optic nerve lesion. Firstly, we

administered siRNA targeting pro-apoptotic ASPP1 and ASPP2 and determined their effect on RGC survival. Secondly, we designed an AAV selectively expressed in RGCs encoding anti-apoptotic iASPP and assessed its neuroprotective properties.

Objectives: 1) To assess the role of p53 in axotomy-induced apoptotic death of RGCs, 2) To evaluate the effect of pro-apoptotic ASPP1/2 knockdown by siRNA on improving RGC survival, 3) To measure the neuroprotective potential of anti-apoptotic iASPP overexpression in RGCs by AAV and 4) To study the molecular pathways of RGC survival mediated by ASPP family members.

Experimental Protocols: 1) Animal model: The optic nerve axotomy model of apoptotic RGC death was carried out in adult Sprague-Dawley rats, 2) Endogenous ASPP expression pattern in the retina was assessed by immunohistochemistry on retinal crossections, 3) Gene expression modulation: siRNA and AAV were delivered by intravitreal injections, 4) Evaluation of RGC survival: RGCs were either retrogradely labeled with Fluorogold or immunolabeled with a cell-specific marker (e.g. Brn3a), and the number of surviving RGCs was counted on flat-mounted retinas, 5) ASPP signaling components were characterized by western blots, qPCR and RT-PCR of retinal lysates.

CHAPTER 2

II. FIRST ARTICLE: "ASPP1/2 REGULATE P53-DEPENDENT DEATH OF RETINAL GANGLION CELLS THROUGH PUMA AND FAS/CD95 ACTIVATION *IN VIVO*"

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ASPP1/2 Regulate p53-Dependent Death of Retinal Ganglion Cells through PUMA and Fas/CD95 Activation *In Vivo*

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Abbreviated title: ASPP1/2 gene silencing protects injured RGCs.

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II.1. ABSTRACT

The transcription factor p53 mediates neuronal death in a variety of stress-related and neurodegenerative conditions. The pro-apoptotic activity of p53 is tightly regulated by the apoptosis-stimulating proteins of p53 (ASPP) family members: ASPP1 and ASPP2. However, whether ASPP1/2 play a role in the regulation of p53-dependent neuronal death in the CNS is currently unknown. To address this, we asked whether ASPP1/2 contribute to the death of retinal ganglion cells (RGC) using *in vivo* models of acute optic nerve damage in mice and rats. Here, we show that p53 is activated in RGCs soon after injury and that axotomy-induced RGC death is attenuated in p53 heterozygote and null mice. We demonstrate that ASPP1/2 proteins are abundantly expressed by injured RGCs, and that siRNA-based ASPP1 or ASPP2 knockdown promotes robust RGC survival. Comparative gene expression analysis revealed that siASPP-mediated downregulation of PUMA, Fas/CD95 and Noxa depends on p53 transcriptional activity. Furthermore, siRNA against PUMA or Fas/CD95 confers neuroprotection, demonstrating a functional role for these p53 targets in RGC death. Our study demonstrates a novel role for ASPP1 and ASPP2 in the death of RGCs, and provides evidence that blockade of the ASPP-p53 pathway is beneficial for central neuron survival after axonal injury.

Keywords: Retinal Ganglion Cell, p53, Apoptosis, Ankyrin Repeat and Proline-Rich Domain-Containing Proteins (ASPP).

II.2. INTRODUCTION

The nuclear transcription factor p53 mediates the apoptosis of post-mitotic neurons exposed to a wide range of insults (Culmsee and Mattson, 2005). Neuronal death induced by p53 has been documented in a variety of neurodegenerative diseases, suggesting a key role for this transcription factor in the regulation of neuronal viability after injury (Chatoo et al., 2011, Chang et al., 2012). Given its critical role in the control of cell death, several mechanisms exist to ensure tight regulation of p53 activity. The level of p53 protein is kept low in most cell types, including neurons (Soussi, 2000), via rapid and continuous degradation following ubiquitination by Mdm2 and MdmX (Wade et al., 2010). Additional control of p53 function is exerted via post-translational modifications such as phosphorylation, acetylation and/or methylation; and by interactions with protein partners (Boehme and Blattner, 2009).

The identification of the apoptosis-stimulating proteins of p53 (ASPP), a family of ankyrin repeat and proline-rich domain-containing proteins, has revealed a new form of p53 regulation. The ASPP family is composed of three members: ASPP1, ASPP2 and iASPP. ASPP2 was identified as a p53-binding protein in a yeast two-hybrid screen (Iwabuchi et al., 1994), and ASPP1 was found in a homology search (Nagase et al., 1998). ASPP1 and ASPP2 enhance p53-dependent death of tumour cells by selectively increasing the ability of p53 to activate pro-apoptotic gene transcription (Lopez et al., 2000, Ao et al., 2001, Samuels Lev et al., 2001, Bergamaschi et al., 2006), whereas iASPP inhibits p53-dependent cell death (Yang, 1999, Bergamaschi et al., 2003). ASPP1 and ASPP2 play a role in tumour suppression by increasing the ability of p53 to induce apoptosis, but not cell cycle arrest, in tumour-derived cell lines (Samuels Lev et al., 2001, Slee et al., 2004). ASPP2 null mice die perinatally and although ASPP2 heterozygous mice survive to adulthood, they display a much higher

propensity for developing tumours compared to wild-type counterparts (Vives et al., 2006). This may have physiological relevance since ASPP1/2 protein levels are reduced in many forms of cancer, a deficit associated with poor patient‟s prognosis (Lossos et al., 2002 , Liu et al., 2004, Agirre et al., 2005, Liu et al., 2005).

ASPP1/2 function has been examined solely in relation to tumour biology, but the role of these proteins in neuronal apoptosis has not been established. To address this, we asked whether ASPP1 or ASPP2 regulate death of adult retinal ganglion cells (RGCs) after axonal injury. RGCs are CNS neurons that undergo a predictable onset and time-course of apoptotic death following optic nerve axotomy. Here, we demonstrate that ASPP1 and ASPP2 proteins are abundantly expressed by intact and injured RGCs, and that depletion of ASPP1 and ASPP2 using short interfering RNAs (siRNAs) promotes RGC survival *in vivo*. Moreover, our data support a critical role for the p53-upregulated-modulator-of-apoptosis (PUMA) and Fas/CD95 in siASPP2-mediated survival of injured RGCs. This study identifies a novel role for ASPP1 and ASPP2 as important regulators of neuronal death in the injured CNS.

II.3. MATERIALS AND METHODS

II.3.1. Experimental animals

Animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care for the use of experimental animals (www.ccac.ca). All surgeries were carried out in adult, female Sprague-Dawley rats (180-200 g) or in p53 knockout mice under general anesthesia (2% Isoflurane, 0.8 liter/min). p53 heterozygote mice (B6; 129S2-

Trp53^{tm1Tyj}/J, Jackson Laboratory, Bar Harbor, ME), were maintained in our animal facility and bred to produce p53 null and wild-type littermate control mice.

II.3.2. Axotomy-induced RGC death assay

Selective RGC death was induced by complete transection (axotomy) of the optic nerve leading to rapid onset and predictable apoptotic loss of these neurons (Berkelaar et al., 1994, Cheng et al., 2002). Prior to axotomy, RGCs were backlabeled by application of Fluorogold (2%, Fluorochrome, Englewood, CO) to the superior colliculus, the primary target of these neurons in the rodent brain. To ensure that all RGCs were fully labeled prior to axonal injury, axotomy was performed 7 days after Fluorogold application. The left optic nerve was transected at 0.5-1 mm from the optic nerve head avoiding damage to the ophthalmic artery. Fundus examination was performed to check the integrity of the retinal circulation after surgery. The right eye was never operated on and served as intact control. At 1 or 2 weeks post-lesion, rats were anesthetised by intraperitoneal injection of 1mL/kg of anaesthetic cocktail (100 mg/mL ketamine, 20 mg/mL xylazine, 10 mg/mL acepromazine) and perfused with 4% paraformaldehyde (PFA); the retinas were removed and flat-mounted vitreal side up on a glass slide for examination of the ganglion cell layer. Fluorogold-labeled neurons were counted within 3 square areas at distances of 1, 2 and 3 mm from the rat optic disc in each of the 4 retinal quadrants for a total of 12 retinal areas. In mice retinas, quantification was performed as in rats but sampled areas were localized at 0.25 mm, 0.625 mm and 1 mm from the optic disc for a total of 12 retinal areas. Microglia and macrophages, that may have incorporated Fluorogold after phagocytosis of dying RGCs, were excluded from our analysis of neuronal survival based on their morphology (Raibon et al., 2002). Fluorescent staining was examined with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC), and

pictures were captured with a CCD video camera (Retiga, Qimaging, Burnaby, BC) and analyzed with Northern Eclipse software (Empix Imaging, Mississauga, ON).

II.3.3. Reverse transcription and quantitative real time PCR (qPCR)

Total RNA was isolated from individual retinas using the RNEasy Mini kit (Qiagen Inc., Valencia, CA) or TRIzol reagent (Invitrogen, Burlington, ON). cDNAs were generated from 0.5 µg-3 µg of total RNA using the MML-V reverse transcriptase (Invitrogen). PCR for p53 was performed using the following primers: p53 forward: 5′-GATGGTGACGGCCTGGCTCCT-3′, p53 reverse: 5′-CTCGAAGCGCTCACGCCCAC -3′, ß-actin forward: 5'-CACCACTTTCTACAATGAGC-3′, ß-actin reverse: 5'-CGGTC AGGATCTTCATGAGG-3′, and the following cycle conditions: 94°C for 1 min, 58°C for 1 min, 72°C for 1 min. Reaction products were separated on agarose gels, visualized under UV light and digitalized using Gel Doc™ EZ System (Biorad, Hercules, CA). Densitometric analysis was performed with ImageJ software (NIH, USA). For p53 target genes, quantitative real-time PCR (qPCR) was performed using the Platinum SYBRGreen SuperMix (Invitrogen) and a real-time PCR apparatus (ABI Prism 7000). Rat primer sets were as follows: PUMA forward: 5‟-CGGAGACAAGAAGAGCAACA-3‟, PUMA reverse: 5‟-TAGTTGGGCTCCAT TTCTGG-3‟, Fas/CD95 forward: 5‟-CCGACAACAACTGCTCAGAA-3‟, Fas/CD95 reverse: 5‟-GGTGCAGTTCGTTTCCACTT-3‟, Bax forward: 5‟-TGCAGAGGATGATTGCTGAC-3‟, Bax reverse: 5‟-GATCAGCTCGGGCACTTTAG-3‟, Noxa forward: 5‟-GGAGTGCACCGG ACATAACT-3‟, Noxa reverse: 5‟-CTCCAATTCTCCGGAGTTGA-3‟, GAPDH forward: 5‟-ATGGGAAGCTGGTCATCAAC-3‟, GAPDH reverse: 5‟-GTGGTTCACACCCATCACAA-3‟. The ASPP2 primers (QT01599402) were purchased from Qiagen Inc. Mouse primer sets were as follows: PUMA forward: 5‟-CAAGAAGAGCAGCATCGA CA-3, PUMA reverse:

5‟-TAGTTGGGCTCCATTTCTGG-3‟, Fas/CD95 forward: 5‟-AAACAAACTGCACCCTGA CC-3‟, Fas/CD95 reverse: 5‟-CAACCATAGGCGATTTCTGG-3‟, Bax forward: 5‟-CACGTGACCGTGGTGCGCCG-3‟: Bax reverse, 5‟-CCGCTCCCAAGCTGCTCCCCG-3‟, Noxa forward: 5‟-CACCGGACATAACTGTGGTT-3‟, Noxa reverse: 5‟-TTGAGCACACTC GTCCTTCA-3‟, HRPT forward: 5‟-ACTGTAATGATCAGTCAACGGG-3‟, and HRPT reverse: 5‟-GGCCTGTATCCAACACTTCG-3‟. ß-actin, HRPT and GAPDH were used as internal standards for data calibration, and the $2^{-\Delta\Delta}$Ct formula was used for the calculation of differential gene expression as described (Chatoo et al., 2009).

II.3.4. Short interfering RNA (siRNA)

The siRNA molecules against ASPP1, ASPP2 and Cy3-labeled siRNA were designed at Quark Pharmaceuticals Inc. The control siRNA against GFP has been described elsewhere (Hamar et al., 2004). All these siRNAs were stabilized by alternating 2‟O-methylation (Czauderna et al., 2003) and were synthesized by BioSpring GmbH (Frankfurt, Germany). The following siRNA sequences (sense strands) for ASPP1 and ASPP2 were tested with similar results, ASPP1: 5‟-GGAGAGAAGCACACTGAAA-3‟, 5‟-CAGCGTTTACATTTCCTAA-3‟, and 5‟-CCGTGTTCTTGAGCAACAA-3‟; ASPP2: 5‟-AGGGAGTGTTTGAATAAGC-3‟, and 5‟-CACCCAGAGAACATTTATT-3‟. The siRNA sequences (sense strands) against PUMA: 5‟-GAGCGGCGGAGACAAGAAGAGUU-3‟; Fas/CD95: 5‟-GUGCAAGUGCAAA CCAGACUU-3‟; and Noxa: 5‟-CAAGGAAAGCUGACGGAGA-3‟, 5‟-GAACAGAAGUGG CUACGAA-3‟, 5‟-CCAUGGAUUUCCUCGGCAA-3‟, 5‟-AAGCAAUGGUCGUCGAGCA-3‟; were purchased from Dharmacon (Thermo Scientific, Lafayette, CO). The fluorescent siRNA used for the visualization of intraocular distribution, sequence 5‟-GUGCCAACCUGA

UGCAGCU-3‟ (sense strand), contained a Cy3 fluorophore at the 3‟ end of the antisense strand.

II.3.5. Intravitreal injections

siRNA against ASPP1, ASPP2, PUMA, Fas/CD95, Noxa, siCy3 or control siGFP (2 µg/µl, total volume: 5 µl) were injected into the vitreous chamber of the left eye using a Hamilton syringe fitted with a 32-gauge glass microneedle. Phosphate buffer saline (PBS) was used as vehicle control. The sclera was exposed and the tip of the needle was inserted at a 45° angle through the sclera and retina into the vitreous space using a posterior approach. This route of administration avoided injury to the iris or lens, which can promote RGC survival (Mansour-Robaey et al., 1994, Leon et al., 2000).

II.3.6. Retinal immunohistochemistry

Animals were perfused transcardially with 4% PFA and retinal cryosections (16 µm) were prepared as previously described (Pernet et al., 2005, Lebrun-Julien et al., 2009). Primary antibodies were added to the retinal sections in blocking solution and incubated overnight at 4°C: phospho-p53 (Ser15) (20 µg/ml, Abcam, Cambridge, MA), ASPP1 (1 µg/ml, Bethyl Laboratories, Montgomery, TX), ASPP2 (0.5 µg/ml, Bethyl Labs) or iASPP (0.5 µg/ml, Bethyl Laboratories). For phospho-p53 (Ser15), retinas were subjected to heat-mediated antigen retrieval by incubating sections in 0.01 M sodium citrate in 0.5% Tween-20 (pH 6) at 85-90^{o}C for 30 min. Blocking peptides (2.5 µg/ml, Bethyl Laboratories) were incubated overnight with ASPP1 or ASPP2 primary antibodies (5:1 ratio) prior to application onto retinal sections. Sections were washed and incubated with secondary antibodies: anti-rabbit IgG (1-8 µg/ml, Cy3, Alexa 594 or Alexa 488, Jackson ImmunoResearch Laboratories

Inc., West Grove, PA). Fluorescent labeling was observed with a microscope Zeiss AxioSkop 2 Plus (Carl Zeiss Canada).

II.3.7. Western blot analysis

Whole fresh retinas were rapidly dissected and homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (20 mM Tris pH 8.0, 135 mM NaCl, 1% NP-40, 0.1% SDS, and 10% glycerol supplemented with protease inhibitors). Protein homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatants were removed and resedimented to yield solubilized extracts. Retinal extracts were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Life Science, Mississauga, ON). Blots were incubated overnight at 4°C with each of the following primary antibodies: phospho-p53 (Ser15) (2 µg/ml, Abcam), ASPP1 (1 µg/ml, Bethyl Laboratories), ASPP2 (0.5 µg/ml, Bethyl Laboratories), iASPP (0.5 µg/ml, Bethyl Laboratories), Bax (1.5 µg/ml, N20, Santa Cruz Biotechnologies, Santa Cruz, CA), PUMA (1 µg/ml, Abcam), Noxa (0.5 µg/ml, Sigma-Aldrich, Oakville, ON), Fas/CD95 (1 µg/ml, BD Transduction Laboratories, San Jose, CA) or β-actin (0.5 µg/ml, Sigma-Aldrich). Membranes were incubated in anti-rabbit or anti-mouse peroxidase-linked secondary antibodies (0.5 µg/ml, Amersham Biosciences, Baie d‟Urfé, QC). Blots were developed with a chemiluminescence reagent (ECL, Amersham Biosciences) and exposed to X-OMAT imaging film (Eastman Kodak, Rochester, NY). Densitometric analysis was performed using Scion Image software (Scion Corporation, Frederick, MD) on scanned autoradiographic films obtained from a series of 3 independent western blots each carried out using retinal samples from distinct experimental groups.

II.3.8. Statistical analyses

Data analysis and statistics were performed using the GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test, or by a Student‟s *t* test.

II.4. RESULTS

II.4.1. Axotomized RGCs die in a p53-dependent manner

p53 protein is abundantly expressed in the developing retina, but it is substantially downregulated during maturation (O'Connor, 2008). To establish whether p53 gene expression or activity increase following optic nerve injury, we carried out biochemical and immunohistochemical analysis of retinal samples after axotomy. While p53 mRNA levels did not change at one (not shown) or three days (Fig. 1A) after injury, a significant increase in injury-induced phosphorylation of p53 at serine 15 (S15), a key phosphorylation target during p53 activation (Dumaz and Meek, 1999, Unger et al., 1999) was readily detected at one day post-lesion (Fig. 1B). Immunostaining of Fluorogold-labeled retinas demonstrated selective phospho-p53 upregulation in RGCs at one day after axotomy (Fig. 1C), a time point that precedes the onset of RGC death in this model (Villegas-Perez et al., 1993, Berkelaar et al., 1994). Phosphorylation of p53 at S15 has been shown to be sufficient to induce apoptosis of human glioma cells and leads to selective increase of the pro-apoptotic p53 targets Fas/CD95 and PUMA (Amano et al., 2009). Consistent with axotomy-induced phosphorylation of retinal p53 at S15, we observed increased protein expression of PUMA and Fas/CD95 at one day after axotomy, whereas levels of Bax and Noxa did not change (Fig. 1D). Of interest,

phospho-p53 (S15), PUMA and Fas/CD95 proteins dropped to basal levels at seven days post-injury (Figs. 1B, D) indicating that axotomy leads to early and transient activation of this pathway.

To address the functional role of increased p53 activity in axotomy-induced RGC death, we analyzed the density of RGCs in p53 heterozygote ($p53^{+/-}$) and null ($p53^{-/-}$) mice subjected to optic nerve injury. RGCs were first labeled by application of the retrograde tracer Fluorogold to the superior colliculus, followed by axotomy and quantification of neuronal survival. Figure 1E shows that 73% of RGCs survived in $p53^{-/-}$ retinas and 65% in $p53^{+/-}$ retinas, while only 50% remained in wild-type retinas at one week post-lesion. The total density of RGCs in non-injured (intact) $p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$ adult retinas was similar indicating that p53 is not required for developmental programmed RGC death, consistent with previous findings (Li et al., 2002). These data demonstrate that optic nerve axotomy leads to activation of p53 in RGCs and that p53 plays a role in axotomy-induced RGC death in a dose dependent manner.

II.4.2. ASPP1 and ASPP2 are expressed by adult RGCs

To characterize the role of ASPP1 and ASPP2 in RGC death, we first determined which retinal cells express them. Retinal immunohistochemistry showed abundant expression of endogenous ASPP1 and ASPP2 in the ganglion cell layer (GCL) (Fig. 2A, K) while other retinal layers were virtually devoid of ASPP1/2. Since displaced amacrine cells account for ~40% of the total number of neurons in the GCL (Perry, 1981), we performed co-localization studies where RGCs were retrogradely labeled with Fluorogold (Fig. 2B, L). All Fluorogold-labeled neurons displayed robust ASPP1 and ASPP2 immunoreactivity (Figs. 2C-F and 2M-P)

indicating that adult RGCs are endowed with high levels of ASPP1/2 proteins. Co-labeling with the nuclear marker DAPI demonstrated that ASPP1 had a nuclear and cytoplasmic (perinuclear) localization within RGCs (Fig. 2H-J), while ASPP2 was primarily found in RGC nuclei (Fig. 2R-T). ASPP1 and ASPP2 blocking peptides resulted in absence of staining (Fig. 2 G, Q) confirming the specificity of the ASPP1 and ASPP2 antibodies.

Following axotomy, there were no detectable changes in the levels or sub-cellular localization of ASPP1, ASPP2 or the anti-apoptotic member iASPP visualized by retinal immunohistochemistry (Fig. 3A). Analysis of protein homogenates at 24 hrs or 48 hrs (Figs. 3 and 4) after axotomy confirmed that ASPP1, ASPP2 and iASPP levels were similar to those in intact, non-injured retinas (Fig. 3B, C). A time-course analysis of ASPP proteins up to five days post-axotomy revealed no change in their levels with respect to control retinas (not shown). Collectively, these data indicate that ASPP1 and ASPP2 are abundantly expressed by intact and axotomized RGCs.

II.4.3. Selective knockdown of retinal ASPP1 or ASPP2 by intravitreal siRNA delivery

To investigate the role of ASPP1/2 proteins in retinal neuron death, we synthesized siRNA sequences against ASPP1 or ASPP2. We first examined whether intraocular siRNA delivery led to effective uptake by adult RGCs using a Cy3-tagged control siRNA. Figure 4 shows that a single intravitreal injection of Cy3-siRNA resulted in robust Cy3 labeling in RGCs as early as 5 hrs after administration (Fig. 4A-F). The co-localization of Cy3 and Fluorogold confirmed that siRNA was rapidly taken up by RGCs after intravitreal delivery (Fig. 4G-I).

To assess the ability of siRNAs to knockdown retinal ASPP1 or ASPP2 protein expression *in vivo*, we carried out western blot analysis of retinal homogenates from eyes that received a single injection of each siRNA at the time of axotomy. Administration of siRNA against ASPP1 (siASPP1) led to a significant reduction of retinal ASPP1 protein at 24 hrs after delivery, while a control siRNA against GFP (siGFP) had no effect (Fig. 4J, K). Importantly, siASPP1 did not reduce the protein levels of the other family members, ASPP2 or iASPP, confirming the specificity of the siRNA. Similarly, siRNA against ASPP2 (siASPP2) selectively depleted retinal ASPP2 protein levels without reducing ASPP1 or iASPP levels (Fig. 4L, M). Quantification of ASPP1 or ASPP2 protein levels at 24 hrs after treatment with siASPP2 or siASPP1, respectively, did not show a compensatory increase in these proteins (Fig. 4K, M, hatched bars). Endogenous levels of ASPP1 and ASPP2 proteins returned to basal at 48 hrs after siRNA delivery (Fig. 4J-M, black bars). Immunohistochemistry of axotomized retinas treated with siASPP1 or siASPP2 confirmed that siRNA-mediated knockdown of ASPP1/2 occurred in RGCs, visualized with Fluorogold (Fig. 4N, O). These data demonstrate that intravitreal delivery of siRNA results in rapid and effective uptake by RGCs, and that siRNAs against ASPP1 and ASPP2 promote transient depletion of ASPP proteins in these neurons.

II.4.4. ASPP1 and ASPP2 knockdown protects RGCs from axotomy-induced death

To determine if ASPP1 and ASPP2 were required for axotomy-induced RGC death, we asked whether targeted siRNA-mediated knockdown promoted RGC survival. For this purpose, RGCs were retrogradely labeled prior to optic nerve transection and intravitreal injections of siRNA against ASPP1 or ASPP2 were performed concomitant with axotomy. Flat-mounted retinas from eyes treated with siASPP1 or siASPP2 consistently displayed

higher densities of Fluorogold-labeled RGCs than those treated with control siGFP (Figs. 5A-D). Quantitative analysis demonstrated that ASPP1 or ASPP2 knockdown resulted in substantial RGC survival relative to vehicle-treated (PBS) or control siGFP-treated eyes (Fig. 5E), with siASPP2 promoting slightly more neuroprotection (79%: 1636 ± 62 RGCs/mm^2) than siASPP1 (69%: 1430 ± 34 RGCs/mm^2) at one week post-injury. All the siRNA sequences against ASPP1 or ASPP2 yielded similar results.

We also examined the effect of siASPP1 and siASPP2 on RGC survival at two weeks after axotomy, a time-point when few RGCs remain alive in the absence of treatment (Lebrun-Julien et al., 2009). Figure 5E shows that only 6% of RGCs survived in animals treated with vehicle or control siGFP (130 ± 7 RGCs/mm^2) whereas 26% RGC survival was observed in animals that received siASPP1 or siASPP2 (542 ± 30 RGCs/mm^2, 509 ± 24 RGCs/mm^2, respectively). The combination of siASPP1 and siASPP2 did not increase RGC survival further (Fig. 5E, black bars) suggesting that ASPP1 and ASPP2 activate redundant pathways to promote RGC death. We conclude that ASPP1 and ASPP2 are required for p53-dependent axotomy-induced death of RGCs in the adult retina.

II.4.5. siASPP2 protects axotomized RGCs through downregulation of the p53 pro-apoptotic targets PUMA and Fas/CD95

To investigate the mechanisms by which ASPP protein knockdown might lead to RGC neuroprotection after optic nerve injury, we examined changes in p53 pro-apoptotic targets at the mRNA and protein levels (Figs. 6, 7). We focused on siASPP2 because it confers enhanced neuroprotection over siASPP1. Real-time qPCR analysis of rat retinal samples at 6 hrs after axotomy and siASPP2 administration, a time-point when siRNA is already present in

RGCs (Fig. 4A-I), revealed downregulation of ASPP2 and the p53 apoptotic targets PUMA, Fas/CD95 and Noxa compared to uninjured or control siRNA-treated retinas (Fig. 6A). There was no change in Bax expression levels. To further substantiate our findings, we performed qPCR in retinal samples from p53 null mice and wild-type littermate controls collected at 6 hrs after axotomy with or without siASPP2 treatment. Overall, the transcript levels of PUMA, Fas/CD95 and Noxa were significantly reduced in non-injured or axotomized p53 null mice with respect to wild-type littermates, suggesting that these genes are transcriptionally regulated by p53 (Figs. 6B, C, E). More importantly, ASPP2 knockdown effectively reduced PUMA, Fas/CD95 and Noxa gene expression in axotomized retinas from p53 wild-type mice but not from p53 knockout mice (Figs. 6B, C, E), while Bax remained unchanged (Fig. 6D). siASPP2 did not reduce PUMA and CD95 expression further relative to the already reduced levels found in p53 null mice. Collectively, these data demonstrate that siASPP2-mediated knockdown of PUMA, Fas/CD95 and Noxa depends on p53 transcriptional activity.

Western blot analysis of axotomized retinal samples at 24 hrs after siRNA administration showed that siASPP2 reduced PUMA and Fas/CD95 protein levels relative to control siGFP (Fig. 7A, B), while Bax and Noxa levels did not change with any of the treatments (Figs. 7C, D). These findings, supported by our observation that PUMA and Fas/CD95 are upregulated following axotomy (Fig. 1D) raised the possibility that they might play a role in the p53-dependent death of axotomized RGCs. To test this, we administered siRNAs against PUMA or Fas/CD95 at the time of axotomy and quantified retrogradely labeled RGCs at one week post-injury. siPUMA or siFas/CD95 effectively downregulated endogenous retinal PUMA or Fas/CD95, respectively (Fig. 7E-G). Importantly, a substantial increase in RGC survival was observed in retinas exposed to siPUMA (77%: 1597 ± 38

RGCs/mm^2, n=4) or siFas/CD95 (69%: 1432 ± 71 RGCs/mm^2, n=4) relative to siGFP-treated controls (Fig. 7H). Unlike PUMA or Fas/CD95, siASPP2-mediated decrease in Noxa transcript levels at 6 hrs after axotomy (Fig. 6) did not correlate with Noxa protein reduction at 24 hrs post-injury (Fig. 7D). Since this discrepancy might simply reflect a difference in the kinetics of Noxa mRNA and protein synthesis (Aikawa et al., 2010, Armstrong et al., 2010), we also used a siRNA against Noxa to assess its role on RGC survival at one week after axotomy. siNoxa effectively reduced endogenous Noxa protein levels (Fig. 7G), but did not result in significant RGC protection (Fig. 7H) suggesting that although Noxa is transcriptionally regulated by p53, it does not play a prominent role in RGC death. We conclude that ASPP2 knockdown protects RGCs via downregulation of the p53 targets PUMA and Fas/CD95, and that these molecules mediate axotomy-induced RGC apoptosis.

II. 5. DISCUSSION

The present study explored the functional role of the p53 co-activators ASPP1 and ASPP2 in the regulation of injury-induced death of adult retinal neurons *in vivo*. Our data support four major conclusions. First, axotomy-induced RGC death is mediated *inter alia* through p53 activation in a gene dose-dependent manner. Second, ASPP1 and ASPP2 proteins are abundantly expressed by intact and injured RGCs. Third, selective siRNA-mediated knockdown of ASPP1 or ASPP2 leads to substantial RGC survival after axonal injury. Fourth, the neuroprotective effect of siASPP2 involves downregulation of the p53 apoptotic targets PUMA and Fas/CD95, and siRNAs against PUMA or Fas/CD95 effectively promote RGC

survival. Our data support a novel, key role of pro-apoptotic ASPP family members in the regulation of retinal neuron death.

Axonal injury is a major cause of neuronal loss in the CNS of adult mammals and it is the primary damaging event in most optic neuropathies, including glaucoma. Using an axotomy model, we found that p53 phosphorylation and upregulation of the p53 pro-apoptotic targets PUMA and Fas/CD95 are markedly increased at one day after injury, a time that precedes the onset of RGC death. In adult rodents, RGCs survive for five days after axotomy and then die abruptly (Villegas-Perez et al., 1993, Berkelaar et al., 1994), therefore this finding suggests that early p53 activation plays a causal role in injury-induced RGC loss. In support of this, our data further demonstrate that p53 deficiency promotes RGC survival after axotomy in a gene dose dependent manner and this is consistent with studies showing that inactivation of the p53 gene attenuates RGC death following ischemia, excitotoxicity or optic nerve crush (Rosenbaum et al., 1998, Li et al., 2002, Park et al., 2008).

ASPP1 and ASPP2 are co-factors that enhance the pro-apoptotic function of p53, but not its ability to regulate cell cycle arrest (Samuels-Lev, 2001). ASPP proteins are known to interact directly with the p53 DNA-binding domain through their ankyrin and SH3 domains, readily increasing its transcriptional activity at pro-apoptotic gene promoters (Gorina and Pavletich, 1996, Patel et al., 2008). We demonstrate that RGCs express high endogenous levels of ASPP1 and ASPP2 that do not change after axotomy, supporting the hypothesis that these neurons become vulnerable to injury-induced activation of p53. It is unlikely that the function of ASPP1 and ASPP2 changes after injury, but rather that they are poised to act as co-factors to facilitate rapid p53-mediated transcriptional activation of PUMA and Fas/CD95

following axotomy. This is consistent with previous studies showing that other p53 co-factors, such as Brn-3b and SP1, can effectively modulate p53 activity without undergoing stress-induced changes in function or expression levels (Budhram-Mahadeo et al., 2006, Dhar et al., 2006, Chatoo et al., 2011).

To address the functional role of ASPP1/2, we used a siRNA-based approach to knockdown ASPP1/2 gene expression *in vivo*. siRNA has been successfully delivered to RGCs via injection into the optic nerve or the superior colliculus (Lingor et al., 2005, Koeberle et al., 2009), but the invasive nature of these approaches greatly limits their application. We chose to deliver siRNA molecules by intravitreal injections which led to effective siRNA uptake by RGCs, as previously demonstrated by us (Ahmed et al., 2011). We cannot rule out that other retinal cells also incorporated siRNA injected into the vitreous chamber; however, we show that this strategy is suitable for silencing genes that are highly enriched in RGCs. Our results show that targeted siRNA successfully downregulated retinal ASPP1 and ASPP2 by ~85-90% at 24 hrs after intravitreal delivery, but this effect was transient since protein levels were restored by 48 hrs. In spite of this short-lived effect, our data demonstrate a strong neuroprotective effect of siASPP1/2 suggesting that there is a window of opportunity soon after axotomy in which ASPP1/2 knockdown counters the pro-apoptotic effect of p53. This is supported by our observation that the levels of phospho-p53, PUMA and Fas/CD95, which are upregulated at 24 hrs after axotomy, drop to basal levels at 7 days post-injury. Therefore, siASPP1/2 treatment to knockdown pro-apoptotic p53 targets during this critical period effectively attenuates RGC loss.

To identify the mechanism by which ASPP1/2 silencing delayed RGC death, we examined mRNA and protein levels of p53 pro-apoptotic targets. Comparative gene expression analysis revealed a substantial downregulation of PUMA, Fas/CD95 and Noxa in axotomized rat retinas derived from eyes treated with siASPP2 but not with control siRNA. Importantly, ASPP2 knockdown effectively reduced PUMA, Fas/CD95 and Noxa expression in axotomized retinas from p53 wild-type but not from p53 null mice, demonstrating that downregulation of these apoptotic genes depends on p53 transcriptional activity. At the protein level, siASPP2 substantially reduced retinal PUMA and Fas/CD95 proteins, while Noxa remained unchanged. Consistent with this, our data show that while targeted siRNA against Noxa failed to promote significant neuroprotection, siRNAs against PUMA or Fas/CD95 knockdown led to robust RGC survival indicating that these molecules play a functional role in axotomy-induced RGC death. PUMA is a BH3-only protein and a potent inducer of cell death, but so far there have been no reports of its role on RGC death. The pro-apoptotic activity of PUMA requires its interaction with anti-apoptotic Bcl-2 family members, localization to the mitochondria, and induction of cytochrome c release leading to caspase activation and cell death (Nakano and Vousden, 2001). Biochemical studies suggest that PUMA regulates mitochondrial outer membrane permeabilization by binding to anti-apoptotic Bcl-2 proteins, which results in derepression and activation of Bax or Bak (Chipuk and Green, 2009). Although we did not detect changes in Bax mRNA or protein levels in the presence of siASPP2, we cannot entirely rule out a decrease in Bax activity following PUMA downregulation. PUMA can release cytosolic p53 from its inactive complex with Bcl-XL to form a PUMA-Bcl-XL complex, allowing p53 to activate Bax (Chipuk et al., 2005).

Therefore, a decrease in PUMA levels could potentially reduce Bax activity by reducing its release from Bcl-XL.

Our data also demonstrate that ASPP2 knockdown leads to downregulation of Fas/CD95 and that siRNA-mediated Fas/CD95 silencing was neuroprotective. Fas/CD95 is a death receptor of the tumour necrosis factor receptor superfamily of single-pass trans-membrane proteins (Ashkenazi, 2002, Peter et al., 2007). Fas/CD95 is activated by Fas ligand (FasL) leading to the recruitment of the adaptor protein FADD (Fas-associated death domain) and activation of caspase 8 (Haase et al., 2008). Analysis of p53 responsive elements in the CD95 gene revealed a role for p53 in Fas/CD95 transcription (Schilling et al., 2009). Furthermore, nuclear ASPP1 can activate p53-induced Fas/CD95 expression (Aylon et al., 2010). FasL has been shown to increase in retinal microglia in a rat model of ocular hypertension (Ju et al., 2006) and FasL-positive autoreactive T cells have been implicated in the loss of RGCs following heat shock protein immunization (Wax et al., 2008). More recently, the membrane-bound form of FasL was implicated in glaucomatous RGC degeneration (Gregory et al., 2011). Collectively, these studies support a role for Fas/CD95 in RGC death. Our data, specifically, demonstrate that Fas/CD95 contributes to ASPP1/2-p53 pro-apoptotic signaling and that Fas/CD95 downregulation promotes marked RGC protection after axonal injury.

Recent studies have unveiled a novel and unexpected role for p53 in the promotion of neurite outgrowth and axonal regeneration that is independent of its pro-apoptotic function (Di Giovanni and Rathore, 2012). Indeed, p53 gene silencing or dominant negative forms of p53 have been shown to inhibit neurotrophin-dependent outgrowth *in vitro* and axonal

regeneration *in vivo* (Di Giovanni et al., 2006, Zhang et al., 2006, Tedeschi et al., 2009). Of interest, p53 acetylation at specific lysine residues is thought to be required for the p53 growth-promoting effects (Tedeschi et al., 2008, Gaub et al., 2010). More recently, viral-mediated overexpression of the histone acetyltransferase p300 in RGCs increased axonal regeneration within the injured optic nerve (Gaub et al., 2011). We have previously demonstrated that signaling pathways that lead to RGC survival may differ from those that promote RGC axon regeneration (Pernet and Di Polo, 2006). Therefore, although our current data demonstrate a pro-apoptotic role of p53 we cannot rule out the possibility that p53 might enhance RGC axon growth through activation of different signaling pathways. In this context, it should be of interest to assess the role of ASPP1/2 on axonal regeneration following optic nerve injury.

In conclusion, we used a loss-of-function approach to identify the role of the p53 activators ASPP1 and ASPP2 in retinal neuron death following optic nerve axotomy. Our data demonstrate a novel and prominent role of the pro-apoptotic ASPP1/2 proteins in the death of RGCs that involves the p53 targets PUMA and Fas/CD95. These findings expand our understanding of the molecular basis of RGC neurodegeneration, and might have implications for the design of strategies for neuroprotection in the injured CNS.

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Figure 1. Axotomized RGCs die in a p53-dependent manner.

(A) RT-PCR analysis revealed that p53 mRNA levels do not change at 1 day or 3 days (shown here) after axotomy with respect to non-injured retinas (Student‟s *t* test, $p>0.05$). (B) In contrast, a significant increase in axotomy-induced phosphorylation of p53 at serine 15 (S15) was readily detected at 1 day after axotomy, but returned to basal levels at 7 days post-lesion (ANOVA, *=$p<0.05$). (C) Retinal immunostaining of Fluorogold-labeled RGCs confirmed that p53 phosphorylation (activation) was detected in these neurons at 1 day after axotomy. Scale: 10 μm. (D) Protein levels of the p53 apoptotic targets PUMA and Fas/CD95 increased at 1 day after axotomy, but returned to normal levels at 7 days post-injury. Bax and Noxa remained unchanged. (E) Analysis of RGC loss quantified at 1 week after axotomy in p53 null, heterozygote and wild-type retinas demonstrated an allelic dose dependency on p53 (ANOVA, ***=$p<0.001$). Data are expressed as RGC densities (RGCs/mm^2; mean ± S.D.).

Figure 2. ASPP1 and ASPP2 are expressed by adult RGCs.

Retinal immunofluorescence demonstrated abundant expression of endogenous ASPP1 (A-G) and ASPP2 (K-Q) in RGCs visualized with the retrograde tracer Fluorogold. DAPI staining showed that ASPP1 is present in RGC nuclei and cytoplasm (perinuclear) (H-J), while ASPP2 is primarily in the nuclei (R-T). ASPP1 and ASPP2 blocking peptides resulted in absence of staining (G, Q) confirming the specificity of the ASPP1 and ASPP2 antibodies. Scale bars: (A-C) and (K-M) = 70 μm; (D-G) and (N-Q) = 50 μm; (H-J) and (R-T) = 10 μm. RPE: Retinal Pigment Epithelium; PS: Photoreceptor Segments; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 3. Expression of ASPP family members after optic nerve axotomy.

(A) The levels or sub-cellular localization of ASPP1, ASPP2 or the anti-apoptotic member iASPP, visualized by retinal immunohistochemistry and Fluorogold (FG) staining, did not change at 48 hrs after optic nerve injury. Scale bar: 10 μm. (B) Analysis of protein homogenates confirmed that ASPP1, ASPP2 and iASPP levels in axotomized retinas collected at 48 hrs were similar to those in intact, non-injured retinas. The lower panel represents the same blot as in the upper panel but probed with an antibody that recognizes β-actin used to confirm equal protein loading. (C) Densitometric analysis of western blots, showing the ratio of ASPP proteins relative to β-actin, confirmed that there is no significant change in protein expression after injury (Student‟s *t* test, $p>0.05$).

Figure 4. Selective knockdown of retinal ASPP1 or ASPP2 by intravitreal siRNA delivery.

Intravitreal delivery of Cy3-tagged siRNA resulted in rapid and effective uptake by RGCs. Lack of Cy3 fluorescence in non-injected control retinas (A-C) contrasted with robust Cy3 labeling in RGCs, visualized with Fluorogold (FG) (D-I), as early as 5 hrs after siRNA administration. (J-M) Intravitreal delivery of siRNA against ASPP1 (siASPP1) led to a significant reduction of retinal ASPP1 protein at 24 hrs after delivery while control siRNA against GFP (siGFP) had no effect (ANOVA, *=$p<0.05$). siASPP1 did not decrease or increase the protein levels of the other family members, ASPP2 or iASPP, confirming the specificity of the siRNA. Similarly, siRNA against ASPP2 (siASPP2) selectively depleted retinal ASPP2 protein levels (ANOVA, *=$p<0.05$) without altering ASPP1 or iASPP levels. Endogenous levels of both ASPP1 and ASPP2 proteins returned to basal at 48 hrs after siRNA delivery. Immunohistochemistry of axotomized retinas at 24 hrs after siASPP1 or siASPP2 administration confirmed that siRNA-mediated knockdown of ASPP1/2 occurred in RGCs, visualized with Fluorogold (N, O). Scale bars: (A-F) = 50 μm; (G-I) = 10 μm; (N-O) = 12 μm. PS: Photoreceptor Segments; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 5. ASPP1 and ASPP2 knockdown protects RGCs from axotomy-induced death. Fluorogold-labeled RGCs in flat-mounted retinas from a representative non-injured eye (A) or axotomized eyes treated with siASPP1 (B), siASPP2 (C) or control siGFP (D) at one week post-injury. Scale bars: 100 μm. (E) Quantitative analysis of RGC survival following intraocular injection of siASPP1 (hatched), siASPP2 (dark grey), combined siASPP1 and siASPP2 (black), control siGFP (light grey) or PBS (white) (ANOVA, ***=$p<0.001$; *=$p<0.05$). The density of RGCs in intact, uninjured Sprague-Dawley rat retinas is shown as reference (open bar). Data are expressed as the mean ± S.E.M.

Figure 6. siASPP2-mediated knockdown of PUMA, Fas and Noxa depends on p53 transcriptional activity.

(A) Real-time qPCR analysis of rat retinal samples at 6 hrs after axotomy and siASPP2 administration revealed that ASPP2 knockdown leads to downregulation of PUMA, Fas/CD95 and Noxa (ANOVA, ***=$p<0.001$; **=$p<0.01$), but not Bax (ANOVA, $p>0.5$) gene expression. (B-E) qPCR of retinal samples from p53 null mice and wild-type littermate controls collected at 6 hrs after axotomy and siASPP2 injection. Transcript levels of PUMA, Fas/CD95 and Noxa were significantly reduced in non-injured or axotomized p53 null mice with respect to wild-type littermates. Moreover, ASPP2 knockdown effectively reduced PUMA, Fas/CD95 and Noxa gene expression in axotomized retinas from p53 wild-type mice, but not from p53 knockout mice (B, C, E) (ANOVA, ***=$p<0.001$; **=$p<0.01$). Bax gene expression remained unchanged (D).

Figure 7. siASPP2 protects RGCs through downregulation of the p53 pro-apoptotic targets PUMA and Fas/CD95.

(A) Western blot analysis of axotomized and injected retinal samples at 24 hrs after siRNA administration show that PUMA and Fas/CD95 protein levels are downregulated compared to control siGFP (A, B; Student‟s *t* test, *=$p<0.05$), whereas Bax and Noxa remained unchanged (C, D; Student‟s *t* test, $p>0.05$). Western blot analysis of axotomized and injected retinal samples at 24 hrs show that siFas/CD95, siPUMA and siNoxa downregulated endogenous retinal Fas/CD95 (E), PUMA (F) and Noxa (G) protein levels, respectively, compared to control siGFP (Student‟s *t* test *=$p<0.05$). (H) A significant increase in RGC survival was observed in retinas exposed to siPUMA (black) or siFas/CD95 (dark grey) with respect to siGFP-treated (light grey) or PBS-treated (white) controls (ANOVA, **=$p<0.01$; *=$p<0.05$). Administration of siNoxa (horizontal lines) did not result in significant RGC survival (ANOVA, $p>0.5$). The density of RGCs in intact, uninjured Sprague-Dawley rat retinas is shown as reference (open bar). Data are expressed as the mean ± S.E.M.

II.7. FIGURES

FIGURE 1

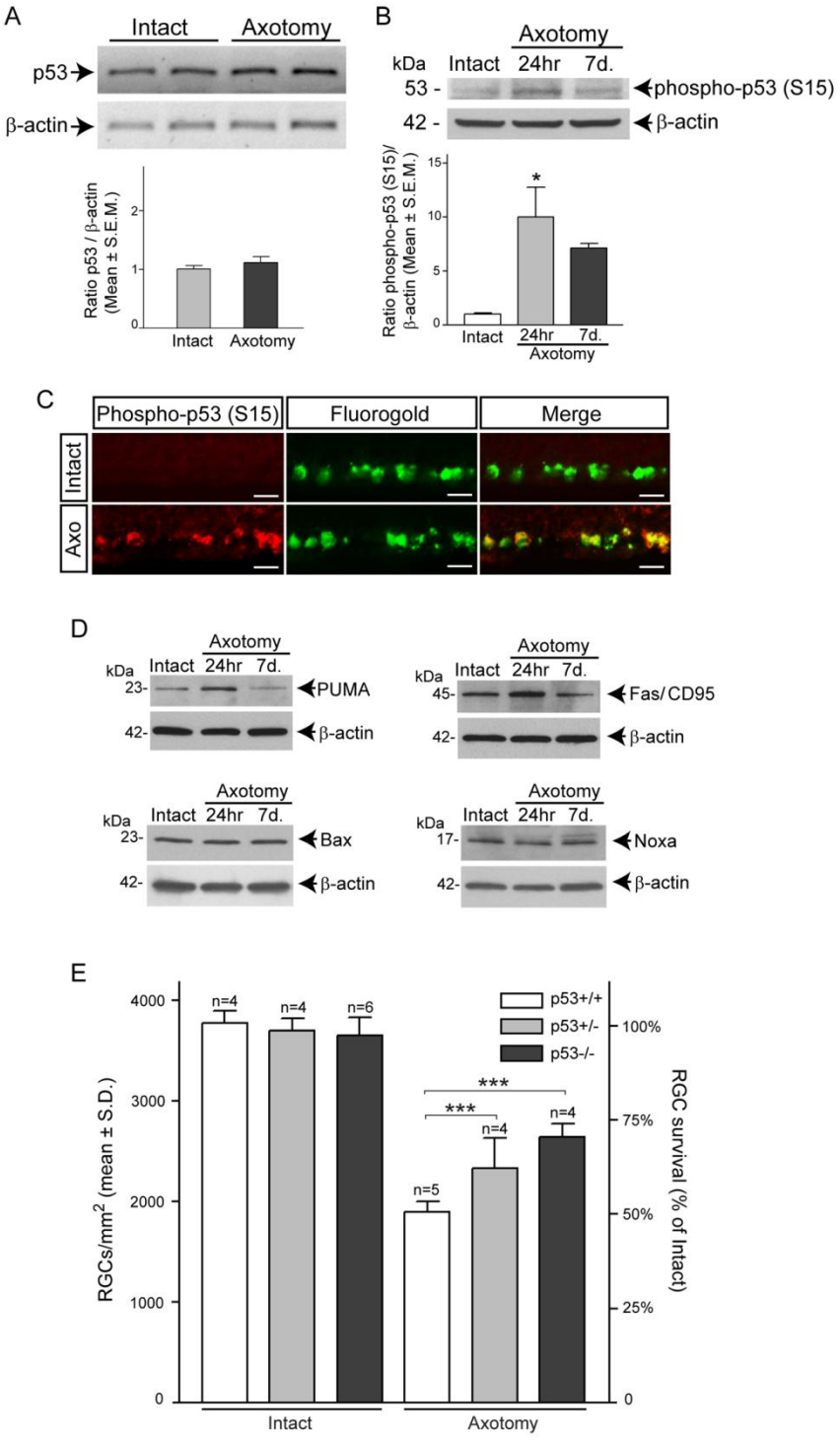


FIGURE 2

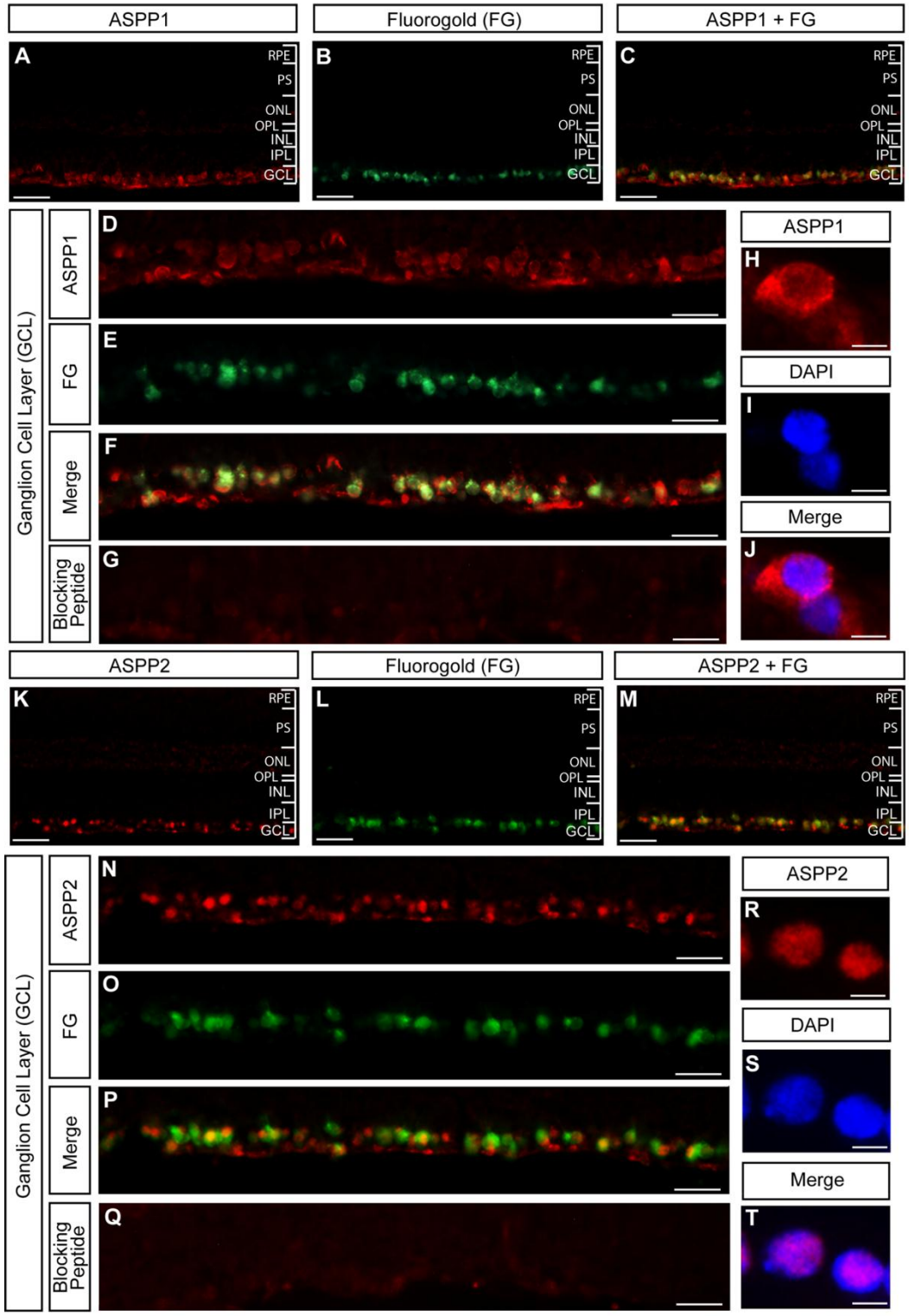

ASPP1
Fluorogold (FG)
ASPP1 + FG
A
B
C
RPE
PS
ONL
OPL
INL
IPL
GCL
Ganglion Cell Layer (GCL)
ASPP1
FG
Merge
Blocking Peptide
D
E
F
G
ASPP1
H
DAPI
I
Merge
J
ASPP2
Fluorogold (FG)
ASPP2 + FG
K
L
M
N
O
P
Q
R
S
T

FIGURE 3

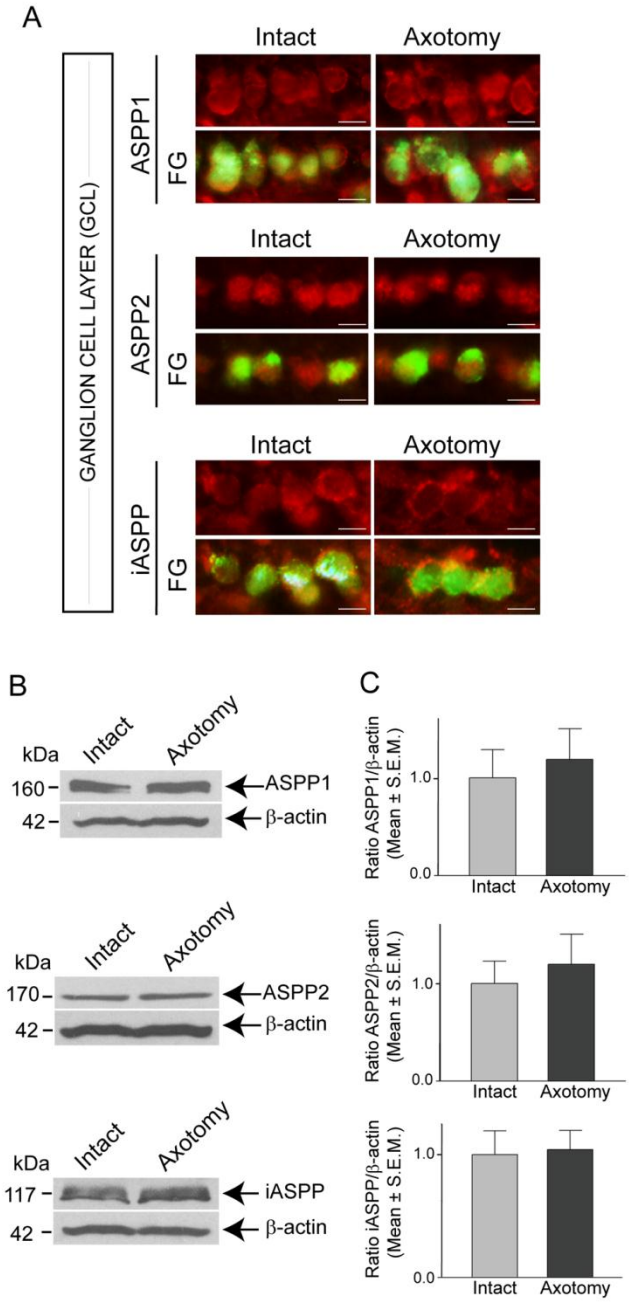

A
Intact
Axotomy
GANGLION CELL LAYER (GCL)
ASPP1
FG
ASPP2
iASPP
B
kDa
160
42
ASPP1
β-actin
170
ASPP2
117
iASPP
C
Ratio ASPP1/β-actin (Mean ± S.E.M.)
Ratio ASPP2/β-actin (Mean ± S.E.M.)
Ratio iASPP/β-actin (Mean ± S.E.M.)
1.0
0.0

FIGURE 4

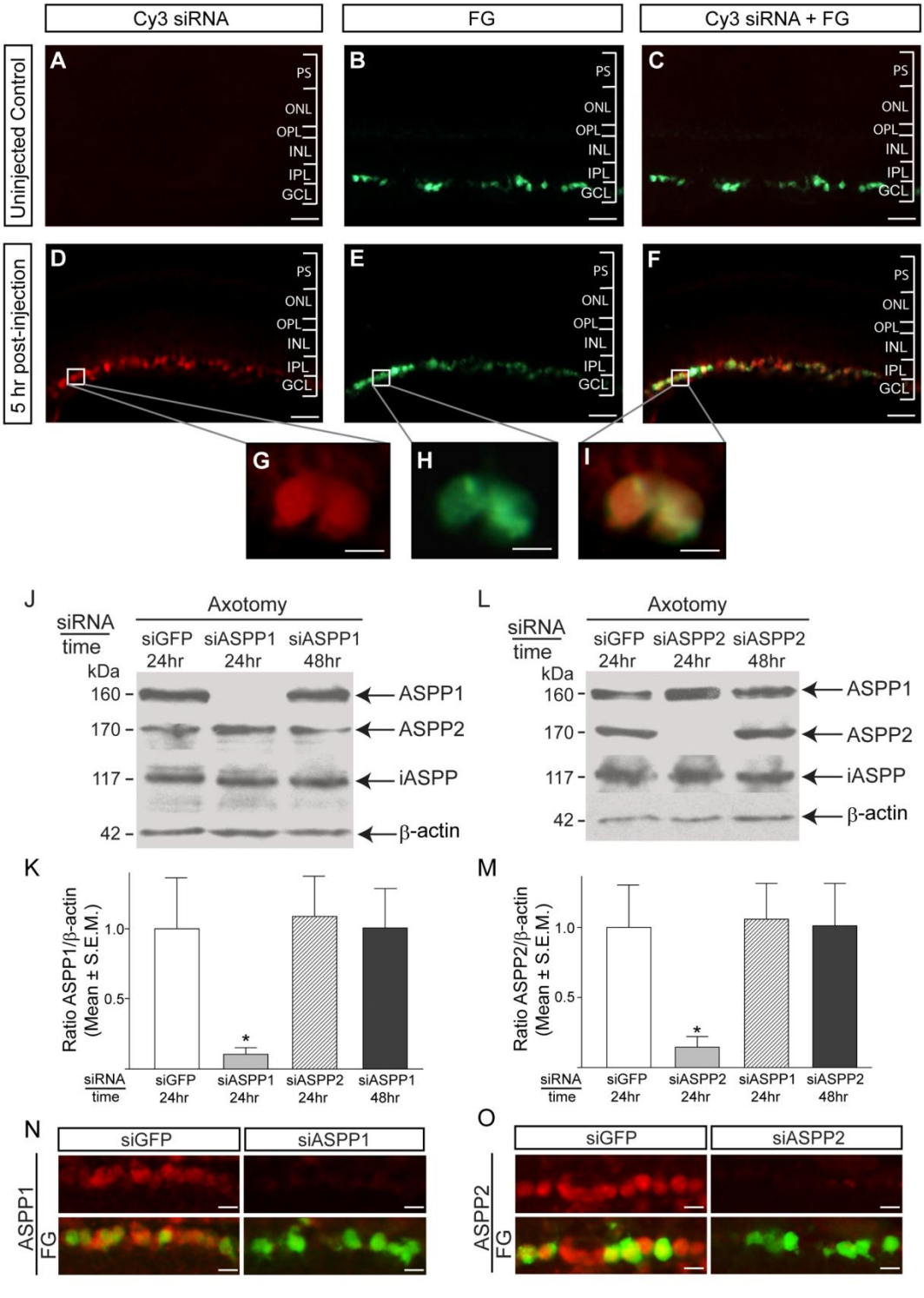

Cy3 siRNA
FG
Cy3 siRNA + FG
Uninjected Control
5 hr post-injection
A
B
C
D
E
F
G
H
I
PS
ONL
OPL
INL
IPL
GCL
J
Axotomy
siRNA
time
siGFP 24hr
siASPP1 24hr
siASPP1 48hr
kDa
160
170
117
42
ASPP1
ASPP2
iASPP
β-actin
L
Axotomy
siRNA
time
siGFP 24hr
siASPP2 24hr
siASPP2 48hr
K
Ratio ASPP1/β-actin (Mean ± S.E.M.)
1.0
0.5
*
siRNA time
siGFP 24hr
siASPP1 24hr
siASPP2 24hr
siASPP1 48hr
M
Ratio ASPP2/β-actin (Mean ± S.E.M.)
siRNA time
siGFP 24hr
siASPP2 24hr
siASPP1 24hr
siASPP2 48hr
N
siGFP
siASPP1
ASPP1
FG
O
siGFP
siASPP2
ASPP2
FG

FIGURE 5

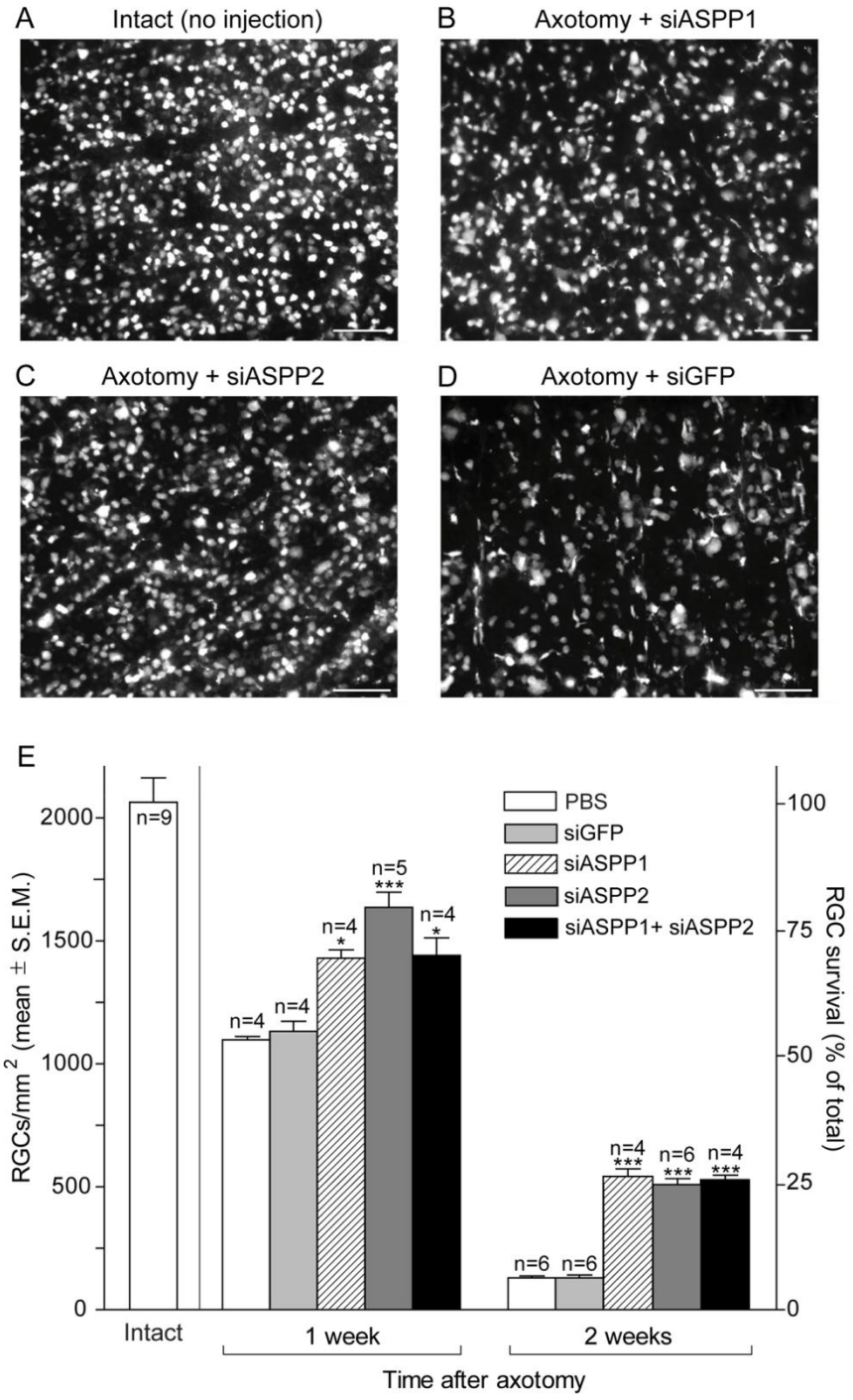

A
Intact (no injection)
B
Axotomy + siASPP1
C
Axotomy + siASPP2
D
Axotomy + siGFP
E
PBS
siGFP
siASPP1
siASPP2
siASPP1+ siASPP2
RGCs/mm 2 (mean ± S.E.M.)
RGC survival (% of total)
2000
1500
1000
500
0
100
75
50
25
0
n=9
n=4
n=4
n=4
*
n=5
***
n=4
*
n=6
n=6
n=4
***
n=6
***
n=4
***
Intact
1 week
2 weeks
Time after axotomy

FIGURE 6

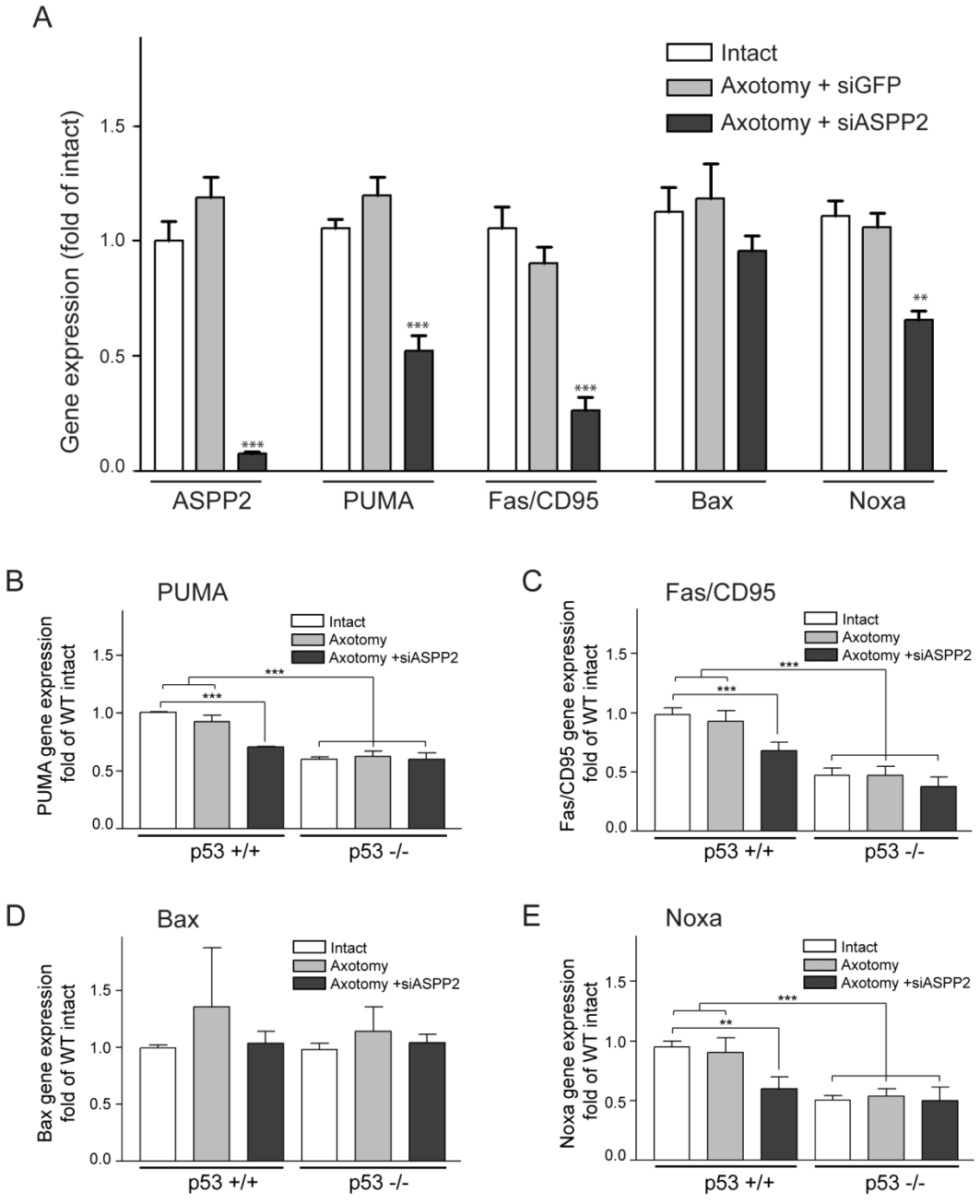
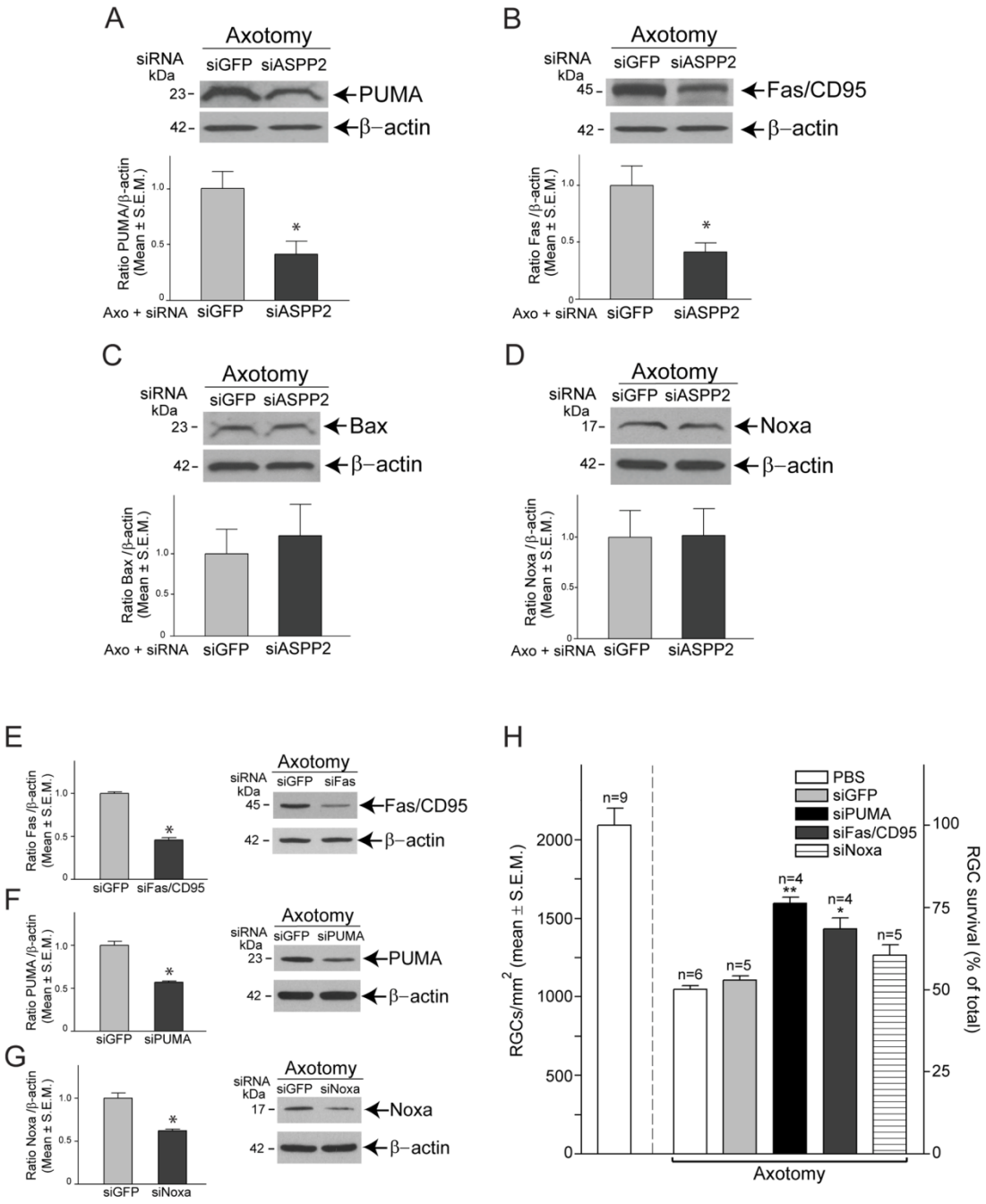

A
Intact
Axotomy + siGFP
Axotomy + siASPP2
Gene expression (fold of intact)
1.5
1.0
0.5
0.0
***
***
***
**
ASPP2
PUMA
Fas/CD95
Bax
Noxa
B
PUMA
PUMA gene expression fold of WT intact
Intact
Axotomy
Axotomy +siASPP2
***
***
p53 +/+
p53 -/-
C
Fas/CD95
Fas/CD95 gene expression fold of WT intact
Intact
Axotomy
Axotomy +siASPP2
***
***
p53 +/+
p53 -/-
D
Bax
Bax gene expression fold of WT intact
Intact
Axotomy
Axotomy +siASPP2
p53 +/+
p53 -/-
E
Noxa
Noxa gene expression fold of WT intact
Intact
Axotomy
Axotomy +siASPP2
***
**
p53 +/+
p53 -/-

FIGURE 7


A
Axotomy
siRNA
kDa
siGFP siASPP2
23-
PUMA
42-
β–actin
Ratio PUMA/β-actin (Mean ± S.E.M.)
1.0
0.5
0
*
Axo + siRNA siGFP siASPP2
B
Axotomy
siRNA
kDa
siGFP siASPP2
45-
Fas/CD95
42-
β–actin
Ratio Fas /β-actin (Mean ± S.E.M.)
1.0
0.5
0
*
Axo + siRNA siGFP siASPP2
C
Axotomy
siRNA
kDa
siGFP siASPP2
23-
Bax
42-
β–actin
Ratio Bax /β-actin (Mean ± S.E.M.)
1.0
0.5
0
Axo + siRNA siGFP siASPP2
D
Axotomy
siRNA
kDa
siGFP siASPP2
17-
Noxa
42-
β–actin
Ratio Noxa /β-actin (Mean ± S.E.M.)
1.0
0.5
0
Axo + siRNA siGFP siASPP2
E
Ratio Fas /β-actin (Mean ± S.E.M.)
1.0
0.5
0
*
siGFP siFas/CD95
Axotomy
siRNA
kDa
siGFP siFas
45-
Fas/CD95
42-
β–actin
F
Ratio PUMA /β-actin (Mean ± S.E.M.)
1.0
0.5
0
*
siGFP siPUMA
Axotomy
siRNA
kDa
siGFP siPUMA
23-
PUMA
42-
β–actin
G
Ratio Noxa /β-actin (Mean ± S.E.M.)
1.0
0.5
0
*
siGFP siNoxa
Axotomy
siRNA
kDa
siGFP siNoxa
17-
Noxa
42-
β–actin
H
RGCs/mm2 (mean ± S.E.M.)
2000
1500
1000
500
0
PBS
siGFP
siPUMA
siFas/CD95
siNoxa
n=9
n=6
n=5
n=4
**
n=4
*
n=5
RGC survival (% of total)
100
75
50
25
0
Axotomy

CHAPTER 3

III. SECOND ARTICLE: INHIBITOR OF APOPTOSIS-STIMULATING PROTEIN OF p53 (iASPP) IS REQUIRED FOR NEURONAL SURVIVAL AFTER AXONAL INJURY

Undergoing minor revisions for resubmission to PLoS ONE

Inhibitor of Apoptosis-Stimulating Protein of p53 (iASPP) is Required for Neuronal Survival After Axonal Injury.

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Number of pages: 33
Number of words: Abstract (204), Introduction (395), Discussion (1119)

The authors declare no conflict of interest.

III.1. ABSTRACT

The transcription factor p53 mediates the apoptosis of post-mitotic neurons exposed to a wide range of stress stimuli. The apoptotic activity of p53 is tightly regulated by the apoptosis-stimulating proteins of p53 (ASPP) family members: ASPP1, ASPP2 and iASPP. We previously showed that the pro-apoptotic members ASPP1 and ASPP2 contribute to p53-dependent death of retinal ganglion cells (RGCs). However, the role of the p53 inhibitor iASPP in the central nervous system (CNS) remains to be elucidated. To address this, we asked whether iASPP contributes to the survival of RGCs in an *in vivo* model of acute optic nerve damage. We demonstrate that iASPP is expressed by injured RGCs and that iASPP phosphorylation at serine residues, which increase iASPP affinity towards p53, is significantly reduced following axotomy. We show that short interference RNA (siRNA)-induced iASPP knockdown exacerbates RGC death, whereas adeno-associated virus (AAV)-mediated iASPP expression promotes RGC survival. Importantly, our data also demonstrate that increasing iASPP expression in RGCs downregulates p53 activity and blocks the expression of pro-apoptotic targets PUMA and Fas/CD95. This study demonstrates a novel role for iASPP in the survival of RGCs, and provides further evidence of the importance of the ASPP family in the regulation of neuronal loss after axonal injury.

Keywords: Retinal Ganglion Cell, Axotomy, Inhibitor of Apoptosis-Stimulating Protein of p53 (iASPP), Apoptotic Cell Death, Adeno-Associated Virus (AAV), Short Interference RNA (siRNA).

III.2. INTRODUCTION

iASPP is the most evolutionarily conserved member of the „Ankyrin-repeat, SH3-domain, and Proline-rich-region containing Protein" (ASPP) family (Bergamaschi et al., 2003), comprised of ASPP1, ASPP2, and iASPP. The first detected form of iASPP, a truncated variant termed RelA-associated inhibitor (RAI), was identified as a nuclear factor kappa beta (NFκB) inhibitor in a yeast two hybrid screen (Yang et al., 1999b). The full-length isoform of iASPP, which is the predominant form of this molecule expressed in cells, was later discovered and shown to carry a C-terminus identical to RAI (Slee et al., 2004). ASPP family members have attracted much attention since their implication in a novel mechanism of p53 apoptotic regulation was identified in cancer cells. During tumorigenesis, pro-apoptotic ASPP1/2 enhance p53-dependent cell death (Lopez et al., 2000, Ao et al., 2001, Samuels Lev et al., 2001, Bergamaschi et al., 2006), while anti-apoptotic iASPP binds to p53 to inhibit its ability to transactivate pro-apoptotic target genes (Yang et al., 1999a, Lopez et al., 2000, Bergamaschi et al., 2003, Bergamaschi et al., 2004, Bergamaschi et al., 2006, Ahn et al., 2009).

Since its discovery, iASPP was shown to be encoded by the Protein Phosphatase 1 Regulatory Subunit 13-Like (PPP1R13L) gene, which is overexpressed in many tumors including acute leukemia (Zhang et al., 2005), breast cancer (Bergamaschi et al., 2003), glioblastoma (Li et al., 2011), ovarian cancer (Jiang et al., 2011), and head and neck squamous cell carcinoma (Liu et al., 2012). Previous studies demonstrated that overexpression of iASPP in a human osteosarcoma cell line increased their resistance to ultraviolet radiation or cisplatin-induced apoptosis, without altering p53 expression (Bergamaschi et al., 2003). Due to its potent inhibitory role of p53 apoptotic activity, iASPP function has been studied

primarily in cancer cells or in the context of tumor biology. However, the role of iASPP in neuronal survival and neurodegeneration is not well understood.

To address this, we asked whether iASPP is implicated in the survival of retinal ganglion cells (RGC) after axonal injury. RGCs are central nervous system (CNS) neurons that undergo a predictable onset of apoptotic death following optic nerve transection (Berkelaar et al., 1994, Cheng et al., 2002). Here, we demonstrate that iASPP is expressed by adult intact and axotomized RGCs. We show that short interference RNA (siRNA)-mediated knockdown of retinal iASPP expression exacerbates RGC death, while iASPP overexpression using serotype 2 adeno-associated virus (AAV) promotes RGC survival *in vivo*. We demonstrate that increased iASPP expression leads to a reduction in p53 apoptotic activity as evidenced by downregulation of p53 phosphoserine 15 (pSer15 p53), and its targets PUMA and Fas/CD95. In summary, our study identifies a novel role for iASPP in the survival of RGCs after acute optic nerve damage, and further supports a critical role of ASPP family members in the regulation of neuronal loss in the injured CNS.

III.3. MATERIAL AND METHODS

III.3.1. Experimental Animals

Animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care for the use of experimental animals (www.ccac.ca). All protocols were approved by the Committee on the Ethics of Use of Experimental Animals at the University of Montreal (Permit Number: 13-018). Surgeries were carried out in adult, female Sprague-Dawley rats (250-275 g), and performed under general anesthesia (2% Isoflurane, 0.8

liter/min). The number of animals used in each experiment is indicated in the results and the legend of the corresponding figure. All efforts were made to minimize the suffering of experimental animals.

III.3.2. Optic nerve axotomy

The left optic nerve was exposed and carefully transected at 1 mm from the optic nerve head avoiding injury to the ophthalmic artery, as previously described (Cheng et al., 2002, Pernet et al., 2005, Wilson et al., 2013). The right eye was never operated on and served as internal control. Fundus examination was performed immediately after axotomy and three days later to check the integrity of the retinal circulation after surgery. Animals showing signs of compromised blood supply were excluded from the study.

III.3.3. Short interfering RNA (siRNA)

The siRNA sequences against iASPP were purchased from Dharmacon (Smartpool, Thermo Scientific, Lafayette, CO) (sense strands): 5‟-CCGCCAAAGUGGACGAAUU-3‟, 5‟-UGACAGGCGGUUCUGACGUU-3‟, 5‟-CCGAAGGCCUGGAACGAGU-3‟, 5‟-UGGUACAGCAGGCGGUGAA-3‟. The control siRNA against GFP was kindly provided by Dr. Elena Feinstein (Quark Pharmaceuticals Inc.) and has been described elsewhere (Hamar et al., 2004).

III.3.4. Recombinant AAV Serotype 2 Vectors

A murine iASPP cDNA containing a c-terminal, myc tag (synthesized by GenScript USA Inc., Piscataway, NJ) was inserted downstream of the Synapsin 1 (Syn1) promoter into an AAV vector plasmid containing bovine growth hormone poly A and AAV serotype 2 terminal repeats. Site-directed mutagenesis of surface-exposed tyrosine residues on AAV2 was done to prevent proteasome-mediated degradation and improve transduction efficiency as

previously described (Zhong et al., 2008). Vectors were packaged, concentrated, and titered using standard methods (Hauswirth et al., 2000). A control vector containing the green fluorescent protein (GFP) gene under control of the same Syn1 promoter was prepared in identical fashion and used as control. The titers of the vector stocks were: 1.07E+13 vector genomes/ml (vg/ml) for hSyn1-iASPP-myc AAV2 Triple Y-F (AAV.iASPP) and 8.23E+12 vg/ml for Syn1-hGFP AAV2 Triple Y-F (AAV.GFP).

III.3.5. Intravitreal injections

siRNA against iASPP or control siGFP (2 μg/μl), as well as AAV encoding iASPP (AAV.iASPP, 1.07E+13 vg/ml) or control GFP (AAV.GFP, 8.23E+12 vg/ml) were injected into the vitreous chamber of the left eye using a Hamilton syringe fitted with a 32-gauge glass microneedle (total volume: 5 μl). The sclera was exposed and the tip of the needle inserted at a 45° angle through the sclera and retina into the vitreous space using a posterior approach. This route of administration avoided injury to anterior eye structures, which can promote RGC survival (Mansour-Robaey et al., 1994, Leon et al., 2000). Surgical glue (Indermill, Tyco Health Care, Mansfield, MA) was used to seal the injection site. Intraocular injection of siRNA was performed at the time of optic nerve axotomy, while injection of AAV was performed two weeks prior to axotomy to allow for AAV-mediated transgene expression to reach a plateau (Cheng et al., 2002, Pernet et al., 2005).

III.3.6. Retinal immunohistochemistry

Animals were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS, pH 7.4). Retinal cryosections (16 μm) were prepared as previously described (Pernet et al., 2005, Lebrun-Julien et al., 2009). For iASPP

immunohistochemistry, retinas were subjected to heat-mediated antigen retrieval by incubating sections in 0.01 M sodium citrate in 0.5% Tween-20 (pH 6) at 85^{o}C for 15 min. The following primary antibodies were added to the retinal sections in blocking solution and incubated overnight at 4°C: RNA binding protein with multiple splicing (RBPMS), Brn3a (1 µg/ml, Santa Cruz Biotechnologies, Santa Cruz, CA), iASPP (1 µg/ml, Bethyl Laboratories, Montgomery, TX), iASPP (1 µg/mL, Clone LXO49.3, 1 µg/ml, Sigma-Aldrich, Saint-Louis, MO), Calretinin (1:1000, Millipore, Billerica, MA), Calbindin (1:10,000, Swant, Switzerland), or c-myc (1 µg/mL, Abcam, Cambridge, MA). For RBPMS, rabbit and guinea pig polyclonal antibodies were generated against the N-terminus GGKAEKENTPSEANLQEEEVR ($RBPMS_{4-24}$) by ProSci Inc. (Powy, CA). Sera were collected following immunization and affinity purified using a RBPMS polypeptide affinity column as described (Rodriguez et al., 2013). Sections were then incubated with secondary antibodies: anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG (1-8 µg/ml, Cy3, Alexa 594, Alexa 488, Alexa 350, Jackson ImmunoResearch Laboratories Inc., West Grove, PA), washed and mounted in anti-fade reagent (SlowFade, Molecular Probes, Eugene, OR). Fluorescent labeling was observed with a Zeiss AxioSkop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC).

III.3.7. Western blot analysis

Whole fresh retinas were rapidly dissected and homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (20 mM Tris pH 8.0, 135 mM NaCl, 1% NP-40, 0.1% SDS, and 10% glycerol supplemented with protease inhibitors). For phosphorylated protein analysis, retinas were homogenized in ice-cold phosphorylation lysis buffer (50 mM

Tris HCl pH 7.4, EDTA 1 mM, NaCl 150 mM, NP40 1%, NaF 5 mM, Na deoxycholate 0.25%, $NaVO_3$ 2 mM, supplemented with protease and phosphatase inhibitors). Protein homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatants removed and resedimented for an additional 10 min to yield solubilized extracts. Retinal extracts (40 µg) were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Life Science, Mississauga, ON). Blots were incubated overnight at 4°C with each of the following primary antibodies: phospho-p53 (Ser15) (2 µg/ml, Abcam), acetyl p53 (Lys 373, Lys 382) (1:100, Millipore), iASPP (0.5 µg/ml, Bethyl Laboratories), iASPP (0.1 µg/ml, Clone LXO49.3, Sigma-Aldrich), Bax (1.5 µg/ml, N20, Santa Cruz Biotechnologies), PUMA (1 µg/ml, Abcam), Noxa (0.5 µg/ml, Sigma-Aldrich), Fas/CD95 (1 µg/ml, BD Transduction Laboratories, San Jose, CA) or β-actin (0.5 µg/ml, Sigma-Aldrich). Membranes were washed and incubated in anti-rabbit or anti-mouse peroxidase-linked secondary antibodies (0.5 µg/ml, Amersham Biosciences, Baie d‟Urfé, QC). Blots were developed with a chemiluminescence reagent (ECL clarity, BioRad, Hercules, CA) and imaged by ChemiDoc MP (BioRad). Densitometric analysis was performed using Image Lab software (BioRad) on scanned nitrocellulose membranes obtained from a series of three independent blots each carried out using retinal samples from distinct experimental groups.

III.3.8. Immunoprecipitation

Retinal extracts of AAV.iASPP or AAV.GFP injected eyes were immunoprecipitated with 2 µg of phosphoserine or control IgG antibodies (Millipore) following Catch and Release version 2.0 kit procedures (Millipore) and processed for Western blot analysis. Briefly, retinal extracts (400 µg) were incubated in continuous rotation for 3 hrs at 4^oC in 500 µl of affinity beads carrying rabbit polyclonal anti-phosphoserine IgG (2 µg, Millipore). The beads were

washed three times with wash buffer (Millipore), and the bound proteins were eluted by treating the beads twice with 70 µl of elution buffer. Detection and identification of immunoprecipitated proteins were performed by Western blots analysis as described above.

III.3.9. Quantification of RGC survival

Rats were euthanized at one or two weeks post-axotomy by transcardial perfusion with 4% PFA and both the left (optic nerve lesion) and right (intact control) retinas were dissected and fixed for an additional 15 min. Brn3a immunodetection on whole-mounted retinas was performed as described (Nadal-Nicolas et al., 2009). Briefly, whole mounted retinas were permeabilized in PBS containing 0.5% Triton X-100 (Fisher, Waltham, MA) by freezing them at -80^{o}C for 15 min, rinsed and incubated overnight at 4^{o}C with goat-anti-Brn3a (0.27 µg/ml, Santa Cruz Biotechnologies, C-20) in blocking buffer (PBS, 2% normal donkey serum, 2% Triton X-100). Retinas were washed and incubated for 2 hrs at room temperature with Alexa Fluor donkey anti-goat IgG (1 µg/ml, Jackson ImmunoResearch Laboratories Inc.). Retinas were then rinsed, mounted vitreal side up, and covered with anti-fade solution (SlowFade, Molecular Probes, Eugene, OR). Brn3a-labeled neurons were counted within three square areas at distances of 1, 2 and 3 mm from the rat optic disc in each of the four retinal quadrants for a total of twelve retinal areas. Fluorescent staining was examined with a Zeiss Axioskop 2 Plus microscope (Carl Zeiss Canada, Kirkland, QC). Images were captured with a CCD video camera (Retiga, Qimaging, Burnaby, BC) and analyzed with Northern Eclipse software (Empix Imaging, Mississauga, ON).

III.3.10. Statistical analyses

Data analysis and statistics were performed using the GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test or Student‟s *t* test.

III.4. RESULTS

III.4.1. iASPP is abundantly expressed by injured RGCs but its activity decreases after axonal damage

To characterize the role of the p53 inhibitor iASPP in RGC death, we first determined its cellular localization in the adult rat retina. Retinal immunohistochemistry showed expression of endogenous iASPP in the ganglion cell layer (GCL) and inner nuclear layer (INL) (Fig. 1A). As displaced amacrine cells account for ~40-50% of the total number of neurons in the rat GCL (Perry, 1981, Schlamp et al., 2013), we performed co-localization studies using antibodies against iASPP and „RNA binding protein with multiple splicing‟ (RBPMS), a selective RGC marker (Kwong et al., 2010, Rodriguez et al., 2013). All RBPMS-positive neurons were immunoreactive for iASPP (Fig. 1B-F), indicating that adult RGCs are endowed with high levels of constitutive iASPP protein. In the INL, iASPP immunolabeling co-localized with calretinin, a marker of amacrine cells, and calbindin, a horizontal cell-specific marker (Fig. 1G-J), indicating that these cells also express iASPP. There was no co-localization between iASPP and PKCα suggesting that iASPP is not expressed by rod bipolar cells (not shown).

In adult rodents, RGCs survive for five days after axotomy and then die abruptly (Villegas-Perez et al., 1993, Berkelaar et al., 1994). We did not detect changes in the levels or cellular localization of iASPP at 24 hrs or 3 days after axotomy (Fig. 2A-C), indicating that iASPP levels are similar in axotomized and non-injured retinas prior to the onset of RGC death. This finding was confirmed by western blot analysis of iASPP protein at 6 hrs, 12 hrs, 24 hrs, 48 hrs, 3 days and 5 days after axotomy (Fig. 2D, E). The phosphorylation of iASPP at serine residues increases its affinity towards p53 thus blocking the transcription of p53 pro-apoptotic target genes (Lu et al., 2013). Therefore, we asked whether iASPP undergoes injury-induced changes in phosphorylation at serine residues. For this purpose, immunoprecipitation (IP) of endogenous phosphoserine proteins was performed on retinal lysates, and the eluates were probed with iASPP antibody to detect endogenous iASPP phosphorylated at serine residues. Phosphoserine IP showed enrichment of retinal iASPP in the intact eye, whereas no co-precipitation of iASPP was observed in axotomized retinas (Fig. 2F). Retinal lysates subjected to IP with an IgG antibody, to control for non-specific interactions, did not show detectable bands (Fig. 2F). Our finding suggests that optic nerve injury reduces iASPP phosphorylation at serine residues, which might compromise its ability to inhibit p53-mediated apoptosis.

III.4.2. Retinal iASPP knockdown exacerbates RGC loss after axonal damage

To elucidate the role of iASPP in retinal neuron death, we first undertook a loss-of-function approach based on siRNA-mediated iASPP knockdown. We previously demonstrated that a single intravitreal injection of siRNA rapidly downregulates target mRNAs in RGCs (Wilson et al., 2013). Analysis of axotomized retinas treated with siRNA against iASPP (si-iASPP) at the time of axotomy revealed effective knockdown of iASPP protein in the GCL as

early as 24 hrs after administration (Fig. 3A, B). Treatment with si-iASPP led to depletion of iASPP from RGCs, visualized with RBPMS (Fig. 3 D-F), whereas a control siRNA against GFP (si-GFP) had no effect (Fig. 3C). Similarly, western blot analysis confirmed robust knockdown of iASPP following si-iASPP administration in injured retinas (Fig. 3 G, H). Next, we asked whether iASPP depletion had an effect on axotomy-induced RGC loss. Quantitative analysis of Brn3a-labeled RGCs demonstrated that siRNA-mediated iASPP downregulation resulted in significantly greater RGC death (63%: 821 ± 68 RGCs/mm^2, n=4) compared to siGFP-treated eyes (48%: 1154 ± 27 RGCs/mm^2, n=4) at one week post-axotomy (Fig. 3I). These results indicate that loss of iASPP exacerbates RGC death following optic nerve injury.

III.4.3. AAV-mediated iASPP overexpression selectively increases iASPP activity in RGCs

AAV serotype 2 vectors were administered by intraocular injection to examine iASPP transgene expression in retinal cells *in vivo*. Retinas were examined two to four weeks following administration of AAV, the time required for optimal transgene expression using this vector (Cheng et al., 2002, Pernet et al., 2005). To distinguish AAV-mediated iASPP from endogenous iASPP, we used an antibody against the c-myc tag present only in the iASPP transgene. Robust c-myc staining was observed in a large number of cells in the GCL of retinas treated with AAV.iASPP, but not in control eyes injected with AAV.GFP (Fig. 4A, B). Co-localization of c-myc with RBMPS confirmed that AAV-transduced iASPP was expressed by RGCs (Fig. 4C-E). Quantification of double-labeled c-myc and RBMPS-positive cells demonstrated that ~85% of RGCs produced virally-encoded iASPP, consistent with previous reports showing high RGC transduction rates following intraocular administration of AAV serotype 2 (Cheng et al., 2002, Pernet et al., 2005). Western blot analysis confirmed virally-

mediated iASPP upregulation in injured retinas, while control AAV.GFP had no effect (Fig. 4 F, G). Next, we asked whether overexpression of iASPP increased its availability to undergo serine phosphorylation after axotomy. IP experiments demonstrated a significant enrichment of iASPP phosphorylated at serine residues in axotomized retinas treated with AAV.iASPP, while no detectable iASPP phosphoserine was observed in AAV.GFP-treated injured retinas (Fig. 4H). Our data demonstrate that AAV-mediated iASPP expression increases the amount of serine phosphorylated iASPP in axotomized retinas.

III.4.4. AAV.iASPP protects RGCs from axotomy-induced death

The widespread expression of AAV-mediated iASPP in RGCs and its ability to activate iASPP *in vivo* prompted us to test its effect on RGC survival. For this purpose, intravitreal injections of AAV.iASPP or AAV.GFP were performed two weeks prior to axotomy and retinas were examined histologically at 7 and 14 days post-lesion to determine the density of surviving RGCs in all retinal quadrants. Flat-mounted retinas from eyes treated with AAV.iASPP showed higher densities of Brn3a-positive RGCs compared to AAV.GFP-treated control retinas (Fig. 5A, B). Quantitative analysis demonstrated that iASPP overexpression resulted in significant RGC survival (77%: 1720 ± 61 RGCs/mm^2, n=5) with respect to eyes that received AAV.GFP (49%: 1094 ± 55 RGCs/mm^2, n=5) at one week post-injury (Fig. 5 C). At two weeks after axotomy, only 9% of RGCs remained in eyes treated with AAV.GFP (221 ± 14 RGCs/mm^2, n=5) whereas 27% of RGCs survived following AAV.iASPP treatment (615 ± 31 RGCs/mm^2, n=4). These data indicate that AAV-mediated iASPP expression delays RGC death after injury and supports the conclusion that iASPP activity promotes RGC survival following axonal injury

III.4.5. iASPP downregulates p53 activity and the expression of pro-apoptotic targets PUMA and Fas/CD95

To investigate the mechanisms by which iASPP overexpression might promote RGC survival, we first examined its effect on p53 post-translational modifications. Acetylation at p53 lysine residues 373 and 382 (Lys373, Lys382) by p300 occurs in the carboxyl-terminal region of p53 and has been correlated with its apoptotic function (Liu et al., 1999, Knights et al., 2006, Yamaguchi et al., 2009). No changes in p53 acetylation at Lys373 and Lys382 were detected 24 hrs after axotomy in retinas treated with AAV.iASPP or control AAV.GFP (Fig. 6 A, B). In contrast, AAV.iASPP markedly inhibited the axotomy-induced increase in phosphoserine 15 (pSer15) p53 (Fig. 6 A, C), a key phosphorylation target during p53 activation (Dumaz and Meek, 1999, Unger et al., 1999). The reduction of pSer15 p53 in retinas overexpressing iASPP prompted us to assess the levels of the p53 pro-apoptotic targets PUMA, Fas/CD95, Bax and Noxa. Western blot analysis of retinal samples showed that AAV.iASPP markedly reduced PUMA and Fas/CD95 protein levels relative to control AAV.GFP (Fig. 6 D, E, F), while Bax and Noxa did not significantly change with any of the treatments (Fig. 6 D, G, H). We conclude that iASPP overexpression leads to downregulation of PUMA and Fas/CD95, suggesting that iASPP protects RGCs by inhibiting the ability of p53 to activate key pro-apoptotic targets.

III.5. DISCUSSION

The critical anti-apoptotic function of iASPP is underscored by its phylogenetic conservation, as it is the most evolutionarily conserved inhibitor of p53 (Bergamaschi et al.,

2003). Our study provides novel insight into the functional role of iASPP in neuronal survival, and allows us to draw the following conclusions. First, iASPP is abundantly expressed by adult RGCs, as well as a subset of amacrine and horizontal cells. Second, although total iASPP levels are not altered by optic nerve injury, phosphoserine iASPP levels, which serve as a readout of iASPP activity, were markedly reduced after axotomy. Third, selective knockdown of iASPP exacerbated RGC death while targeted iASPP overexpression increased phosphoserine iASPP levels and promoted RGC survival. Finally, we showed that AAV-mediated iASPP expression resulted in reduced p53 activity and rapid downregulation of pro-apoptotic targets PUMA and Fas/CD95. These data reveal a critical role for iASPP in the survival of CNS neurons following axonal injury.

We report constitutive expression of iASPP in RGCs, amacrine and horizontal cells of the adult rat retina. Although we previously showed that the expression of pro-apoptotic ASPP family members, ASPP1 and ASPP2, is restricted to the ganglion cell layer (Wilson et al., 2013), another p53 inhibitor, MDM2, is also expressed by amacrine and horizontal cells in adult mice (Xu et al., 2009). MDM4, which is structurally similar to MDM2, is also constitutively expressed in the adult retina (Guo et al., 2008), suggesting that complementary mechanisms are in place to ensure a tight regulation of p53 pro-apoptotic activity in retinal cells. Our finding that selective knockdown of iASPP by siRNA exacerbates RGC death after axonal injury is consistent with recent findings showing that downregulation of endogenous iASPP expression increases apoptosis in tumors of different origin including lung, breast, and prostate cancer as well as leukemia (Liu et al., 2008, Liu et al., 2009, Zhang et al., 2011, Li et al., 2012). Indeed, the inhibition of iASPP has been proposed as a novel strategy for treating tumors affected by deregulation of p53 function. Of interest, our observation that AAV-

mediated iASPP increases RGC survival following axotomy resembles the conferred resistance of iASPP-overexpressing cancer cells to chemotherapeutic drugs including placlitaxel (Jiang et al., 2011), and cisplatin (Bergamaschi et al., 2003). Thus, our complementary loss-of-function and gain-of-function experiments reveal a close parallel between the strong anti-apoptotic role of iASPP in cancer cells and that reported here for adult RGCs.

Our data show that retinal iASPP protein levels were not altered after optic nerve axotomy, similar to pro-apoptotic ASPP family members ASPP1 and ASPP2 (Wilson et al., 2013). Unlike cancer or stroke models in which the total level of ASPP has been shown to vary, this finding supports that, instead, iASPP phosphorylation is markedly reduced after axonal injury suggesting loss of iASPP activity in damaged neurons. The phosphorylation of iASPP at serine residues Ser84 and Ser113 sites was found to increase iASPP binding affinity to p53 (Lu et al., 2013). PhosphoSitePlus (Hornbeck et al., 2012), a curated protein phosphorylation site database identified by large scale Mass Spectrometry screening from various tissues and cell lines, reports the identification of at least 9 other serine phosphorylation residues in iASPP in addition to the Ser84 and Ser113 sites. Although not residue-specific, our phosphoserine immunoprecipitation assay demonstrates that iASPP is endogenously phosphorylated in the intact retina and that the level of iASPP serine phosphorylation is markedly reduced following optic nerve injury. These data suggest that, in the axotomized eye, the affinity of retinal iASPP towards p53 is reduced and, as such, may contribute to tilting the fate of injured neurons towards death. This hypothesis is strengthened by our finding that phosphoserine iASPP increases in AAV.iASPP-treated retinas, further supporting the conclusion that the affinity of iASPP towards p53 is greater in retinas

overexpressing iASPP thereby blocking the apoptotic effect of p53 and enhancing cell survival.

We previously demonstrated that RGCs die in a p53-dependent manner following axonal damage and that although total p53 expression levels did not change after injury, critical post-translational modifications occurred *in vivo* (Wilson et al., 2013). We detected phosphorylation of p53 at serine 15, which has been shown to increase the ability of p53 to recruit CBP/p300 acetyltransferase (Lambert et al., 1998). However, we did not detect changes in acetylation of p53 on Lys 373 and Lys 382 soon after axotomy, which is consistent with a previous study in which neither acetylation at Lys373 nor p300 acetyltransferase levels were altered in RGCs 24 hrs after optic nerve crush (Gaub et al., 2011). Therefore, although acetylation at Lys373 and Lys382 are known to be involved in fine-tuning the p53 stress response (Krummel et al., 2005), these modifications do not appear to play a critical role in early changes associated with RGC death. In agreement with this, the loss of p53 acetylation at its C terminus by CBP/p300 was not required for p53 transactivation in an acetylation-deficient missense mutant mouse model (Krummel et al., 2005).

Along with an increased affinity towards p53, phosphorylated iASPP has been reported to reduce pro-apoptotic gene transcription (Lu et al., 2013). Indeed, iASPP phosphorylation at Ser84 and Ser113 resulted in reduced transcriptional activity of p53 targets PUMA, Bax and PIG3 compared to wild-type ASPP in melanoma cells (Lu et al., 2013). Although we did not detect a change in Bax protein levels following axotomy and AAV-iASPP administration, we cannot rule out a role for Bax in our model as Bax activation results in its translocation from the cytosol to the mitochondria, and not necessarily altered transcription rates or protein levels (Wolter et al., 1997, Schellenberg et al., 2013). We do however show that AAV-iASPP

significantly increases the levels of phosphoserine iASPP, which coincided with reduced levels of PUMA and Fas/CD95, leading to neuronal survival. Of interest, siRNA-mediated knockdown of PUMA or Fas/CD95 resulted in substantial RGC protection after optic nerve injury [18]. Decreased PUMA and Fas/CD95 expression may rescue RGCs by affecting intrinsic and extrinsic apoptotic pathways, respectively. Active PUMA, a BH3-only Bcl-2 family member and critical mediator of p53-dependent apoptosis (Jeffers et al., 2003), may act indirectly on pro-apoptotic Bcl-2 family members by relieving the inhibition imposed by anti-apoptotic members (Kim et al., 2009, Ren et al., 2010). Fas/CD95, a death receptor that triggers apoptosis when bound by Fas ligand after recruiting the adapter protein FADD (Fas-associated death domain) and pro-caspase 8 (Medema et al., 1997), is weakly expressed in the intact rodent retina (Kim and Park, 2005, Wax et al., 2008). The expression of Fas/CD95 markedly increases in the ganglion cell layer as well as microglia during glaucomatous damage (Kim and Park, 2005, Ju et al., 2006). Furthermore, FADD is upregulated in RGCs subjected to ocular hypertension (Ju et al., 2006), and FasL-expressing microglia can induce apoptotic RGC death in a spontaneous mouse glaucoma model (Gregory et al., 2011). The reduction of Fas/CD95 levels reported here following iASPP.AAV administration may effectively decrease the activation of death receptor apoptotic pathways mediated by FasL, thereby increasing RGC survival. Overall, our data suggest that iASPP expression in RGCs is required as a molecular checkpoint to ensure that p53 activity is kept low and under tight control in healthy cells. Specifically, iASPP is likely to inhibit the ability of p53 to stimulate pro-apoptotic retinal targets, including PUMA and Fas/CD95, thus preventing or attenuating p53-dependent neuronal death.

In conclusion, we identify a novel role for the highly conserved p53 inhibitor iASPP in the survival of retinal neurons subjected to axonal injury. Our findings expand our current understanding of the role of the ASPP family of p53 regulators in neurodegeneration which could prove beneficial for the design of strategies aimed at curtailing neuronal loss in the injured CNS.

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Figure 1. Adult RGCs express iASPP.

Endogenous retinal iASPP was detected by immunofluorescence in the ganglion cell layer (GCL) and inner nuclear layer (INL) (A, C, D, and G). iASPP staining in RGCs was confirmed using the RGC-specific marker RBPMS (D-F). iASPP was also detected in amacrine and horizontal cells, visualized with calretinin (H,J, arrows) and calbindin (I,J, arrows), respectively. Scale bars: (A-C) = 50 μm; (D-J) = 20 μm. PS: Photoreceptor Segments; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 2. iASPP protein and phosphoserine levels after axotomy.

Retinal iASPP expression and localization did not change at 24 hrs or 3 days after optic nerve injury compared to intact eyes (A-C). Scale bar: 10 μm. Analysis of protein homogenates from axotomized retinas collected at 6, 12, 24, 48 hrs, 3 and 5 days confirmed that iASPP levels were similar to those in intact, non-injured retinas. The lower panel represents the same blot as in the upper panel but probed with an antibody that recognizes β-actin used to confirm equal protein loading (D). Densitometric analysis of western blots, showing the ratio of iASPP protein relative to β-actin, confirmed that there is no significant change in protein levels after injury (E) (ANOVA, $p>0.05$). Phosphoserine immunoprecipitation (IP) of intact and axotomized retinas probed with iASPP antibody revealed a decrease in phosphoserine iASPP at 24 hrs after axotomy. IP of retinal homogenates with an IgG antibody was included as control for non-specific interactions (F). ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 3. Selective siRNA knockdown of iASPP exacerbates axotomy-induced RGC death.

A significant reduction of iASPP in the GCL was observed by immunohistochemistry of axotomized retinas at 24 hrs after intravitreal delivery of siRNA against iASPP (si-iASPP) compared to intact retinas, while control siRNA against GFP (siGFP) had no effect (A-C). RBPMS labeling confirmed that siRNA-mediated knockdown of iASPP occurred in RGCs (D-F). Scale bars: (A-C) = 50 μm; (D-F) = 15 μm. Western blot analysis confirmed that intravitreal delivery of si-iASPP led to marked reduction of retinal iASPP protein at 24 hrs after delivery, while siGFP had no effect (G, H; Student‟s T-test, ***=$p<0.001$). Quantitative analysis of RGC survival at one week after axotomy following intraocular injection of si-iASPP (black), or control siGFP (grey) (n=4/group, ANOVA, *=$p<0.05$). The density of RGCs in intact, uninjured Sprague-Dawley rat retinas is shown as reference (open bar). Data are expressed as the mean ± S.E.M. ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 4. Targeted overexpression of iASPP in RGCs increases iASPP phosphoserine levels post-axotomy.

AAV-mediated iASPP expression was distinguished from endogenous iASPP with an antibody against the c-myc tag encoded only in iASPP transgenes. Robust c-myc labeling was observed in the GCL of retinas that received AAV.iASPP, but not in control eyes injected with AAV.GFP (A, B). Selective expression of AAV-mediated iASPP in RGCs was confirmed using the RGC marker RBPMS (C-E). Scale bars: (A-B) = 50 μm; (C-E) = 15 μm. Immunoblotting and densitometric analyses confirmed that intravitreal delivery of AAV.iASPP led to significant overexpression of iASPP protein while AAV.GFP had no effect (F, G; Student‟s T-test, ***=$p<0.001$). Phosphoserine immunoprecipitation of retinas probed with an iASPP antibody reveals abundant iASPP phosphoserine levels in axotomized retinas (24 hrs) treated with AAV.iASPP but not with control AAV.GFP (H). PS: Photoreceptor Segments; ONL: Outer Nuclear Layer; OPL: Outer Plexiform Layer; INL: Inner Nuclear Layer; IPL: Inner Plexiform Layer; GCL: Ganglion Cell Layer.

Figure 5. AAV-mediated iASPP overexpression increases RGC survival.

Brn3a-labeled flat-mounted retinas from axotomized eyes demonstrate higher RGC densities following treatment with AAV.iASPP (A) than with AAV.GFP (B) at one week post-injury. Scale bars: 100 μm. Quantitative analysis of RGC survival following axotomy and intraocular injection of AAV.iASPP (black) or control AAV.GFP (grey) (ANOVA, ***=$p<0.001$) at one and two weeks post-injury (C). The density of RGCs in intact, uninjured Sprague-Dawley rat retinas is shown as reference (open bar). Data are expressed as the mean ± S.E.M.

Figure 6. AAV.iASPP inhibits p53 activation and downregulates retinal PUMA and Fas/CD95 levels.

Western blot analysis of axotomized retinal samples show that p53 phosphoserine15 (pSer15) levels are reduced in AAV.iASPP-treated retinas compared to control AAV.GFP at 24 hrs post-axotomy (A, C; ANOVA, *=$p<0.05$). Acetyl p53 levels remained unchanged (A, B; ANOVA, $p>0.05$). The p53 apoptotic targets PUMA and Fas/CD95 protein levels decrease in retinas treated with AAV.iASPP compared to AAV.GFP-treated control retinas (D, E, F; ANOVA, ***=$p<0.005$, **=$p<0.001$), whereas Bax and Noxa remained unchanged (D, G, H; ANOVA, $p>0.05$).

III.7. FIGURES

FIGURE 1.

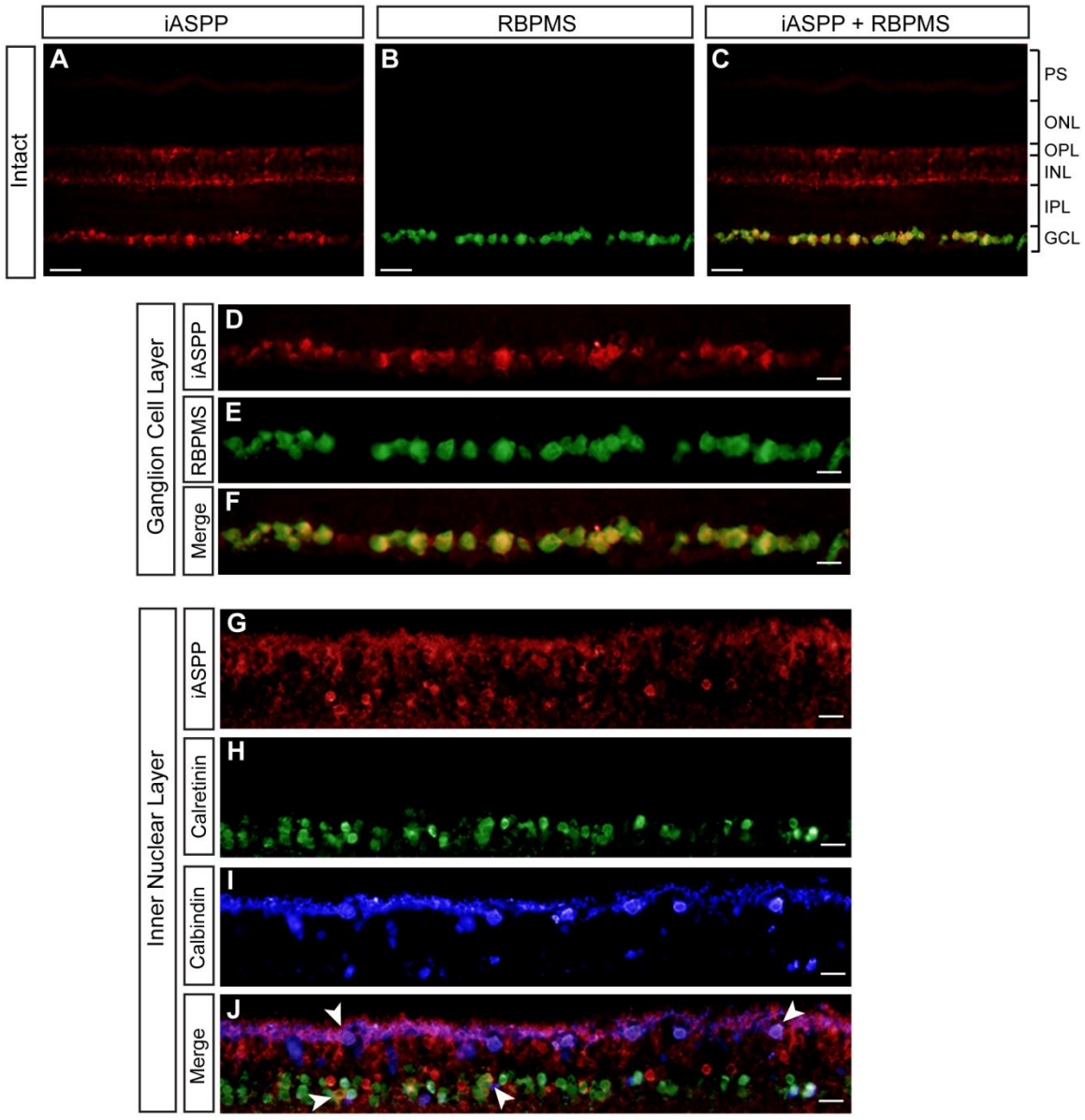


FIGURE 2.

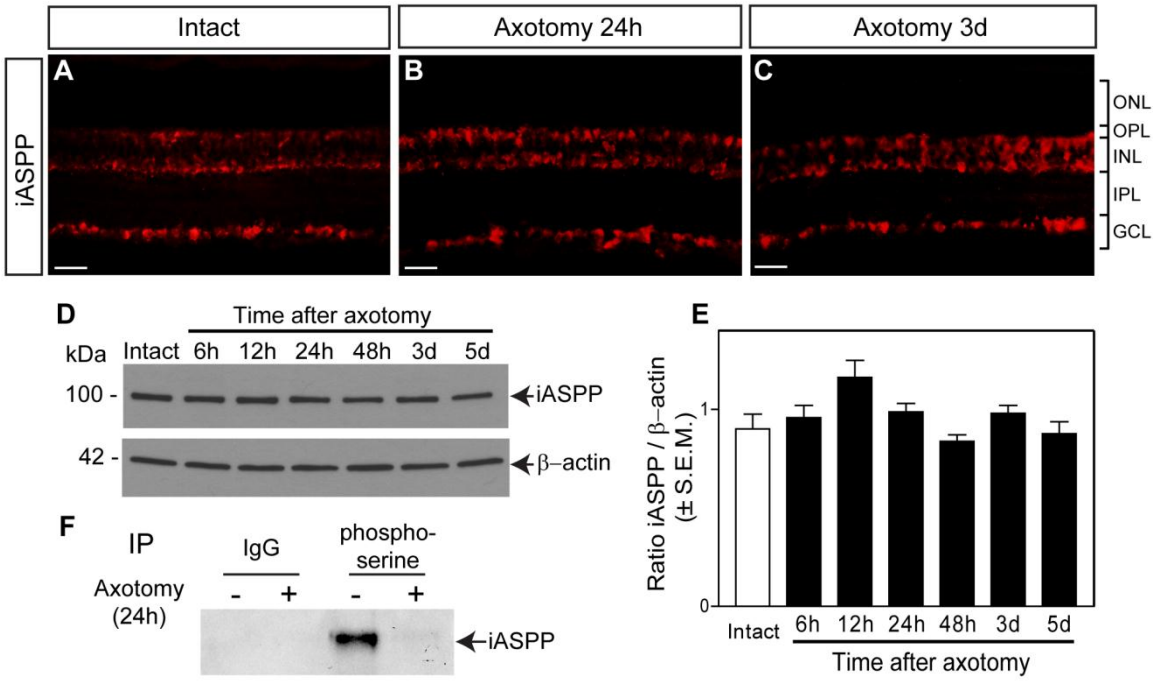

Intact
Axotomy 24h
Axotomy 3d
iASPP
A
B
C
ONL
OPL
INL
IPL
GCL
D
Time after axotomy
kDa
Intact
6h
12h
24h
48h
3d
5d
100 -
42 -
iASPP
β–actin
E
Ratio iASPP / β–actin (± S.E.M.)
1
0
Intact
6h
12h
24h
48h
3d
5d
Time after axotomy
F
IP
IgG
phospho-serine
Axotomy (24h)
-
+
-
+
iASPP

FIGURE 3.

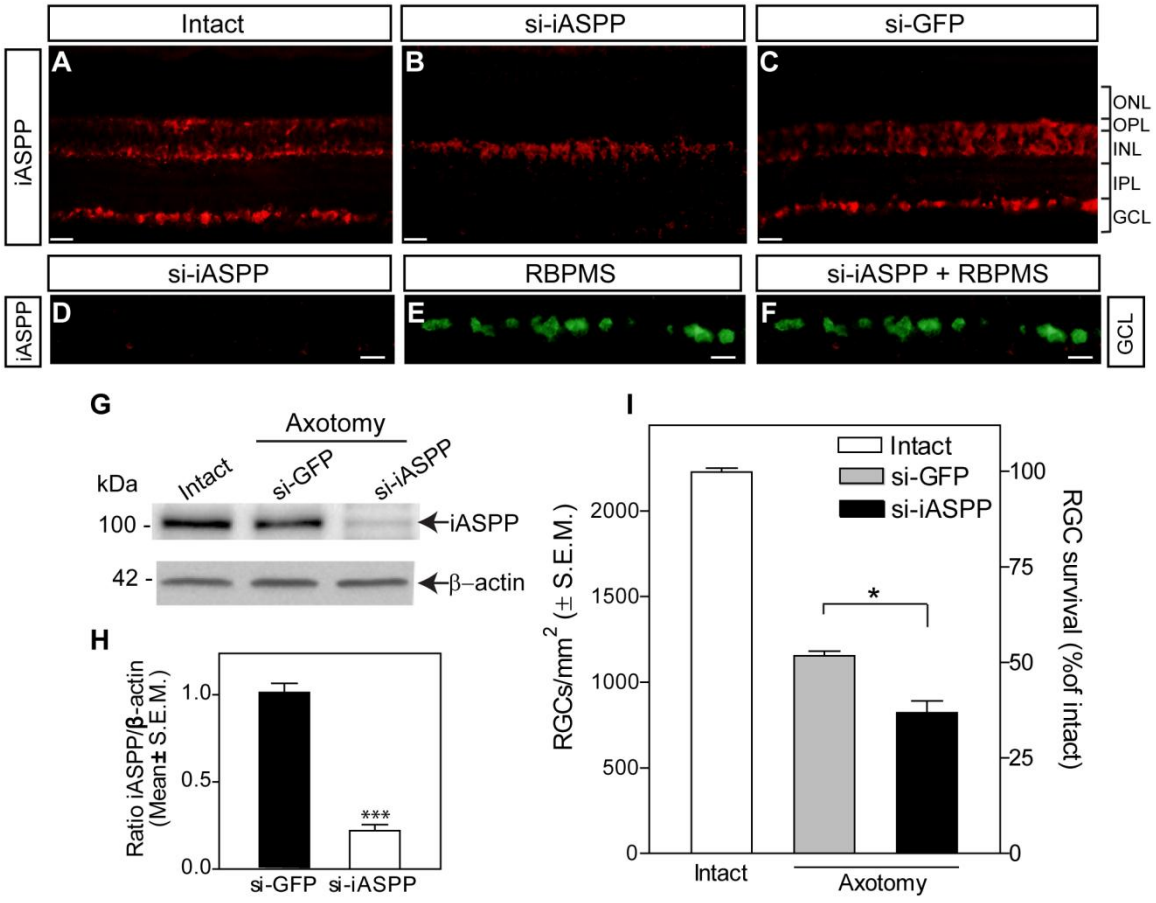

Intact
si-iASPP
si-GFP
A
B
C
iASPP
ONL
OPL
INL
IPL
GCL
si-iASPP
RBPMS
si-iASPP + RBPMS
D
E
F
iASPP
GCL
G
Axotomy
kDa
Intact
si-GFP
si-iASPP
100 -
iASPP
42 -
β–actin
H
Ratio iASPP/β-actin (Mean± S.E.M.)
1.0
0.5
0.0
***
si-GFP
si-iASPP
I
Intact
si-GFP
si-iASPP
RGCs/mm2 (± S.E.M.)
2000
1500
1000
500
0
*
100
75
50
25
0
RGC survival (%of intact)
Intact
Axotomy

FIGURE 4.

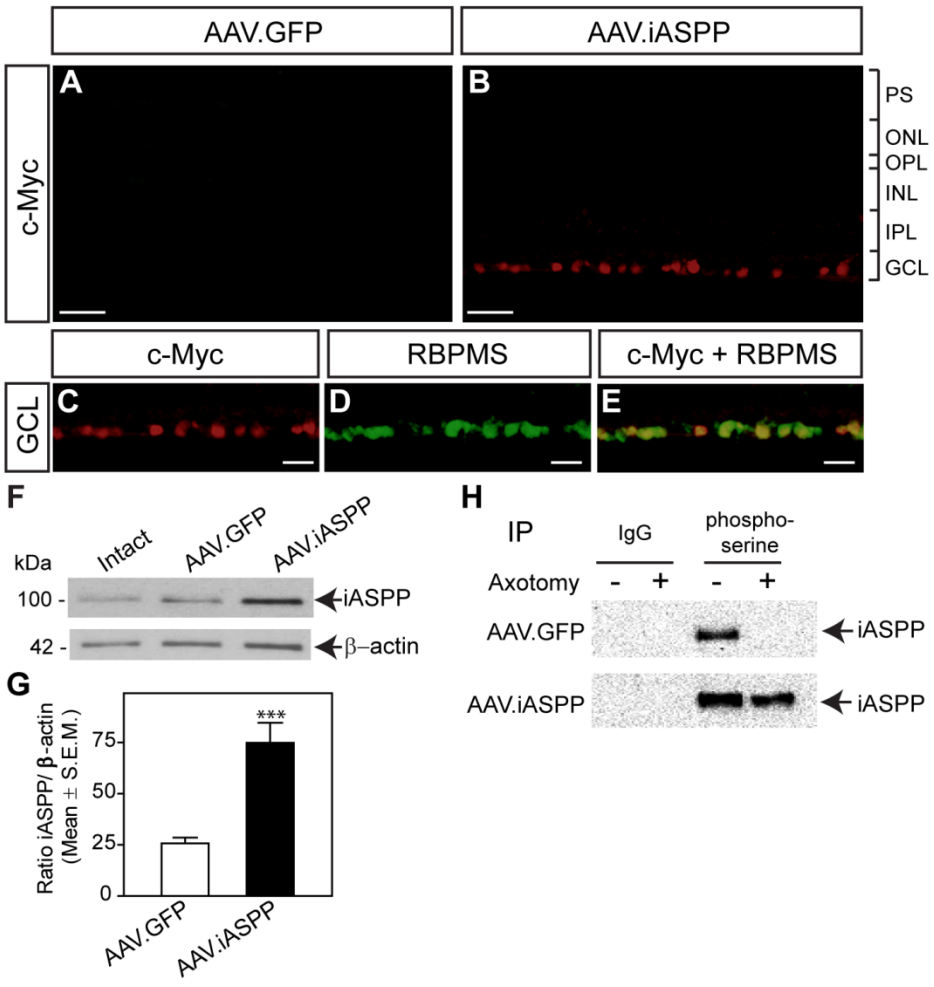

AAV.GFP
AAV.iASPP
A
B
c-Myc
PS
ONL
OPL
INL
IPL
GCL
c-Myc
RBPMS
c-Myc + RBPMS
GCL
C
D
E
F
kDa
Intact
AAV.GFP
AAV.iASPP
100 -
iASPP
42 -
β–actin
G
Ratio iASPP/ β-actin
(Mean ± S.E.M.)
75
50
25
0
***
AAV.GFP
AAV.iASPP
H
IP
IgG
phospho-
serine
Axotomy
- + - +
AAV.GFP
iASPP
AAV.iASPP
iASPP

FIGURE 5.

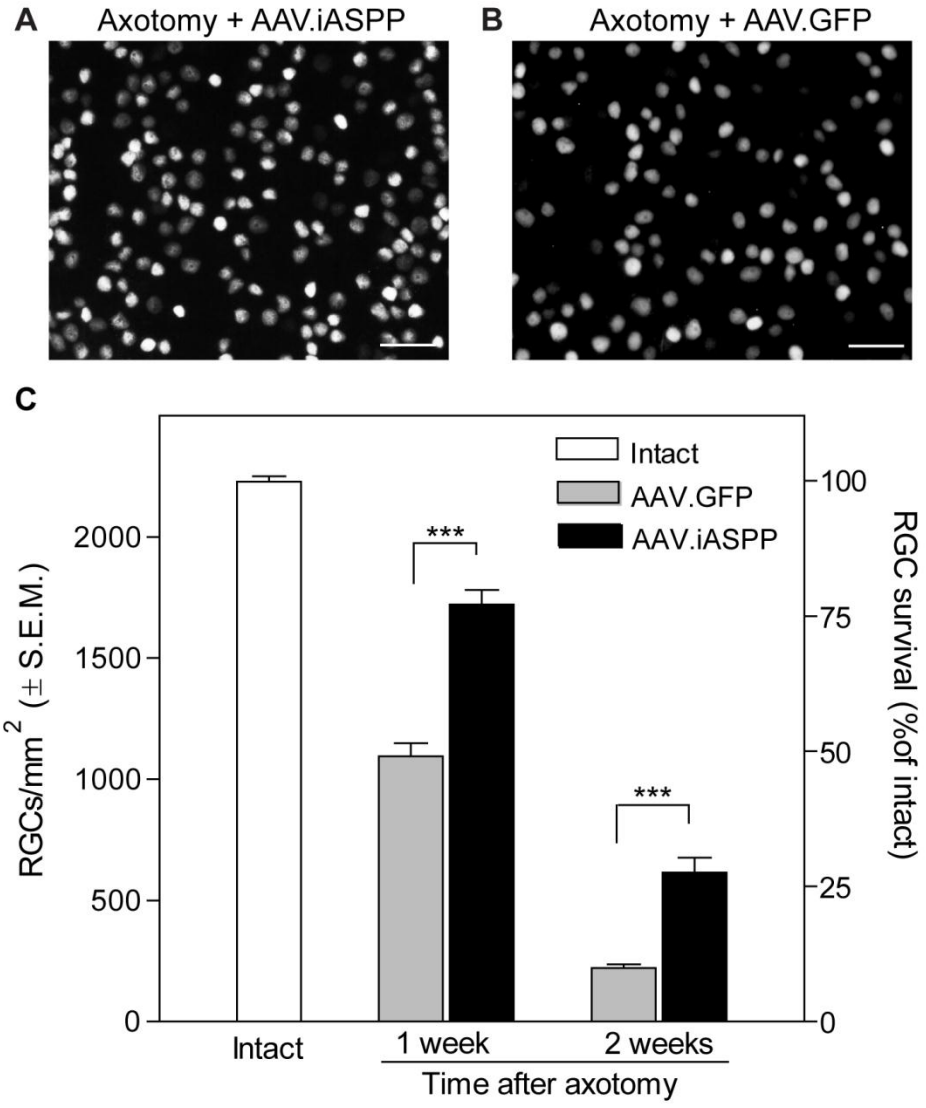
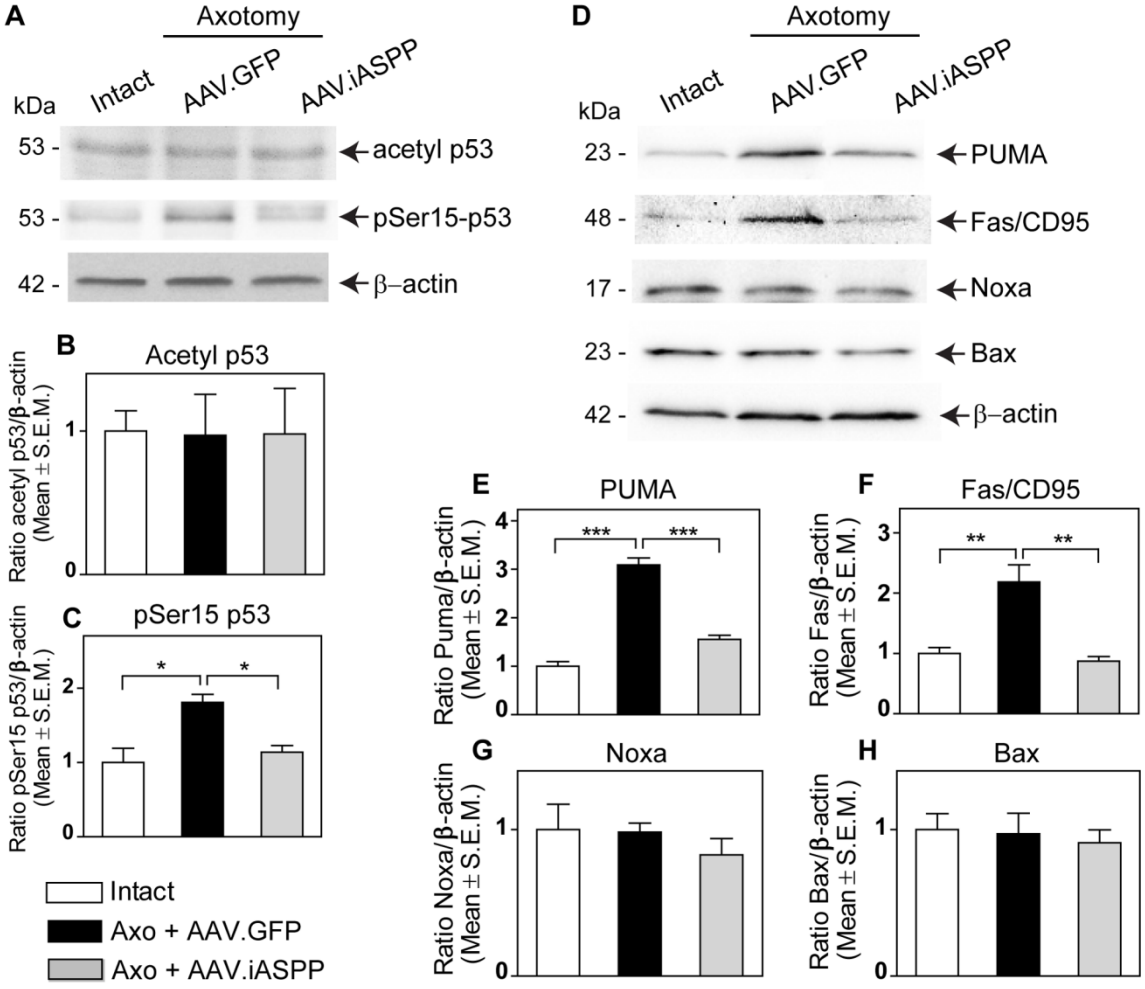

A
Axotomy + AAV.iASPP
B
Axotomy + AAV.GFP
C
Intact
AAV.GFP
AAV.iASPP
***
***
2000
1500
1000
500
0
RGCs/mm2 (± S.E.M.)
100
75
50
25
0
RGC survival (%of intact)
Intact
1 week
2 weeks
Time after axotomy

FIGURE 6.


A
Axotomy
kDa
Intact
AAV.GFP
AAV.iASPP
53 -
acetyl p53
53 -
pSer15-p53
42 -
β–actin
B
Acetyl p53
Ratio acetyl p53/β-actin (Mean ± S.E.M.)
C
pSer15 p53
Ratio pSer15 p53/β-actin (Mean ± S.E.M.)
Intact
Axo + AAV.GFP
Axo + AAV.iASPP
D
Axotomy
kDa
Intact
AAV.GFP
AAV.iASPP
23 -
PUMA
48 -
Fas/CD95
17 -
Noxa
23 -
Bax
42 -
β–actin
E
PUMA
Ratio Puma/β-actin (Mean ± S.E.M.)
F
Fas/CD95
Ratio Fas/β-actin (Mean ± S.E.M.)
G
Noxa
Ratio Noxa/β-actin (Mean ± S.E.M.)
H
Bax
Ratio Bax/β-actin (Mean ± S.E.M.)

CHAPTER 4

IV. GENERAL DISCUSSION

IV.1. THE ROLE OF P53 IN RGC APOPTOTIC DEATH

In the first article presented in this thesis (Chapter 2), we explored the role of p53 regulators ASPP1 and ASPP2 in apoptotic death of RGCs. We first assessed whether p53 was implicated in axotomy-induced apoptotic RGC death. Heretofore, the role of p53 in apoptotic death of RGCs had never been clearly demonstrated. Although it was surmised that p53 played a key role in the apoptosis of RGCs in glaucoma (Nickells, 1999), and despite the fact p53 implication had been demonstrated in NMDA-induced excitotoxic and hypoxic/ischemic death of RGCs (Joo et al., 1999, Li et al., 2002), its role had not been clearly shown in an apoptotic death model. As p53 protein and mRNA levels are not significantly upregulated following optic nerve injury or in glaucomatous models (Levkovitch-Verbin, 2006), the role of p53 was not as evident as in excitotoxic or hypoxic/ischemic paradigms. We showed upregulation of p53 phosphoserine 15, a key apoptotic p53 post-translational modification site, in RGCs following optic nerve axotomy. We also showed marked increase of p53 downstream targets Fas/CD95 and PUMA following axotomy. Most importantly, optic nerve axotomy in p53 knockout, heterozygote and wild-type mice revealed a tight correlation between p53 gene expression and RGC survival. Indeed, increased RGC survival was observed in p53 heterozygote and knockout mice compared to wild type mice. The survival rate of RGCs in $p53^{-/-}$ following optic nerve lesion was not 100%, but 73%, demonstrating that although p53 is an important contributor to apoptotic RGC death, it is not the sole pathway involved.

IV.2. THE ROLE OF ASPP FAMILY MEMBERS IN RGC SURVIVAL

IV.2.1. Retinal expression pattern of ASPP family members

Our findings presented in Chapters 2 and 3 revealed that pro-apoptotic ASPP1 and ASPP2 are solely expressed in the ganglion cell layer in the adult rat retina, whereas anti-apoptotic iASPP is found in the ganglion cell layer as well as in the inner nuclear layer. Interestingly, it is the expression pattern of iASPP that most closely resembles that of p53 mRNA. Indeed, although p53 protein is not detectable in the intact adult retina, in situ hybridization revealed that p53 mRNA is expressed in the inner nuclear layer and ganglion cell layer of adult rats (Shin et al., 1999). Furthermore, other p53 interacting proteins have been shown to have similar expression patterns to ASPP1/2 or iASPP. Pro-apoptotic Bax mRNA is solely expressed in the ganglion cell layer (Shin et al., 1999), similarly to ASPP1/2. Anti-apoptotic Bcl-2 mRNA is expressed in the inner nuclear layer and in the ganglion cell layer (Shin et al., 1999), similar to iASPP. It would, however, be oversimplifying to state that pro-apoptotic and anti-apoptotic p53-interacting proteins have distinct expression patterns, as p53 interacting proteins Brn3a and Brn3b, both confined to the ganglion cell layer (Nadal-Nicolas et al., 2012), have both anti-apoptotic and pro-apoptotic roles, respectively (Budhram Mahadeo et al., 2006).

The presence of both pro-apoptotic and anti-apoptotic proteins in the ganglion cell layer of an intact retina fits the hypothesis that apoptosis is a default pathway that needs to be kept under control to allow cell survival. Thus, the levels of iASPP in the ganglion cell layer might be necessary to promote neuronal survival because iASPP inhibits p53 from binding to ASPP1 and ASPP2, also expressed in RGCs, thus blocking the apoptotic cascade.

IV.2.2. Putative role of ASPP family members in ocular neuropathies

In an ischemic model, p53 upregulation was detected in the inner nuclear layer as well as the ganglion cell layer after a 60-minute period of ischemia (Rosenbaum, 1998). Following ischemic insults, neurons in the inner nuclear layer and ganglion cell layer undergo necrotic and apoptotic degeneration (Joo et al., 1999). Of interest, the necroptotic protein RIP3, which switches TNF-α induced cell death from apoptosis to necrosis (Zhang et al., 2009), has a similar expression pattern to iASPP as it was also found in amacrine and horizontal cells as well as in the ganglion cell layer (Huang et al., 2013). As p53 is implicated in necrosis in addition to apoptosis (Vaseva et al., 2012), it is possible that iASPP may also regulate necrosis. Consistent with this, downregulation of iASPP by siRNA resulted in an increase of apoptosis and necrosis in an MCF-7 breast cancer cell line expressing wild-type p53 (Wang, 2010). Could iASPP be involved in the necrotic events occurring in the ischemic retina? Further investigation of iASPP in ocular neuropathies is warranted. It would be of great interest to assess the implication of iASPP in glaucoma. Indeed, iASPP is an inhibitor of NF-kβ, a transcription factor commonly upregulated in glaucoma. Therefore, the effect of NF-kβ inhibition by iASPP in glaucoma would be highly relevant. Of interest, a cohort of POAG patients were associated with p53-PRO, a *TP53* sequence polymorphism resulting in a proline at amino-acid 72 position in the p53 protein (Wiggs et al., 1998). Normally, iASPP preferentially inhibits the p53-PRO form and not the arginine variant p53-ARG (Bergamaschi et al., 2006), and yet in this group of glaucoma patients the apoptotic activity of p53-PRO was enhanced (Wiggs et al., 1998). It would be of interest to assess if this enhancement of p53-PRO activity is due to a reduction in iASPP activity.

The roles of ASPP1 and ASPP2 should also be assessed in glaucoma, as they might play novel roles in disease progression. ASPP2 can directly interact and inhibit pro-survival Bcl-2 and Bcl-XL (Katz et al., 2008), and, interestingly, the levels of Bcl-2 and Bcl-XL are significantly reduced in models of glaucoma and optic nerve lesion, but not following excitotoxic injury (Levkovitch Verbin et al., 2010, Levkovitch Verbin et al., 2013). It would be useful to assess whether ASPP2 levels are upregulated in animal models of ocular hypertension and in human glaucoma.

IV. 2.3. Regulation of ASPP family apoptotic activity by phosphorylation

The most common type of post-translational modification in signal transduction is protein phosphorylation (reviewed in (Ubersax and Ferrell, 2007)). It has been estimated that 30% of all cellular proteins are phosphorylated on at least one residue (Pinna and Ruzzene, 1996, Cohen, 2000). ASPP1, ASPP2 and iASPP belong to this family of proteins whose functions can be regulated by the addition of a γ-phosphate group, as the phosphorylation of ASPP family members have been shown to play a key role in their apoptotic activity. Indeed, the phosphorylation of ASPP1 and ASPP2 increases their pro-apoptotic activity, whereas iASPP phosphorylation enhances its anti-apoptotic activity (Aylon et al., 2010, Godin Heymann et al., 2013, Lu et al., 2013). It was shown that ASPP1 can be phosphorylated by the tumor suppressor Large Tumor Suppressor 2 (Lats2), which increases p53 binding to pro-apoptotic Fas/CD95, PUMA and Bax protomoters (Aylon et al., 2010). Futhermore, ASPP2 was recently shown to be phosphorylated by Ras/MAPK, and this serine phosphorylation event enhances ASPP2 pro-apoptotic activity by upregulating p53-mediated transactivation of Bax (Godin Heymann et al., 2013). iASPP anti-apoptotic activity is also regulated by its phosphorylation status, as iASPP phosphorylation by Cyclin B1/CDK1 increases its affinity

towards p53 (Lu et al., 2013). We demonstrated that iASPP phosphorylation status is affected by optic nerve injury in Chapter 3 of this thesis. As apoptotis-specific phosphorylation events abound (Dix et al., 2012), it would be interesting to assess whether ASPP proteins belong to the apoptotic phosphoproteome of glaucoma.

IV.2.4. Summary of the role of ASPP family members in RGC apoptosis

The ASPP family is comprised of both initiators and inhibitors of cell death, similarly to other gene families expressing members with dichotomous roles such as the Bcl-2 superfamily and the TNF superfamily (Gross et al., 1999a, Aggarwal, 2003). Indeed, these families express both pro-apoptotic and anti-apoptotic members. Families of genes including members with distinct and opposing functions allows for careful orchestration of cell fate. Indeed, the cell‟s ability to regulate the expression levels of anti-apoptotic and pro-apoptotic proteins assures a checkpoint in the switch between cell survival and programmed cell death pathways. Furthermore, protein family members have similar protein domains thus similar ligand binding sites, and by acting on the same proteins the inhibitory family members can counteract the pro-apoptotic actions of their family members in a targeted manner. They help maintain the balance between cell life and cell death via competitive interactions with each other.

The roles of ASPP family members ASPP1, ASPP2 and iASPP were assessed in the intact and injured retina, revealing a novel role for these proteins in the apoptotic pathway induced by axonal damage after optic nerve axotomy. Figure 8 summarizes our model of ASPP family pathways implicated in intact and apoptotic events occurring in RGCs following optic nerve lesion.

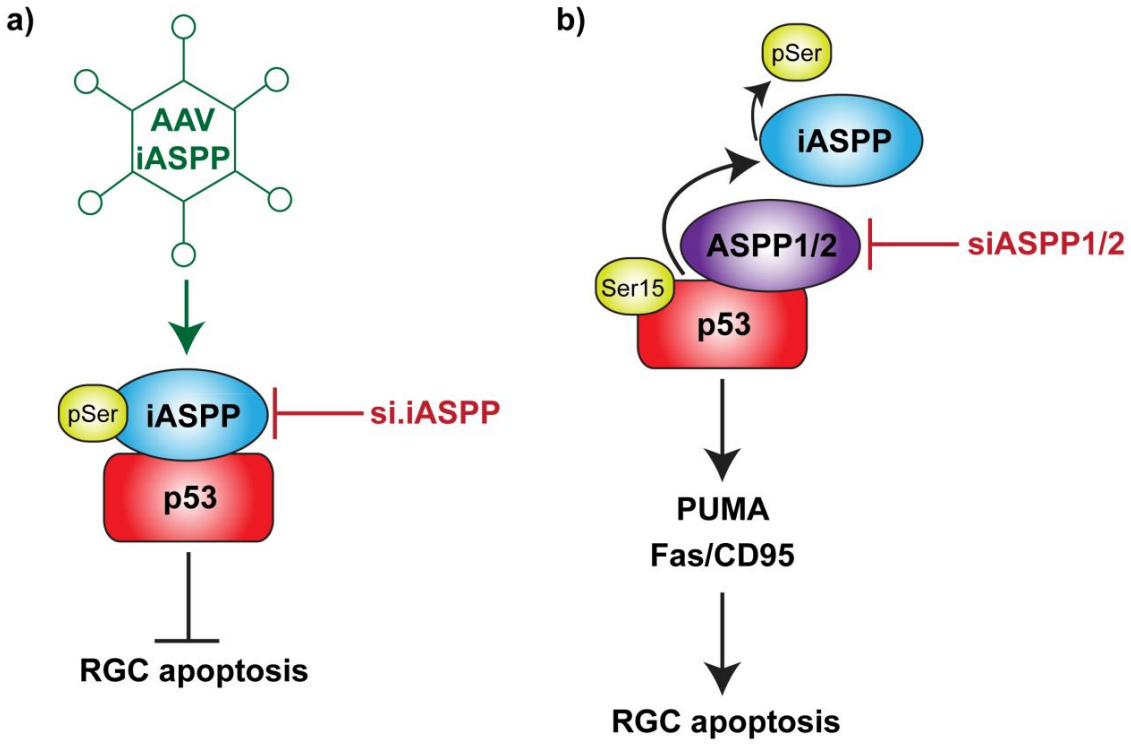


Figure 8. Model of ASPP family signaling in intact and injured RGCs. a) In the intact retina, the phosphorylation of iASPP at one or more serine sites promotes iASPP-p53 interaction, inhibiting p53 apoptotic function. iASPP overexpression by AAV (green arrow) in the axotomized retina promotes serine phosphorylation of iASPP and consequently inhibits p53 apoptotic function, increasing RGC survival. Knockdown of iASPP by siRNA (red arrow) exarcerbates RGC death. b) In the axotomized retina, p53 is phosphorylated at its Ser15 site, a key apoptotic activation site. iASPP serine phosphorylation is lost and iASPP is displaced by pro-apoptotic ASPP1/2. Pro-apoptotic p53 downstream targets PUMA and Fas/CD95 are upregulated, resulting in RGC death. Knockdown of ASPP1 and ASPP2 by siRNA (red arrow) reduces PUMA and Fas/CD95 activation levels and increases RGC survival. (Image generated by Ariel Wilson).

IV.3. NOVEL ROLE OF PUMA IN RGC APOPTOSIS

In the first chapter of this thesis, we uncovered a role for PUMA in RGC apoptosis. PUMA is a BH3-only Bcl-2 family member and a critical mediator of p53-dependent apoptosis (Jeffers et al., 2003). It is known that mitochondrial apoptotic signaling pathways are critically dependent on the interaction between BH3-only proteins and their pro-survival counterparts (Youle and Strasser, 2008). Relevantly, other Bcl-2 family members have been shown to play an important role in RGC survival, such as Bax (Isenmann et al., 1999, Semaan et al., 2010), Bim (McKernan and Cotter, 2007), Bcl-XL (Levkovitch Verbin et al., 2013) and Bcl-2 (Levkovitch Verbin et al., 2013). Furthermore, PUMA promotes neuronal death induced by trophic factor deprivation, endoplasmic reticulum stress, ischemia and oxidative stress (Reimertz et al., 2003, Steckley et al., 2007, Niizuma et al., 2009, Galehdar et al., 2010, Ambacher et al., 2012). The data shown in this thesis allow us to now add axonal injury to the list of cellular stresses leading to PUMA activation and neuronal death in the CNS.

PUMA acts indirectly on the Bcl-2 family members Bax and/or Bak by relieving the inhibition imposed by antiapoptotic members. Bax is an essential effector responsible for the mitochondrial outer membrane permeabilization, and requires activation by PUMA (Kim et al., 2009, Ren et al., 2010). PUMA thus liberates activated Bax to translocate to the mitochondrial membrane and mediate the release of AIF, SMAC and cytochrome C, leading to caspase activation. As PUMA knockdown by siRNA reduced cytokine-induced Bax activation and translocation to the mitochondria (Gurzov et al., 2010), we surmise that reduced levels of PUMA observed in the context of iASPP overexpression and axotomy could diminish Bax activation levels following optic nerve injury, tilting the fate of injured RGC towards survival. Therefore, although we did not detect modulations of Bax levels following

AAV.iASPP administration and axotomy, Bax apoptotic activity could indirectly be affected by PUMA downregulation.

Furthermore, PUMA has the ability of disrupting the interaction between cytosolic p53 and Bcl-XL, an anti-apoptotic Bcl-2 family member present in the GCL (Medearis et al., 2011). This p53-Bcl-XL disruption by PUMA liberates p53 to directly activate pro-apoptotic Bcl-2 members Bax and Bak (Follis et al., 2013). It is of interest that the pro-apoptotic role of PUMA uncovered in an optic nerve axotomy model may be implicated in glaucomatous neurodegeneration, as siRNA or AAV-mediated regulation of the ASPP proteins influences PUMA protein levels, and therefore activity.

IV.4. USE OF AAV AND SIRNA TO REGULATE RGC SURVIVAL IN VIVO

The robust RGC survival rates following administration of siRNA to knockdown pro-apoptotic ASPP1 or ASPP2, or administration of AAV encoding iASPP to overexpress anti-apoptotic iASPP reflects the neuroprotective possibilities afforded by gene therapy tools. Although many studies target the apoptotic pathway with the common goal of rescuing RGCs from apoptotic death, the use of AAV and siRNA in our studies had the additional advantage of allowing the identification of the function of ASPP family members in axotomy-induced RGC death.

Our findings highlight the advantages of using gene therapy strategies that target multiple anti-apoptotic pathways, as this appears to be a promising avenue to increase RGC survival after axonal damage. One such pathway is neurotrophin-regulated signaling. There is substantial evidence that neurotrophic factors suppress the intrinsic apoptotic cascade by activating intracellular survival signals. Upon binding to their cognate tyrosine kinase

receptors, most neurotrophic factors stimulate two primary pro-survival signaling pathways: the extracellular signal-regulated kinase 1/2 (ERK1/2) and the phosphatidylinositol-3 kinase pathways (Kaplan and Miller, 2000). Some factors, such as CNTF, can also activate the Janus kinase/signal transducer and activator of transcription 3 (Peterson et al., 2000). AAV-mediated gene transfer of constitutively active extracellular signal-regulated kinase kinase 1 (ERKK1/2), the obligate upstream activator of ERK1/2, resulted in robust survival rates of RGCs in both axotomy and ocular hypertension models of optic nerve injury (Pernet et al., 2005, Zhou et al., 2005).

In contrast to protein tyrosine kinases that promote neuronal survival, there are several pro-apoptotic protein kinases that contribute to RGC death, such as the c-Jun N-terminal kinase (Bessero et al., 2010). c-Jun, activated by c-Jun N-terminal kinase phosphorylation, mediates transcription of pro-apoptotic genes and has been shown to be upregulated in rat and monkey models of glaucoma (Hashimoto et al., 2005, Levkovitch-Verbin et al., 2005). siRNA-mediated gene expression knockdown of c-Jun in RGCs resulted in a threefold increase in RGC survival after optic nerve lesion (Lingor et al., 2005). These studies support our findings that administration of siRNA or AAV may be beneficial to modulate the activity of signaling intermediaries and promote RGC neuroprotection after injury.

The intravitreal injection of siRNA or AAV might be considered as of non-selective delivery of gene therapy, as the injected genetic material is diffused into the vitreous chamber of the eye to reach the retina. The main caveats of this approach are the need to increase the concentration of the injected siRNA or AAV to assure that an adequate quantity reaches the retina, and that it might produce undesirable effects on other ocular tissues, however the AAV

promoter assures only the targeted cells will express the transgene. The advantage of this approach is that it results in diffuse distribution of siRNA or AAV across the retina.

The therapeutic potential of viral vectors as a treatment for glaucoma has mainly focused on adeno-associated vectors as opposed to retroviruses, lentiviruses, herpes simplex viruses or adenoviruses. Insofar as the main limitation of AAVs is a relatively small packaging capacity, the main advantage is that AAVs are non-inflammatory and non-pathogenic (Thomas et al., 2003, Wilson and Di Polo, 2012). Other viral vectors are either less efficient at transducing RGCs, elicit immunogenic responses, or lack clinical safety data (Balaggan and Ali, 2012). There is currently substantial clinical experience with AAV subretinal injections to target photoreceptors and retinal pigmented epithelial cells, as well as pre-clinical experience with AAV intravitreal injections to target RGCs. Indeed, AAV subretinal injections were tested in human patients in a clinical trial of Leber‟s Congenital Amaurosis (LCA) (Cideciyan et al., 2009, Cideciyan, 2010, Jacobson et al., 2012, Cideciyan et al., 2013) and resulted in improved vision. Not to be confused with LCA, Leber Hereditary Optic Neuropathy (LHON), also described by Theodore Leber, is another ocular pathology for which AAV therapy was tested. LHON is associated with mitochondrial DNA mutations including the nicotidamide adenine dinucleotide dehydrogenase subunit IV (ND4) gene (reviewed in (Yen et al., 2006)). In pre-clinical studies, RGCs were targeted by intravitreal injection of AAVs encoding ND4. Intravitreal injection of ND4.AAV in rodent, nonhuman primate and ex vivo human eyes resulted in expression of ND4 in RGCs in the ex vivo human eye, rescue of the Leber Hereditary Optic Neuropathy (LHON) mouse model, and no serious adverse reactions in the primates followed up for 3 months (Koilkonda et al., 2014). Clinical testing of an intravitreal injection of AAV.ND4 in LHON patients with mutated ND4 is planned (Lam et al., 2014).

IV.5. GENERAL CONCLUSIONS

The medical need for neuroprotective therapies for glaucoma is undeniable. In the last 10 years, there has been tremendous progress in the use of gene therapy strategies not only to understand the molecular basis of RGC death, but also to stimulate the survival and regeneration of these neurons in a variety of preclinical models of optic nerve injury. The work presented in this thesis introduces the novel role of the ASPP pathway in RGC apoptotic death induced by optic nerve axotomy. We used nonviral and viral approaches to modulate RGC gene expression: siRNA, which is a novel approach in the retinal field, and an AAV-2 vector which has the ability to mediate infection of a large number of adult RGCs *in vivo* with relatively high specificity. The ASPP family represent a key molecular pathway that dictates the fate of RGCs after optic nerve injury. ASPP1, ASPP2 and iASPP are candidate molecules that have emerged as promising therapeutic targets, and further investigation into their role in glaucoma will determine if targeting this pathway is a promising avenue for several optic neuropathies including glaucoma.

Current glaucoma therapies involve the IOP lowering interventions, including medicated eye drops, laser treatment or surgery. IOP remains the only modifiable and clinically validated risk factor that has been shown to slow the progression of glaucomatous loss of vision (Chang and Goldberg, 2012). But lowering IOP is only partially effective and does not address the underlying susceptibility of RGCs to glaucomatous death. Gene therapy is a potential therapeutic approach that is inching closer to reality in the treatment of glaucoma (Borras, 2012). As ASPP proteins were initially discovered and characterized in cancer cells, cells whose death – and not survival – is the ultimate goal, current drug design strategies are

not targeting pro-apoptotic ASPP1/2, but anti-apoptotic iASPP. Peptide inhibitors of iASPP have been patented (Lu et al., 2008), but no pharmaceutical inhibitors of ASPP1 or ASPP2 have been designed. Our findings therefore suggest new targets for gene expression and gene silencing approaches. A caveat to testing siRNAs in glaucomatous eyes is that Toll-Like-Receptor (TLR) signaling is upregulated in human glaucoma (Luo et al., 2010), and siRNAs of sizes over 21 nucleotides have been shown to activate TLR3, resulting in off-target effects including retinal degeneration (Kleinman et al., 2012). The ASPP siRNAs used in our studies were of 19-20 nucleotides in length, below the threshold of TLR activation.

Another important issue relates to the effect of long-term modification of gene expression, such as that provided by AAV, in glaucoma. For example, neurotrophic factor supplementation by gene therapy is a promising strategy to promote RGC survival in several models of optic nerve damage, but the long-term effect and safety of increased diffusible neurotrophin levels in the retina are currently unknown. Sustained AAV-mediated expression of CNTF or BDNF leads to changes in the dendritic structure of transduced RGCs (Harvey et al., 2009). Therefore, if administration of AAV.iASPP is neuroprotective in glaucoma, it will be important to establish whether the dendritic structure of RGCs is modified, and whether these morphological changes have a tangible effect on RGC electrophysiological function.

Whether ASPP proteins are modulated in human glaucoma is unknown, and whether they are acting in other ocular tissues remains to be seen. If the ASPP family is implicated in other neurodegenerative diseases, our work will be relevant as it demonstrated the potential of ASPP regulation by AAV or siRNA to promote neuronal survival. In conclusion, the studies presented in this thesis have demonstrated that regulation of ASPP proteins is beneficial for

neuroprotection of injured RGCs, and we delineated their molecular pathways. The challenge now is to assess how this knowledge can be translated into potential gene therapies for neuroprotection in glaucoma.

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APPENDIX A

CONTRIBUTION TO THE ARTICLES

For the article **"ASPP1/2 Regulate p53-Dependent Death of Retinal Ganglion Cells through PUMA and Fas/CD95 Activation *in Vivo*"** (Chapter 2 in this thesis), I played a key role in the acquisition, analysis and interpretation of the data. My supervisor was responsible for the conception of the study. We wrote the article together. The study was principally performed on Sprague Dawley rats, with the exception of a few transgenic mice. I performed all the rat surgeries (retrograde Fluorogold labelling, intraocular injections, optic nerve axotomies) as well as the immunohistochemistry experiments, western blotting and RGC survival quantification on rat and mice retinas. The laboratory of Dr. Philip Barker provided the reverse transcription PCR results, representing 1 of the 61 panels comprising the 7 figures of this paper. The laboratory of Dr. Bernier performed the qPCR results, representing 5 of the 61 panels of the article. My colleague Barbara Morquette performed surgeries on the transgenic mice, and her contribution appears on 2 of the 61 pannels of the article. I provided the data for 54 of the 61 pannels of the article, and on the 7 remaining panels I performed the animal surgeries or survival quantification. I performed all the statistical analyses and worked alongside my supervisor on the data interpretation.

For the article **"Inhibitor of Apoptosis-Stimulating Protein of p53 (iASPP) is Required for Neuronal Survival After Axonal Injury"** (Chapter 3 in this thesis), I played a key role in the acquisition, analysis and interpretation of the data. My supervisor was responsible for the conception of the study. We wrote the article together. I performed all the animal surgeries as well as the immunohistochemistries, westerns and survival quantifications. The laboratory of Dr. Hauswirth provided us with the iASPP.AAV, and the laboratory of Dr. Brecha provided us with the RBPMS antibody. I performed all the statistical analysis and worked alongside my supervisor on the data interpretation.

APPENDIX B

PLOS ONE ACADEMIC EDITOR RESPONSE TO ARTICLE SUBMISSION

PONE-D-14-00867
Inhibitor of Apoptosis-Stimulating Protein of p53 (iASPP) is Required for Neuronal Survival After Axonal Injury.
PLOS ONE

Dear Dr. Di Polo,

Thank you for submitting your manuscript for review to PLOS ONE. After careful consideration, we feel that your manuscript will likely be suitable for publication if it is revised to address the points below. Therefore, my decision is "**Minor Revision**."

We invite you to submit a revised version of the manuscript that addresses the following points:

The authors should include some technical clarifications about the choice of antibodies and the extent of AAV transduction and siRNA transfection in the retina. The discussion should address the main questions from reviewer 1 and questions 3 from reviewer 1. Additional references about the rate of RGC death after optic nerve axotomy should be included.

We encourage you to submit your revision within forty-five days of the date of this decision.

When your files are ready, please submit your revision by logging on to http://pone.edmgr.com/ and following the Submissions Needing Revision link. Do not submit a revised manuscript as a new submission. Before uploading, you should proofread your manuscript very closely for mistakes and grammatical errors. Should your manuscript be accepted for publication, you may not have another chance to make corrections as we do not offer pre-publication proofs.

If you would like to make changes to your financial disclosure, please include your updated statement in your cover letter.
In addition, when submitting your revision please include the following items:

* A rebuttal letter that responds to each point brought up by the academic editor and reviewer(s). This letter should be uploaded as a 'Response to Reviewers' file.
* A clean revised manuscript as your 'Manuscript' file.
* A marked-up copy of the changes made from the previous article file as a 'Revised

Manuscript with Track Changes' file.

If you choose not to submit a revision, please notify us.

Yours sincerely,

Pedro Gonzalez, Ph.D.
Academic Editor
PLOS ONE