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P53 Target Genes in the Regulation of Neuronal Apoptosis

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P53 Target Genes in the Regulation of Neuronal Cell Death

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ABSTRACT

The role of the tumour suppressor p53 in neuronal cell death following acute injury is well documented. However, the transcriptional targets upregulated by this transcription factor and required to mediate its apoptotic function have not been identified. Although Bax has previously been reported as a direct target for p53, its expression levels are not elevated in neurons. I set out to find key p53 targets involved in injury-induced apoptosis in neurons. Using data generated from DNA microarray analysis of total RNA isolated from neurons undergoing p53-induced apoptosis as the basis for my search, I proceeded to identify two novel p53 target genes. The first of these was Apaf1, a gene crucial for the activation of caspases via the mitochondrial apoptotic pathway. Apaf1 was upregulated in several models of p53-mediated neuronal cell death including the *in vivo* model of middle cerebral artery occlusion. Electrophoretic mobility shift assay revealed that p53 could directly bind to two p53 consensus binding sites located within the Apaf1 promoter, and luciferase reporter assays using Apaf1 promoter-luciferase constructs indicated that p53 could directly activate the Apaf1 promoter via both p53 sites. In the absence of Apaf1, neurons were significantly more resistant to apoptosis induced by DNA damage as compared to their wildtype counterparts. These results established Apaf1 as an important transcriptional target for p53 in the regulation of neuronal apoptosis. The second gene of interest was the proapoptotic gene SIVA. SIVA mRNA was induced in models of p53-dependent apoptosis in cultured neurons as well as in stroke injury *in vivo*. Its expression was sufficient to induce apoptosis in cultured neurons. Analysis of the SIVA gene identified two p53 consensus binding sites located within intron 1 along with two E2F sites within the SIVA promoter. Electrophoretic mobility shift assay demonstrated that

both transcription factors could bind to their respective consensus binding sites, and luciferase reporter assays indicated that E2F and p53 could induce activity from the SIVA promoter. These findings indicated that the proapoptotic gene, SIVA, was a direct transcriptional target for both p53 and E2F1. Altogether, my research identified two novel transcriptional targets for p53 in the modulation of neuronal apoptosis.

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LIST OF ABBREVIATIONS

53BP	p53 binding protein
Aβ	amyloid β
Ab	antibody
Ad	adenovirus
AD	activation domain
AD	Alzheimer's disease
ADP	adenosine di-phosphate
AIF	apoptosis inducing factor
ALDH4	aldehyde dehydrogenase 4
ALS	amyotrophic lateral sclerosis
Apaf1	apoptotic protease activating factor 1
APO1	apoptosis antigen-1
araC	cytosine arabinoside
ARF	alternate reading frame
ASPP	apoptotic-stimulating proteins of p53
ATM	ataxia telangiectasia mutated
ATP	adenosine tri-phosphate
ATR	ataxia telangiectasia mutated
BAI1	brain angiogenesis inhibitor 1
Bcl	B-cell lymphoma
BH3	Bcl-2 homology domain 3

B-gal	beta-galactosidase
BS	binding sequence
BSA	bovine serum albumin
c	complementary
C	contralateral
C12orf15	chromosome12 open reading frame 15
c-Myb	cellular homolog avian myeloblastosis virus
C-terminal	carboxyl terminal
CABC1	chaperone-activity of bc1 complex in S. pombe (ABC1)-like
Campto	camptothecin
CARM1	co-activator-associated arginine methyltransferase 1
Casp3	caspase 3
Casp7	caspase 7
CBP	cyclic AMP response element-binding (CREB) binding protein
CBS	consensus binding sequence
CDK	cyclin-dependent kinase
Chk	checkpoint kinase
CK	casein kinase
CLIC	chloride intracellular channel
CMV	cytomegalovirus
CNS	central nervous system
COL18A1	precursor for the antiangiogenic factor endostatin
Comp	competition

COP1	constitutively photomorphogenic 1
COX2	cyclooxygenase 2
cpm	counts per minute
d	deoxy
DA	dalton
DBD	DNA binding domain
DcR	decoy receptor
DDB2	damage-specific DNA-binding protein
DDR1	discoidin domain receptor 1
DHFR	dihydrofolate reductase
DISC	death-inducing-signaling-complex
DM	double transactivation mutant
DME	Dulbecco's Modified Eagle's
DNA	deoxyribonucleic acid
DNA-PK	DNA-activated protein kinase
DR	death receptor
DTT	1,4-dithiothreitol
E	embryonic day
E2F	E2 promoter binding factor
EDTA	ethylenediamine tetra-acetic acid
EF-1α	elongation factor-1alpha
EGTA	ethylene glycol-O,O'-bis-{2-amino-ethyl]-N,N,N',N',-tetraacetic acid
EMSA	electrophoretic mobility shift assay

ERK	extracellular signal-regulated kinase
FACT	chromatin transcriptional elongation factor
FADD	Fas-associated with death domain
FDXR	ferredoxin reductase
g	gram
GADD45	growth arrest and DNA damage-inducible gene #45
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GITR	glucocorticoid-induced TNF receptor
GML	glycosylphosphatidylinositol-anchored molecule-like protein gene
GPX	glutathione peroxidase 1
GSNO	S-nitrosogluthathione
h	hours
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HD	Huntington's disease
HDAC	histone deacetylases
HDM2	human double minute 2
Hi95	hypoxia induced gene #95
HIC-1	hypermethylated in cancer-1
HIPK2	homeodomain-interacting protein kinase 2
Hrs	hours
Htt	Huntingtin
I	ipsilateral

IAP	inhibitor of apoptosis protein
IGF-BP3	insulin-like growth factor binding protein 3
IKIP	I Kappa B kinase interacting protein
IR	δ- irradiation
JNK	c-Jun N-terminal protein kinase
JMY	junction-mediating and regulatory protein
k	kilo
KA	kainic acid
l	litre
LacZ	beta-galactosidase
luc	luciferase
MAPK	mitogen-activated protein kinase
MAP4	microtubule associated protein 4
MAP4K4	mitogen-activated protein 4 kinase 4
MCAO	middle cerebral artery occlusion
Mcl-1	myeloid cell leukemia sequence 1
MDM2	mouse double minute 2
MEF	mouse embryo fibroblast
μ	micro
mX	milli
M	molar
mHtt	mutant huntingtin
min	minute

Mn	manganese
MOI	multiplicity of infection
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
mSin3a	mammalian Sin3a
mt	mitochondrial
MUG	4-methylumbelliferyl-D-galactoside
MUT	mutated
n	nano
N-terminal	amino terminal
NES	nuclear export signal
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
OHDA	hydroxydopamine
P2XM	P2X specifically expressed in skeletal muscle
p53AIP1	p53-dependent apoptosis-inducing protein1
p53CABC1	chaperone-activity of bc1 complex in *S. pombe* (ABC1)-like
p53DINP1	p53-dependent damage-inducible nuclear protein 1
p53R2	p53 inducible ribonucleotide reductase small subunit 2 homologue
p53RDL1	p53-regulated receptor for death and life
p53RFP	p53-inducible RING-finger protein
PA26	p53-activated gene 26
PAC1	phosphotase of activated cells 1

PAG608	p53-activated gene 608
PAR	poly (ADP)-ribose
PARP	poly (ADP)-ribose polymerase
PBS	phosphate buffered saline
PC	pheochromocytoma
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD	Parkinson's disease
Peg3/Pw1	Paternally expressed gene 3
PERP	p53 apoptosis effector related to PMP-22
PFT	pifithrin –α
pfu	plaque forming units
PGK	phosphoglycerate kinase
PIDD	p53-induced protein with death domain
PIG	p53-inducible gene
PIKK	phosphoinositide-3-kinase-related-kinase
Pin1	peptidyl-prolyl cis/trans isomerase 1
Pirh2	p53 induced RING-H2
PKC	protein kinase C
PLK3	polo-like kinase 3
PML	promyelocytic leukaemia
PML-NB	promyelocytic leukaemia nuclear bodies

PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
Pol	polymerase
PPIase	praline isomerase
pRb	retinoblastoma protein
PRG3	p53-responsive gene 3
PRKAB1	protein kinase, AMP-activated, beta 1 non-catalytic subunit
PRMT1	protein arginine methyltransferase 1
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTPRV	protein tyrosine phophatase, receptor type V
PUMA	p53-upregulated modulator of apoptosis
QA	quinolic acid
QS	glutamine serine
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RT	reverse transcription
s	second
S.D.	standard deviation
S-phase	synthesis phase
SAH	SIVA amphihelical domain
SAK	Snk/Plk-akin kinase
SCN3B	sodium channel subunit 3 beta

Set9	SET domain-containing histone methyltransferase 9
SH3	Src homology-3
Siah1	seven in absentia homologue 1
SN	substantia nigra
SOD	superoxide dismutase
SSC	sodium chloride-sodium citrate
STAG1	Stromal antigen 1
Strap	stress-responsive activator of p300
SUMO	small ubiquitin-like modifier
SV40	simian virus-40
TA	transactivation domain
TAF	TATA-binding protein associated factor
tBid	truncated Bid
TBI	traumatic brain injury
TBP	TATA-binding protein
TdT	terminal transferase
THSA	thromboxane synthase
TNF	tumour necrosis factor
TNFR	TNF receptor
TPBS	tris-phosphate buffered solution
TPR	tetratricopeptide repeat
TRAF4	TNFR-associated factor 4
TRAIL	TNF-related apoptosis-inducing ligand receptor

TSP1	thrombospondin-1
TUNEL	terminal deoxynucleotide transferase biotin-dUTP Nick End Labeling
UTP	uradine tri-phosphate
UV	ultra-violet
Wig-1	wildtype p53-induced gene 1
Wip1	wildtype p53-induced phosphotase 1
Xm	metre
XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4
Zac-1	zinc finger protein which regulates apoptosis and cell cycle arrest 1

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PREFACE

In accordance with the guidelines established by the School of Graduate Studies and Research (SGSR) of the University of Ottawa, the experimental findings presented in this thesis are comprised of published journal articles.

The experimental findings of this thesis are preceded by an overview of our current understanding of p53 function as a tumour suppressor and of its role in neuronal cell death along with the contradictions and complications which are currently present in the field (Chapter 1). The subsequent chapters (2 and 3) are two manuscripts describing novel p53 target genes upregulated in neurons after injury. These experimental findings are summarized and discussed in relation to the relevant literature in Chapter 5.

Each of the articles included in this thesis is prefaced with a Statement of Contributions of Collaborators, as required by the SGSR (University of Ottawa). Portions of this thesis are reprinted with permission from: The Journal of Cell Biology and the Journal of Biological Chemistry. Copies of official letters granting permission to reproduce the articles are included in Appendix A.

Chapter 1. Introduction to p53 function

The aim of the introductory chapter of this thesis is to provide the reader with a broad understanding of p53 and an overview of its role in neuronal apoptosis. As an important tumour suppressor, most of our understanding of p53 is based on research done in non-neuronal cells. Its study in neurons is still relatively new. Thus, this chapter will often describe what is known from research done in non-neuronal cells with the inclusion of that from research focused on neuronal cells where possible.

I. p53 Tumour Suppressor

I-I. Introduction

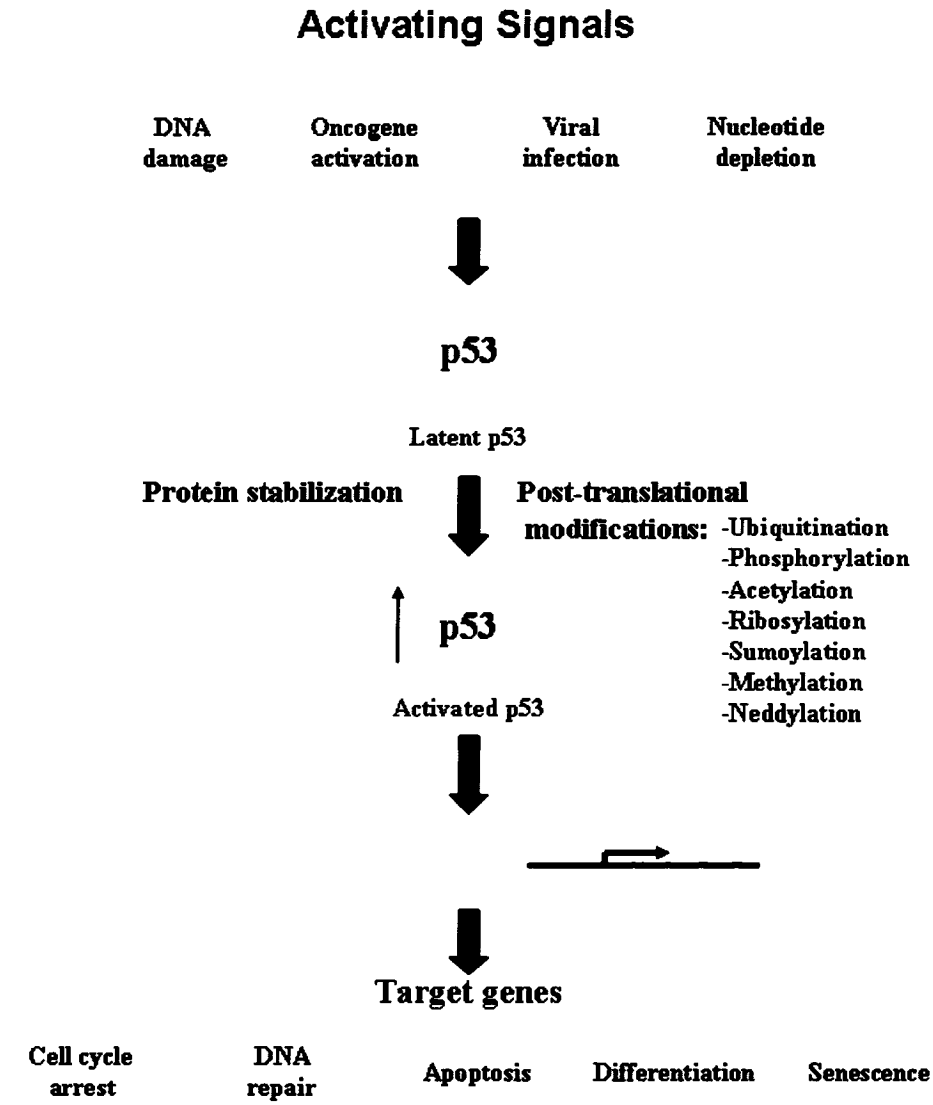
p53, the product of the gene, TP53, was first discovered in 1979 (DeLeo, Jay et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). It was identified as a protein interacting with the simian virus 40 (SV40) large T antigen. The designation, p53, resulted from the protein's apparent 53 kDa molecular weight (DeLeo, Jay et al. 1979). Since its discovery, it has become one of the most studied molecules with over 30,000 research articles written. The extreme level of interest in this molecule comes from the fact that p53 is the most frequently altered protein in human cancers. In fact, approximately 50% of all human malignancies harbour mutations or deletions in the TP53 gene (Hollstein, Sidransky et al. 1991; Hainaut and Hollstein 2000). In the majority of the remaining cancer cases, p53 activity is compromised by alternative mechanisms (Vogelstein, Lane et al. 2000). Although the observed expression of p53 in cancerous

cells originally lead people to believe it was an oncogene (Crawford 1983), further research confirmed its role as a powerful tumour suppressor. A tumour suppressor is a gene whose product can regulate cell cycle. If conditions are not optimal for cell cycle progression, as for example DNA is damaged, it can inhibit the further division of cells within the affected tissue. What distinguishes an oncogene from a tumour suppressor is that mutation of the oncogene results in a "gain of function" or the inability of the gene product to be controlled leading to tumour formation whereas the mutation of the tumour suppressor results in a "loss of function", thus, loosing its ability to control cell division and allowing tumour development. Of note, p53-deficient mice were prone to developing spontaneous tumours thereby confirming its role as a tumour suppressor (Donehower, Harvey et al. 1992).

p53 is often referred to as the 'guardian of the genome' (Lane 1992) because it controls a complex signaling pathway which has evolved to maintain genome integrity and to protect multicellular organisms from cancer development that can be initiated from a broad range of cellular stresses. These include DNA damage, oncogene activation, viral infection and ribonucleotide depletion (Vogelstein, Lane et al. 2000; Vousden and Lu 2002). p53 suppresses tumour formation mainly through the induction of cell cycle arrest or apoptosis but it also contributes to cellular processes such as DNA repair, differentiation and senescence, also important for tumour suppression (Fig.1.1).

The mechanisms by which p53 accomplishes all of its biological functions are still not fully understood but it is believed that it functions primarily as a potent transcription factor. It binds to p53 responsive elements in a sequence-specific manner

Fig 1.1: Overview of p53 function. p53 is maintained in a latent state. Following cellular stresses such as DNA damage, oncogene activation, viral infection and nucleotide deprivation, p53 is activated through protein stabilization and post-translational modifications. Once activated, p53 binds to p53 responsive elements in a sequence-specific manner within target genes inducing their expression. Upregulation of p53 targets is required for its ability to suppress tumour formation by mechanisms including cell cycle arrest, apoptosis, DNA repair, senescence or differentiation.



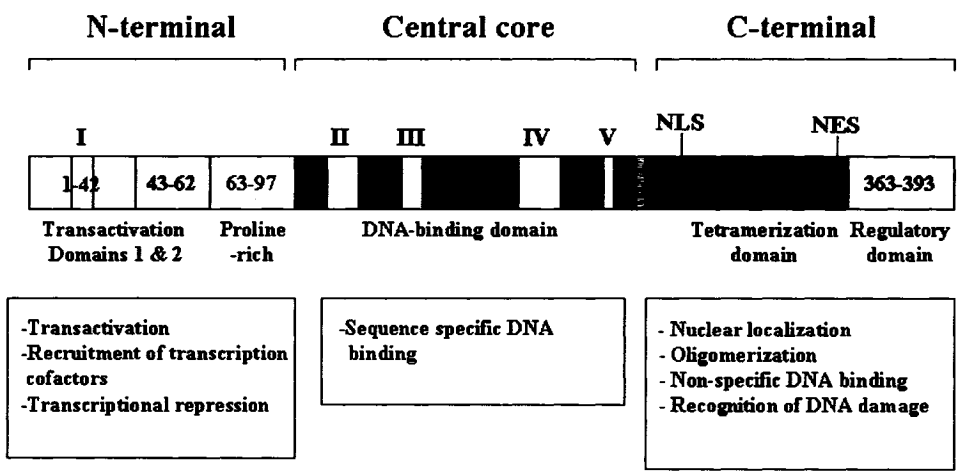
Modified from: Michalak et al., Biochem Biophys Res Commun, (2005), 331(3):786-798.

within the genome and activates the transcription of the corresponding genes. The proteins encoded by the p53 target genes are responsible for delineating its biological effects. These effects are not induced at random but only when cells are exposed to stress. Thus, p53 expression is strictly regulated. Only under these stressed conditions does p53 become activated. Activation is characterized by a marked increase in the cellular abundance of p53 molecules through protein stabilization as well as by post-translational modifications which also confer increased capabilities to modulate gene expression and alter cell phenotype. Thus, when a cell is altered in such a way as to predispose it to become cancerous, p53 is activated to either inhibit cell cycle progression to allow the repair of the damaged cell or to eliminate the affected cell altogether and thereby prevent its expansion into a cancerous tumour. The variety of stresses that can activate a protective response from p53 indicates its importance as a central tumour suppressor and may explain why it is found mutated in almost all types of cancer.

I-II. p53 – Structure

The identification and comparison of p53 from several species led to the characterization of a number of features common to all p53 proteins as well as the presence of 5 blocks of highly conserved gene homology (Soussi and May 1996) (Fig. 1.2). Similar to other transcription factors, p53 is a modular protein which can be divided into three regions: (1) the N-terminal activation domain; (2) the central core DNA binding domain; and, (3) the C-terminal regulatory domain (Arrowsmith and Morin 1996; Soussi and May 1996; Lane and Hupp 2003).

Fig. 1.2: Diagram of p53 structure. The p53 protein is a modular protein. It is divided into three regions: the N-terminal, the central core and the C-terminal, and it contains five boxes of conserved homology (I-V). The N-terminal contains two contiguous activation domains which are responsible for transcriptional activation through binding with the basal transcription machinery and a proline-rich domain which may be required for transcriptional repression. The central core is the most highly conserved region and is responsible for sequence specific DNA binding. It harbours the majority of the p53 mutations found in cancer. The C-terminal regulates nuclear localization through the nuclear localization signal, oligomerization through the tetramerization domain and non-specific DNA binding and recognition of DNA damage through the regulatory domain.



Modified from: Yee & Vousden, Carcinogenesis, (2005), 26(8):1317-22.

i) N-terminal

The N-terminal region is subdivided into the transcriptional activation domain and the proline-rich domain. The transcriptional activation domain is composed of two contiguous transcriptional transactivation subdomains: activation domain 1 within residues 1-42 (human numbering) and activation domain 2 within residues 43-62 (Zhu, Zhang et al. 2000). The transcriptional activation domain of p53 is not specific as activity is still retained following substitution by equivalent regions from other transcription factors (Pietenpol, Tokino et al. 1994). The transactivation functions of p53 are mediated by the binding of several proteins to the transcriptional activation domain such as the coactivators, TATA-associated factors (Lu and Levine 1995; Thut, Chen et al. 1995) and CBP/p300 (Scolnick, Chehab et al. 1997). The binding of these regulators is within the highly conserved BoxI. Partial deletion of BoxI retains p53 transcriptional activity but abrogates its capacity to bind transcriptional cofactors (Marston, Crook et al. 1994; Thut, Chen et al. 1995; Scolnick, Chehab et al. 1997), thus delineating the significance of Box1 (Soussi and May 1996). That transcriptional activity is conserved in this mutant, however, suggests that it is the overall structure of the transactivation domain and not the sequence which is important for transcriptional activity (Soussi and May 1996). Indeed, in human cancers, no mutations have been found in the transactivation domain. The proline-rich SH3-target domain (residues 63-97) contains five repeats of the amino acid sequence PXXP (where X is any amino acid) and, although its function is not fully understood, it is believed to be involved in the induction of proapoptotic target genes

(Walker and Levine 1996; Sakamuro, Sabbatini et al. 1997; Venot, Maratrat et al. 1998) and the transcriptional repression of survival genes (Venot, Maratrat et al. 1998).

ii) DNA-binding domain

The most important function of p53 is believed to be as a sequence-specific transcriptional activator. p53 recognizes specific promoters containing a consensus sequence consisting of two repeats of RRRC(A/T)(T/A)GYYY, where R and Y are a purine and pyrimidine, respectively, separated by a spacer region of up to 13 nucleotides (el-Deiry, Kern et al. 1992; Bourdon, Deguin-Chambon et al. 1997; el-Deiry 1998). The central core DNA binding domain (residues 98-292) is indispensable for p53 activity. Providing it is in the right conformation, the DNA binding domain binds tightly to these consensus sequences allowing p53 to activate the promoter through the interaction of the N-terminal activation domain with basal transcription factors (Kohn and Pommier 2005). The importance of the core domain for p53 function is underscored by the fact that it is the most evolutionary conserved domain with the presence of four of the five conserved boxes (BoxII-V) (Soussi, Caron de Fromentel et al. 1990) and that the loss of sequence-specific DNA binding impedes p53-dependent growth suppression which is the most characteristic feature of mutant p53 proteins (Vogelstein, Lane et al. 2000; Bullock and Fersht 2001; Soussi and Beroud 2001). Furthermore, unlike the N-terminal transactivation domain, the DNA binding domain is very sensitive to mutations and, in human cancers, approximately 80% of all mutations identified in p53 occur within this region (Lu 2005).

iii) C-terminal domain

The C-terminal regulatory domain (residues 300-393) has been demonstrated to perform at least three biological functions: nuclear localization, tetramerization, and both non-specific DNA binding and recognition of primary DNA damage (Soussi and May 1996). These are achieved through the nuclear localization signal within residues 300-323, the tetramerization domain within residues 324-355 and the C-terminal basic domain within residues 363-393.

Wild-type p53 exists predominantly as a tetramer (Friedman, Chen et al. 1993) or more appropriately a "dimer of dimers" (McLure and Lee 1998). Although many reports have demonstrated that p53 deleted of its tetramerization domain can still bind to DNA and stimulate transcription (Bargonetti, Manfredi et al. 1993; Pavletich, Chambers et al. 1993; Shaulian, Zauberman et al. 1993; Sang, Chen et al. 1994; McLure and Lee 1998), tetrameric p53 is the most effective for transactivation (Halazonetis and Kandil 1993; Hainaut, Hall et al. 1994; Pietenpol, Tokino et al. 1994; Tarunina, Grimaldi et al. 1996; McLure and Lee 1998; Weinberg, Veprintsev et al. 2004). McLure and Lee (McLure and Lee 1998) proposed the following model for the binding of tetrameric p53 to its consensus sequences: one dimer of the tetramer binds first to one half-site of the consensus DNA binding site; this increases the probability of the second dimer binding to the adjacent half-site; and, once bound, the dimers interact independently of the tetramerization domain to stabilize binding (McLure and Lee 1998). Thus, they suggested that the tetramerization domain may function to pre-organize the DNA binding domains in such a way as to preferentially bind the p53 consensus sequences.

The tetramerization domain is fairly robust as very few mutations have been found in human cancers within this domain. A nuclear export signal (NES) is found within the tetramerization domain and it is believed that the oligomeric status of p53 regulates its availability to the export machinery (Stommel, Marchenko et al. 1999). In the tetrameric conformation, the NES is buried and inaccessible resulting in p53 nuclear retention whereas, in the monomeric and dimeric forms, the NES is exposed and p53 is subject to nuclear export.

The C-terminal basic domain is a negative regulatory domain thought to mediate sequence-specific DNA binding. It is believed that the C-terminal basic domain interacts and inhibits the core domain. Following activation, post-translational modifications triggers a conformational change exposing the core domain allowing for sequence-specific DNA binding (Yakovleva, Pramanik et al. 2002). This model was born from *in vitro* evidence demonstrating that sequence-specific DNA binding was induced by deletion of the C-terminal basic domain or by factors which targeted the C-terminal basic domain (Hupp, Meek et al. 1992; Hupp and Lane 1994; Hupp, Sparks et al. 1995). Current research, however, has called this model into question as NMR studies have shown that there is no conformational difference between the inactive non-DNA binding and the active DNA binding forms of p53 and that there was no interaction between the C-terminal basic domain and any other domains of p53 (Ayed, Mulder et al. 2001). Instead, it is suggested that the non-specific DNA binding activity of the C-terminal basic domain prevents the core domain from binding with specific DNA sequences (Yakovleva, Pramanik et al. 2001). Another controversial function regulated by the non-

specific DNA binding activity of the C-terminal basic domain is recognition and repair of damage DNA (Albrechtsen, Dornreiter et al. 1999). This is believed to be possible through interactions with components of the DNA repair and recombination machinery and the 3'→ 5' exonuclease activity of the core DNA binding domain (Albrechtsen, Dornreiter et al. 1999).

I-III. p53 Family Proteins

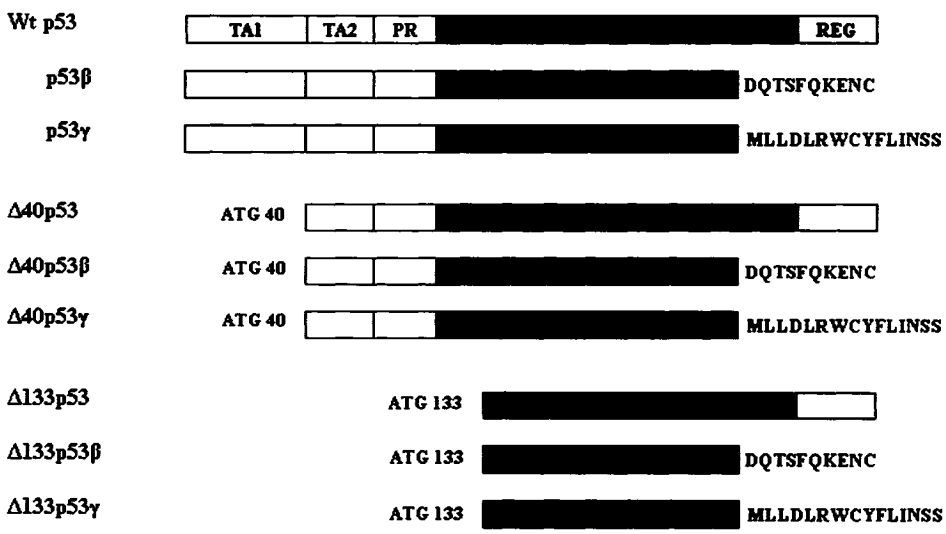
For many years, it was believed that p53 was an "orphan tumour suppressor" (Courtois, de Fromentel et al. 2004). However, recently, two novel family members were identified and named p73 (Kaghad, Bonnet et al. 1997) and p63 (Schmale and Bamberger 1997; Osada, Ohba et al. 1998; Trink, Okami et al. 1998; Yang, Kaghad et al. 1998; Zeng, Zhu et al. 2001), respectively. p73 and p63 are considered to be ancestors of p53. Sequence analysis of p53-like genes in lower organisms has demonstrated that these resemble p73 and p63 more closely than p53 (Kaghad, Bonnet et al. 1997; Yang, Kaghad et al. 1998). It is suggested that p53 has evolved from a template modeled after p73 and p63 to contend with the stress induced by DNA damage in multi-cellular organisms (Kaghad, Bonnet et al. 1997; Schmale and Bamberger 1997; Yang, Kaghad et al. 1998). p73 and p63 are highly homologous to p53 in terms of important protein domain structure and conformation (Courtois, de Fromentel et al. 2004). All three have a highly conserved N-terminal transactivation domain, DNA binding domain and C-terminal oligomerization domain (Moll and Slade 2004). Thus, p73 and p63 function similarly to p53 in that they

can oligomerize, bind to DNA, transactivate p53 target genes and induce cell cycle arrest or apoptosis (Jost, Marin et al. 1997; Yang, Kaghad et al. 1998). Their role in tumour suppression is supported by the fact that as with p53, disruption of p73 and p63 predisposes mice to spontaneous tumour development (Flores, Sengupta et al. 2005). p73 and p63 do also have unique physiological roles as they appear to play an important role in pathways related to development and differentiation (Courtois, de Fromentel et al. 2004; Moll and Slade 2004).

p73 and p63, have two promoters, one in the 5' untranslated area and one within intron 3. These give rise to proteins that contain a transactivation domain (TAp73 and Tap63) and to proteins that do not (ΔNp73 and ΔNp63), respectively. ΔNp73 and ΔNp63 function in a dominant-inhibitory fashion inhibiting full-length members of the p53 family (Pozniak, Radinovic et al. 2000; Yang, Walker et al. 2000; Fillippovich, Sorokina et al. 2001). To add to the complexity of p73 and p63, alternative splicing at the C-terminal of both p73 and p63 and at the N-terminal for p73 can generate several different isoforms. For p63, six different mRNA variants encoding six different proteins isoforms have been observed. For p73, fourteen different protein isoforms have been characterized. However, there potentially could exist several more as it is estimated that p73 can express at least 35 mRNA variants of which 28 different protein isoforms could be encoded (Bourdon, Fernandes et al. 2005). These different isoforms are believed to vary in their transcriptional activity (Jacobs, Walsh et al. 2004) or may act as modulators of full-length p53 proteins (Courtois, de Fromentel et al. 2004).

To date, p53 was considered much simpler than its family counterparts. It was believed to have one promoter which gave rise to full-length p53 as well as a 40 kDA dominant-negative alternative splice form, ΔNp53 (Moll and Slade 2004) which lacked the first transcriptional activation domain as a result of internal initiation of translation at codon 40 (Courtois, Verhaegh et al. 2002; Yin, Stephen et al. 2002) or alternative splicing of intron 2 (Matlashewski, Pim et al. 1987). Current research, however, has demonstrated that p53 gene structure is similar to those of p73 and p63. As with p73 and p63, the p53 gene contains an alternate promoter and transcribes multiple splice forms (Bourdon, Fernandes et al. 2005). The internal promoter was identified within intron 4 and gives rise to a N-truncated p53 protein initiated at codon 133 which lacks the transactivation domain, the proline-rich domain as well as part of the DNA-binding domain (Bourdon, Fernandes et al. 2005). Splice variants arise from alternatively spliced intron 9. In all, three variants were described: α, which corresponds to the complete splice of intron 9; β, which gives rise to a truncated p53 protein lacking the oligomerization domain and terminating with 10 additional amino acids coded from an additional exon within intron 9; and, γ, which is similar to β but terminates with an additional 15 amino acids (Bourdon, Fernandes et al. 2005). Thus, theoretically, it is believed that p53 can transcribe 9 different isoforms (Fig. 1.3). All isoforms are expressed in normal tissue at the mRNA level, however there appears to be some tissue specificity in the pattern of expression demonstrating a certain regulation in isoform expression (Bourdon, Fernandes et al. 2005). Much work is now needed to understand the roles that each isoform will play in fulfilling the biological function of p53.

Fig. 1.3: Putative p53 protein isoforms. The human p53 gene encodes nine putative mRNA isoforms which are all expressed in normal tissue. Δ40p53 isoforms arise as a result of internal initiation of translation at codon 40 or through alternative splicing of intron 2. Δ133p53 isoforms are generated from an internal promoter within intron 4. β and γ variants are engendered through alternative splicing of an additional exon (exon 9b) found within intron 9.



Modified from: Bourdon et al., Genes Dev, (2005), 19(18):2122-37.

II. p53 Regulation and Transcriptional Activation

II-I. Introduction

In healthy cells including neurons, p53 expression and function is tightly controlled. In fact, it has a half-life of only 5-20 minutes (Oren, Maltzman et al. 1981; Rogel, Popliker et al. 1985). This rapid protein turnover ensures that p53 is held in a latent form that is unable to bind specifically to DNA at very low concentrations within the cell and is only triggered when cells are stressed or damaged. This is critical for normal cell growth and development as elevated p53 levels could interfere with cell cycle progression and cell survival. Even in postmitotic neurons, overexpression of p53 still has detrimental effects (Slack, Belliveau et al. 1996). Following DNA damage and other types of stress, an accumulation of active p53 is observed in the nucleus. The rapid increase in nuclear p53 levels is predominantly attributed to the stabilization of the protein and not to *de novo* synthesis thereby advantageously providing the cell with a timely response to genotoxic and other stresses (Oren 1999). The upregulation of p53 mRNA, however, was observed in some models of neuronal injury (Morrison, Kinoshita et al. 2003). The regulation and activation of p53 is achieved through a complex mechanism involving multiple genes (Vogelstein, Lane et al. 2000). To get a better understanding of this mechanism the following sections will give an overview of the pathways leading to p53 activation, the regulators of p53 expression in healthy cells, the post-translational modifications leading to p53 stabilization and activation, the cofactors

and coactivators involved in p53 activation, the conformational change required for sequence specific DNA binding and other factors regulating p53 activity.

II-II. Pathways activating p53

P53 is activated following DNA damage or oncogene activation in order to maintain genome integrity and to protect multicellular organisms from cancer development. It is generally recognized that p53 activation is controlled by at least three independent pathways (Vogelstein, Lane et al. 2000). Two of these pathways are initiated by DNA damage: the first being induced by double-strand breaks as generated by ionizing radiation while the second being driven by damage resulting from chemotherapeutic agents and ultraviolet light (UV). Following genotoxic stress, signaling is regulated by two separate kinase families, the phosphoinositide-3-kinase-related kinases (PIKKs), which include ataxia telangiectasia mutated (ATM), ataxia telangiectasia related (ATR) and dsDNA-activated protein kinase (DNA-PK), and the checkpoint kinases made up of checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) (for review see (McGowan and Russell 2004)). Activation of ATM and ATR by ionizing radiation and ultraviolet light, respectively, leads to phosphorylation and activation of their immediate substrates, Chk2 and Chk1, respectively. Both ATM/Chk2 and ATR/Chk1 pathways phosphorylate p53 following DNA damage leading to increased stability and activity (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Hirao, Kong et al. 2000).

In neurons, ATM is required for p53 activation and neuronal cell death in response to DNA damage (Herzog, Chong et al. 1998; Chong, Murray et al. 2000; Lee, Chong et al. 2001; Keramaris, Hirao et al. 2003). It has been shown to play a vital role in the regulation of p53 activation/stabilization and neuronal death following DNA damage induced by inhibitors of topoisomerase I and II. Primary cortical neurons deficient for ATM were protected from DNA damage induced cell death. This may be independently of Chk2 as its deficiency had no affect on p53 stability or apoptosis in these death paradigms (Keramaris, Hirao et al. 2003). Neuronal apoptosis induced by ionizing irradiation in mouse embryos also required ATM (Herzog, Chong et al. 1998). Embryonic mice deficient for ATM were resistant to ionizing radiation and demonstrated reduced levels of p53 accumulation in several regions of the brain (Herzog, Chong et al. 1998), indicating that the ATM gene is upstream of p53. Other signaling pathways must also lie upstream of p53, however, as deficiency for p53 provided significantly more protection to ionizing radiation and camptothecin treatment than did ATM-deficiency (Xiang, Kinoshita et al. 1998; Chong, Murray et al. 2000; Giovanni, Keramaris et al. 2000; Morris, Keramaris et al. 2001). The ATM-related protein (ATR)/Checkpoint1 (CHK1) pathway is a candidate although it has not yet been studied in the brain.

Uncontrolled proliferation caused by expression of oncogenes such as Ras, Myc or adenovirus E1A leads to the induction of the third pathway known to activate p53. This pathway is regulated by the protein, $p19^{ARF}$ ($P14^{ARF}$ in humans), which is a direct target for the transcription factor E2F-1, thus, providing a link between the p53 pathway and the retinoblastoma (pRb) tumour suppressor protein pathway (Lowe 1999; Sherr and

Weber 2000). $p19^{ARF}$ directly binds and inhibits the p53-negative regulator MDM2 (see following section) allowing for p53 stabilization and activation (reviewed in (Sherr and Weber 2000)). Interestingly, although a crucial mediator of cell cycle progression, E2F-1 has been shown to induce apoptosis through both p53-dependent and –independent pathways (Ginsberg 2002). That E2F-1 could induce p53-dependent apoptosis in $p19^{ARF}$ –deficient mice and cells suggested that the link between Rb/E2F-1 and p53 went beyond simply $p19^{ARF}$ (Rogoff, Pickering et al. 2002; Russell, Powers et al. 2002; Tolbert, Lu et al. 2002; Tsai, MacPherson et al. 2002). Indeed, E2F-1 was reported to induce phosphorylation of p53 (Rogoff, Pickering et al. 2002) and this could be through the aforementioned DNA damage activated kinases, ATM and Chk2, which have recently been shown to be up-regulated by E2F-1 (Berkovich and Ginsberg 2003; Rogoff, Pickering et al. 2004).

In neurons, we have demonstrated that E2F-1 can induce apoptosis independently of p53 (O'Hare, Hou et al. 2000). They do, however, cooperate in certain models of cell death. For example, the apoptosis observed in the central nervous system of whole embryo pRB-deficient mice was abrogated in the absence of p53 (Macleod, Hu et al. 1996) or E2F-1 (Tsai, Hu et al. 1998). Research focused on exploring the relationship between RB/E2F-1 and p53 in regulating apoptosis will augment our understanding of the mechanisms involved.

II-III. Regulators of p53 expression in healthy cells

The key antagonist of p53 is the oncogenic mouse double minute 2 (Mdm2, Hdm2 in humans) protein. Mdm2 is a direct binding partner for p53 (Momand, Zambetti et al. 1992; Oliner, Kinzler et al. 1992) and as such exercises its control over p53 in two ways: first, it inactivates p53 function (Momand, Zambetti et al. 1992; Chen, Marechal et al. 1993; Chen, Wu et al. 1996; Haupt, Barak et al. 1996), and second, it targets p53 for degradation (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). The blocking of p53 transcriptional activity by Mdm2 is achieved through its interaction with the N-terminal transactivation domain which is thought to interfere with the recruitment of the components of the basal transcription machinery (Oren 1999). Additionally, Mdm2, which has p53-specific E3 ubiquitin protein ligase activity (Honda, Tanaka et al. 1997), can inhibit p53 function by inducing mono-ubiquitination at multiple C-terminal lysine residues (Lai, Ferry et al. 2001) including Lys370, 372, 373, 381, 382, 386 respectively (Michael and Oren 2003). This results in the exposure of the nuclear export signal (NES) of p53 which allows the export of p53 from the nucleus to the cytoplasm away from its site of action (Li, Brooks et al. 2003; Kohn and Pommier 2005). Degradation of p53 is also regulated by Mdm2-mediated poly-ubiquitination of p53 (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). This occurs either in the nucleus or in the cytoplasm (Yu, Geyer et al. 2000; Xirodimas, Stephen et al. 2001; Joseph, Zaika et al. 2003) via the 26S proteosome (Maki, Huibregtse et al. 1996).

The interplay between Mdm2 and p53 goes one step further. The Mdm2 gene is a direct transcriptional target for p53 thus forming a negative feedback loop. The physiological importance of the Mdm2-p53 loop is reinforced by the observation that

Mdm2-deficient mice are embryonic lethal due to unregulated p53 activity but can be rescued if generated on a p53-null background (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995; de Rozieres, Maya et al. 2000). Furthermore, inhibition of Mdm2-p53 interaction is a key target for many stress signals in the activation of p53 (Chene 2003).

In neurons, the role of MDM2 has not been fully established although p53-MDM2 complexes have been observed (Tan, Qu et al. 2000). In one study, hypoxia was shown to induce the nuclear accumulation of p53 and the down-regulation of MDM2 in primary cortical neurons demonstrating a role for MDM2 in p53 regulation (Zhu, Mao et al. 2002). In other research, however, elevated levels of p53 expression were detected even though there was accumulation of MDM2 and the formation of p53-MDM2 complexes in p53-positive neurons undergoing apoptosis (Tan, Qu et al. 2000; Tan, Tu et al. 2001; Tan, Sankar et al. 2002). These apoptotic neurons, alternatively, exhibited a marked decrease in free ubiquitin suggesting a novel mechanism of p53 stabilization in neurons involving decreased levels of free ubiquitin and independent of MDM2 expression.

Although Mdm2 is the most important regulator of p53 degradation through the ubiquitin system, other E3 (or E3-like) ubiquitin ligases for p53 have been identified. These include Mdm4 (Migliorini, Lazzerini Denchi et al. 2002), constitutively photomorphogenic 1 (COP1) (Dornan, Wertz et al. 2004) and Pirh2 (Leng, Lin et al. 2003). Interestingly, as with Mdm2, COP1 and Pirh2 are also p53-inducible genes. In

conclusion, ubiquitin-mediated degradation plays a crucial role in maintaining low levels of p53 expression in healthy cells.

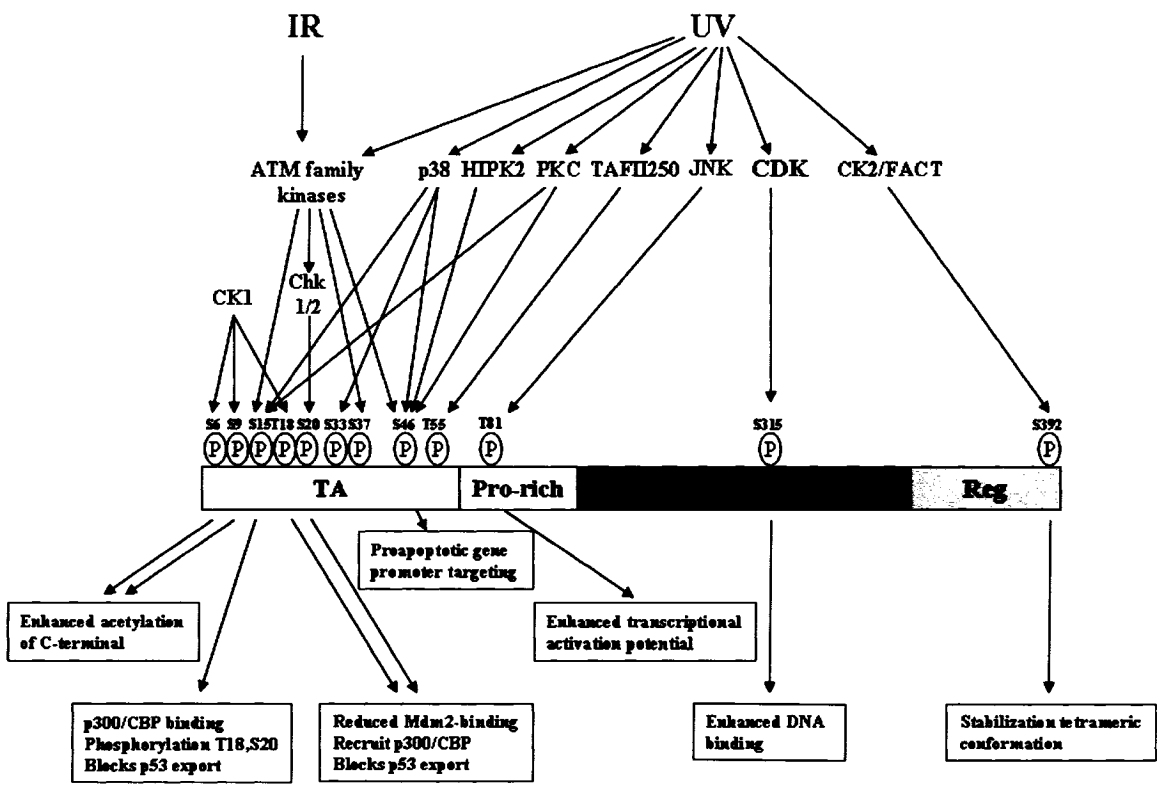
II-III. Post-translational modifications

The activation of p53 is achieved mainly through post-translational modifications such as phosphorylation, acetylation, poly (ADP) rybosylation, sumoylation and methylation to the N-terminal and C-terminal domains. The plethora of signals that converge on p53 to regulate its expression and activation demonstrate the importance of p53 as a tumour suppressor. Significant redundancies are evident as, for example, p53 can be phosphorylated on the same residue by different protein kinases and a specific protein kinase can phosphorylate several residues on p53. The existence of these redundancies could function as a 'fail safe' mechanism (Bode and Dong 2004) to elicit the appropriate physiological response. The complexity of the p53 network observed in non-neuronal cells is probably also reflected in neurons. The post-tanslational modifications observed to p53 may be different depending on the neuronal population, the type and severity of the injury and may change during development and aging (Coultas and Strasser 2000). Evidence of post-translational modifications of p53 in neurons following injury is not extensive, however, there are indications that these do occur (Daily, Barzilai et al. 1999; Lee, Chong et al. 2001; Martin, Kaiser et al. 2001; Keramaris, Hirao et al. 2003). The most studied mechanisms of p53 activation come from research following injury induced by DNA damage. Therefore, the following sections will highlight important post-translational modifications observed in this model.

i) Phosphorylation

p53 can be phosphorylated on at least a dozen sites with the highest density of these found in the N-terminal domain (Fig. 1.4) (for review see (Brooks and Gu 2003; Xu 2003)). Activation of ATM by exposure to DNA damaging agents leads to p53 phosphorylation by ATM on ser15, (Banin, Moyal et al. 1998; Canman, Lim et al. 1998). Ser15 is a prime example of one of the redundancies present in the regulation of p53 as not only is it phosphorylated by ATM, it can also be phosphorylated by ATR (Tibbetts, Brumbaugh et al. 1999), DNA-PK (Shieh, Ikeda et al. 1997), extracellular signal-regulated kinases (ERK) (She, Chen et al. 2000; Lee, Kim et al. 2005), p38 MAPK protein kinase (p38) (She, Chen et al. 2000) and protein kinase C (PKC) delta (Lee, Kim et al. 2005). Phosphorylation of ser15 allows the subsequent phosphorylations of ser20 by ATM activated Chk2 and of thr18 by casein kinase 1 (CK1) (Dumaz, Milne et al. 1999; Hirao, Kong et al. 2000; Saito, Yamaguchi et al. 2003). Both of these residues are believed to be critical for p53 stability as they are located within the Mdm2 binding site and it is suggested that they can independently interfere with p53-Mdm2 binding thereby disrupting p53 degradation (Sakaguchi, Saito et al. 2000; Dumaz, Milne et al. 2001; Bean and Stark 2002; Jabbur and Zhang 2002). Since the N-terminal is crucial for p53 binding to Mdm2, it is not surprising that, in an effort to disrupt this interaction and stabilize p53, several residues within this domain are key targets for phosphorylation following DNA damage. It should be noted, however, that p53 stabilization has also been described without the phosphorylation of these two residues (Wu, Earle et al. 2002).

Fig. 1.4: DNA damage-induced phosphorylation of p53. The principal residues within the p53 protein that are phosphorylated following DNA damage are shown. The kinases responsible for these phosphorylations are indicated with arrows to the appropriate residues. Boxed text describes the reported consequences of phosphorylation of specific residues.



Modified from: Meek, DNA Repair, (2004), 3(8-9):1049-56.

The phosphorylation of the N-terminal residue, ser46, following severe DNA damage appears to be a key requirement for apoptosis. Phosphorylated ser46 is thought to influence p53 promoter targeting towards proapoptotic genes such as p53-dependent apoptosis-inducing protein 1 (p53AIP1) (Oda, Arakawa et al. 2000) and phosphatase and tensin homolog deleted on chromosome ten (PTEN) (Mayo, Seo et al. 2005). Several kinases have demonstrated the ability to phosphorylate p53 on Ser46 including p38 (Bulavin, Saito et al. 1999), the homeodomain-interacting protein kinase 2 (HIPK2) (D'Orazi, Cecchinelli et al. 2002; Hofmann, Moller et al. 2002) and protein kinase C delta (Yoshida, Hanshao et al. 2005).

The phosphorylation of N-terminal residues is required in some instances for the subsequent modifications to the C-terminal. For example, phosphorylation of ser15, thr18 and/or ser20 promotes the recruitment of the transcription cofactors p300 and cyclic AMP response element-binding (CREB) protein (CBP) to the N-terminal domain (Kohn and Pommier 2005). Through their histone acetyl-transferase activity, they go on to acetylate p53 on several lysine residues of the C-terminus (Liu, Scolnick et al. 1999).

The phosphorylation of C-terminal residue can also influence p53 activity. ser392 is believed to be an important target for the activation of p53 function as phosphorylation by kinases such as p38 (Huang, Ma et al. 1999) is believed to stabilize p53 tetrameric conformation (Sakaguchi, Sakamoto et al. 1997). Furthermore, the phosphorylation of ser315 by a cyclin-dependent kinase pathway following UV-induced DNA damage was reported to be important for stimulating p53-dependent transcription (Blaydes, Luciani et al. 2001).

In neurons, phosphorylation of several residues has been reported. Primary cortical neurons treated with the DNA damaging agent, camptothecin, was phosphorylated on ser15 in an ATM-dependent manner (Keramaris, Hirao et al. 2003). Neuronal apoptosis induced by ionizing irradiation in mouse embryos was characterized by the ATM-dependent phosphorylation of ser15 (Herzog, Chong et al. 1998) and the subsequent phosphorylation of ser 18 (Lee, Chong et al. 2001). Both p38 and protein kinase C have also demonstrated the ability to phosphorylate ser15 in neurons (Zhu, Mao et al. 2002; Lee, Kim et al. 2005). Phosphorylation of ser392 was observed along with increased DNA binding in damaged neurons of the lateral geniculate nucleus in a cortical injury model (Martin, Kaiser et al. 2001). Thus, in neurons, phosphorylation of p53 appears to play an important a role in regulating p53 activity.

ii) Acetylation

Acetylation is a post-translational modification of the C-terminal and is regulated by CREB-binding protein (CBP)/p300 and p300/CBP-associated factor (PCAF) (for review see (Brooks and Gu 2003; Xu 2003)). CBP/p300 and PCAF both have an inherent acetyl-transferase function. Acetylation by CBP/p300 competes with ubiquitination by Mdm2 for the same lysine residues namely lys370, lys372, lys373, lys381, lys382 and lys386 (Rodriguez, Desterro et al. 2000). A shift in the balance towards acetylation initiated by N-terminal phosphorylation following DNA damage can inhibit MDM2-mediated ubiquitination leading to p53 stabilization (Xu 2003). The acetylation of p53 at these lysines are also believed to increase sequence-specific DNA binding following

DNA damage, thus, augmenting transcriptional activation (Luo, Li et al. 2004). PCAF acetylates p53 at lys320 following DNA damage and this is believed to result in the enhanced sequence-specific DNA binding of p53 as well (Liu, Scolnick et al. 1999). In neurons, p53 and p300 are known to interact following genotoxic stress (Culmsee, Siewe et al. 2003) suggesting that perhaps p300-mediated acetylation is also an important post-translational modification in this cell type.

iii) Poly (ADP) ribosylation

The poly (ADP) ribosylation of p53 through activation of poly (ADP-ribose) polymerase (PARP) 1 appears to play an important role in p53 activation and regulation following DNA damage (Pieper, Verma et al. 1999). PARP1 is a nuclear protein that is activated following DNA damage to participate in DNA repair. It catalyses the addition of long branched chains of poly (ADP)-ribose (PAR) from its substrate nicotinamide adenine dinucleotide (NAD) to a number of acceptor proteins including p53. Poly (ADP) ribosylation targets three domains in the p53 protein including one in the C-terminal oligomerization domain and two in the sequence-specific core DNA binding domain (Malanga, Pleschke et al. 1998). p53 poly (ADP) ribosylation and stabilization was observed in neurons undergoing MPTP-induced apoptosis (Mandir, Simbulan-Rosenthal et al. 2002) as well as following DNA damage induced by homocysteine (Kruman, Culmsee et al. 2000). Neurons deficient for PARP-1 or exposed to PARP-1 inhibitors exhibited a considerable downregulation of p53 and were protected from DNA damaging agents, oxidative stress, NMDA toxicity and *in vivo* models of stroke, brain trauma,

Parkinson's disease and epilepsy (Mandir, Przedborski et al. 1999; Skaper 2003). The mechanism by which the accumulation of poly (ADP)-ribosylated p53 leads to neuronal apoptosis is not clear based on the fact that poly (ADP)-ribosylated p53 fails to bind consensus sequences and is, thus, unable to transactivate proapoptotic target genes (Malanga, Pleschke et al. 1998). However, some believe that at later times, after accumulation, p53 becomes deribosylated at which point its ability to transactivate proapoptotic genes is re-established (Mandir, Simbulan-Rosenthal et al. 2002).

iv) Sumoylation and methylation

Other C-terminal modifications include SUMOylation and methylation. The SUMO pathway is a ubiquitin-like modification system. SUMO and ubiquitin share a common 3-dimensional structure and SUMOylation involves the conjugation of SUMO to target proteins (Muller, Ledl et al. 2004). SUMOylation of p53 at lys386 by SUMO-1 is thought to stimulate p53 transcriptional activation (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999; Muller, Berger et al. 2000), however, this is still controversial as mutation of lys386 to an arginine enhanced p53-mediated transactivation as did the overexpression of a SUMO-isopeptidase which cleaves SUMO from p53 (Muller, Ledl et al. 2004). Methylation of p53 at lys372 by Set9 methyltransferase sequesters p53 into the nucleus and positively affects its stability (Chuikov, Kurash et al. 2004). The consequence of sumoylation or methylation to p53 activation/stabilization has not been determined in neurons.

II-IV Cofactors/coactivators

Regulation of p53 stabilization and activation is highly influenced by post-translational modifications, however, a growing number of cofactors have been identified to be important for p53 activity (reviewed in (Coutts and La Thangue 2005)). The transcriptional coactivators, CBP/p300, are key regulators of RNA polymerase II-mediated transcription. They promote gene transcription by linking transcription factors to the basal transcription machinery and by modifying chromatin and, as previously mentioned, transcription factors through acetylation (Grossman 2001). Thus, in addition to acetylating C-terminal lysine residues on p53, they stimulate p53 transcriptional activity by binding to its transactivation domain and linking it to the basal transcription machinery (Avantaggiati, Ogryzko et al. 1997; Gu, Shi et al. 1997; Lill, Grossman et al. 1997; Scolnick, Chehab et al. 1997). The interaction of CBP/p300 with p53 appears to involve the cofactors Strap and junction-mediating and regulatory protein (JMY). Strap, a protein believed to regulate protein complex assembly through its six tetratricopeptide (TPR) repeats, increases p53 levels and half-life by recruiting p300 and JMY (Shikama, Lee et al. 1999) thus, facilitating the acetylation of p53 leading to enhanced p53-dependent apoptosis (Demonacos, Krstic-Demonacos et al. 2001). Strap phosphorylation by ATM is essential for its stabilization and nuclear accumulation demonstrating its role in the DNA damage response (Demonacos, Krstic-Demonacos et al. 2004). Through its C-terminal proline-rich domain which is vulnerable to alternative splicing, JMY has been suggested to influence p53 promoter targeting. In the presence of the proline-rich domain,

proapoptotic transcriptional targets are favoured over cell cycle arrest targets (Shikama, Lee et al. 1999). Other cofactors implicated in remodeling chromatin structure and known to be involved in p53 transcriptional activation are the arginine methyltransferases, coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltrasferase 1 (PRMT1). Both have been shown to bind p53 and co-operate with p300 to induce p53 target genes during the p53 response (An, Kim et al. 2004).

Other proteins which have a positive role in p53 regulation and activation in non-neuronal cells are numerous. Promyelocytic leukaemia (PML) protein, a direct p53 target (de Stanchina, Querido et al. 2004), may also be involved in p53 regulation by recruiting p53 to PML nuclear bodies (PML-NBs) (Guo, Salomoni et al. 2000). PML-NBs are thought to function as sites for the formation of multi-subunit complexes from where post-translational modification of nuclear proteins can occur (Dellaire and Bazett-Jones 2004). Indeed, CBP and HIPK2, which as mentioned above are responsible for p53 acetylation and phosphorylation, respectively, are both recruited along with p53 to PML-NBs following DNA damage (D'Orazi, Cecchinelli et al. 2002; Hofmann, Moller et al. 2002). The apoptotic-stimulating proteins of p53 (ASPP1 and ASPP2/53BP2) enhance p53 transcriptional activity by directly interacting with the DBD of p53 (Iwabuchi, Bartel et al. 1994; Iwabuchi, Li et al. 1998; Samuels-Lev, O'Connor et al. 2001). ASPP proteins enhanced p53's ability to bind and induce promoters of proapoptotic genes and not other targets (Samuels-Lev, O'Connor et al. 2001). Another p53 DBD interactor is 53BP1 (Iwabuchi, Bartel et al. 1994). It is also believed to function as a p53 transcriptional coactivator (Iwabuchi, Li et al. 1998). In response to DNA damage, 53BP1 is

phosphorylated by ATM and localizes to discrete nuclear foci (Schultz, Chehab et al. 2000; Rappold, Iwabuchi et al. 2001) where it may recruit p53 or other substrates to these damaged areas where ATM can subsequently phosphorylate them (Wang, Matsuoka et al. 2002).

II-V Conformational change

Although controversial, the activation of p53's function as a sequence specific transcription factor is believed to necessitate a conformational change in the protein to reveal the DNA binding domain hidden by the C-terminal portion of the protein (Hupp, Meek et al. 1992; Bayle, Elenbaas et al. 1995; Wolkowicz, Elkind et al. 1995; Wolkowicz, Peled et al. 1995). Recent research has demonstrated that the phosphorylation of ser315 along with that of the N-terminal residues, ser33 and thr81, (and ser46 with δ-irradiation) promoted the recruitment of the proline isomerase (PPIase), Pin1 (Wulf, Liou et al. 2002; Zacchi, Gostissa et al. 2002; Zheng, You et al. 2002). PPIases are chaperone enzymes which have recently been shown to regulate important cellular proteins such as cdc25c and Myc through the induction of conformational changes mediated by specific phosphorylation events (Mantovani, Gostissa et al. 2004). PPIases bind to sites comprising of a phosphorylated serine or threonine residue, followed by a proline residue and switches the peptide bond from the *cis* to the *trans* conformation and vice versa (Mantovani, Gostissa et al. 2004). Thus, it is suggested that upon recruitment to p53, Pin1 catalyses a conformational changes in p53 enhancing its

stability and transactivation activity (Wulf, Liou et al. 2002; Zacchi, Gostissa et al. 2002; Zheng, You et al. 2002).

II-VI. Other factors influencing p53 activity

Calcium-dependent neutral proteases, calpains, have been shown to mediate p53 activation following DNA damage (Sedarous, Keramaris et al. 2003). Co-treatment of primary cortical neurons with calpain inhibitors or deficiency of the small regulatory calpain subunit, capn4, which is required for proper calpain activity, in differentiated stem cell cultures resulted in substantial reduction in p53 induction and resistance to DNA damage induced cell death (Sedarous, Keramaris et al. 2003). The mechanism by which calpains regulate p53 induction is not understood although it has been proposed to perhaps involve the NF-κB pathway (Sedarous, Keramaris et al. 2003). Calpains are known to cleave the negative regulator of the NF-κB pathway, IκB (Han, Weinman et al. 1999; McDonald, Mota-Filipe et al. 2001), and NFκB has been shown to directly regulate transcriptional activation of p53 (Wu and Lozano 1994; Hellin, Calmant et al. 1998; Pei, Nakanishi et al. 1999; Zhang, Youn et al. 2001). In neurons exposed to DNA damage, apoptosis was mediated by NF-κB through a p53-dependent mechanism as inhibition of NF-κB protected cultured cortical neurons from DNA damage-induced apoptosis and reduced p53 transcripts and activity (Aleyasin, Cregan et al. 2004).

This section has attempted to give an overview of the complex mechanism mediating the regulation and activation of p53. Many genes are involved in this process.

They may be involved in the initiation of the p53 pathway, the induction of post-translational modifications on p53, thus affecting its expression and influencing its transcriptional activity, the targeting of specific promoters by direct binding to p53 or the mediation of p53 conformational change required for sequence specific DNA binding. It is thus evident that p53 is heavily regulated and this regulation may be required to ensure that p53 is only activated in situations of severe cellular stress.

III. P53-mediated Apoptosis

III-I. Apoptosis

Programmed cell death (PCD) or apoptosis is an evolutionarily conserved biological process of cell-suicide and is an essential part of life for multicellular organisms (Kerr, Wyllie et al. 1972). It plays a crucial role in development and tissue homeostasis (Prindull 1995). During development, many cells are produced in excess. These eventually are eliminated by apoptosis which contributes to the sculpting of organs and tissues (Meier, Finch et al. 2000). Apoptosis must be precisely balanced and regulated as failure to do so results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Thompson 1995). Classical morphological features of apoptosis include nuclear and cytoplasmic condensation, intranucleasomal DNA cleavage, nuclear membrane breakdown and plasma membrane blebbing and the formation of apoptotic bodies (Arends and Wyllie 1991).

III-II. Apoptotic pathways

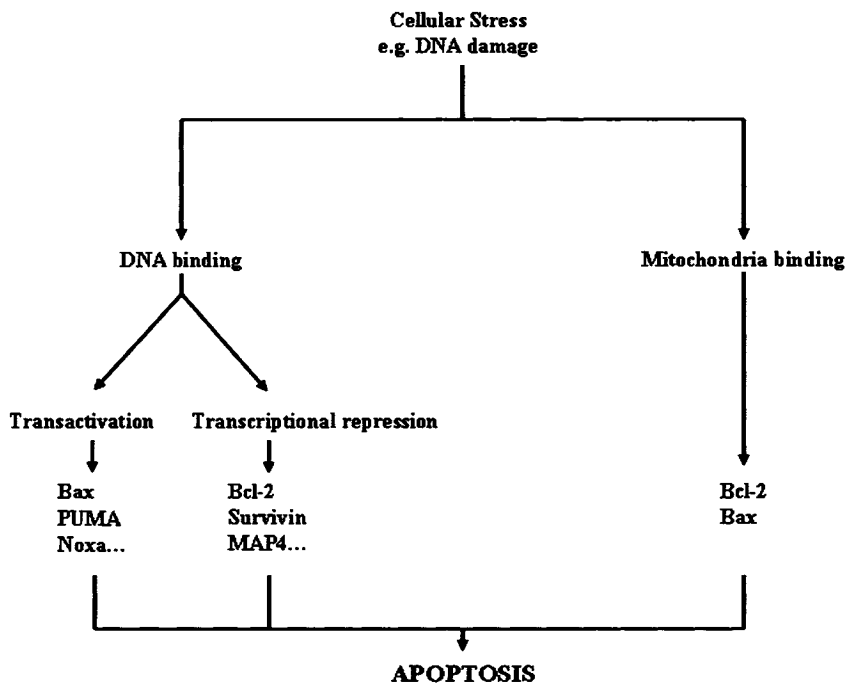
Apoptosis occurs through a highly ordered sequence of signaling cascades. Two main pathways of apoptosis have been described – the 'extrinsic' or death receptor-initiated pathway, and the 'intrinsic' or mitochondrial pathway (for review see (Jin and El-Deiry 2005)). Both pathways lead to the activation of the aspartate-specific cysteine proteases, or caspases, which are believed to be the downstream mediators of apoptosis (Haupt, Berger et al. 2003). The extrinsic apoptotic pathway is initiated by the activation of plasma membrane bound death receptors from the tumour necrosis factor (TNF) receptor family. These include TNF-receptor 1 (TNFR1), apoptosis antigen-1 (APO1/FAS/CD95) and TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1/DR4). The binding of extrinsic ligands to the death receptor, results in the trimerization of the receptors and the recruitment of death domain containing adaptor molecules such as Fas-associated with death domain (FADD) to the cytoplasmic tail. This allows for the formation of the death-inducing-signaling-complex (DISC) which leads to the recruitment and activation of the initiator caspase, caspase-8 and the subsequent activation of the effector caspase, caspase 3. The intrinsic pathway is associated with the permeabilization of the outer mitochondrial membrane and the release of mitochondrial factors such as cytochrome c into the cytoplasm. The mitochondrial pathway is regulated by the Bcl-2 family of proteins as they control mitochondrial permeability and release of mitochondrial factors. Upon release, cytochrome c along with

ATP (or dATP) binds to APAF-1 triggering its oligomerization and the formation of the apoptosome allowing for the subsequent recruitment and activation of the initiator caspase, caspase9. Caspase 9 then goes on to activate the effector caspase, caspase 3. In both pathways, activation of caspase 3 leads to the induction of apoptosis. The extrinsic and intrinsic pathways were considered, until recently, as two distinct and separate pathways. However, recent research has discovered that there is crosstalk between the two pathways. For example, activated caspase 8 from the extrinsic pathway can cleave Bid, a BH3-only member of the Bcl-2 family. Cleaved or truncated Bid (tBid) then translocates to the mitochondria and initiates mitochondrial permeabilization and release of cytochrome c (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998).

III-III. Mechanisms of p53-mediated apoptosis

The mechanisms by which p53 mediates apoptosis are not fully understood but they appear to be dependent on the cell type and the nature of the insult (for review see (Ryan, Phillips et al. 2001)). There is strong evidence supporting the role of p53 as a transcription factor in the induction of cell death. However, there is some controversy whether the transcription function of p53 is indispensable for the induction of cell death as even in the presence of transcription and translation inhibitors, p53-dependent apoptosis is still observed in certain cell types (Caelles, Helmberg et al. 1994) and overexpression of p53 transactivation mutants still promoted cell death in several cancer cell lines (Haupt, Rowan et al. 1995; Ryan and Vousden 1998; Kokontis, Wagner et al.

Fig. 1.5: Mechanisms of p53-mediated apoptosis. The mechanisms through which p53 induces apoptosis include those involving DNA binding such as transcriptional activation of pro-apoptotic genes and transcriptional repression of pro-survival genes along with a direct function at the mitochondria through interactions with the Bcl-2 family members.



Modified from: Slee et al., Oncogene, (2004), 23(16):2809-18.

2001). Furthermore, no one target gene product appears to be absolutely required for p53-mediated apoptosis in all cell types (Haupt, Berger et al. 2003). Thus, mechanisms other than transcriptional activation such as transcriptional repression and transcription-independent mechanisms must account for the ability of p53 to induce apoptosis at least in some cells (for review see (Bates and Vousden 1999; Oren 2003)) (Fig 1.5).

III-III-a. Transcriptional activation

p53 is best known as a sequence specific transcription factor and it is believed that p53 exercises its biological function primarily through transcriptional upregulation of target genes. This is underscored by the fact that in cancer, most tumour derived mutants of p53 are defective in transactivation (Raycroft, Wu et al. 1990; Zambetti, Bargonetti et al. 1992). Two distinct activation domains (TA1 and TA2) have been identified within the N-terminal domain of p53. Overexpression studies using p53 proteins harbouring inactivating deletions or mutations within TA1 have demonstrated that the transcriptional function of TA1 is vital for the induction of apoptosis in certain cellular systems (Attardi, Lowe et al. 1996; Chao, Saito et al. 2000; Jimenez, Nister et al. 2000; Nister, Tang et al. 2005), although not in others (Haupt, Rowan et al. 1995; Chen, Ko et al. 1996; Ding, Lin et al. 2000). Mutations in TA2 have been shown to reduce apoptotic activity and arrest cells in the G_1 phase of the cell cycle (Zhu, Zhang et al. 2000). As well, small molecular compounds and several peptides have been shown to restore mutant p53 transcriptional activity by re-establishing wild-type p53 conformation allowing it to induce apoptosis in cancer cells (Foster, Coffey et al. 1999; Bykov, Issaeva et al. 2002). The relative

importance of transcriptional activation to p53-mediated apoptosis has triggered the search for proapoptotic genes that are direct targets for p53 transcriptional activation. This search has identified many genes that can be activated by p53 and that can mediate apoptosis (Vousden and Lu 2002; Fridman and Lowe 2003; Yu and Zhang 2005) (discussed in section III-IV).

III-III-b. Transcriptional repression

Recent research has shown that the transcriptional repression function of p53, in addition to its transcriptional activation function, may be important for the induction of apoptosis (Miyashita, Harigai et al. 1994; Murphy, Hinman et al. 1996; Roperch, Alvaro et al. 1998). Studies looking at p53 gene regulation during apoptosis and cell cycle progression observed that 80% of responsive genes were transcriptionally repressed by p53 (Mirza, Wu et al. 2003; Kho, Wang et al. 2004). Downregulation of p53-repressed genes by RNAi was sufficient to induce apoptosis (Kho, Wang et al. 2004; Li, Tan et al. 2005) and their overexpression was shown to inhibit apoptosis (Wang, Szekely et al. 1993; Murphy, Hinman et al. 1996; Hoffman, Biade et al. 2002; Mirza, McGuirk et al. 2002; Li, Tan et al. 2005). Additionally, deletion of the proline-rich domain of p53, which has been shown to be required for transcriptional repression (Venot, Maratrat et al. 1998), abrogated p53's ability to induce apoptosis without affecting its ability to transactivate target genes such as Bax (Walker and Levine 1996; Sakamuro, Sabbatini et al. 1997; Venot, Maratrat et al. 1998). Similar to the transcriptional upregulation of proapoptotic targets which would drive apoptosis, the repression of anti-apoptotic genes

could also favour a shift in the balance toward an apoptotic fate. Indeed, p53 has been shown to repress several anti-apoptotic targets and this could play an important role in p53-mediated cell death. Both Bcl-2 and Bcl-X_L, two anti-apoptotic members of the Bcl-2 family, have been shown to be actively repressed by p53 (Haldar, Negrini et al. 1994; Miyashita, Harigai et al. 1994; Miyashita, Krajewski et al. 1994; Sugars, Budhram-Mahadeo et al. 2001). Survivin, a member of the inhibitor of apoptosis protein (IAP) family which fulfills its anti-apoptotic role by inhibiting the caspases, has also recently been identified as a direct target for transcriptional repression by p53 (Hoffman, Biade et al. 2002; Mirza, McGuirk et al. 2002). Other survival factors trans-repressed by p53 include microtubule associated protein MAP4 and presinilin-1 (Murphy, Hinman et al. 1996; Roperch, Alvaro et al. 1998).

The mechanisms by which p53 regulates transcriptional repression have not been completely delineated. Transcriptional repressors are believed to function through three different mechanisms: 1) disruption of the functions of other transcription factors; 2) impedance of the basal transcriptional machinery and; 3) alteration of chromatin structure at the promoters of target genes by recruiting proteins such as histone deacetylases (Ho and Benchimol 2003). Examples of all three mechanisms have been observed in p53-mediated transcriptional repression. In the first instance, p53 has been shown to inhibit ets-1-dependent transcription of thromboxane synthase (THSA), a factor promoting invasion and resistance to apoptosis in gliomas, by direct interaction with ets-1 (Kim, Gunther et al. 2003). Furthermore, it is suggested that p53 binding to limited amounts of transcriptional cofactors, p300 and CBP, may be sufficient to inhibit activity from other

transcriptional factors such as CREB or NF-κB (Wadgaonkar, Phelps et al. 1999). Secondly, p53 can bind to TATA-binding proteins (TBP) and certain TBP-associated factors (TAFs) and suppress initiation of transcription (Seto, Usheva et al. 1992; Truant, Xiao et al. 1993; Horikoshi, Usheva et al. 1995; Thut, Chen et al. 1995; Farmer, Colgan et al. 1996). It has been demonstrated *in vitro* that the interaction of TBP with p53 can inhibit TBP binding to a TATA-containing DNA fragment (Ragimov, Krauskopf et al. 1993). Finally, in many instances transcriptional repression of anti-apoptotic genes by p53 involves the association of p53 with the histone deacetylases (HDACs) mediated through the interaction of p53 with the corepressor protein mSin3a (Sin3) (Murphy, Ahn et al. 1999). It is believed that the interaction of p53 and Sin3 targets HDACs to the promoters of p53-repressed genes where they effectuate the deacetylation of histones and create a chromatin environment not suitable for transcription (Murphy, Ahn et al. 1999; Hoffman, Biade et al. 2002). This mechanism has been observed in the transcriptional repression of Bcl-2 (Lai, Lin et al. 2005), MAP4 (Murphy, Ahn et al. 1999) and SAK (Li, Tan et al. 2005).

III-III-c. Transcription-independent mechanisms of p53-mediated apoptosis

The ability of p53 to induce apoptosis independently of transcription was discovered over ten years ago (Caelles, Helmberg et al. 1994). The mechanism by which this occurs is not completely understood, however, recent research has shed some light on this phenomenon and it appears that some p53 protein is found at the mitochondria at the onset of p53-dependent apoptosis. This was first described in cancer cell lines treated

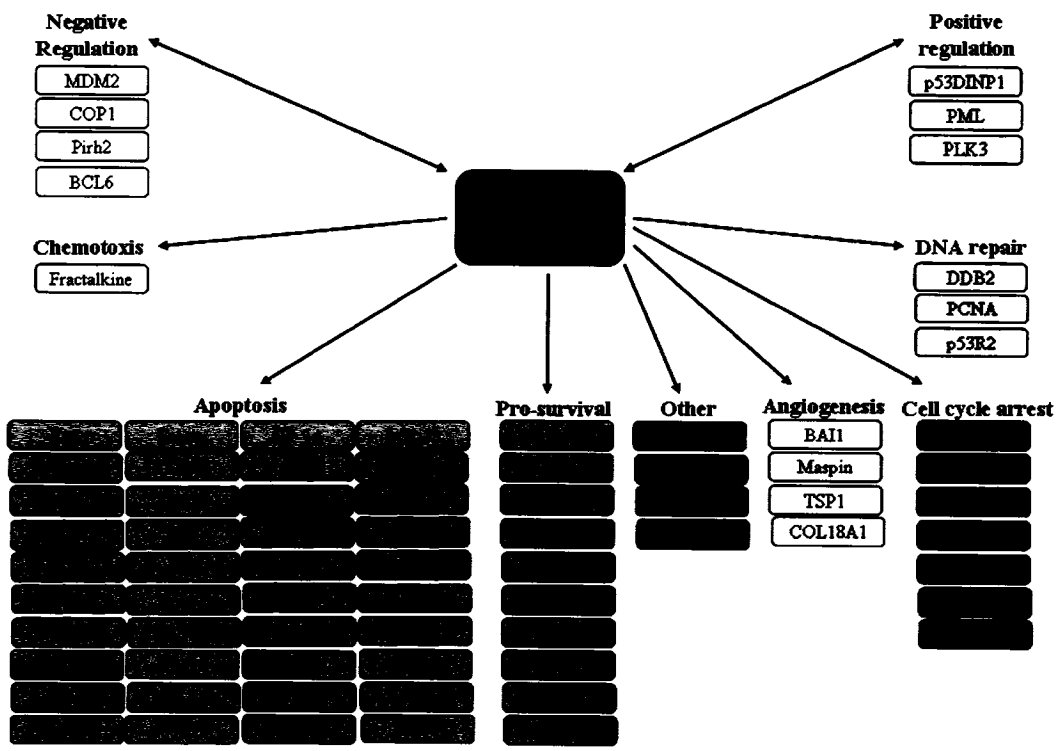
with DNA damaging agents (Marchenko, Zaika et al. 2000). Later, in functional studies of a polymorphism at codon 72 of p53 that substitutes arginine for proline, it was found that $p53^{R72}$ had greater proapoptotic activity then its $p53^{P72}$ counterpart and that this correlated to its ability to co-localize to the mitochondria rather than to its function as a transcription factor (Dumont, Leu et al. 2003). Thus, an extranuclear role for p53 at the mitochondria represented a plausible means by which p53 regulated transcription-independent apoptosis (for review see (Erster and Moll 2005; Moll, Wolff et al. 2005; Schuler and Green 2005; Yee and Vousden 2005)). Substantiating a role for p53 at the mitochondria was research establishing that p53 could function in a manner similar to a BH3-only protein, both as an enabler and an activator (Yee and Vousden 2005). As an enabler, p53 directly interacted and inhibited the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L, permitting the release of Bak from Bcl- X_L, thereby allowing for Bak-mediated mitochondrial outer membrane permeabilization (Mihara, Erster et al. 2003). As an activator, direct interaction between Bak and p53 initiated the release of Bak from the anti-apoptotic myeloid cell leukemia sequence 1 (Mcl-1) resulting in Bak oligomerization and mitochondrial outer membrane permeabilization (Leu, Dumont et al. 2004). It's activator role was also reported in experiments with Bax (Chipuk, Kuwana et al. 2004). *In vivo* evidence of mitochondrial accumulation of p53 was demonstrated in several tissues including thymus, spleen and testis of mice subjected to whole body γ-irradiation (Erster, Mihara et al. 2004) and in kidney of rats following ischemic injury (Kelly, Plotkin et al. 2003), thus, lending a biological relevance to p53 localization to the mitochondria.

Translocation of p53 to the mitochondria occurs very rapidly. It was observed in the thymus and the spleen within 30 minutes of treatment with γ-irradiation and this engendered a p53-dependent activation of caspase 3 within 1 hour and the subsequent induction of apoptosis within 2 hours after irradiation (Erster, Mihara et al. 2004). The kinetics for p53-mediated transcriptional-dependent apoptosis are much slower as mRNA for p53-target gene, PUMA, only begins to be detected in irradiated thymus after 2 hours and peaks after 3 hours (Erster and Moll 2005). This has lead to the proposed hypothesis that accumulation of p53 at the mitochondria initiates a quick apoptotic response to DNA damage while waiting for the slower transcription-dependent mechanism to take over (Erster, Mihara et al. 2004). Whether this holds true in neurons is still yet to be determined.

III-IV. p53 targets regulating apoptosis

The search for p53 target genes has uncovered many candidates (Fig. 1.6) and some have been shown to regulate apoptosis. Many more could still be discovered as it is estimated that 400 or more p53 target genes may exist based on the degenerative nature of the p53 DNA-binding site (el-Deiry 1998). Most of our current understanding of how p53 fulfills its biological functions comes from the identification of effector genes whose expression is upregulated by p53 in non-neuronal cells. Unlike cell cycle arrest which appears to rely on the transcriptional upregulation of several well characterized genes such as $p21^{Waf1}$, 14-3-3 σ and growth arrest and DNA-damage inducible gene # 45

Fig. 1.6: p53-target genes. A schematic summary of p53-target genes sorted in conjunction with their physiological roles and/or their observed roles in the p53 pathway.



Modified from: Nakamura Y., Cancer Science, (2004), 95(1):7-11.

(GADD45) (el-Deiry 1998), p53-mediated apoptosis is not as well defined and may be regulated by multiple complex mechanisms involving many genes (Zhao, Gish et al. 2000). To date no one gene appears to modulate p53-dependent apoptosis in all cell types. A gene that may be crucial for p53-mediated apoptosis in one cell type may be dispensable in another. Thus, it will be critical to identify p53 targets genes involved in p53-mediated neuronal cell death as they may differ from those observed in non-neuronal cells.

Some p53 target genes are directly involved in the apoptotic pathways. Others have distinct functions but their disruption affects the induction of cell death. p53-mediated apoptosis is believed to progress primarily through the intrinsic pathway (Fridman and Lowe 2003), thus, it is not surprising that several genes known to regulate cell death at the mitochondrial level are direct targets for p53. Members of the extrinsic pathway, however, are also represented. Several proapoptotic members of the Bcl-2 family have been identified as p53 targets, the first of these was Bax, the multi-domain protein required for the permeabilization of the outer mitochondrial membrane (Miyashita and Reed 1995). Three members of the BH3-only proteins crucial for the regulation of the multi-domain Bcl-2 family members have, also, been shown to be directly regulated by p53. These include PUMA (Han, Flemington et al. 2001; Nakano and Vousden 2001; Yu, Zhang et al. 2001), Noxa (Oda, Ohki et al. 2000) and Bid (Sax, Fei et al. 2002). Another direct target for p53 is the mitochondrial protein, p53AIP1 (Oda, Arakawa et al. 2000). Overexpression of p53AIP1 resulted in apoptosis characterized by loss of mitochondrial membrane potential and the release of cytochrome c (Matsuda,

Yoshida et al. 2002). This occurred through an unknown mechanism but could be inhibited by Bcl-2 overexpression. Direct interaction with Bcl-2 was observed (Matsuda, Yoshida et al. 2002). As mentioned previously, p53AIP1 induction required the phosphorylation of p53 at ser46 which is mediated by the nuclear protein, p53DINP1, another p53 target (Okamura, Arakawa et al. 2001). Down-regulation of p53DINP1 with an antisense oligonucleotide impaired apoptosis triggered by DNA damage (Okamura, Arakawa et al. 2001). Several death receptors of the extrinsinc pathway such as FAS (also known as APO-1 or CD95) (Owen-Schaub, Zhang et al. 1995), death receptor 4 (DR4/TRAIL-R1) (Guan, Yue et al. 2001) and death receptor 5 (DR5/Killer) (Wu, Burns et al. 1997; Takimoto and El-Deiry 2000) have been shown to be upregulated by p53. Their contribution to p53-dependent apoptosis is an ongoing debate as they can often signal apoptosis independently of p53 (O'Connor, Harris et al. 2000). p53-induced protein with death domain (PIDD), a protein implicated in the activation of caspase 2 through formation of the PIDDosome (Tinel and Tschopp 2004), was identified as a target for p53 (Lin, Ma et al. 2000). The physiological relevance of PIDD to p53-dependent apoptosis has not been tested. Apoptotic signaling through the intrinsic and extrinsic pathways leads to downstream activation of the caspases. Until recently no caspases were believed to be direct targets for p53. Caspase 6, however, appears to be a p53 target and may represent the most downstream effector of apoptosis to be regulated by p53 (MacLachlan and El-Deiry 2002).

Other p53 targets have been shown to play a role in apoptosis. p53 apoptosis effector related to PMP-22 (PERP) is a transmembrane protein upregulated during p53-

dependent apoptosis but not cell cycle arrest (Attardi, Reczek et al. 2000). It regulates apoptosis in a cell type-specific manner as work performed with PERP-deficient mice afforded some protection to thymocytes from γ-irradiation (Ihrie, Reczek et al. 2003). p53-inducible genes (PIGs), such as PIG3 (Contente, Dittmer et al. 2002) and PIG8/ei24 (Gu, Flemington et al. 2000), are believed to play a role in the redox state of the cell and specifically to regulate increased apoptosis-dependent production of reactive oxygen species leading to mitochondrial dysfunction (Polyak, Xia et al. 1997). These may play roles in the induction of apoptosis at other organelles as well, as PIG8 has recently been suggested to mediate apoptosis stemming from the endoplasmic reticulum through regulation of Bcl-2 activity at this organelle (Zhao, Ayer et al. 2005). Two other p53 target genes localized at the endoplasmic reticulum, the sodium channel subunit 3 beta (SCN3B) and Scotin are also believed to modulate apoptosis. SCN3B was upregulated following DNA damage in cancer cell lines and adenoviral-mediated expression of SCN3B enhanced apoptosis induced by anticancer agents. In certain cancer cell lines, its overexpression was sufficient to induce apoptosis (Adachi, Toyota et al. 2004). Forced expression of Scotin was sufficient to induce apoptosis in cancer cell lines and disruption of Scotin expression by anti-sense oligonucleotide protected cells from UV-induced apoptosis (Bourdon, Renzing et al. 2002).

Many genes have been identified as p53 targets involved in the regulation of apoptosis. The disruption of these genes, however, has not been able to fully recapitulate the effects observed from p53-deficiency. p53-dependent apoptosis is often impaired to varying degrees in these knock-outs but never to the same extent as that seen in the p53

knock-out. This would suggest that p53-dependent apoptosis is orchestrated by multiple genes. It should be noted that knock-outs for all p53-targets have not yet been generated. One gene, PUMA, however, appears to have a well defined role in p53-mediated apoptosis. PUMA is believed to be essential for p53-mediated apoptosis in many cell types (Jeffers, Parganas et al. 2003; Villunger, Michalak et al. 2003). Experiments using PUMA-knockout mice demonstrated that loss of PUMA afforded similar deficiencies in the apoptotic program as that observed in p53-knockout mice. This was especially evident in the apoptosis induced by γ-irradiation in thymocytes and induced by oncogenes in MEFs (Jeffers, Parganas et al. 2003; Villunger, Michalak et al. 2003). Thus, PUMA is the first p53 target gene to offer comparable protection from apoptosis upon deletion as that of p53 deletion. Although the protection offered by PUMA-deficiency was substantial it did not, however, fully equal the level of protection observed with p53-deficiency suggesting that other proapoptotic factors are also at play.

IV. P53 in Neuronal Apoptosis

IV-I. Introduction

Investigators have only recently begun to focus on a role for p53 in neuronal apoptosis. This was prompted by evidence indicating that p53 was a potent inducer of cell death and that some models of neuronal injury lead to DNA damage, an important model of p53 activation in cancer research. In the central nervous system (CNS),

apoptosis plays an important function. It is crucial for development, is required for the effective removal of damaged neurons following injury and is observed in neurodegenerative disorders. Thus, the study of a potential role for p53 in neuronal cell death was a natural progression.

IV-II. p53 in neuronal injury

Over the past ten years, accumulated evidence demonstrating a role for p53 in the apoptosis of postmitotic neurons (for review see (Morrison and Kinoshita 2000; Morrison, Kinoshita et al. 2003; Culmsee and Mattson 2005)) has come through two streams of studies. First of all, documented increases in p53 expression both at the mRNA and at the protein level have been observed in a large number of studies involving both *in vivo* and *in vitro* models of injury (Morrison, Kinoshita et al. 2003). Secondly, forced expression of p53 was sufficient to induce apoptosis in several postmitotic neuronal populations including sympathetic (Slack, Belliveau et al. 1996), hippocampal (Jordan, Galindo et al. 1997) and cortical neurons (Xiang, Hochman et al. 1996).

In vivo, accumulation of p53 was observed in a wide variety of acute injury models including adrenalectomy (Schreiber, Sakhi et al. 1994), ischemia (Chopp, Li et al. 1992; Li, Chopp et al. 1994; Joo, Choi et al. 1999; Tomasevic, Kamme et al. 1999; Watanabe, Ohta et al. 1999), excitotoxicity (Sakhi, Bruce et al. 1994; Hughes, Alexi et al. 1996; Sakhi, Sun et al. 1996; Qin, Chen et al. 1999; Wang, Qin et al. 1999; Nakai, Qin et al. 2000; Tan, Sankar et al. 2002), ionizing radiation (Herzog, Chong et al. 1998) and traumatic brain injury (TBI) (Kaya, Mahmood et al. 1999; Muir, Raghupathi et al. 1999;

Napieralski, Raghupathi et al. 1999). Of these, excitotoxicity has demonstrated a very strong correlation with induction of p53 (Morrison and Kinoshita 2000; Morrison, Kinoshita et al. 2003). Excitotoxicity involves the sustained activation of glutamate receptors by glutamate receptor agonists such as glutamate, kainic acid (KA) and N-methyl-D-aspartate (NMDA). The molecular mechanism linking excitotoxicity to p53 activation is believed to involve DNA damage. Neuronal injury mediated by excitotoxicity has been linked to increased production of reactive oxygen species (ROS) (Ankarcrona, Dypbukt et al. 1995; Dugan, Sensi et al. 1995; Reynolds and Hastings 1995; Schinder, Olson et al. 1996) and the accumulation of single-stranded DNA breaks (Didier, Bursztajn et al. 1996). Higher levels of p53 were reported using a KA seizure model (Sakhi, Bruce et al. 1994; Sakhi, Sun et al. 1996). In rodent models, seizures produced by direct injection of KA are associated with a well characterized progression of neuronal cell death especially to neurons within the CA3 hippocampal region (Strain and Tasker 1991). The increased expression of p53 occurred in neurons exhibiting morphological signs of damage (Sakhi, Bruce et al. 1994). In similar research, the intrastriatal injection of either N-methyl-D-aspartate (NMDA), quinolic acid (QA) or KA stimulated significant accumulation of p53 in striatal neurons (Qin, Chen et al. 1999; Wang, Qin et al. 1999; Nakai, Qin et al. 2000). Studies using TBI, revealed increased p53 mRNA levels as early as 6 hours following treatment in the affected neurons of the cortex, the hippocampus and the thalamus (Napieralski, Raghupathi et al. 1999). Similarly, ischemia-induced cell death of cortical and striatal neurons through middle cerebral artery occlusion is associated with a significant increase in p53 mRNA and

protein in the penumbral region within 24 hours of treatment (Chopp, Li et al. 1992; Watanabe, Ohta et al. 1999). The penumbral region is the area surrounding the ischemic core and undergoes a delayed cell death taking 2-5 days (Linnik, Zobrist et al. 1993; Li, Chopp et al. 1995; Li, Chopp et al. 1995; Li, Chopp et al. 1995; Siesjo, Katsura et al. 1995). Death in this region is believed to be responsible for the major cause of neurological defect following stroke (Olsen, Bruhn et al. 1986). Both TBI and ischemia are believed to mediate neuronal apoptosis through activation of multiple interdependent molecular pathways which are triggered by increased levels of extracellular excitatory amino acids, especially glutamate (Arundine and Tymianski 2004). As with excitotoxicity, TBI and ischemia result in a prolonged activation of glutamate receptors which can lead to elevated levels of ROS and subsequent DNA damage (Arundine and Tymianski 2004).

In vitro models of neuronal injury have also demonstrated increased levels of p53 expression corroborating the results from *in vivo* studies. Excitotoxicity induced by treatment with glutamate significantly increased expression of p53 mRNA and protein in cultured cerebellar granule neurons (Uberti, Belloni et al. 1998; Chen and Chuang 1999). Upregulation of p53 protein was observed in cultured cortical neurons following exposure to hypoxic conditions, which mimics the ischemic insult induced by middle cerebral artery occlusion *in vivo*. Hypoxia-exposed neurons displayed changes in cellular morphology and DNA fragmentation consistent with apoptosis and the increases in p53 protein was dependent on the severity and the duration of the hypoxic treatment (Banasiak and Haddad 1998). DNA damage induced by genotoxic agents or ionizing

radiation (Wood and Youle 1995; Enokido, Araki et al. 1996; Jordan, Galindo et al. 1997; Johnson, Xiang et al. 1998) was also a potent stimulus for enhancing p53 expression. Substantial evidence exists indicating that most DNA-damaging agents can induce apoptosis in postmitotic neurons including sympathetic, cortical, hippocampal and cerebellar neurons. Examples of genotoxic agents used to induce neuronal cell death include cytosine arabinoside (araC) (Winkelman and Hines 1983; Wallace and Johnson 1989; Martin, Wallace et al. 1990; Tomkins, Edwards et al. 1994; Anderson and Tolkovsky 1999; Chen and Chuang 1999), DNA topoisomerase II inhibitors such as etoposide (Enokido, Araki et al. 1996), cisplatin (Windebank 1999) and the topoisomerase I inhibitor camptothecin (Morris and Geller 1996), the latter, which we have used extensively in our laboratory. The importance of DNA damage as an initiator of neuronal apoptosis via p53 is underscored by the observation of massive neuronal apoptosis in mice deficient for DNA repair proteins, DNA ligase IV and XRCC4, and its subsequent rescue by p53 deficiency (Frank, Sharpless et al. 2000; Gao, Ferguson et al. 2000).

The physiological relevance of p53 to these *in vivo* and *in vitro* models of neuronal injury has been evaluated using p53-deficient neurons derived from these mice and inhibitors of p53 expression or p53 function. P53-deficiency protected neurons from *in vivo* models of neuronal injury including focal ischemia (Crumrine, Thomas et al. 1994), ionizing radiation (Wood and Youle 1995; Herzog, Chong et al. 1998; Chong, Murray et al. 2000), kainate-induced seizures (Morrison, Wenzel et al. 1996), metamphetamine-induced neurotoxicity (Hirata and Cadet 1997) and adrenalectomy

(Sakhi, Gilmore et al. 1996). In cultured neurons, p53-deficiency rescued cells from many toxic insults including DNA damaging agents (Enokido, Araki et al. 1996; Araki, Enokido et al. 1998; Xiang, Kinoshita et al. 1998; Anderson and Tolkovsky 1999; Chen, Saunders et al. 1999; D'Sa-Eipper, Leonard et al. 2001; Morris, Keramaris et al. 2001), ionizing radiation (Enokido, Araki et al. 1996; Johnson, Xiang et al. 1998), excitotoxicity (Xiang, Hochman et al. 1996; Uberti, Belloni et al. 1998; Chen and Chuang 1999), and hypoxia (Banasiak and Haddad 1998; Halterman, Miller et al. 1999). Antisense oligonucleotides have been used successfully to protect neurons from excitotoxicity (Uberti, Belloni et al. 1998; Lakkaraju, Dubinsky et al. 2001), DNA damage (Chen, Saunders et al. 1999) and hypoxia (Banasiak and Haddad 1998). The compound pifithrin-α (PFT), which can inhibit p53 transcriptional activity, protected cultured neurons from DNA damage and excitotoxicity and intraperitoneal injection of pifithrin-α in mice reduced ischemic brain injury induced by middle cerebral artery occlusion (Culmsee, Zhu et al. 2001). P53-deficiency does not, however, protect neurons from all death stimuli as death of cerebellar granule neurons was still observed following potassium withdrawal (Enokido, Araki et al. 1996) and following treatment with methylazoxymethanol (Wood and Youle 1995). Taken together, the evidence suggests that p53 plays an important role in neuronal cell death following acute neuronal injury.

IV-III. p53 in neurodegenerative disease

Although the above evidence builds a strong case for the involvement of p53 in acute neuronal apoptosis, its role in neurodegenerative disease is somewhat more controversial. A common sequence of events delineates the biochemical pathway leading to neuronal cell death despite the fact that neurodegenerative disorders may be triggered by different genetic or environmental factors. It involves excitotoxic and oxidative mechanisms (Beal 1996; Markesbery 1997; Shaw and Ince 1997; Grunewald and Beal 1999), both of which could lead to DNA damage and potentially to activation of p53 (Miller, Pozniak et al. 2000). Hence, similar to the studies in acute neuronal injury, much of the evidence describing a role for p53 in neurodegenerative diseases comes from observations of elevated levels of p53 in diseased tissue (Morrison, Kinoshita et al. 2003). This has been documented in brain tissue obtained from patients diagnosed with a neurodegenerative disorder and from animal models simulating human neurodegenerative diseases. The strongest evidence for the involvement of p53 in neurodegenerative diseases, however, is that observed from studies in Parkinson's disease (PD) and Huntington's disease (HD).

Parkinson's disease results primarily from the death of dopaminergic neurons in the substantia nigra pars compacta (Dauer and Przedborski 2003). The favoured *in* vivo model of PD is that induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) because it is thought to reproduce the pathophysiology, anatomy and biochemistry of PD (Mandir, Simbulan-Rosenthal et al. 2002). Dopaminergic neuron death induced by MPTP has been associated with the harmful production of ROS (Schmidt and Ferger 2001), DNA damage (Mandavilli, Ali et al. 2000; Wang, Shimoji et al. 2003), and the

accumulation and activation of p53 (Duan, Zhu et al. 2002; Mandir, Simbulan-Rosenthal et al. 2002). The physiological relevance of p53 to this model of PD is evoked by the protection offered by p53-deficiency (Trimmer, Smith et al. 1996) and by pharmacological inhibitors of p53 (Duan, Zhu et al. 2002). The requirement of p53 to induce cell death was also described in an *in vitro* model of PD (Biswas, Ryu et al. 2005). In neuronal PC12 cells treated with 6-hydroxydopamine (6-OHDA), a drug used to mimic the neurodegeneration observed in PD, the upregulation of p53 protein was observed (Blum, Wu et al. 1997) and p53 activation correlated with the ATM-dependent phosphorylation of p53 on ser15 (Nair 2006). The ability of p53 to induce cell death in neuronal PC12 cells treated with 6-OHDA was dependent on the upregulation of the p53-inducible gene PUMA and inhibition of p53 protected neurons from 6-OHDA (Biswas, Ryu et al. 2005; Nair 2006).

Huntington's disease is a genetically dominant degenerative disorder caused by a mutation in the huntingtin (Htt) gene which is characterized by expanded polyglutamines. This results in production of a misfolded huntingtin protein (mHtt) which cannot be easily degraded and accumulates in the nucleus where it forms inclusion bodies with deleterious effects to striatal neurons (La Spada and Morrison 2005). Elevated levels of p53 were observed in striatal neurons cultured from mHtt knockin mice (Trettel, Rigamonti et al. 2000) as well as in PC12 cells overexpressing mHtt (Bae, Xu et al. 2005). In the latter study, higher p53 levels corresponded to increased p53 transcriptional activity as measured by upregulation of the several p53 target genes including Bax and

PUMA (Bae, Xu et al. 2005). Deletion of p53 protected primary cortical neurons from mHtt induced apoptosis and reduced retinal degeneration in the mHtt transgenic fly (Bae, Xu et al. 2005). Furthermore, p53-deficiency ameliorated behavior abnormalities in the mHtt transgenic mouse (Bae, Xu et al. 2005). Taken together, the evidence indicates that p53 could play an important role in the death of neurons associated with Parkinson's disease and Huntington's disease.

The role of p53 in other neurological diseases such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) is much less well defined. Patients suffering from AD have shown increased p53 protein levels in neurons exhibiting signs of morphological damage consistent with apoptosis (de la Monte, Sohn et al. 1997; de la Monte, Sohn et al. 1998). Other research looking at changes of p53 levels in the brains of patients with AD, however, suggest that p53 immunoreactivity is observed only in glial cells and that apoptosis in neurons of AD patients may occur through a p53-independent pathway (Kitamura, Shimohama et al. 1997). Amyotrophic lateral sclerosis (ALS) which results in selective death of upper motor neurons in the cerebral cortex and lower motor neurons in the brainstem and spinal chord has also been linked to increased levels of p53 (de la Monte, Sohn et al. 1998; Gonzalez de Aguilar, Gordon et al. 2000; Martin 2000). Mutations in superoxide dismutase (SOD) 1 account for 20% of all familial forms of ALS (Sathasivam and Shaw 2005) and transgenic mice expressing the human G93A superoxide dismutase (SOD) 1 mutation when crossbred to p53-knockout mice did not show any neuroprotective effect in the absence of p53 (Kuntz, Kinoshita et al. 2000;

Prudlo, Koenig et al. 2000). Thus, more research will be required to fully understand the role of p53 in these two neurodegenerative diseases.

IV-IV. p53 in neuronal development

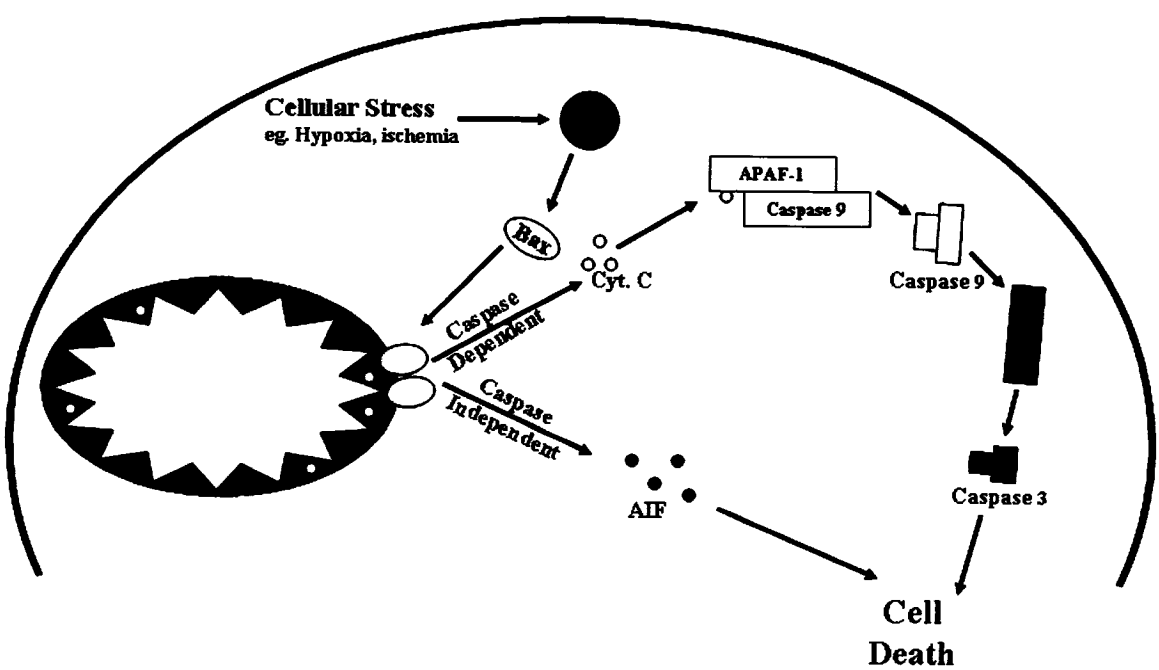
During development of the CNS, it is believed that an excess of progenitor cells and postmitotic neurons are produced (Miller, Pozniak et al. 2000). Subsequently, the cells that have not fully differentiated or have not made the appropriate connections with their target cells are eliminated through programmed cell death (Miller, Pozniak et al. 2000). The importance of apoptosis to brain development is underscored by the observations that deficiency of certain key apoptotic genes resulted in increased cell numbers in the brains of the corresponding mutant mice and this with deleterious effects. Mice deficient for caspase 9 (Kuida, Haydar et al. 1998), caspase 3 (Kuida, Zheng et al. 1996) and Apaf-1 (Cecconi, Alvarez-Bolado et al. 1998; Yoshida, Kong et al. 1998) all exhibited brain malformations from an expanded progenitor population due to failed apoptosis. These mice died embryonically or shortly after birth. A role for p53 in brain development was, originally, not suspected as mice carrying a null mutation in the p53 gene were viable. These mice, however, were prone to developing tumours and only survived to early adulthood (Donehower, Harvey et al. 1992). Upon further examination, it was discovered that a significant portion of the p53 -/- embryos died during embryogenesis (Armstrong, Kaufman et al. 1995; Sah, Attardi et al. 1995). They were of similar phenotype to the Apaf-1- and caspase 9-deficient embryos in that they had severe craniofacial malformations and brain overgrowth due to impaired apoptosis during

development. Thus, from these observations, it was suggested that apoptosis mediated by p53 plays a role in regulating cell numbers during development of the CNS. p53 has also been described to play a crucial role in regulating apoptosis of sympathetic neurons during the development of the peripheral nervous system (PNS) (Aloyz, Bamji et al. 1998). Sympathetic neurons deficient for p53 were protected from apoptosis induced by nerve growth factor withdrawal (NGF) and p75 neurotrophin receptor activation (Aloyz, Bamji et al. 1998).

IV-V. P53-mediated pathway of neuronal apoptosis

Currently, in non-neuronal cells, it is believed that p53-mediated apoptosis functions primarily through the intrinsic pathway and that the extrinsic pathway is only used to enhance the apoptotic response (Fridman and Lowe 2003). In neurons, there is strong evidence suggesting that the intrinsic pathway plays a major role (Fig. 1.7). Following DNA damage induced by camptothecin, we and others have shown that neurons undergo apoptosis through a mechanism consisting of Bax translocation (Morris, Keramaris et al. 2001), cytochrome c release (Stefanis, Park et al. 1999; Keramaris, Stefanis et al. 2000) and caspase activation (Stefanis, Park et al. 1999; Keramaris, Stefanis et al. 2000). This was also observed following death induced by glutamate, Aβ and oxidative stress (Paradis, Douillard et al. 1996; Dargusch, Piasecki et al. 2001; Polster and Fiskum 2004). We have also demonstrated that overexpression of p53 induces apoptosis in cultured neurons through a Bax-dependent activation of caspase 3

Fig. 1.7: p53-mediated pathways of neuronal cell death. Cellular stress signals activate p53. This is followed by the translocation of Bax to the mitochondria and the release of cytochrome c into the cytoplasm. Upon release, cytochrome c binds to Apaf-1 initiating the formation of the apoptosome and allowing for the subsequent recruitment and activation of initiator and effector caspases. Apoptosis thus ensues. Inhibition of caspase activation triggers a caspase-independent delayed cell death involving mitochondrial release and nuclear translocation of apoptosis inducing factor (AIF).


Cellular Stress
eg. Hypoxia, ischemia
Bax
Cyt. C
APAF-1
Caspase 9
Caspase 9
Caspase Dependent
Caspase Independent
AIF
Caspase 3
Cell Death

(Cregan, MacLaurin et al. 1999). The importance of Bax to p53 mediated-neuronal cell death is evident by the observations that Bax-deficiency protected neurons from p53 overexpression as well as from ionizing radiation and camptothecin (Xiang, Hochman et al. 1996; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999; Chong, Murray et al. 2000; Morris, Keramaris et al. 2001). Caspase-deficiency or the inhibition of caspase activity does not afford the same protection, suggesting caspase-independent mechanisms may also be at play (Johnson, Xiang et al. 1998; Johnson, Kinoshita et al. 1999; Yu, Wang et al. 2002; Fukuda, Fukuda et al. 2004). We (Cregan, Fortin et al. 2002; Cheung, Melanson-Drapeau et al. 2005) and others (Dawson and Dawson 2004; Hong, Dawson et al. 2004) have shown that caspase-independent mechanisms of p53-mediated neuronal cell death involve mitochondrial release and nuclear translocation of apoptosis inducing factor (AIF). While p53 can induce caspase-dependent and caspase-independent pathways, the upstream mechanisms are not fully understood.

IV-VI. Mechanisms of p53-mediated neuronal apoptosis

The mechanisms by which p53 mediates apoptosis in neurons are not fully understood. The contributions of p53 transcriptional activation, p53 transcriptional repression and p53 transcription-independent mechanisms of apoptosis have not been completely elaborated but it is believed that transcriptional activation is the key regulator. We have found that transcriptional activation is essential for p53-mediated neuronal cell death (Cregan, Arbour et al. 2004). From i*n vitro* studies, we observed that expression of

a transactivation mutant or a DNA-binding mutant p53 protein was incapable of inducing apoptosis in cultured neurons. This was corroborated in studies exploring the responsiveness of p53-deficient neurons to DNA-damage induced apoptosis following re-introduction of either wild-type p53 or various deletion/point mutants of p53. Neurons expressing either a p53 protein deficient for transcriptional activity or for DNA-binding activity were protected form DNA-damage induced apoptosis to the same extent as p53-deficient controls (Cregan, Arbour et al. 2004). Furthermore, neurons cultured from QS homozygous mice, which harbour a mutation in the first activation domain of p53 (Jimenez, Nister et al. 2000), were also as equally protected from DNA-damage induced apoptosis as neurons cultured from p53-deficient mice (Cregan, Arbour et al. 2004). These results clearly suggest a crucial role for p53 transcriptional activation in the induction of neuronal cell death which sets them apart from many other cell types that can still undergo apoptosis independently of p53 transcriptional activity.

V. Statement of Problems and Objectives

Although there has been extensive research examining p53-mediated apoptosis, the bulk of this work has been done to elucidate its role as a tumour suppressor in non-neuronal cells. Various mechanisms exist by which p53 can mediate apoptosis but its capacity as a transcription factor is believed to be the principal mechanism by which it fulfills this function. Observations that cells cultured from mice expressing a

transactivation-defective mutant p53 were resistant to p53-mediated apoptosis further reinforced this hypothesis. Given this evidence and the fact that little is known regarding the mechanisms by which p53 could induce apoptosis in neuronal cells,

I hypothesized that p53 induces neuronal apoptosis by transcriptional activation of key proapoptotic genes.

The search for p53 target genes that could regulate apoptosis identified many candidate genes, mostly in non-neuronal cells. At the onset of this research, very few p53 targets had been identified in neurons other than Bax, a proapoptotic member of the Bcl-2 family. We and others, however, demonstrated that Bax expression was not induced during neuronal apoptosis. The primary objective of my research, therefore, was as follows:

To identify p53 target genes which are important for the regulation of injury-induced apoptosis in neurons.

Fortin, A. *et al.* (2001) APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. **J.Cell Biol.** 155(2): 207-16.

This first manuscript describes Apaf1 as a direct target for the transcription factor p53 and characterizes its role in neuronal cell death.

The experiments in this manuscript were performed by A. Fortin, assisted occasionally by Dr. S.P. Cregan with the exception of microarray analysis which was performed by Dr. S.P. Cregan and J.G. MacLaurin and middle cerebral artery occlusion which was performed by Dr. C.S. Thompson from the laboratory of Dr. A. Hakim. Technical advice was provided by JG MacLaurin for gel shift experiments and by N. Kushwaha from the laboratory of Dr. A.R. Albert for luciferase reporter assays. Dr. E.S. Hickman from the laboratory of Dr. K. Helin cloned the Apaf1 promoter used in the luciferase repoter assays. Dr. F. Cecconi provided the Apaf1 mice used in these experiments. Dr. D.S Park provided feedback. Reagents were provided as outlined in the text. This manuscript was written under the guidance and editorial assistance of Dr. S.P. Cregan and Dr. R.S. Slack.

APAF1 IS A KEY TRANSCRIPTIONAL TARGET FOR P53 IN THE REGULATION OF NEURONAL CELL DEATH

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Key Words: apoptosis; neurodegeneration; neurons; bax; caspase 3

ABSTRACT

P53 is a transcriptional activator which has been implicated as a key regulator of neuronal cell death after acute injury. We have shown previously that p53-mediated neuronal cell death involves a Bax-dependent activation of caspase 3; however, the transcriptional targets involved in the regulation of this process have not been identified. In the present study, we demonstrate that p53 directly upregulates Apaf1 transcription as a critical step in the induction of neuronal cell death. Using DNA microarray analysis of total RNA isolated from neurons undergoing p53-induced apoptosis a 5-6 fold upregulation of Apaf1 mRNA was detected. Induction of neuronal cell death by camptothecin, a DNA-damaging agent that functions through a p53-dependent mechanism, resulted in increased Apaf1 mRNA in p53 positive, but not p53 deficient neurons. In both in vitro and in vivo neuronal cell death processes of p53-induced cell death, Apaf1 protein levels were increased. We addressed whether p53 directly regulates Apaf1 transcription via the two p53 consensus binding sites in the Apaf1 promoter. Electrophoretic mobility shift assays demonstrated p53-DNA binding activity at both p53 consensus binding sequences in extracts obtained from neurons undergoing p53-induced cell death, but not in healthy control cultures or when p53 or the p53 binding sites were inactivated by mutation. In transient transfections in a neuronal cell line with p53 and Apaf1 promoter-luciferase constructs, p53 directly activated the Apaf1 promoter via both p53 sites. The importance of Apaf1 as a p53-target gene in neuronal cell death was evaluated by examining p53-induced apoptotic pathways in primary cultures of Apaf1-deficient neurons. Neurons treated with camptothecin were significantly protected in the absence of Apaf1 relative to

those derived from wild type littermates. Together, these results demonstrate that Apaf1 is a key transcriptional target for p53 that plays a pivotal role in the regulation of apoptosis after neuronal injury.

INTRODUCTION

Apoptosis is a biological process that plays a crucial role in nervous system development and injury. During development, cell death is essential for the regulation of neuronal cell number as well as protection against the propagation of aberrant cells (Henderson 1996). In the mature nervous system, inappropriate cell death is implicated as an underlying defect in many types of neurodegeneration (Portera-Cailliau, Hedreen et al. 1995; Smale, Nichols et al. 1995)as well as in acute neurological insults (Li, Sharov et al. 1995; Nitatori, Sato et al. 1995; Rink, Fung et al. 1995). Therefore, understanding the molecular events triggering apoptosis is an important step towards the development of effective treatment strategies for such neurological diseases.

The p53 tumor suppressor gene is involved in the regulation of apoptosis in several death paradigms. In oncogenesis, p53 plays an essential role in preventing the propagation of DNA-damaged cells and controlling aberrant cell cycle regulation (for review see (Prives and Hall 1999)). In the mature nervous system, p53 has been implicated as a key regulatory molecule following neuronal injury (for review see (Hughes, Alexi et al. 1997)). Enhanced expression of p53 has been observed in injured neurons prior to cell death induced by focal ischemia (Li, Chopp et al. 1994; McGahan, Hakim et al. 1998), excitotoxicity (Sakhi, Sun et al. 1996; Xiang, Hochman et al. 1996), and hypoxia (Banasiak and Haddad 1998). Furthermore, we have shown that p53 overexpression itself is sufficient to trigger apoptosis in postmitotic neuronal cultures (Slack, Belliveau et al. 1996; Cregan, MacLaurin et al. 1999). Although the mechanisms by which p53 induces apoptosis in proliferating cells are becoming elucidated, those

involved in the induction of p53-mediated neuronal cell death appear to be distinct and are poorly understood.

The signaling cascade induced by p53 is complex and likely differs depending on the type of tissue examined (for review see (Prives and Hall 1999)). In postmitotic neurons, we and others (Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999; Keramaris, Stefanis et al. 2000) have demonstrated that p53-induced cell death involves a Bax-dependent caspase 3 activation, suggesting that these molecules are important determinants in neuronal cell death after injury. However, although Bax was shown to play a crucial role in p53-induced apoptosis, there was little or no induction of Bax protein during neuronal cell death (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999). Presently little is known regarding the transcriptional targets for p53 that are important in the regulation of neuronal cell death.

Recent studies have demonstrated the involvement of caspase activation in the regulation of neuronal cell death both during development and after injury. For example, the absence of Apaf1, caspase 9, or caspase 3 results in severe craniofacial malformations with dramatically enhanced neuronal cell numbers in developing mouse embryos (Kuida, Zheng et al. 1996; Cecconi, Alvarez-Bolado et al. 1998; Kuida, Haydar et al. 1998; Yoshida, Kong et al. 1998). These gross developmental defects were attributed to failed apoptosis in the developing nervous system. Furthermore, caspases have been implicated in neuronal cell death induced by acute injury (Gillardon, Bottiger et al. 1997; Hara, Friedlander et al. 1997; Cheng, Deshmukh et al. 1998; Endres, Namura et al. 1998; Ni, Wu et al. 1998). Since our previous studies (Cregan, MacLaurin et al. 1999; Keramaris,

Stefanis et al. 2000), as well as those of others, have shown that caspase activation is a key determinant in the molecular cascade by which p53 induces neuronal cell death, we examined the role of Apaf1 in this pathway. In this study, we show that Apaf1 is a direct target induced by p53 and that Apaf1 plays a pivotal role in the regulation of neuronal apoptosis after injury.

MATERIALS AND METHODS

Transgenic Mice

Apaf1-deficient transgenic mice have been described previously (Cecconi, Alvarez-Bolado et al. 1998) and were maintained on a C57BL6 background to maintain genetic uniformity. Apaf1 null mice were genotyped by PCR as described previously. The primers for the wild type and knockout Apaf1 alleles were 5'-AGATAGCCTAGGGGGTGCAT-3' (sense) and 5'-ATCAGTTTCCAAT-CGCTGCT-3' (antisense). Conditions were set as follows: 94°C, 5 min (1 cycle); 94°C, 1.5 min, 66°C, 1 min, 72°C, 1.5 min (30 cycles); 72°C, 10 min (1 cycle). p53-deficient transgenic mice were obtained from The Jackson Laboratory and were maintained on a C57BL6 background to maintain genetic uniformity. P53-deficient mice were genotyped by PCR. The primers for the wild type were 5'-GTATCTGGAAGACAGGCAGAC-3' (sense) and 5'-TGTACTTGTAGTGGATGGTGG-3' (antisense) and for the knockout p53 alleles were 5'-TTCCTCGTGCTTTACGGTATC-3' (sense) and 5'-TATACTCAGAGCCGGCCT-3' (antisense). Conditions were set as follows: 94°C, 5 min (1 cycle); 94°C, 1 min; 55°C, 1 min (30 cycles); 72°C, 1 min; 72°C, 10 min (1 cycle).

Primary Cortical Neuron Cultures

Cortical neurons were cultured from dissociated cortices of embryonic day 14.5 mice, as described previously (Xiang, Hochman et al. 1996) with certain modifications. Cortices from individual embryos were dissected and incubated for 25 minutes at 37°C in 1x

Hank's balanced salt solution (GIBCO BRL) containing 0.50 mg/ml trypsin. Trypsinization was stopped by incubating with 0.2 mg/ml trypsin inhibitor (Boehringer) and 0.2 mg/ml DNase I (Boehringer) for 2 minutes at 25°C. Cells were pelleted and triturated in Neurobasal medium (GIBCO BRL) containing 0.2 mg/ml trypsin inhibitor and 0.25 mg/ml DNase1. The cell suspension was centrifuged and the pellet was resuspended in Neurobasal medium containing B-27 supplement, N-2 supplement, 0.5 mM glutamine and 0.05 units/ml-0.05mg/ml penicillin-streptomycin (GIBCO BRL). Cells were plated in either Nunc 4-wells (3×10^5 cells/well) or 35x10mm (1.5×10^6 cells) dishes (GIBCO BRL) coated with poly-d-lysine (Sigma-Aldrich). Cortices from each embryo were cultured individually and remaining tissue was used for genotyping after which the appropriate cultures were selected for experimentation.

Murine SN48 cells, which were derived by fusing septal cells from postnatal day 21 mice to N18TG2 neuroblastoma cells (Lee, Hammond et al. 1990), were maintained in DME supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. Cells were grown to 50-60% confluence, and the media was replaced 12 h before transfection by calcium phosphate coprecipitation as described previously (Storring, Charest et al. 1999).

Semiquantitative RT-PCR Analysis

Total RNA was isolated from cells using Tripure isolation reagent according to the manufacturer's instructions (Boehringer). Pilot experiments were done to determine the linear range of amplification with respect to amount of starting template and PCR cycles.

2 ng of total RNA was used for cDNA synthesis and targeted gene amplification using the SuperScript One-Step RT-PCR kit (GIBCO-BRL). cDNA synthesis was carried out at 48°C for 45 min followed by a 2 min initial denaturation step at 94°C. This was followed by 35 cycles (Apaf1) or 25 cycles (GAPDH) at 94°C for 30 s, 56-58°C for 30 s, and 72°C for 1 min. Primers were designed to amplify nucleotides 582-1352 of the Apaf1 transcript and 139-740 of the GAPDH transcript.

Recombinant adenovirus infection

Recombinant adenoviral vectors carrying the human p53, DNA binding mutant p53-173L or LacZ expression cassettes were constructed, purified and titered as described previously (Cregan, MacLaurin et al. 2000). All experiments were performed at a MOI of 20 pfu/cell. Recombinant adenivoral vectors were added to cell suspensions immediately before plating.

Surgical Procedures

All animal procedures conformed to guidelines endorsed by the Medical Research Council of Canada and were approved by the Animal Care Committee of the University of Ottawa. Male C57BL/6 mice weighing 20 – 22 grams were subjected to 2 h of middle cerebral artery occlusion as described (Nagasawa and Kogure 1989). Mice were anaesthetized with a mixture of 30% oxygen, 70% nitrous oxide containing 1.5% halothane. The left common carotid artery was exposed through a ventral midline incision in the neck and permanent ligature placed around the external carotid artery. A

temporary ligature was placed around the left common carotid artery and a microaneurysm clip placed across the internal carotid artery. A silicon coated -8-0 nylon suture was inserted into the external carotid artery through an incision in the arterial wall, the microaneurysm clip removed, and the suture advanced approximately 9 mm to the origin of the middle cerebral artery. The suture was left in place for 2 h, then withdrawn and the ligature around the common carotid artery was removed. The wound was then sutured with topical application of bupivicaine HCL (0.5 mg/ml).

Cell Viability Assays

Cell survival was measured by two methods, LIVE/DEAD staining and TUNEL assay. At the times indicated, neuronal viability was determined using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes) following manufacturer's instructions. Representative samples were photographed using Zeiss Axiovert 100 with a Northern Eclipse Sony power HAD 3CCD color video camera. TUNEL labelling was used to visualise cells with fragmented DNA. Cells were harvested 24 h after camptothecin treatment and fixed in 4% paraformaldehyde for 20 min, washed in three changes of PBS, and then incubated for 1 h at 37°C with 75 µl of a cocktail (Boehringer) consisting of 0.5 µl terminal transferase, 0.95 µl biotin-16-dUTP, 6.0 µl $CoCl_2$, 15.0 µl 5x TdT buffer, and 52.55 µl distilled water. The reaction was stopped by incubation in 4x SSC buffer followed by three washes in PBS. Cells were then labeled with a streptavidin Cy2 secondary antibody (Jackson Immunoresearch Laboratories) for 45 min at room temperature and counterstained with Hoechst 33258 (1 µg/µl) for 5 minutes. The fraction

of TUNEL-positive cells as a percentage of total cell number was determined. A minimum of 500 cells was scored for each treatment and the data represents the mean and S.D. from 3 independent experiments.

Western Blot Analysis

Tissue was extracted in lysis buffer (50 mM Hepes [pH 7.8], 250 mM KCl, 0.1 M EDTA, 0.1 M EGTA, 10% glycerol, 0.1% NP-40, 1.0 mM DTT, 0.5 mM PMSF, 5 μg/mL aprotinin, 2 μg/mL leupeptin, 0.4 mM sodium vanadate) and aliquots containing 40μg of protein were separated on a 10% acrylamide gel and transferred to a nitrocellulose membrane. After blocking for 2 h with 5% skim milk, membranes were incubated for 1 h with either a rat monoclonal antibody directed against Apaf1 (1:500; Chemicon International, Inc.) or a goat polyclonal antibody directed against actin for standardization (Santa Cruz Biotechnologies Inc.). After three washes with TPBS (25 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.9% NaCl, 0.1% Tween-20), membranes were incubated for 1 h at 25°C with the appropriate secondary antibody, washed five times for 5 min each in TPBS and then developed by an enhanced chemiluminescence system according to the manufacturer's instructions (Perkin Elmer).

Immunohistochemistry

Mice were anaesthetized by an intraperitoneal injection of sodium pentobarbital and transcardially perfused with 5 ml of saline followed by 5 ml of 4% paraformaldehyde in phosphate buffer. Brains were removed and postfixed overnight in 4% paraformaldehyde

followed by 48 hours in 10% sucrose in 0.01M phosphate-buffered saline. 20-μm thick cryostat sections were cut and processed as free floating sections as described (Shu, Ju et al. 1988). The anti-Apaf1 antibody (MAB3505) was obtained from Chemicon International, Inc.

EMSAs

EMSAs were performed on total protein extracts as described (33), with certain modifications. In brief, cells were harvested, centrifuged and extracted in lysis buffer (100 mM Hepes [pH 7.4], 5 mM MgCl2, 2.5 mM EDTA, 20% glycerol, 0.5 M KCl, 0.5 mM PMSF, 0.1% NP-40, 5 μg/mL aprotinin, 2 μg/mL leupeptin, and 20 μM sodium orthovanadate) and assayed by the method of Bradford (Bio-Rad protein assay reagent). 10-20 μg of total cell lysate was incubated with an excess of indicated ^{32}P-labeled double-stranded DNA probes (60,000 cpm/0.2 ng of DNA). Oligonucleotides used included 5'-ATGGAGACATGTCTGGAGACCCTAGGA-CGACAAGCCC-3' (BS2) and 5'-ATGGAGGCACGTCCCCAGCGACAGCAGGCTC-3' (BS1) corresponding to the p53 binding consensus sequences located between –739 to –765 and –572 to –604, respectively, of the Apaf1 promoter. Oligonucleotides 5'-ATGGAGAAATTTC-TGGAGACCCTAGGACGAAAATCCC-3' (BS2-MUT) and 5'-ATGGAGGAACTTCCCC-AGCGACAGAAGTCTC-3' (BS1-MUT) containing mutations within the corresponding p53 consensus sequences were also used as indicated. The binding reaction (25 μl) was carried out at room temperature for 20 min in binding buffer (50% glycerol, 250 mM KCl, 100 mM HEPES [pH 7.4], 5 mM DTT, 5 mg/mL

BSA, and 0.5% Triton X-100) with 0.1 μg sonicated herring sperm DNA and 1 uL of p53 Pab421 monoclonal antibody (Ab-1, Oncogene Research Products). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide for BS1 and BS2 was added to the binding reaction and incubated for 20 min prior to the addition of labeled probe. Furtermore, supershifts were performed with two different p53-specific antibodies, including FL393 and Pab243 (Santa Cruz Biotechnology, Inc.) Complexes were resolved on a 5% polyacrylamide, 1X Tris–Glycine gel, dried, and visualized by autoradiography.

Apaf1-promoter Luciferase Reporter Assays

The Apaf1 luciferase reporter construct (pGL3b-Apaf1) was generated by subcloning the Apaf1 promoter (-871 to +208) into the HindIII site of pGL-3 basic (Promega). Promoter deletion constructs were generated by deleting from the 5' end with Erase-a-Base nucleotide kit (Promega) (Moroni, Hickman et al. 2001). The truncated construct pGL3b-Apaf1ΔBS2, missing the most 5' p53 consensus binding site, contains sequence -715 to +208 and pGL3b- Apaf1ΔBS1/ ΔBS2, missing both putative sites, contains sequence -396 to +208. SN48 cells were transfected by calcium phosphate precipitation (Storring, Charest et al. 1999) using 15 μg of luciferase construct, 5 μg/plate of either the pCMV p53, the DNA binding mutant pCMV p53-173L or the empty pCMV vectors, and 5 μg/plate of pCMV LacZ vector as an internal standard. After 14-16 h, cells were passaged into three wells of a 6-well dish/10-cm plate, and incubated for 36 h with fresh medium before assaying for luciferase activity. All plasmids used for transfection were purified using Maxiprep columns (QIAGEN) and quantified by spectrophotometric

analysis. Luciferase assays were performed after 36 h, at which time cells were washed once with PBS and lysed in the wells with 200 μL/well of reporter lysis buffer (Promega). Cells were collected by scraping and were subjected to one freeze-thaw cycle followed by centrifugation. Supernatants were collected and assayed for luciferase activity using a BioOrbit 11250 luminometer. A portion of the harvested cell extract (10%) was assayed for β-galactosidase activity based on the conversion of 4-methylumbelliferyl-D-galactoside (MUG) (Sigma-Aldrich) to the highly fluorescent molecule methylumbelliferone. Briefly, 30 μl of cell extract was incubated in the dark with 30 μl of 0.3 mM MUG, 15 mM Tris-HCl (pH 8.8) for 30 min after which time a stop solution was added (300mM glycine, 15 mM EDTA, pH 11.2). After addition of 2 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), fluorescence was quantified using a Perkin-Elmer LS50 luminescence spectrofluorometer at 350-nm excitation and 450-nm emission settings. The ratio of luciferase to β-galactosidase activity was determined in triplicate samples and normalized to vector-transfected extracts. All data are presented as the mean ± S.D. of at least three independent experiments.

RESULTS

Apaf1 is induced during p53-mediated neuronal cell death

We have shown previously that adenoviral-mediated delivery of p53 can induce apoptosis in postmitotic neurons (Slack, Belliveau et al. 1996) through a Bax-dependant mechanism (Cregan, MacLaurin et al. 1999). However, unlike with other cell types (Miyashita and Reed 1995), little upregulation of the putative transcriptional target, Bax was observed (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999). In the present study, we used DNA microarray analysis to identify potential transcriptional targets for p53 involved in the regulation of neuronal cell death. Cortical neurons were infected at a multiplicity of infection (MOI) of 20 with recombinant adenovirus vectors carrying an expression cassette for either full length human p53 (Ad-p53) or a DNA binding mutant (Ad-p53-173L) which was shown previously to be transcriptionally inactive (Rowan, Ludwig et al. 1996) and ineffective at inducing neuronal cell death (unpublished data). Microarray analysis of RNA extracted at 48 h postinfection revealed a 5-6 fold increase in Apaf1 transcript levels in cells expressing wild-type p53 relative to controls (Table I). In contrast, transcript levels of various members of the caspase family did not change (Table I).

To confirm the microarray data, we analyzed Apaf1 mRNA levels by reverse transcription (RT)-PCR. As shown in Fig. 1A, neurons transduced with Ad-p53 exhibited a significant increase in Apaf1 mRNA levels in comparison with uninfected cells or cells

Table I. Microarray analysis of gene induction during p53-mediated neuronal cell death.

Microarray analysis of RNA extracted from neurons 48 h after infection with either Ad-p53 or Ad-p53-173L. Fold induction represents the ratio of gene expression in cells transduced with Ad-p53 versus Ad-p53-173L. Accession numbers indicate the sequence used as probes in the microarray analysis. Data represents the average of two independent determinations.

Symbol	Accession No.	Fold change	D-call	Description
APAF1	AF064071	5.2	I	Apoptotic protease activating factor 1
Casp1	L28095	1.0	NC	Caspase 1; interleukin-1β converting enzyme
Casp2	NM007610	-1.1	NC	Caspase 2; apoptosis-related cysteine protease
Casp3	NM009810	-1.1	NC	Caspase 3; apoptosis-related cysteine protease
Casp6	Y13087	1.0	NC	Caspase 6; apoptosis-related cysteine protease
Casp7	U67321	1.0	NC	Caspase 7; apoptosis-related cysteine protease
Casp8	AJ007749	1.0	NC	Caspase 8; apoptosis-related cysteine protease
Casp9	AB019600	-1.2	NC	Caspase 9; apoptosis-related cysteine protease
Casp11	Y13089	1.0	NC	Caspase 11; apoptosis-related cysteine protease
Casp12	Y13090	1.0	NC	Caspase 12; apoptosis-related cysteine protease
Casp14	AF092997	1.3	NC	Caspase 14; apoptosis-related cysteine protease

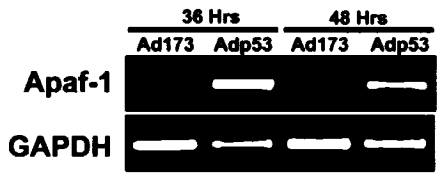
Detection (D)-call: I, increase; NC, no change.

infected with the inactive DNA binding mutant, Ad-p53-173L. This increase in Apaf1 mRNA levels was evident within 36 hours of Ad-p53-infection and remained elevated at 48 h. To determine whether Apaf1 could be upregulated in response to endogenous p53

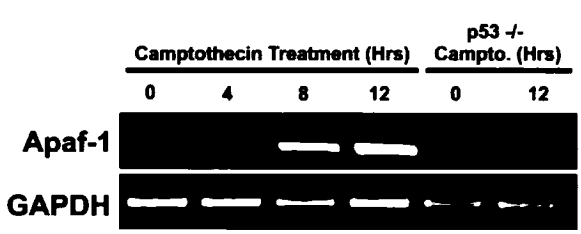
Fig. 1. p53-mediated induction of Apaf1 mRNA in neurons.

(A) RNA was extracted from neurons 36 or 48 h after infection with Ad-p53 or Ad-p53-173L and analyzed for Apaf1 or GAPDH expression using semiquantitative RT-PCR. **(B)** RNA was extracted from wild-type or p53-deficient neurons at the indicated times following treatment with 10 μM camptothecin and analyzed for Apaf1 or GAPDH expression using semiquantitative RT-PCR.

A


36 Hrs
48 Hrs
Ad173
Adp53
Ad173
Adp53
Apaf-1
GAPDH

B


Camptothecin Treatment (Hrs)
p53 -/-
Campto. (Hrs)
0
4
8
12
0
12
Apaf-1
GAPDH

activity, we treated neurons with the DNA-damaging agent camptothecin, which has been shown previously to induce p53-dependent neuronal cell death (Xiang, Kinoshita et al. 1998). As shown in Fig. 1B, camptothecin induced a time-dependent increase in Apaf1 mRNA levels beginning ~8 h after treatment. To confirm that the induction of Apaf1 expression was due to p53, the levels of Apaf1 mRNA were examined in p53-deficient neurons treated with camptothecin. Unlike samples derived from wild-type littermates, Apaf1 mRNA levels did not increase in p53-deficient neurons, thereby confirming the requirement of p53 for Apaf1 induction.

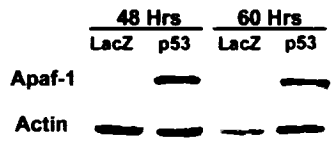
To determine whether the increase in Apaf1 mRNA levels was accompanied by an increase in protein expression, we conducted Western analysis on neurons undergoing p53-induced apoptosis. Cell extracts were obtained from neurons infected at 20 MOI with Ad-p53 or the control vector Ad-LacZ and Apaf1 protein expression was examined. A significant increase in Apaf1 protein levels was evident at 48 and 60 hours following infection with Ad-p53 relative to control (Fig. 2A). We next examined Apaf1 protein levels in camptothecin-induced apoptosis that activates endogenous p53 using wild-type and p53-deficient cortical neurons. A time-dependent increase in Apaf1 protein levels was found in wild-type neurons treated with camptothecin, whereas Apaf1 protein levels did not increase in p53-deficient neurons treated under identical conditions (Fig. 2B).

To determine whether Apaf1 induction occurs in neuronal injury, models in which the involvement of p53 has been demonstrated previously, we examined mice subjected to ischemia via middle cerebral artery occlusion (McGahan, Hakim et al. 1998; Watanabe, Ohta et al. 1999). Mice were subjected to 2 h focal ischemia followed by

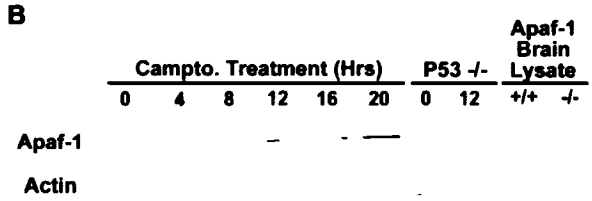
Fig. 2. p53-mediated induction of Apaf1 protein in neurons.

(A) Protein was extracted from neurons 48 or 60 h after infection with Ad-p53 or Ad-p53-173L and assayed for Apaf1 levels by Western blot analysis. **(B)** Protein was extracted from wild-type or p53-deficient neurons at the indicated times after treatment with 10 μM camptothecin and assayed for Apaf1 levels by Western blot analysis. Specificity of the Apaf1 antibody is demonstrated by lack of immunoreactivity in extracts derived from Apaf1 knockout brain and loading is standardized with actin.

A


48 Hrs
60 Hrs
LacZ
p53
LacZ
p53
Apaf-1
Actin

B


Campto. Treatment (Hrs)
P53 -/-
Apaf-1 Brain Lysate
0
4
8
12
16
20
0
12
+/+
-/-
Apaf-1
Actin

reperfusion for 24 h. This procedure generates an infarct in the striatum and cortex on the side of the brain ipsilateral to the occluded middle cerebral artery. Immunohistochemical staining of ischemic mice brain demonstrated increased Apaf1 immunoreactivity in the infarct region relative to the respective contralateral hemisphere (Figure 3A). To corroborate immunohistochemical results Western analysis was performed to measure Apaf1 protein levels from brain tissue after ischemia. The cortex and striatum were removed at 24 h posttreatment, protein was extracted, and Apaf1 protein levels were examined. In support of our immunohistochemical results, a significant increase in the level of Apaf1 expression was evident in the ipsilateral cortex and striatum (Fig. 3B). Consistent with our in vitro results, p53-mediated neuronal injury in vivo also results in the induction of Apaf1 protein.

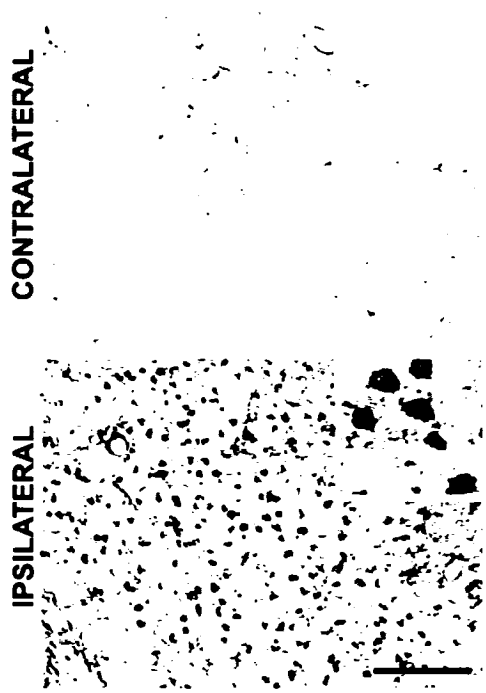
Apaf1 is a direct transcriptional target of p53 in neurons

To determine whether Apaf1 is a direct transcriptional target for p53 in neuronal apoptosis, we examined the Apaf1 promoter recently characterized in the Helin laboratory (Moroni, Hickman et al. 2001). Analysis of the Apaf1 promoter sequence revealed the existence of two putative p53 consensus binding sites located at –572 to – 604 (p53 BS1) and –739 to – 765 (p53 BS2) relative to the transcription initiation site (Fig. 4A). To determine whether p53 can interact with these consensus elements, oligonucleotides derived from these proposed binding sites were synthesized and used in electrophoretic mobility shift assays (EMSAs). Protein extracts were examined from

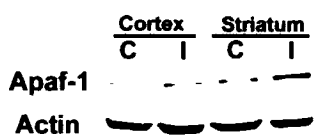
Fig. 3. Induction of Apaf1 in neurons after focal ischemia in mice.

Mice were subjected to 2 h of middle cerebral artery occlusion followed by 24 h of reperfusion. **(A)** Sections from ipsilateral and contralateral forebrain were prepared and immunostained for Apaf1. **(B)** Western blot analysis of Apaf1 expression in contralateral (C) versus ipsilateral (I) cortex and striatum after focal ischemia.

A


CONTRALATERAL
IPSILATERAL

B


Cortex
Striatum
C
I
C
I
Apaf-1
Actin

neurons undergoing p53-induced apoptosis, including: (a) treatment with camptothecin and (b) direct adenovirus-mediated p53 gene delivery. 48 h after infection with an adenovirus vector carrying wild-type p53 or the DNA binding mutant p53-173L, protein was extracted and examined by EMSA. EMSA demonstrates that neuronal extracts exhibit p53-DNA binding activity at both putative binding sites, although greater activity was found on BS1 relative to the BS2 (Fig. 4B). Furthermore, specific DNA binding activity to BS1 was observed with cell extracts prepared from camptothecin-treated neurons, suggesting that endogenous p53 can also interact with these binding sites (Fig. 4C). The specificity of p53 binding from neuronal extracts is supported by several control experiments: (a) the p53 DNA binding mutant (p53-173L) did not exhibit binding to either recognition site; (b) mutation of four key residues within each proposed p53 binding sites on the Apaf1 promoter (Fig. 4A) abolished p53 binding activity; (c) DNA binding activity could be competed out by incubation with excess unlabeled probes; (d) the bands were supershifted by the addition of two different p53-specific antibodies (Fig. 4D); and, (e) no DNA binding activity was seen in extracts obtained from p53-deficient neurons treated under identical conditions (Fig. 4D).

The ability of p53 to activate the Apaf1 promoter in neurons was further examined by using a luciferase reporter assay regulated by the Apaf1 promoter. Three different Apaf1-luc reporter constructs were tested, including the full length Apaf1 promoter (-871 to +208), a truncated promoter missing one p53 recognition sequence (-715 to +208), and a truncated Apaf1 promoter deleted for both proposed p53 recognition sequences (-396 to +208; Fig. 5A). Cultured neural cell lines cotransfected with the

Fig. 4. Specific binding of p53 to Apaf1 promoter elements in neuronal extracts.

(A) Comparison of p53 consensus binding sequence (p53 CBS, El-Deiry et al. 1992) with two putative p53 recognition sequences located in the Apaf1 promoter (Apaf1 BS1 and BS2). The sequence of the corresponding mutated versions of these oligonucleotides (Apaf1 BS1-mut and BS2-mut) used in the electrophoretic mobility shift assays are also indicated. **(B)** Protein was extracted from neurons 48 h after infection with Ad-p53 or Ad-p53-173L and p53 binding activity to the Apaf1 promoter elements was assayed by electrophoretic mobility shift assay. Binding reactions were carried out with neuronal extracts (10 μg protein) and the indicated oligonucleotides in the presence of p53 antibody (pAb1). **(C)** Cell extracts (20 μg protein) obtained from untreated neurons or neurons exposed to camptothecin (10 μM) for 12 h were tested for p53 binding activity to the Apaf1 promoter elements as described above. **(D)** Specificity of p53 binding activity to the Apaf1 promoter was examined in p53+/+ and p53-/- neurons treated with camptothecin. Supershifts with two antibodies directed against p53 were carried out on p53+/+ neurons to further confirm the presence of p53 binding to the Apaf1 promoter.

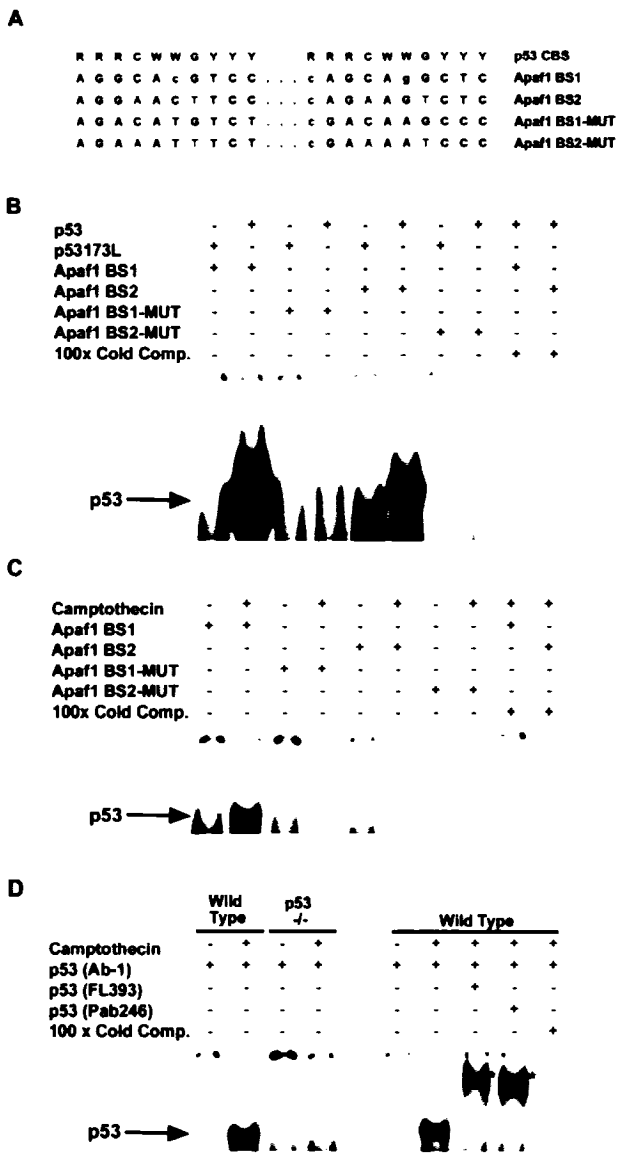
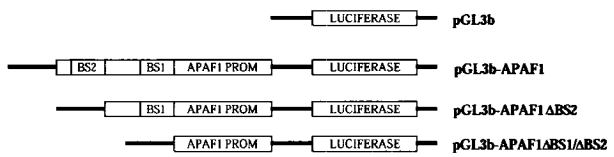

A
R R R C W W G Y Y Y R R R C W W G Y Y Y p53 CBS
A G G C A c G T C C . . . c A G C A g G C T C Apaf1 BS1
A G G A A C T T C C . . . c A G A A G T C T C Apaf1 BS2
A G A C A T G T C T . . . c G A C A A G C C C Apaf1 BS1-MUT
A G A A A T T T C T . . . c G A A A A T C C C Apaf1 BS2-MUT
B
p53
p53173L
Apaf1 BS1
Apaf1 BS2
Apaf1 BS1-MUT
Apaf1 BS2-MUT
100x Cold Comp.
p53
C
Camptothecin
Apaf1 BS1
Apaf1 BS2
Apaf1 BS1-MUT
Apaf1 BS2-MUT
100x Cold Comp.
p53
D
Wild Type
p53 -/-
Wild Type
Camptothecin
p53 (Ab-1)
p53 (FL393)
p53 (Pab246)
100 x Cold Comp.
p53

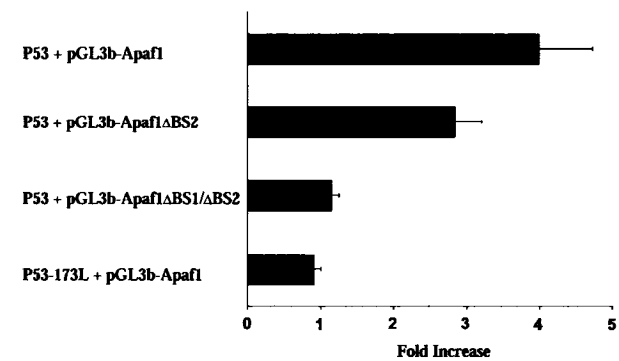
Fig. 5. Activation of the Apaf1 promoter by p53 in a neuronal cell line.

(A) P53 responsiveness of the Apaf1 promoter was tested using luciferase reporter constructs (pGL3b; Promega) consisting of the luciferase gene fused to an Apaf1 promoter fragment containing both p53 binding sites (BS1 and BS2), or truncated promoter fragments deleted for one or both p53 recognition sequences. **(B)** SN48 cells were cotransfected with the indicated luciferase reporter construct, a CMV-β-gal reporter construct, and an expression plasmid for either wild-type p53, DNA binding defective p53-173L, or empty vector as control. Luciferase activity was measured in cell lysates obtained 48 h after transfection and normalized to β-galactosidase activity. Fold increase indicates the ratio of normalized luciferase activity of each Apaf1 promoter construct in the presence of p53 expression vector versus empty vector control. Data represent the mean and standard error of triplicate samples from three independent experiments.

A.


LUCIFERASE
pGL3b
BS2
BS1
APAF1 PROM
LUCIFERASE
pGL3b-APAF1
BS1
APAF1 PROM
LUCIFERASE
pGL3b-APAF1 ΔBS2
APAF1 PROM
LUCIFERASE
pGL3b-APAF1ΔBS1/ΔBS2

B.


P53 + pGL3b-Apaf1
P53 + pGL3b-Apaf1ΔBS2
P53 + pGL3b-Apaf1ΔBS1/ΔBS2
P53-173L + pGL3b-Apaf1
0
1
2
3
4
5
Fold Increase

intact Apaf1 promoter construct and wild-type p53 exhibited a fourfold increase in luciferase activity (Fig. 5B). In contrast, no induction of luciferase activity occurred upon cotransfection with the DNA binding mutant p53-173L. Deletion of BS2 from the Apaf1 promoter resulted in ~ 25% decrease in p53-induced luciferase activity and deletion of both p53 recognition sites essentially abolished all p53-induced promoter activity. Together, our EMSA results demonstrating DNA binding activity at p53 consensus sites in extracts derived from neurons undergoing p53-induced apoptosis, as well as the direct activation of the Apaf1-promoter by p53 show that Apaf1 is a direct target for p53 in the regulation of neuronal cell death.

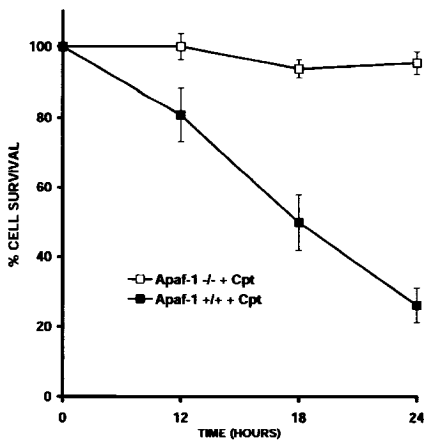
Apaf1 plays an important role in the regulation of p53-mediated neuronal cell death

To determine whether Apaf1 plays an important role in the regulation of neuronal cell death, we treated wild-type and Apaf1-deficient neurons with camptothecin, a DNA damaging agent known to induce neuronal cell death through a p53-dependent mechanism (Xiang, Kinoshita et al. 1998). Primary cortical neurons were cultured from E14.5 Apaf1-deficient embryos in parallel to their corresponding wild-type littermates. After 24 h in vitro, neurons were treated with camptothecin and cell survival was examined after 0, 12, 18, and 24 h. In Apaf1 +/+ neurons, loss of cell viability became apparent at ~12 h after camptothecin treatment and by 24 h only ~25% of neurons survived (Fig. 6). In contrast, Apaf1 -/- neurons treated with camptothecin remained

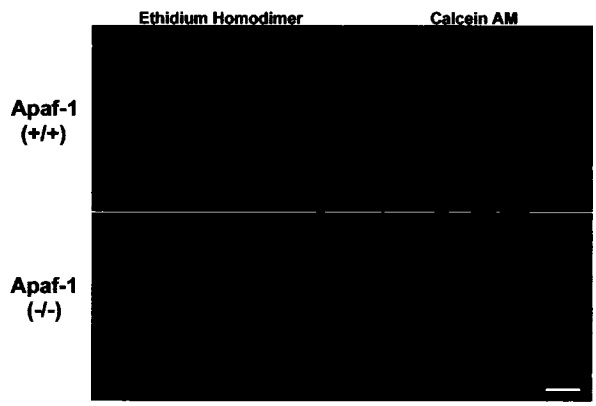
Fig. 6. Apaf1-deficient neurons exhibit increased resistance to p53-mediated cell death.

(A) Cortical neurons obtained from Apaf1-deficient mice or wild-type littermates were treated with 10 μM camptothecin and cell survival was determined by Live/Dead assay (Molecular Probes) at the indicated times. Survival is reported as percentage of corresponding untreated control cultures. Data represents the mean values obtained from independent cultures involving three separate Apaf1 knockout mice and matching wild-type littermates and error bars indicate standard deviation of the mean. **(B)** Cortical neurons from Apaf +/+ and Apaf -/- littermates were treated with camptothecin and after 24 h neurons were stained in a Live/Dead assay. Live cells exhibit positive staining for calcein AM activity (green fluorescence), whereas dead cells stain positive for ethidium homodimer (red fluorescence). Bar, 100 μm.

A.


100
80
60
40
20
0
% CELL SURVIVAL
Apaf-1 -/- + Cpt
Apaf-1 +/+ + Cpt
0
12
18
24
TIME (HOURS)

B.


Ethidium Homodimer
Calcein AM
Apaf-1
(+/+)
Apaf-1
(-/-)

viable throughout this time frame, such that by 24 h there was little difference in cell survival relative to untreated controls.

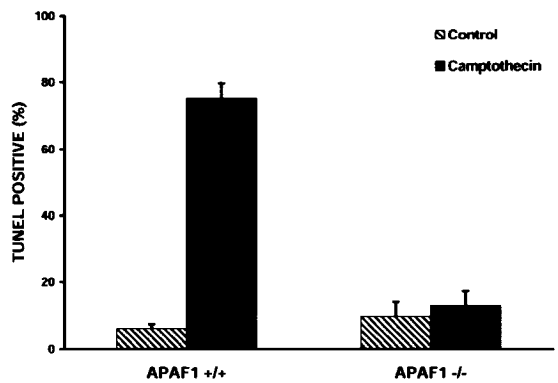
To determine whether this enhanced survival of Apaf1-deficient neurons was associated with a decrease in apoptotic cell death, Apaf1 +/+ and Apaf1 -/- neurons were treated with camptothecin and the frequency of TUNEL-positive cells was determined after 24 h. In wild-type neurons, camptothecin treatment resulted in a significant increase in the number of TUNEL-positive cells (~70%) relative to untreated controls (Fig. 7A). Immunofluorescence revealed that TUNEL-positive cells exhibited the typical pyknotic nuclear morphology (Fig. 7B). In contrast, camptothecin treatment of Apaf1 null neurons did not reveal a significant increase in TUNEL-positive cells. This suggests that within the 24 h time frame examined, in which the majority of wild-type cells have undergone apoptosis, Apaf1-deficient neurons were resistant to DNA-damage induced cell death.

In summary, the results of our studies demonstrate that Apaf1 is a direct transcriptional target regulated by p53 and that it plays a prominent role in the execution of p53-mediated neuronal cell death.

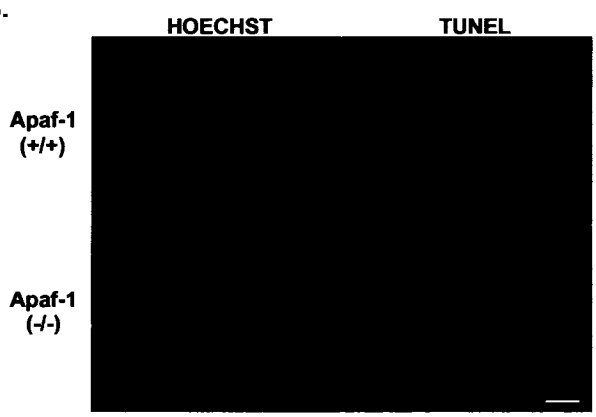
Fig. 7. Apaf1-deficiency decreases p53-mediated apoptosis in neurons.

(A) Cortical neurons obtained from Apaf1-deficient mice or wild-type littermates were treated with 10 μM camptothecin and the extent of apoptotic cell death was determined by Tunel assay. Data represents the mean and standard deviation of three independent experiments. **(B)** Cortical neurons from Apaf1 +/+ and Apaf -/- littermates were treated with camptothecin and after 24 h cells were fixed, stained for Tunel, and counterstained with Hoechst. Bar, 100 μm.

A.


100
80
60
40
20
0
TUNEL POSITIVE (%)
Control
Camptothecin
APAF1 +/+
APAF1 -/-

B.


HOECHST
TUNEL
Apaf-1
(+/+)
Apaf-1
(-/-)

DISCUSSION

In postmitotic neurons, the mechanisms by which p53 induces apoptosis are not well understood and appear to be distinct from those in proliferating cells. We and others have shown that p53-mediated cell death in mature neurons works through a Bax-dependent mechanism (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999). It has been shown previously that p53-mediated cell death can function through transcriptional induction of the Bax gene (Miyashita and Reed 1995); however, we and others (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999) have shown that neurons undergoing p53-induced apoptosis require Bax without significant upregulation of Bax protein levels. The question as to the transcriptional targets for p53 in neuronal cell death remains unknown. Therefore, we set out to conduct a DNA microarray analysis in search of p53-responsive genes important in the regulation of neuronal cell death and have identified Apaf1 as a potential target. In the present study, we examined the role of Apaf1 in the regulation of p53-induced neuronal cell death. Apaf1 is a molecule thought to link the mitochondrial death-signaling events to the initiation of the caspase cascade by forming, along with cytochrome c and dATP, an apoptosomal complex that recruits and activates caspase 9 (Li, Nijhawan et al. 1997; Hu, Benedict et al. 1999). Caspase 9, in turn, goes on to activate other effector caspases such as caspase 3. Accordingly, in the present study we have shown: (a) that Apaf1 is a direct regulatory target for p53 in neuronal cell death and

(b) that the upregulation of Apaf1 by p53 plays an important role in the apoptosis-signaling cascade after neuronal injury.

Several lines of evidence suggest that p53 is a key player in the regulation of neuronal cell death in acute neurological disease (for review see (Hughes, Alexi et al. 1997)). Studies have demonstrated that p53 protein levels are upregulated following excitotoxicity, hypoxia, and ischemia (Xiang, Hochman et al. 1996; Banasiak and Haddad 1998; McGahan, Hakim et al. 1998). Mice carrying a p53 null mutation exhibited almost complete protection against glutamate or kainic acid–induced excitotoxic injury (Morrison, Wenzel et al. 1996; Johnson, Xiang et al. 1998). Similarly, ischemic brain damage was reduced in the absence of functional p53 (Crumrine, Thomas et al. 1994). Furthermore, we and others (Slack, Belliveau et al. 1996; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999) have shown that enforced expression of p53 alone is sufficient to trigger the apoptotic cascade in postmitotic neurons. Taken together, the observations that: (a) p53 is upregulated after acute neuronal injury, (b) p53 overexpression is sufficient to induce apoptosis in neurons, and (c) the loss of functional p53 reduces neuronal cell death following ischemia and excitotoxicity, strongly implicate this molecule as a key regulator of the death cascade in injured neurons.

The mechanism by which p53 regulates transcription is complex and varies depending on the cell type and can lead to either growth arrest or apoptosis (for review see (Prives and Hall 1999)). Although many of the transcriptional targets involved in

p53-mediated cell cycle arrest have been identified (for review see (el-Deiry 1998)) those involved in the regulation of apoptosis have not been well defined. Several p53 target genes capable of inducing cell death have been identified, including Bax (Miyashita and Reed 1995), NOXA (Oda, Ohki et al. 2000), AIP-1α (Oda, Arakawa et al. 2000) and PUMA (Nakano and Vousden 2001; Yu, Zhang et al. 2001); however, their role in p53-mediated cell death appears to be cell type dependent. For example, although Bax appears to be important for p53-induced cell death in neurons (Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999) and certain tumor cell lines (Miyashita and Reed 1995), it does not appear to be required in p53-mediated thymocyte cell death (Brady, Salomons et al. 1996). Likewise, although the induction of AIP-1 appears to play a role in p53-mediated cell death of Saos-2 osteosarcoma cells (Oda, Arakawa et al. 2000) it does not seem to function in p53-mediated cell death of several colorectal cancer cell lines (Yu, Zhang et al. 2001). The proteins encoded by these genes have all been reported to target the mitochondria and initiate caspase activation, suggesting that they may all function through Apaf-1, which has been identified as a critical cofactor in the mitochondrial caspase activation pathway (Li, Nijhawan et al. 1997; Cecconi, Alvarez-Bolado et al. 1998; Hu, Benedict et al. 1999). Indeed, we have shown here that Apaf1 plays an important functional role in p53-mediated cell death in neurons.

Our studies using DNA microarray analysis have identified Apaf1 as a target for p53 in the regulation of neuronal cell death. The Apaf1 promoter contains two p53 consensus sites, and p53 DNA binding activity is dramatically enhanced when neurons are induced to undergo apoptosis. p53-mediated Apaf1 promoter activation is robust in

neuronal cell lines and Apaf1 transcripts are induced in wild-type, but not p53 null, neurons undergoing DNA-damage induced apoptosis. Indeed, Apaf1 protein is enhanced after cerebral ischemia, an injury model previously shown to involve p53 (Li, Chopp et al. 1994; McGahan, Hakim et al. 1998; Watanabe, Ohta et al. 1999). Furthermore, the present studies demonstrate that, not only is Apaf1 a direct transcriptional target for p53 in neurons, but that Apaf1 upregulation is an important event leading to the demise of injured neurons.

Recently, transcriptional regulation of the APAF1 gene was reported by Moroni et al. (2001). APAF1 was identified as an E2F1 target gene using DNA microarray analysis and characterization of the APAF1 promoter revealed the existence of both E2F1 and p53 response elements. Both E2F1 and p53 are known to cooperate in certain neuronal cell death paradigms; for example, pRB-deficient mouse embryos exhibit widespread apoptosis within the central nervous system and this cell death is suppressed in the absence of p53 (Macleod, Hu et al. 1996) or E2F1 (Tsai, Hu et al. 1998). Although E2F1 is known to induce p53 stabilization (Bates, Phillips et al. 1998) the Helin laboratory has demonstrated that E2F1 can also activate the apoptotic machinery directly. Furthermore, we have shown previously that E2F1 can induce cell death in postmitotic neurons in the absence of p53 (O'Hare, Hou et al. 2000). Thus, it appears that E2F1 and p53 can also induce caspase activation and cell death independent of one another, with one possible mechanism being through Apaf1 induction.

The studies of Moroni et al., (2001) also identified potential p53 response elements within the APAF1 promoter and demonstrated APAF1 promoter activation in

tumour cell lines overexpressing p53. Our studies extend this work by demonstrating that endogenous p53 is capable of activating the APAF1 promoter in models of neuronal injury. Our results reveal that primary neurons induced to die by DNA damage or after ischemia in vivo exhibit a robust upregulation of APAF1. Furthermore, we demonstrate using EMSAs from neuronal extracts that endogenous p53 is capable of binding wild-type but not mutated p53 response elements on the APAF1 promoter. Parallel studies with p53-deficient neurons confirmed that this binding activity is due to p53 and not other p53 family genes such as p73. Finally, we have demonstrated that APAF1 plays an important functional role in p53-mediated neuronal cell death. Thus, the results of our study establish a novel mechanism by which p53 induces neuronal cell death.

The importance of the caspase-signaling cascade has been demonstrated in many models of neuronal injury as well as neurodegeneration. For example, caspase 3 activation has been demonstrated in traumatic brain injury and inhibition of caspase activity was shown to reduce post traumatic apoptosis and improve neurological function (Yakovlev, Knoblach et al. 1997). Induction of levels and activity of caspase 3 has been demonstrated in the hippocampus following transient global forebrain ischemia (Gillardon, Bottiger et al. 1997; Ni, Wu et al. 1998), and, caspase inhibitory peptides have been reported to block neuronal cell death in several models of ischemia (Hara, Friedlander et al. 1997; Cheng, Deshmukh et al. 1998; Endres, Namura et al. 1998). Indeed the IAP's (the inhibitor of apoptosis protein family) that have been shown previously to be potent inhibitors of caspase activity, exert neuroprotective effects when expressed in neurons induced to die by ischemia (for review see (Robertson, Crocker et

al. 2000)). Thus, the caspase family of cysteine proteases appears to play an important role in the execution of neuronal cell death, and identification of upstream targets of this cascade is critical for the development of therapeutic strategies for the treatment of acute neuronal injury. In this regard, future studies will be conducted to investigate the role of APAF1 as an upstream regulator of the caspase cascade in in vivo models of neuronal injury using APAF1 conditional knockout mice.

In summary, the results of these studies identify a key mechanism of p53 action in the regulation of neuronal cell death. First, we show that Apaf1 mRNA is upregulated in response to exogenous and endogenous p53 following neuronal injury. Second, we show that Apaf1 protein is upregulated in neurons undergoing P53-induced apoptosis. Third, we demonstrate through EMSA and luciferase reporter assays that p53 directly transactivates the Apaf1 promoter in neuronal cells. Finally, our results show that Apaf1 is an important target for p53 that plays a pivotal role in the regulation of neuronal apoptosis.

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This manuscript establishes SIVA as a direct transcriptional target for p53 and E2F1 in neurons

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THE PROAPOPTOTIC GENE SIVA IS A DIRECT TRANSCRIPTIONAL TARGET FOR THE TUMOR SUPPRESSORS P53 AND E2F1.

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ABSTRACT

The p53 tumor suppressor gene is believed to play an important role in neuronal cell death in acute neurological disease and in neurodegeneration. The p53 signaling cascade is complex, and that the mechanism by which p53 induces apoptosis is cell type-dependent. Using DNA microarray analysis, we have found a striking induction of the proapoptotic gene, SIVA. SIVA is a proapoptotic protein containing a death domain and interacts with members of the tumor necrosis factor receptor family as well as anti-apoptotic Bcl2 family proteins. SIVA is induced following direct p53 gene delivery, treatment with a DNA-damaging agent camptothecin, and stroke injury *in vivo*. SIVA up-regulation is sufficient to initiate the apoptotic cascade in neurons. Through isolation and analysis of the SIVA promoter, we have identified response elements for both p53 and E2F1. Like p53, E2F1 is another tumor suppressor gene involved in the regulation of apoptosis, including neuronal injury models. We have identified E2F consensus sites in the promoter region, whereas p53 recognition sequences were found in intron1. Sequence analysis has shown that these consensus sites are also conserved between mouse and human SIVA genes. Electrophoretic mobility shift assays reveal that both transcription factors are capable of binding to putative consensus sites, and luciferase reporter assays reveal that E2F1 and p53 can activate transcription from the SIVA promoter. Here, we report that the proapoptotic gene, SIVA, which functions in a broad spectrum of cell types, is a direct transcriptional target for both tumor suppressors, p53 and E2F1.

INTRODUCTION

The p53 tumor suppressor gene is believed to play an important role in neuronal cell death in acute neurological disease (Li, Chopp et al. 1994; Sakhi, Bruce et al. 1994; Morrison, Wenzel et al. 1996; Xiang, Hochman et al. 1996; Banasiak and Haddad 1998; McGahan, Hakim et al. 1998) and in neurodegeneration (Zhang, McLaughlin et al. 2002). Studies have demonstrated that p53 protein levels are upregulated following excitotoxicity, hypoxia, and ischemia (Sakhi, Bruce et al. 1994; McGahan, Hakim et al. 1998; Halterman, Miller et al. 1999) and that forced up-regulation of p53 alone is sufficient to cause neuronal cell death (Slack, Belliveau et al. 1996; Xiang, Hochman et al. 1996; Johnson, Kinoshita et al. 1999; Miller, Pozniak et al. 2000). Consistent with this, mice carrying a p53 null mutation exhibit reduced brain damage following excitotoxicity and stroke (Crumrine, Thomas et al. 1994; Morrison, Wenzel et al. 1996; Xiang, Hochman et al. 1996). Finally, recent studies have shown that p53-blocking peptides are neuroprotective following acute brain injury and may serve as potential therapeutic agents (Culmsee, Zhu et al. 2001). Taken together, these studies demonstrate the importance of p53 as a key apoptotic factor following neuronal injury and underscores the necessity to uncover the mechanisms by which p53 induces the death of postmitotic neurons.

The p53 signaling cascade is complex, and it has become increasingly clear that the mechanisms by which p53 induces apoptosis vary depending on the tissue type (reviewed by Prives and Hall (Prives and Hall 1999)). For example, recent studies have demonstrated that, in certain cell types, p53 can induce apoptosis exclusively at the mitochondrial level through direct interaction with Bcl-2 family proteins (Mihara, Erster

et al. 2003). In contrast, other cell types such as postmitotic neurons exposed to DNA-damaging agents require the transcriptional activation domain of p53 for death to occur (Cregan et al. unpublished). In postmitotic neurons, we and others (Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999; Keramaris, Stefanis et al. 2000) have demonstrated that p53-mediated cell death involves a Bax-dependent, caspase3 activation involving induction of APAF1. In proliferating cells, Bax was shown to be a direct target for p53 (Miyashita and Reed 1995), however no significant p53-mediated Bax up-regulation was found in neurons (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999). Although we have found that p53 transcriptional activity is essential for the induction of neuronal cell death, the downstream targets responsible for Bax activation remain unknown. To identify the regulatory targets by which p53 induces neuronal cell death, we conducted DNA microarray analysis using postmitotic neurons undergoing p53-mediated apoptosis. Using this approach, we and others have previously identified APAF1 as a key intermediate in the apoptotic signaling cascade that is directly activated by p53 (Fortin, Cregan et al. 2001; Moroni, Hickman et al. 2001; Cregan, Fortin et al. 2002). We have now used DNA microarray analysis to identify p53 target genes involved in neuronal injury and have consistently found a striking induction of the proapoptotic gene, SIVA.

SIVA is a proapoptotic protein that was originally identified through its association with the cytoplasmic tail of CD27, a member of the tumor necrosis factor receptor (TNFR) superfamily (Camerini, Walz et al. 1991; Gravestein, Blom et al. 1993; Smith, Farrah et al. 1994; Prasad, Ao et al. 1997). CD27 lacks a death domain, thus yeast-2-hybrid assays were performed to identify the mechanism by which CD27 may

induce apoptosis (Agematsu, Kobata et al. 1994; Kobata, Agematsu et al. 1994; Hintzen, Lens et al. 1995; Jacquot, Kobata et al. 1997). SIVA, the CD27-interacting protein was found to contain a death domain homology region, a box-B-like ring finger, and a zinc finger-like domain (Prasad, Ao et al. 1997). More recently SIVA was also found to interact with an additional TNFR family protein, GITR (Spinicelli, Nocentini et al. 2002). Consistent with its structural domains, forced expression of SIVA has been shown to induce apoptosis when expressed in a number of different cell lines (Prasad, Ao et al. 1997).

In addition to its interaction with TNFR superfamily members, SIVA has also been shown to interact with anti-apoptotic Bcl-2 family members (Xue, Chu et al. 2002). There are two SIVA splice variants, SIVA-1 and SIVA-2, of which SIVA-1 retains exon2 which is believed to be critical for apoptotic activity (Yoon, Ao et al. 1999), although a recent study suggests that both splice forms can induce apoptosis in a similar fashion (Py, Slomianny et al. 2004). Exon2 is comprised of an amphipathic helical structure, known as the SAH domain, which is structurally similar to the BH3 domain of Bcl-2 family proteins (Xue, Chu et al. 2002). Consistent with an endogenous interaction with Bcl-2 family proteins, SIVA has been localized to the cytoplasm and mitochondria and was recently shown to directly bind to Bcl-X_L through this amphipathic domain. Mutation of the SAH domain prevents interaction with anti-apoptotic Bcl-2 family proteins and abrogates its ability to induce apoptosis (Xue, Chu et al. 2002). This, as well as studies demonstrating interactions with TNFR family proteins, suggests that SIVA may function through multiple mechanisms that may be dependent on the cell type and apoptotic stimulus. Consistent with a key role in regulating apoptosis in tumor cells, SIVA is up-

regulated in response to UV radiation and oxidative stress in a number of cell types (Cao, Ren et al. 2001; Xue, Chu et al. 2002). Recently, SIVA was identified through DNA microarray analysis as a p53-induced and DNA damage-induced gene following treatment of colon carcinoma cells with a topoisomerase I inhibitor (Daoud, Munson et al. 2003). In addition, examination of certain types of cancer has revealed a down-regulation of the SIVA gene along with p53 suggesting that SIVA itself may have a potential role as a tumor suppressor due to its proapoptotic function (Okuno, Yasutomi et al. 2001).

The role of the proapoptotic gene, SIVA, in regulating the death of neuronal cells has not yet been explored. Using DNA microarray analysis to identify p53 target genes involved in neuronal cell death, we have identified SIVA as a p53 target. In addition to its proapoptotic role in the immune system and in tumor cells, we now demonstrate that SIVA also functions in injury-induced apoptosis of postmitotic neurons. By isolation and analysis of the SIVA promoter, we have identified response elements for both E2F1 and p53. Like p53, E2F1 is a tumor suppressor gene (Field, Tsai et al. 1996; Yamasaki, Jacks et al. 1996) involved in the regulation of apoptosis and its involvement has been demonstrated in a number of neuronal injury models (Park, Morris et al. 1998; Giovanni, Keramaris et al. 2000; Hou, Callaghan et al. 2000; O'Hare, Hou et al. 2000; Osuga, Osuga et al. 2000; Park, Morris et al. 2000; Wang, Corbett et al. 2002). We have identified E2F consensus sites in the promoter region, whereas recognition sequences for p53 were found in the first intron. Furthermore, these consensus sites are also conserved in the human SIVA gene where E2F sites were also located within the promoter and p53 sites were again found in the first intron. Electrophoretic mobility shift assays revealed

that both transcription factors are capable of binding their putative consensus sites, and luciferase reporter assays revealed that E2F1 and p53 can activate transcription of the SIVA promoter at these sites. Here, we report that the proapoptotic gene SIVA is a direct transcriptional target for both tumor suppressors, p53 and E2F1.

MATERIAL & METHODS

Primary Neuronal Cultures and Cell Lines

Cortical and cerebellar granule neurons were cultured as described previously (Cregan, MacLaurin et al. 1999; Fortin, Cregan et al. 2001). Murine SN48 cells were maintained in Dulbecco's modified Eagle's medium (Wisent Inc., St. Bruno, Quebec, Canada) supplemented with 10% fetal calf serum (Wisent Inc.) at 37°C in 5% CO_2 (Fortin, Cregan et al. 2001). To culture progenitor cells, the epidermal ectoderm was removed from E12.5 mouse embryos, and the telencephalic neuroepithelia was dissected and transferred to a 1.5-mL Eppendorf tube containing 400 µl of serum-free stem cell media with 10 ng/ml basic fibroblast growth factor and 2 µg/ml heparin as previously described (Reynolds, Tetzlaff et al. 1992; Tropepe, Sibilia et al. 1999). Neuroepithelia were mechanically dissociated, and single cells were plated at a density of 50,000 cells/ml in uncoated 60-mm Nunclon plates (Invitrogen). Primary neurospheres were expanded for 3-4 days. 7 days post-plating, the neurospheres were pelleted and all but 1-2 ml media was removed. Neurospheres were triturated to generate a single cell suspension that was centrifuged and resuspended in Neurobasal medium containing B-27 supplement, N-2 supplement, 0.5 mM L-glutamine, 20ng/ml platelet-derived growth factor, 1% nondialized fetal bovine serum and 50 units/ml penicillin/streptomycin (Invitrogen). Cells were plated in Nunc 4-well (2×10^5 cells/well) dishes (Invitrogen) coated with poly-L-ornithine (Sigma-Aldrich).

Recombinant Adenovirus Infection and Camptothecin Treatment

cDNA for SIVA was a kind gift from Dr. Kanteti V.S. Prasad (Prasad, Ao et al. 1997). cDNA for wild type p53, p53-ΔV (deletion of conserved box V: residues 270-286), and p53-173L (point mutation at residue 173 to Leu) were kind gifts from Dr. Karen Vousden (Crook, Marston et al. 1994; Rowan, Ludwig et al. 1996). The p53 double transactivation mutant p53-DM (mutations L22E, W23S, W53F and E54S) was a kind gift from Dr. Xinbin Chen (Zhu, Zhang et al. 2000). Recombinant adenoviral vectors carrying the SIVA-GFP, GFP, and wild type or mutant p53 expression cassettes were constructed, purified, and titred as described previously (Cregan, MacLaurin et al. 2000). Prior to use, all recombinant adenovirus vectors were tested and confirmed to be wild type-free. All experiments were performed at a multiplicity of infection (m.o.i.) of 50 plaque-forming units/cell. Recombinant adenoviral vectors were added to cell suspensions immediately before plating for primary neuronal cultures and 24 h following plating for progenitor cell cultures. Cortical neurons were treated with 10 μM camptothecin (Sigma-Aldrich) 2 days after plating. For viability tests the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide survival assay (Cell Titer Kit, Promega, Madison, WI) that measures the mitochondrial conversion of the tetrazolium salt to a blue formazan salt was used as described previously (Slack, Belliveau et al. 1996).

DNA Microarray Analysis

For p53 microarray analysis, cortical neurons were infected at an m.o.i. of 20 with recombinant adenovirus vectors carrying an expression cassette for either a full-length

human p53 (Ad-p53), a transcriptionally defective p53 (Ad-p53-DM) or a DNA-binding mutant p53 (Ad-p53-ΔV). For E2F1 microarray analysis, neural precursor cells were infected at 50 m.o.i. with adenoviral vectors carrying E2F1 (Ad-E2F1) or the control vector Ad-GFP. Total RNA was extracted at 48 (p53) or 72 (E2F1) h post-infection using Tripure isolation reagent according to the manufacturer's instruction (Roche Diagnostics). RNA was sent to the Ottawa Genome Centre Affymetrix GeneChip Facility for analysis.

Surgical Procedures

All animal procedures conformed to guidelines endorsed by the Canadian Institutes of Health Research and were approved by the Animal Care Committee of the University of Ottawa. Male C57BL/6 mice (Jackson Laboratories, Bar Harbour, ME) weighing 20–22 g were subjected to 2 h of middle cerebral artery occlusion as previously described (Fortin, Cregan et al. 2001). Following reperfusion for 48 or 72 h, brains were removed and the striatum and cortex from the ipsilateral and contralateral sides were dissected separately and tissue was flash frozen using liquid nitrogen prior to protein extraction.

Semiquantitative RT-PCR Analysis

Total RNA was isolated from cells using Tripure isolation reagent according to the manufacturer's instructions (Roche Diagnostics). Pilot experiments were done to determine the linear range of amplification with respect to quantity of starting template and PCR cycles using mouse specific-primers: SIVA forward (5'-CGCCCATCGCTTGT TCATCGTG-3') and SIVA reverse (5'-CCGCAGCCCCAGCAGGTGTAT-3'). 6-12 ng of total RNA was used for cDNA synthesis and targeted gene amplification using the

SuperScript One-Step RT-PCR kit (Invitrogen). cDNA synthesis was carried out at 50°C for 30 min followed by a 2-min initial denaturation step at 94°C. This was followed by 35 cycles (SIVA) or 25 cycles (glyceraldehydes-3-phosphate dehydrogenase) at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min. Primers were designed to amplify nucleotides 329-437 of the SIVA transcript and 139–740 of the glyceraldehydes-3-phosphate dehydrogenase transcript. The resulting product was sequenced and confirmed to be SIVA.

Western Blot Analysis

Western blot analysis was performed as described previously (Cregan, MacLaurin et al. 1999) with antibodies against p53 (1C12, Cell Signaling Technology, Beverly, MA) and β-actin (SC-1616, Santa Cruz Biotechnologies, Santa Cruz, CA) as a loading control.

Electrophoretic Mobility Shift Assay

EMSAs were performed on total protein extracts as described previously (Fortin, Cregan et al. 2001), with the following modifications. In brief, cells were harvested, centrifuged, and extracted in lysis buffer and assayed by the method of Bradford (Bio-Rad Laboratories protein assay reagent). 10–20 μg of total cell lysate was incubated with an excess of indicated ^{32}P-labeled double-stranded DNA probes (60,000 cpm/0.2 ng of DNA). Oligonucleotides used included 5'-AGTCTAGACATGGCCTGGCGTCGTGG

CTTGTTT-3' (p53-BS1) and 5'-GTCTA-TGCAAGCCTGGACATGAGT-3' (p53-BS2) corresponding to the p53 binding consensus sequences located between +752 to +780 and

+897 to +917, respectively, and 5'-CAGAGCCTTCAGGCTTTTCGCGCGCT-3' (E2F-BS1) and 5'-CGCCCTTGG-CCTTTTCCCGCGCC-3' (E2F-BS2) corresponding to the E2F binding consensus sequences located between –392 to –372 and –296 to -280, respectively from the transcription start site (+1) referenced from the longest published SIVA sequence (GenBank™ accession number AF033114). The binding reaction (25 μl) was carried out at room temperature for 20 min in binding buffer with 0.1 μg sonicated herring sperm DNA, and, for p53 binding, 1 μL of p53 Ab-1 monoclonal antibody was added to the binding buffer (OP03L; Oncogene Research Products). To control for binding specificity, a 100-fold excess of unlabeled oligonucleotide for BS1 and BS2 (p53 and E2F) was added to the binding reaction, and the mixture was incubated for 20 min before the addition of labeled probe. Furthermore, supershifts were performed with a p53-specific antibody FL393 (Santa Cruz Biotechnology, Inc.) and an E2F1-specific antibody C20-X (Santa Cruz Biotechnology, Inc.). Complexes were resolved on a 5% polyacrylamide, 1x Tris-Glycine gel, dried, and visualized by autoradiography.

SIVA Promoter Luciferase Reporter Assays

The SIVA luciferase reporter construct (pGL3b-SIVA) was generated by subcloning a murine SIVA gene fragment (-440 to +1770) containing the putative promoter, exon1 and intron1 into the SmaI site of pGL-3 basic (Promega). P53 binding site deletion constructs were generated by excising p53-BS1 (+752 to +780) with BsmB1 and PvuII, p53-BS2 (+897 to +917) with PvuII and Eco*R*1, and p53-BS1 and p53-BS2 (+752 to +917) with BsmB1 and EcoR1. SN48 cells were transfected by calcium phosphate precipitation as previously described (Fortin, Cregan et al. 2001) with some modifications. Briefly 15

μg/plate of luciferase construct, 3 μg/plate of either the pCMV-p53, the pCMV-E2F1, the DNA binding mutant pCMV-p53-173L, or the empty pCMV vectors, and 2 μg/plate of pPGK-LacZ vector as an internal standard. After 4 h, cells were passaged into three wells of a 6-well dish/10-cm plate (Sarstedt, Inc., Newton, NC) and incubated for 20 h with fresh medium supplemented with 40mM Hepes buffer (Sigma-Aldrich) before assaying for luciferase activity. After 24 h cells were washed once with phosphate-buffered saline and lysed in the wells with 200 μl/well of 1X reporter lysis buffer (Promega). Cells were collected by scraping and were subjected to one freeze–thaw cycle followed by centrifugation. Supernatants were collected and assayed for luciferase activity using a BioOrbit 11250 luminometer. A portion of the harvested cell extract was assayed for β-galactosidase activity based on the conversion of 4-methylumbelliferyl-D-galactoside (Sigma-Aldrich) to the highly fluorescent molecule methylumbelliferone. Cell extract (10 μl) was incubated in the dark with 30 μl of 0.3 mM 4-methylumbelliferyl-D-galactoside, 15 mM Tris-HCl, pH 8.8, for 30 min after which time a stop solution was added. After addition of 2 ml of Z-buffer, fluorescence was quantified using a PerkinElmer LS50 luminescence spectrofluorometer at 350-nm excitation and 450-nm emission settings. The ratio of luciferase to β-galactosidase activity was determined in triplicate samples and normalized to vector-transfected extracts.

RESULTS

SIVA Is Induced during p53-mediated Neuronal Cell Death

Although previous studies in proliferating cells have identified Bax as a direct transcriptional target for p53 (Miyashita and Reed 1995), we and others have found no induction of Bax in response to p53 in postmitotic neurons (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999). Presently, little is known regarding the molecules that regulate neuronal cell death in the p53 apoptotic cascade. To identify the mechanism by which p53 induces the death of postmitotic neurons, DNA microarray analysis was performed using Affymetrix gene chip arrays. This analysis was conducted in primary cortical neurons by direct p53 overexpression using adenoviral vector-mediated gene delivery of a GFP control vector (Ad-GFP), wild type p53 (Ad-p53), a transactivation defective mutant (Ad-p53-DM) and a p53 DNA-binding mutant (Ad-p53-ΔV) that fails to induce apoptosis. To verify the reliability of the GeneChip data, three known p53 responsive genes were used as a basis for comparison including APAF1 (Fortin, Cregan et al. 2001; Moroni, Hickman et al. 2001), PERP (Ihrie, Reczek et al. 2003), and Bax (Miyashita and Reed 1995). Evaluation of previously characterized p53 responsive genes revealed a reproducible 3.5-fold induction of APAF1, a 6.8-fold increase in PERP, but no significant change in Bax mRNA expression in neurons expressing wild type p53 (Table I). Furthermore, no induction of SIVA mRNA was found in response to mutant p53 lacking either the DNA binding domain or both transactivation domains. These data suggest that SIVA may serve as an important p53 target gene involved in several injury-induced modes of neuronal cell death.

Table 1: SIVA is a p53-inducible gene in neurons.

Microarray analysis of RNA extracted from primary cortical neurons 48H after infection with either Ad-p53, the transcriptionally defective mutant Ad-p53-DM or the DNA binding mutant Ad-p53-ΔV. "Fold change" represents the ratio of gene expression in cells transduced with Ad-GFP versus either Ad-p53 or Ad-p53mutants. Accession numbers indicate the sequence used as probes in the microarray analysis. Data represents the average of two independent determinations.

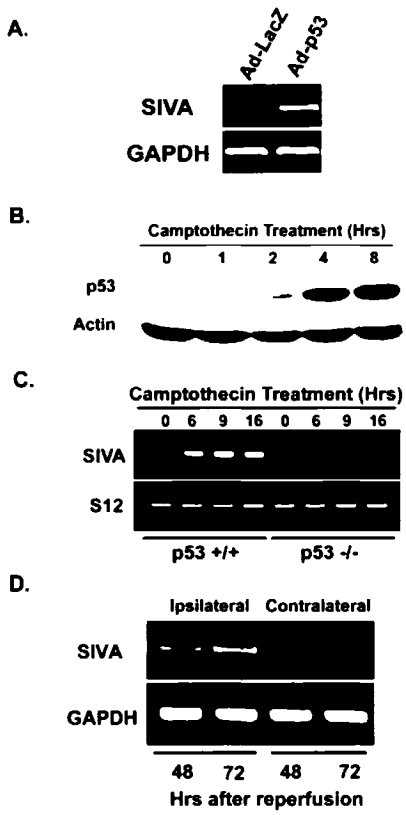
Symbol	Accession No.	Fold Change p53	D-Call p53	Fold Change DM	D-Call DM	Fold Change ΔV	D-Call ΔV
SIVA	NM_009684	4.4	I	1.8	N/C	1	N/C
BAX	BC018228	1.7	N/C	1	N/C	1.2	N/C
PERP	BF456404	6.82	I	1.48	MI	1.67	N/C
APAF-1	AF064071	3.5	I	0.6	N/C	1.9	N/C

Detection (D)-Call: I - Increase, MI - Moderate Increase, N/C - No Change.

To confirm results obtained with DNA microarray analysis, we examined SIVA mRNA levels by reverse transcription (RT)-PCR under similar conditions. Consistent with DNA microarray data, primary cerebellar granule neurons infected at 50 m.o.i. with Ad-p53 exhibited a significant increase in SIVA mRNA levels relative to cells infected with Ad-LacZ (Fig. 1A). This increase in SIVA mRNA levels was evident as soon as p53 became stably expressed, usually at 36 - 48 hours following Ad-p53 infection (results not shown). To verify that SIVA could also be up-regulated in response to endogenous p53 activity, we treated cortical neurons with 10 μM camptothecin a DNA-damaging agent that has

Fig. 1. p53-mediated induction of SIVA mRNA in neurons.

(A) SIVA expression was analyzed using semiquantitative RT-PCR from RNA extracted from cerebellar granule neurons 48 hours after infection with Ad-p53 or Ad-LacZ. **(B)** Protein was extracted from cortical neurons at the indicated times following treatment with 10 μM camptothecin and assayed for p53 levels by Western blot analysis. **(C)** RNA was extracted from cortical neurons at the indicated times after treatment with 10 μM camptothecin and SIVA expression was analyzed using semiquantitative RT-PCR. **(D)** SIVA expression was analyzed using RT-PCR from RNA extracted from ipsilateral and contralateral forebrain from mice subjected to 2 hours of middle cerebral artery occlusion and following the indicated hours of reperfusion. Figures are a representative of three (n=3) independent experiments.


A.
Ad-LacZ
Ad-p53
SIVA
GAPDH
B.
Camptothecin Treatment (Hrs)
0 1 2 4 8
p53
Actin
C.
Camptothecin Treatment (Hrs)
0 6 9 16 0 6 9 16
SIVA
S12
p53 +/+
p53 -/-
D.
Ipsilateral
Contralateral
SIVA
GAPDH
48 72 48 72
Hrs after reperfusion

been shown previously to induce p53-dependent death (Xiang, Kinoshita et al. 1998). Cortical neurons treated with camptothecin responded with a rapid increase in p53 protein that was first detectable at 2 h and continued to increase for 4 h after which the levels became stabilized. (Fig.1B) In wild type neurons, camptothecin caused SIVA induction as early as 6 h following treatment demonstrating that SIVA up-regulation is an early event in the apoptotic cascade co-incident with the increase in p53 protein (Fig.1C). This induction, however, was not observed in p53-deficient cells treated under identical conditions (Fig. 1C). Finally, to ask whether SIVA induction occurs *in vivo* following neuronal injury in which the involvement of endogenous p53 has previously been demonstrated (McGahan, Hakim et al. 1998; Watanabe, Ohta et al. 1999), we examined the brains of mice subjected to a model of focal ischemia. Brains were removed from mice subjected to 2 h middle cerebral artery occlusion followed by 48 and 72 h of reperfusion. This procedure generates an infarct in the striatum and cortex on the side of the brain ipsilateral to the occluded middle cerebral artery. Extracts from brain tissue revealed that SIVA mRNA is induced in the affected ipsilateral forebrain relative to the control tissue obtained from the contralateral hemisphere (Fig.1D). Together, these results indicate that increased levels of SIVA transcript correlate directly with p53 activation and suggest the possibility that this proapoptotic gene may be a direct transcriptional target of p53 in the regulation of neuronal cell death.

Up-regulation of SIVA is Sufficient to Induce Cell Death in Neural Precursor Cells and Postmitotic Neurons

Before exploring the possibility that p53 may directly activate the SIVA gene, we first determined whether SIVA up-regulation alone would be sufficient to induce the death of primary neural precursor cells and postmitotic neurons. A recombinant adenovirus vector was constructed carrying an expression cassette with full length SIVA-1 tagged with GFP. Primary cerebellar granule neurons or neural precursor cells were infected at 50 m.o.i. with Ad-SIVA-GFP or a control adenoviral vector carrying GFP only. SIVA expression was verified by Western blot analysis (data not shown). Postmitotic neurons infected with Ad-SIVA-GFP underwent apoptosis that was first evident by 72 h post-infection. The rate of cell death progressed such that by 120 h more than 72% of the SIVA expressing cerebellar granule neurons had died relative to only 33% of control cultures (Fig.2A). Similarly, increased expression of SIVA was also sufficient to induce the death of neural precursor cells treated under identical conditions (Fig. 2B). Cell death was evident by 72 h post infection and progressed rapidly leaving only 23% of the cells surviving at 120 h. These results demonstrate that SIVA is up- regulated in response to p53 following neuronal injury and that SIVA induction alone is sufficient to activate the apoptotic cascade in neural tissue.

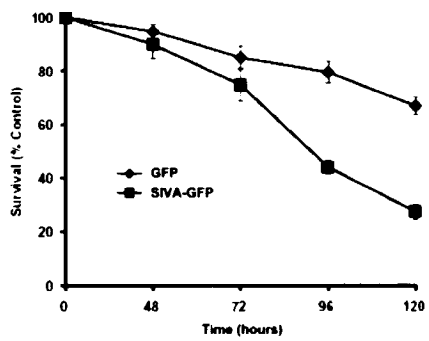
The Regulatory Sequences of the SIVA Gene Contain p53 and E2F1 Response Elements

To understand the mechanism by which the SIVA gene is regulated, we used the PromoterInspector (Genomatix) software to identify the putative promoter region for the SIVA gene. Analysis of the SIVA promoter sequence with MatInspector (Genomatix) software revealed the existence of two putative E2F consensus binding sites (Slansky and

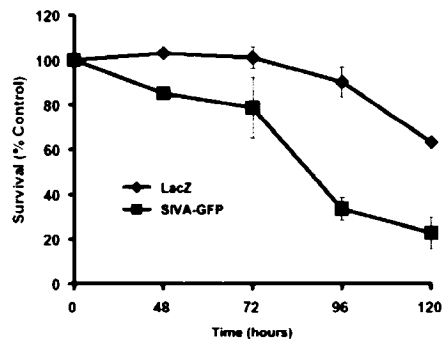
FIG. 2. ***SIVA* expression is sufficient to induce neuronal cell death.**

Neuronal cells were infected with 50 m.o.i. of Ad-*SIVA*-GFP or Ad-GFP/Ad-LacZ control. Survival was measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at the indicated times and shown as a percentage of uninfected controls. *A*, cerebellar granule neurons. Data represent the mean ± S.E. from four ($n = 4$) independent experiments. *B*, neural precursor cells. Data represent the mean ± S.E. from three ($n = 3$) independent experiments.

A.


100
80
60
40
20
0
Survival (% Control)
GFP
SIVA-GFP
0
48
72
96
120
Time (hours)

B.


120
100
80
60
40
20
0
Survival (% Control)
LacZ
SIVA-GFP
0
48
72
96
120
Time (hours)

Farnham 1996) at –392 to -372 bp and –296 to -280 bp and two putative p53 consensus sequences (Levine 1997) within intron 1 at 752-780 bp and 897-917 bp (Fig. 3A). These are referenced from the transcription start site (+1) which refers to the longest published sequence of SIVA (GenBank™ accession no. AF033114). Because the SIVA gene exhibited a striking induction during p53-mediated neuronal apoptosis, we assayed whether p53 was able to bind the consensus sites and activate transcription on the SIVA promoter. Oligonucleotides corresponding to the p53 consensus sites were synthesized, and electrophoretic mobility shift assays (EMSAs) were performed. Protein was extracted from neurons 48 h following adenovirus-mediated p53 delivery. The EMSA demonstrates that neuronal extracts exhibit p53–DNA binding activity at both putative binding sites (Fig. 3B). The specificity of p53 binding at these consensus sites was supported by two control experiments: (a) DNA binding activity could be competed out by incubation with excess unlabeled probes and, (b) the complexes were supershifted by the addition of a p53-specific antibody. The results of these experiments demonstrate that p53 can bind the two p53 consensus sites in the first intron of the SIVA gene.

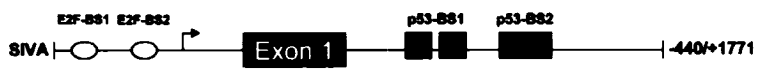
p53 Can Activate Transcription from the SIVA Promoter

Although the above experiments demonstrate that p53 can directly bind the putative consensus sequences, we next asked whether p53 binding could activate transcription from the SIVA promoter. To test this, primers were designed to isolate 550 bp of the SIVA promoter as well as exon1 and intron1, which contains the p53 response elements. This insert was isolated by PCR and cloned into the pGL3b luciferase reporter plasmid. To examine whether p53 could directly activate transcription, four different SIVA-

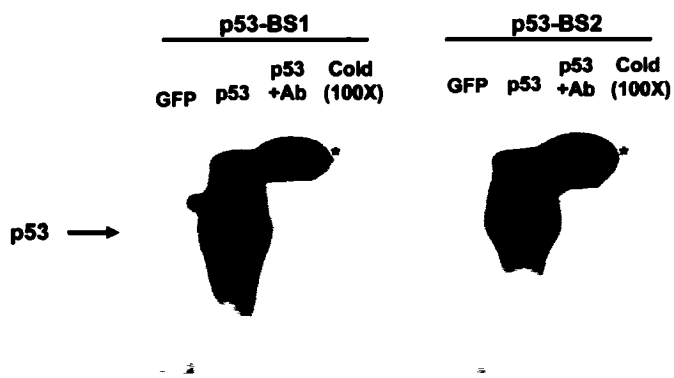
Fig. 3. Specific binding of p53 to SIVA promoter elements in neuronal extracts.

(A) Map of the SIVA fragment isolated from mouse genomic DNA containing two putative E2F consensus binding sequences within the promoter region and two putative p53 consensus binding sequences within intron1. **(B)** EMSA: Protein was extracted from cerebellar granule neurons 48 hours after infection with Ad-p53. p53 binding activity to the SIVA p53 response elements was assayed by electrophoretic mobility shift assay. Binding reactions were carried out with neuronal extracts (10μg protein) and the indicated oligonucleotides in the presence of p53 antibody (Ab1). Supershift with an antibody directed against p53 (FL393) was carried out to further confirm the composition of the complex on the SIVA p53 response elements.

A.


E2F-BS1 E2F-BS2
SIVA
Exon 1
p53-BS1
p53-BS2
-440/+1771

B.

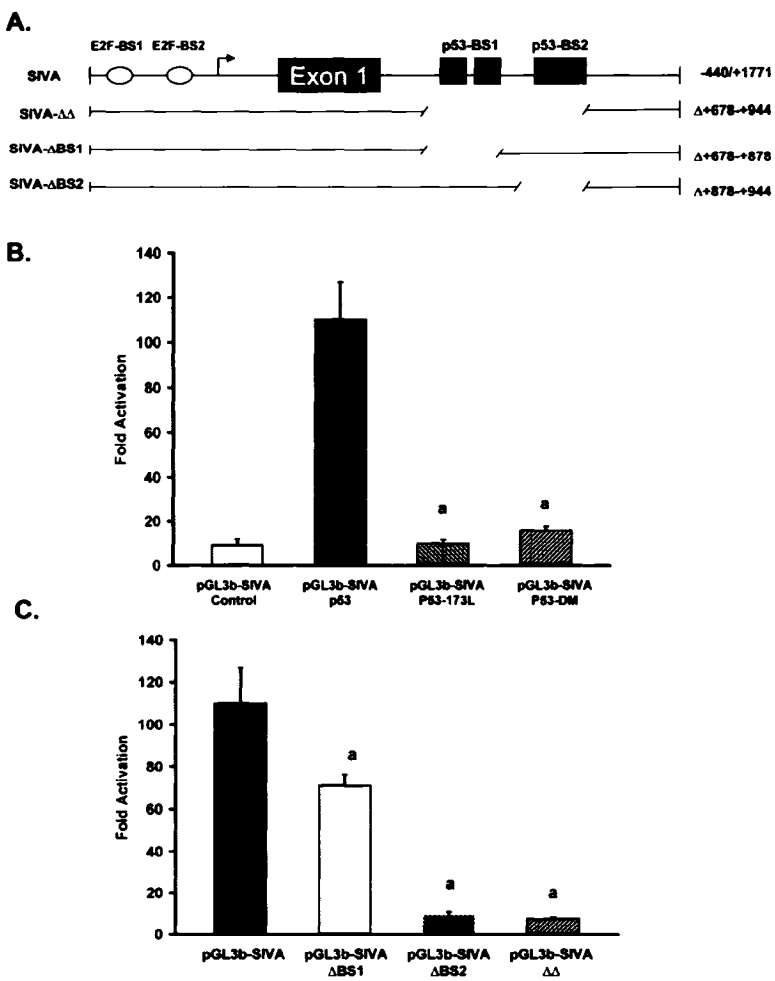

p53-BS1
p53-BS2
GFP
p53
p53 +Ab
Cold (100X)
GFP
p53
p53 +Ab
Cold (100X)
p53

luciferase reporter constructs were tested (Fig. 4A), including the full length SIVA insert (pGL3b-SIVA), a truncated insert missing the first p53 consensus sequence (pGL3b-SIVA-ΔBS1), a truncated insert missing the second p53 recognition sequence (pGL3b-SIVA-ΔBS2), and a truncation deleting both proposed p53 recognition sequences (pGL3b-SIVA-ΔΔ). Cultured SN48 neuronal cell lines were transfected with one of the SIVA promoter/luciferase constructs, a plasmid containing PGK-LacZ to control for transfection efficiency, and one of following plasmids containing wild type p53, mutant p53 or empty vector. Cells cotransfected with the intact SIVA luciferase construct (pGL3b-SIVA) and wild type p53 exhibited a dramatic 12-fold increase in luciferase activity (Fig. 4B). As controls, we used p53 mutants that failed to induce SIVA mRNA, based on DNA microarray analysis (Table I). In contrast to wild type p53, there was no induction of luciferase activity following cotransfection with the DNA binding mutant p53-173L or the double mutant (p53-DM) lacking both transactivation domains of p53 (Fig.4B). To determine the responsiveness of the individual p53 DNA binding sites, cells were cotransfected with each of the SIVA promoter constructs carrying deletions in the p53 consensus sites together with an expression vector for wild type p53. Deletion of p53-BS1 exhibited a 35% reduction in luciferase activity, however, still retaining activity. In contrast, deletion of p53-BS2 alone or deletion of both binding sites revealed complete loss of p53 responsiveness (Fig.4C). From these results, we conclude that the SIVA gene is a direct transcriptional target for p53, and, of the two p53 consensus sites, BS2 is the most important to confer p53 responsiveness.

SIVA is Directly Induced by E2F1

Fig. 4. Activation of the SIVA promoter by p53 in a neuronal cell line.

(A) p53 responsiveness of the SIVA gene was tested using luciferase reporter constructs (pGL3b; Promega) consisting of the luciferase gene fused to a DNA fragment containing both p53 binding sites (BS1 and BS2), or truncated fragments deleted for one or both p53 recognition sequences. SN48 cells were cotransfected with the indicated luciferase reporter construct, a PGK-LacZ reporter construct, and **(B)** an expression plasmid for either wild type p53, DNA binding-defective p53-173L, transactivation mutant p53-DM or empty vector as control or **(C)** an expression plasmid for either wild type p53 or empty vector. Luciferase activity was measured in cell lysates obtained 24 hours after transfection and normalized to β-galactosidase activity. Fold increase indicates the ratio of normalized luciferase activity of each SIVA construct in the presence of p53 expression vector versus empty vector control. Data represent the mean and standard error of triplicate samples from five (n=5) independent experiments (except 'a' which represents two (n=2) independent experiments).


A.
E2F-BS1 E2F-BS2
p53-BS1
p53-BS2
SIVA
Exon 1
-440/+1771
SIVA-ΔΔ
Δ+678-+944
SIVA-ΔBS1
Δ+678-+878
SIVA-ΔBS2
Δ+878-+944
B.
Fold Activation
140
120
100
80
60
40
20
0
a
a
pGL3b-SIVA Control
pGL3b-SIVA p53
pGL3b-SIVA P53-173L
pGL3b-SIVA P53-DM
C.
Fold Activation
140
120
100
80
60
40
20
0
a
a
a
pGL3b-SIVA
pGL3b-SIVA ΔBS1
pGL3b-SIVA ΔBS2
pGL3b-SIVA ΔΔ

Because putative E2F response elements were found on the SIVA promoter and E2F1 has previously shown to be a key proapoptotic protein involved in neuronal injury (Park, Morris et al. 1998; Giovanni, Keramaris et al. 2000; Hou, Callaghan et al. 2000; O'Hare, Hou et al. 2000; Osuga, Osuga et al. 2000; Park, Morris et al. 2000; Wang, Corbett et al. 2002), we asked whether E2F1 could also directly modulate transcription of the SIVA gene in neurons. We first addressed whether E2F1 had any effect on SIVA-1 mRNA levels by conducting DNA microarray analysis and RT-PCR. Consistent with previous results, E2F1 induced mRNA expression for c-Myb (10.5-fold), DHFR (7.9-fold), caspase3 (2.9-fold), and APAF1 (2.5-fold). In addition, our results revealed a 2.8-fold induction of SIVA-1 mRNA in response to E2F1 (Table II). Results from DNA microarray analysis were confirmed by RT-PCR, which also demonstrated a dramatic induction of SIVA-1 mRNA in response to E2F1 (Fig.5A). To ask whether E2F1 was capable of binding the SIVA-1 promoter at the two consensus sequences, EMSA was performed as for p53. Our results show that E2F1 is capable of binding both consensus E2F sites on the SIVA promoter (Fig.5B). Again, specificity was confirmed by competition with unlabelled probe and supershift with an E2F1 specific antibody.

Table II: SIVA is an E2F1-inducible gene in neurons.

Microarray analysis of RNA extracted from neural precursor cells 48 h after infection with either Ad-GFP or Ad-E2F1. "Fold change" represents the ratio of gene expression in cells transduced with Ad-GFP versus Ad-E2F1. Accession numbers indicate the sequence used as probes in the microarray analysis.

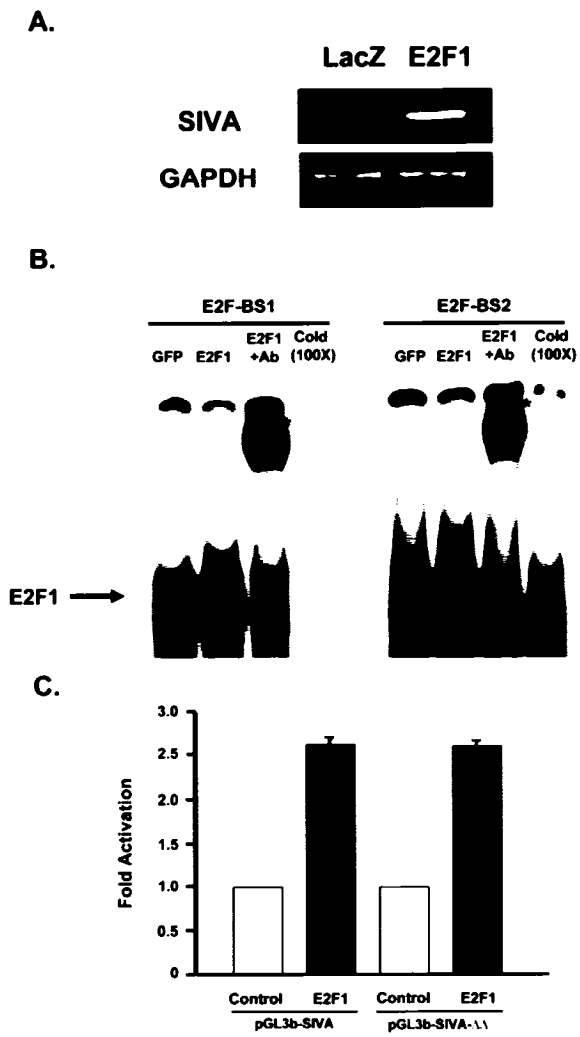
Symbol	Accession No.	Fold Change	D-Call
SIVA	NM_009684	2.8	I
BAX	BC018228	1.8	N/C
APAF-1	AF064071	2.5	I
cMyb	M12848	10.5	I
DHFR	J00388	7.9	I
CASP3	U54803	2.9	I
CASP7	U67321	2.5	I

Detection (D)-call: I, increase; MI, moderate increase; N/C, no change.

Although our results demonstrate that E2F1 could bind the E2F consensus sites on the SIVA promoter, we next examined whether E2F1 could activate transcription. Luciferase reporter assays were performed with the -440 to 1771 bp fragment containing the SIVA promoter, the first exon and intron as described above as well as the luciferase construct pGL3b-SIVA-ΔΔ missing both p53 DNA consensus binding sequences. SN48 cells were cotransfected with pGL3b-SIVA or pGL3b-SIVA-ΔΔ along with expression plasmids for E2F1 or empty control vector and LacZ. Our assays revealed that E2F1 was capable of inducing a 2.6-fold activation of the SIVA promoter relative to the control vector alone (Fig. 5C). Deletion of both p53 consensus binding sites from the luciferase construct did not alter the ability of E2F1 to induce luciferase activity from the SIVA promoter demonstrating that SIVA can be regulated independently of p53. Based on our findings, that E2F1 can induce SIVA mRNA in neurons, the SIVA promoter contains E2F consensus sequences, E2F1 is capable of binding these sites, and the SIVA promoter is

Fig. 5. SIVA is a direct target for E2F1.

(A) RNA was extracted from cerebellar granule neurons 72 hours after infection with Ad-E2F1 or Ad-LacZ and analyzed for SIVA or GAPDH expression using semiquantitative RT-PCR. **(B)** Protein was extracted from cerebellar granule neurons 72 hours after infection with Ad-E2F1. E2F1 binding activity to the SIVA promoter was assayed by electrophoretic mobility shift assay. Binding reactions were carried out with neuronal extracts (10 μg protein) and the indicated oligonucleotides. Supershift with an antibody directed against E2F1 (C20X) was carried out to further confirm the presence of E2F1 binding to the SIVA promoter. **(C)** SN48 cells were cotransfected with the indicated luciferase reporter construct (pGL3b-SIVA contains the intact SIVA promoter; pGL3b-SIVA-ΔΔ contains the SIVA promoter lacking both p53 consensus binding sites), a PGK-LacZ reporter construct, and an expression plasmid for wild type E2F1 or empty vector as control. Luciferase activity and fold increase were determined as above. Data represent the mean and standard error of triplicate samples from three (n=3) independent experiments.


A.
LacZ
E2F1
SIVA
GAPDH
B.
E2F-BS1
E2F-BS2
GFP
E2F1
E2F1 +Ab
Cold (100X)
E2F1
C.
Fold Activation
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
E2F1
pGL3b-SIVA

activated-2.6 fold by E2F1, we conclude that SIVA is also a direct transcriptional target for E2F1 in postmitotic neurons.

E2F1 and p53 Regulatory Elements are Conserved in the Human SIVA Gene

Similar to the mouse, analysis of the promoter region of the human SIVA gene with the Genomatix software revealed four E2F consensus binding sequences but no p53 binding sites in the promoter region (Fig. 6A). Further analysis of the human Siva gene also uncovered putative p53 consensus binding sequences in introns 1, 2 and 3 (Fig. 6B) which are also similar to those found in mouse genomic DNA.

In summary, the results of our studies demonstrate that the proapoptotic gene SIVA is induced following p53-mediated neuronal injury and that SIVA induction alone is sufficient to induce the death of neurons. Moreover, we have cloned the SIVA promoter and identified response elements for E2F1 and p53. By EMSA and luciferase reporter assays, we show that both transcription factors are capable of binding their respective consensus sites and activating transcription on the SIVA promoter. Taken together, our results show that the proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressor genes p53 and E2F1.

Fig. 6. Putative E2F1 and p53 consensus binding sequences are located in human SIVA gene.

DNA consensus binding sites within the human SIVA gene were identified using Genomatix software. **(A)** Four putative E2F binding sites fitting the general consensus site TTTSSCGC (Slansky and Farnham 1996) were identified in a potential promoter region. **(B)** Putative p53 binding sites closely resembling the p53 consensus binding half site, RRRCWWGYYY (Levine 1997), were identified in introns 1, 2 and 3.

A.

E2F DNA Binding Sites

Consensus	**T T T S S C G C** **S=C or G**
Mouse promoter	
E2F-BS1	**T T T C G C G C**
E2F-BS2	**T T T C C C G C**
Human promoter	
E2F-BS1	**G T T C C C G C**
E2F-BS2	**T T T C C C C C**
E2F-BS3	**C T T C C C G C**
E2F-BS4	**T T T C C C G C**

B.

P53 DNA Binding Sites

Consensus	**R R R C W W G Y Y Y (N=0...13) R R R C W W G Y Y Y** **R=A or G** **W=A or T** **Y=C or T**
Mouse Intron1	
p53 - BS1	**A G A C A T G G C C tggcgtcg T G G C T T G T T T**
p53 - BS2	**A C T C A T G T C C A G G C T T G C A T**
Human Intron1	**G C A C A A G C C T tggt T G T C T G G A G G**
Human Intron2	**A T G C A T G C G T G T G C A T G T A G**
Human Intron3	**G T A C T T G G C A G G G C A T G T C T**

DISCUSSION

A dramatic induction of the SIVA gene resulting from p53-mediated apoptosis prompted us to pursue the regulation of the SIVA gene in neuronal injury. Previous studies have revealed that SIVA is induced by oxidative stress (Cao, Ren et al. 2001) and p53-mediated death induced by genotoxic agents (Daoud, Munson et al. 2003). Studies with colon carcinoma have revealed that SIVA is up-regulated in response to treatment with a DNA-damaging agent, topotecan, and that cells lacking p53 fail to induce SIVA (Daoud, Munson et al. 2003). Our studies with neuronal cell types also revealed a considerable induction of SIVA in response to forced expression of p53, in response to up-regulation of endogenous p53 induced by camptothecin, and following stroke injury *in vivo*. Using p53 deletion mutants, our results show that the transactivation domains at residues 22 and 23, and 53 and 54, as well as the DNA binding domains of p53 are essential for SIVA to be induced. These observations strongly suggested to us that SIVA may be a direct transcriptional target for p53. Isolation and analysis of the regulatory sequences of the SIVA gene, including the promoter and the first exon and intron revealed two p53 response elements within the first intron. Luciferase reporter assays revealed a striking 12-fold induction of the SIVA gene in response to wild type p53. To determine which of the consensus sites were most important to confer p53 responsiveness deletion analysis was performed. Deletion mutations of each of the p53 consensus sites revealed that the second p53 binding site, p53-BS2, was most important and was most likely responsible for p53-mediated SIVA activation. The fact that deletion of this second recognition sequence resulted in complete loss of activity is consistent with this

interpretation. As a p53 responsive gene, SIVA was shown to be induced by many injury models involving p53, not only in neuronal cells but also in tumor cells, and cells of the immune system. Taken together, SIVA is a direct transcriptional target for p53 that is induced in a broad scope of tissue types.

SIVA induction was also found in response to E2F1, another proapoptotic transcription factor (Giovanni, Keramaris et al. 2000). E2F1 is a cell cycle regulatory gene that, when overexpressed in cells, can induce S-phase entry or apoptosis (Nevins 1992; Kowalik, DeGregori et al. 1995; Asano, Nevins et al. 1996; Wu, Classon et al. 1996; Fueyo, Gomez-Manzano et al. 1998). Mice deficient in E2F1 develop tumors in a number of tissues including uterine, lung and lymphatic, thus demonstrating a tumor suppressor function for E2F1 (Field, Tsai et al. 1996; Yamasaki, Jacks et al. 1996). We and others have shown E2F1 plays a prominent role in the regulation of neuronal cell death (Park, Morris et al. 1998; Giovanni, Wirtz-Brugger et al. 1999; MacManus, Koch et al. 1999; Giovanni, Keramaris et al. 2000; Park, Morris et al. 2000). Specifically, neurons deficient for E2F1 are more resistant to injury by β-amyloid (Giovanni, Keramaris et al. 2000), oxygen glucose deprivation (Gendron, Mealing et al. 2001), K+ deprivation (O'Hare, Hou et al. 2000), and mice lacking E2F1 show significant protection from stroke injury (MacManus, Koch et al. 1999). Because stroke injury has been shown to involve both E2F1 and p53, it is not surprising that we find a significant induction of SIVA gene expression in brains following focal ischemia. Interestingly, the presence of both E2F and p53 response elements on the promoter of a proapoptotic gene has been previously shown. Moroni et al. have demonstrated that the promoter of APAF1 is regulated by both p53 and E2F1 (Moroni, Hickman et al. 2001). Recently, the BH3-only proteins PUMA

and Noxa known to be direct p53 targets, have also been shown to be regulated by E2F1 (Hershko and Ginsberg 2004). It appears that genes like APAF1, PUMA, Noxa and SIVA are part of a growing list of genes that may be important in regulating the death response initiated by these tumor suppressors.

E2F1-mediated apoptosis is thought to proceed through both p53-dependent and p53-independent pathways. Thus, the potential for the convergence of both the p53 and E2F1 pathways to induce neuronal cell death exists following brain injury. Recent research, in proliferating cells, has demonstrated that interaction of E2F1 with the p53 pathway could involve transcriptional up-regulation of E2F1 target genes such as p14/p19ARF which affect p53 accumulation (Bates, Phillips et al. 1998; Robertson and Jones 1998), E2F1- induced phosphorylation of p53 (Rogoff, Pickering et al. 2002) or direct E2F1-p53 complex formation (Hsieh, Yap et al. 2002). All are believed to enhance the apoptotic activity of p53. In neurons, SIVA regulation could be complemented by this synergy between E2F1 and the p53 pathways. Not all models of neuronal cell death, however, require both pathways. β-amyloid-evoked neuronal cell death, which is mediated by E2F1, does so in a p53-independent manner (Giovanni, Keramaris et al. 2000). We have also shown here that, following DNA damage, SIVA is not up-regulated in p53-deficient neurons. Western blot analysis demonstrated that E2F1 was not induced at early timepoints following treatment with camptothecin and was only upregulated after 16 h, long after apoptotic events have been initiated (data not shown). These results suggest that E2F1 is not involved in the regulation of SIVA in this model of neuronal injury. Whether SIVA regulation is an independent event or a consequence of cross-talk between these two apoptotic pathways is likely a function of the initiating mechanism.

Although SIVA has been shown to play a role in regulating apoptosis through the TNFR signaling cascade, the involvement of SIVA in neuronal injury has not yet been described. SIVA contains a death domain and therefore functions in TNFR signaling in cells of the immune system (Prasad, Ao et al. 1997). More recently SIVA has also been shown to interact with GITR, another member of the TNFR superfamily expressed in T cells. Although SIVA was first characterized in cells of the immune system and was thought to be involved in T cell homeostasis, expression analysis revealed that SIVA is expressed in most tissues (Yoon, Ao et al. 1999). In addition to normal tissues, SIVA is also expressed in tumor cells exposed to genotoxic agents that induce a p53 death response. Since CD27 is expressed primarily in T and B cells, the mechanism by which SIVA signals apoptosis in other cell types such as neurons or tumor cells remains unknown. Presently, it is unknown whether SIVA interacts with any TNFR family members expressed in the nervous system. There are a number of TNFR subtypes expressed in CNS neurons, including TNFR1, p55-TNFR, TNFRII and the p75 low affinity neurotrophin receptor (Martin-Villalba, Herr et al. 1999). It has also been reported that TNF signaling is increased in stroke and is believed to have a major impact on the extent of brain injury (Barone and Feuerstein 1999; Martin-Villalba, Herr et al. 1999). Based on studies in the immune system it is likely that SIVA may also be involved in TNF signaling following injury, however the interacting TNF targets in the CNS remain unknown.

In addition to interactions with members of the TNFR super family, SIVA-1 has also been shown to directly interact with antiapoptotic members of the Bcl-2 family proteins. SIVA has no BH3-like domain, however the site of interaction with Bcl-X_L was

found to be through a 20 amino acid long amphipathic helix known as the SAH domain (Xue, Chu et al. 2002). Disruption of this interaction by mutation of this site mitigated the apoptotic response. Thus, it is believed that SIVA may induce cell death by sequestering antiapoptotic Bcl-2 family proteins and thereby allowing the proapoptotic family members to function. The molecules with which SIVA interacts to induce apoptosis may vary depending on the cell type, and future studies should elucidate its mechanism of action in the CNS.

In summary, SIVA is a proapoptotic gene that is expressed in a broad scope of tissues, including cells of the immune system, tumor cells and in the CNS. SIVA is induced in response to p53 and E2F1, two tumor suppressor genes that function in cell cycle regulation and apoptosis. Up-regulation of SIVA in itself is sufficient to induce the apoptotic cascade implicating its importance in injury-induced apoptosis. Indeed, a number of tumor tissues have revealed significantly low levels of SIVA suggesting that SIVA may also play an important role in protection against cancer. By isolation of the mouse SIVA promoter, we have identified consensus binding sites for both p53 and E2F1 and shown that these factors are able to bind their respective sites. Moreover, we have shown that p53 and E2F1 binding can activate the SIVA promoter demonstrating that SIVA is a direct transcriptional target for these tumor suppressor genes. Presently, a SIVA-deficient mouse has not been developed, however, future studies involving deletion of SIVA will reveal the extent of its involvement in different modes of cell death. The fact that SIVA is highly induced by p53 and E2F1 in a broad spectrum of cell types, and that SIVA up-regulation is sufficient to induce the death cascade speak to its

importance not only in protection against tumorigenesis but also in brain damage following acute injury.

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Chapter 4. Summary and Discussion

Implications of Reported Findings

Summary of findings

In Chapter 2, I demonstrate that Apaf1 is a key target for p53 in the regulation of neuronal apoptosis. Along with our collaborators (the Helin laboratory), we were the first to report this finding. I observed that Apaf1 mRNA and protein was induced in primary cultured neurons following expression of p53 and treatment with the DNA damaging agent, camptothecin. In an *in vivo* model of focal ischemia, middle cerebral artery occlusion, Apaf1 protein was also upregulated in the cortex and striatum of the affected ipsilateral forebrain. p53 protein isolated from primary neuronal cultures expressing p53 or treated with camptothecin was able to bind to oligonucleotides designed from p53 consensus binding sequences located in the Apaf1 promoter. As well, p53 could induce activity from the Apaf1 promoter in a neuronal cell line. Finally, Apaf1 deficiency protected primary neurons from apoptosis induced by DNA damage. These studies not only show that Apaf1 is a direct target, they also demonstrate its importance in the physiological context of acute brain injury.

In Chapter 3, I describe a novel p53 target, the previously characterized proapoptotic gene SIVA. SIVA expression was upregulated in primary neuronal cultures expressing p53, following treatment with camptothecin and in an *in vivo* model of focal ischemia. Adenoviral-mediated expression of SIVA was sufficient to induce cell death in cultured neurons and neural precursor cells. These results initiated the search for p53 consensus binding sequences within the SIVA gene. Two sites were found within intron1. p53 protein isolated from primary neuronal cultures was able to bind to oligonucleotides designed from each consensus sequence. A fragment of the SIVA gene containing a

putative promoter, exon1 and intron1 was cloned and p53 was able to induce activity from a luciferase reporter construct containing this regulatory sequence in a neuronal cell line. Interestingly, I also demonstrate that the SIVA gene is a direct target for the transcription factor E2F1, thus adding to the growing list of genes regulated by both p53 and E2F1. These studies suggest that SIVA may be an important target in the regulation of neuronal cell death after acute brain injury.

Apaf1-deficient neurons succumb to a delayed caspase-independent cell death

Although I have shown that Apaf1-deficiency protected neurons from DNA damage induced neuronal cell death, this was within the first 24 hours. Further research extending the timecourse demonstrated that there was a delayed onset of apoptosis (Cregan, Fortin et al. 2002). Apaf1 is a key regulator of caspase activation via the intrinsic pathway through formation of the apoptosome. Apaf1-deficiency abrogated caspase activation in neurons expressing p53 directly or following DNA damage (Cregan, Fortin et al. 2002). Thus, the delayed neuronal cell death observed was independent of caspase activity. Evidence of caspase-independent neuronal cell death has previously been reported in excitotoxic cell death (Johnson, Kinoshita et al. 1999; Lankiewicz, Marc Luetjens et al. 2000) and in experimental models of stroke (Rideout and Stefanis 2001; Zhan, Wu et al. 2001). Furthermore, models of neuronal cell death known to involve caspase activation displayed a delayed cell death upon inhibition of caspase activity (Miller, Moulder et al. 1997; Stefanis, Park et al. 1999; D'Mello, Kuan et al. 2000; Keramaris, Stefanis et al.

2000; Selznick, Zheng et al. 2000) much like neurons deficient for Apaf1. We have shown that caspase-independent neuronal cell death functions through a mechanism that involves apoptosis-inducing factor (Cregan, Fortin et al. 2002). The importance of Apaf1 to p53-mediated neuronal cell death lies in its requirement for the initiation of caspase activity via the intrinsic pathway.

Physiological relevance of SIVA to p53-mediated neuronal cell death

To identify that an inducible proapoptotic target of p53 is important for neuronal cell death would involve demonstrating several criteria. First, the target should be upregulated in models of p53-mediated neuronal cell death. Second, p53 consensus binding sequences should be located within regulatory elements of the target gene and p53 should induce activity from these sites. Third, expression of the target gene should be sufficient to induce neuronal cell death or enhance the apoptotic response. Finally, disruption of the p53 target gene should offer protection from p53-mediated models of neuronal cell death. I have been able to demonstrate that SIVA answers to the first three requirements. Demonstrating the physiological importance of SIVA to p53-mediated neuronal cell death, however, will require the fourth.

Future research in the lab should focus on determining whether SIVA is required for p53-mediated neuronal cell death. The generation of a SIVA knock-out mouse would be the ideal tool. *In vitro* experiments on neurons cultured from SIVA-deficient mice subjected to DNA damage could establish the requirement for SIVA if its deletion provides protection from apoptosis compared to wild-type counterparts. More

importantly, testing for neuroprotection in *in vivo* models of focal ischemia would be possible, thus, measuring the true physiological relevance of SIVA. To date no SIVA knock-out mouse is available. Depletion of SIVA expression by small interfering RNA could serve to weigh the relative importance of SIVA to p53-mediated neuronal cell death. Future studies will reveal the importance of SIVA in different apoptotic paradigms involved in acute brain injury.

p53 target genes regulating neuronal apoptosis

At the onset of my research few p53 targets had been identified in models of neuronal cell death. Bax, a key regulator of neuronal cell death (Xiang, Kinoshita et al. 1998; Cregan, MacLaurin et al. 1999; Keramaris, Stefanis et al. 2000), was identified as a direct transcriptional target for p53 (Miyashita and Reed 1995) , however, we (Cregan, MacLaurin et al. 1999) and others (Johnson, Xiang et al. 1998; Xiang, Kinoshita et al. 1998) had failed to detect increased induction of Bax protein in neurons during apoptosis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was upregulated in a p53-dependent manner in cerebellar granule neurons subjected to DNA damage and suppression of GAPDH by anti-sense oligonucleotides protected cerebellar granule neurons from DNA damage induced apoptosis (Chen, Saunders et al. 1999). Whether GAPDH is actually a direct target for p53 is still to be determined. Thus, very little information existed about transcriptional targets of p53 that might mediate neuronal cell death.

Since then, several genes, including the ones described in this thesis, have been shown to mediate neuronal apoptosis. As with Apaf1, other genes regulating the intrinsic pathway are direct targets for p53. These include the BH3-only proteins PUMA, Noxa, and Bid which were first identified in non-neuronal cells. We have demonstrated that PUMA and Noxa are upregulated in neurons following injury induced by DNA damage and p53 overexpression (Cregan, Arbour et al. 2004), however, only direct expression PUMA and not Noxa is sufficient to induce neuronal cell death (Cregan, Arbour et al. 2004). It has also been observed that PUMA is required for DNA damage induced apoptosis in sympathetic neurons (Wyttenbach and Tolkovsky 2006). In both studies, PUMA deficiency conferred protection from DNA damage induced apoptosis (Cregan, Arbour et al. 2004; Wyttenbach and Tolkovsky 2006). Further, PUMA deficiency protected developing neurons from radiation (Jeffers 2003). Noxa was identified as a critical mediator of p53-dependent axotomy-induced motor neuron death in adult mice as it demonstrated the largest upregulation of all BH3-only proteins examined and its deficiency suppressed axotomy-induced apoptosis (Kiryu-Seo, Hirayama et al. 2005). Bid-deficiency protected neurons form *in vitro* and *in vivo* models of ischemia (Plesnila, Zinkel et al. 2001). Another gene, the zinc finger transcription factor Peg3/Pw1 which is believed to boost p53-mediated apoptosis by promoting Bax translocation from the cytosol to the mitochondria (Deng and Wu 2000) was induced in neurons following DNA damage (Johnson, Wu et al. 2002) and ischemia/hypoxia (Yamaguchi, Taniguchi et al. 2002). Overexpression of a dominant negative Peg3/Pw1 inhibited neuronal cell death in these studies (Johnson, Wu et al. 2002; Yamaguchi, Taniguchi et al. 2002) demonstrating a key role in this type of injury.

Members of the extrinsic pathway may also play a role in p53-mediated neuronal cell death. Upregulation of Fas and Fas-mediated activation of caspase 8 has been observed in several models of neuronal cell death including trophic factor withdrawal, cerebral ischemia and spinal chord ischemia (Martin-Villalba, Herr et al. 1999; Matsushita, Wu et al. 2000; Raoul, Pettmann et al. 2000). In neurons, it is believed that caspase 8 activation leads to the cleavage of Bid and the subsequent activation of the intrinsic pathway (Culmsee and Mattson 2005). It is not clear whether death receptor-mediated neuronal cell death is fully dependent on p53 (Culmsee and Mattson 2005), however, as they can be regulated independently of p53. The extent of its contribution to neuronal cell death is not known as Fas-deficiency had a greater protective antiapoptotic effect towards glia than neurons in a spinal chord injury model (Casha, Yu et al. 2005).

p53 apoptosis effector related to PMP-22 (PERP) has been shown to be regulated by p53 and to play a role in neuronal cell death (Attardi, Reczek et al. 2000). Its expression was induced in developing neurons following DNA damage (Ihrie, Reczek et al. 2003) and in dopaminergic neurons in the substatia nigra (SN) following intrastriatal injection of 6-hydroxydopamine (Iwata, Nomoto et al. 2004). PERP-deficiency protected developing neurons of the subventricular zone from γ-irradiation induced apoptosis and death initiated by inappropriate proliferation in the Retinoblastoma (Rb)-deficient embryo (Ihrie, Reczek et al. 2003). The mechanism by which PERP regulates apoptosis is not understood (Ihrie, Reczek et al. 2003).

Although it has been questioned whether GAPDH is a direct target for p53, increased GAPDH expression has been observed following focal ischemia induced by middle cerebral artery occlusion (Tanaka, Mochizuki et al. 2002). GAPDH has been

implicated in several neurodegenerative diseases including Huntington's (Senatorov, Charles et al. 2003), Parkinson's (Tsuchiya, Tajima et al. 2005) and Alzheimer's (Sunaga, Takahashi et al. 1995) disease. How GAPDH contributes to apoptosis has not been fully deciphered but involves its translocation from the cytoplasm to the nucleus where it may undergo structural and functional changes (Chen, Saunders et al. 1999). This translocation may be regulated by another p53-target gene, the E3 ubiquitin ligase Siah1 (Hara, Agrawal et al. 2005). Siah1 and GAPDH appear to have a mutually beneficial relationship. Siah1 harbours a nuclear localization signal and its binding to GAPDH has been shown to mediate the nuclear translocation of GAPDH in stressed cells. In turn, GAPDH binding to Siah1 leads to Siah1 stabilization which facilitates its degradation of nuclear proteins and the promotion of cell death (Hara, Agrawal et al. 2005). In developing neurons, RNAi directed towards GAPDH and Siah1 prevented cell death induced after treatment with NMDA, GSNO or etoposide (Hara, Agrawal et al. 2005). The interaction between GAPDH and Siah1 is initiated by the *S*-nitrosylation of GAPDH following induction of oxidative stress. It has therefore been suggested that GAPDH functions as a sensor to detect and signal changes within the cell, and fulfills an apoptotic function through its close association with SIAH1, where it may influence the specific targets selected by Siah1 for degradation in the initiation of cell death (Hara, Agrawal et al. 2005). Interestingly, mutant Huntingtin whose N-terminal fragment translocates to the nucleus and causes neurotoxicity in Huntingtin's disease, does so by forming a ternary complex with GAPDH and Siah1 (Bae, Hara et al. 2006).

The list of p53 target genes that play a role in neuronal cell death is continuously growing. Still many genes identified in non-neuronal cells such as the mitochondrial

proteins p53AIP1 and p53-inducible genes (PIGs) have not been studied in neurons. Furthermore, given the estimates that p53 may have several hundred targets, novel genes regulating neuronal cell death may yet to be discovered and this will be crucial to completely understand the mechanisms by which p53 induces neuronal apoptosis.

It is hard to conceive why p53 should target so many proapoptotic genes. One can predict that one possibility is that the induction of p53 targets is dependent on cell type and on death stimulus. This is beautifully illustrated by the BH3-only proteins PUMA and Noxa. We have demonstrated that both PUMA and Noxa are upregulated following expression of p53 and after DNA damage, however, only the upregulation of PUMA and not Noxa is sufficient to induce cell death in cultured cerebellar granule neurons (Cregan, Arbour et al. 2004). Thus, Noxa is not a major regulator of apoptosis in this context. This is in sharp contrast with the model of axotomy-induced motor neuron cell death in the adult mouse. In this death paradigm, Noxa is a key mediator of the apoptotic cascade, whereas PUMA is dispensable (Kiryu-Seo, Hirayama et al. 2005). This evidence builds a strong case for the influence of cell type and death stimulus on the upregulation of proapoptotic targets by p53. It is also possible that many targets are required to introduce a certain redundancy into the system ultimately enabling the elimination of damaged cells even if certain key molecules were to become non-functional. p53 can upregulate several death receptors as well as several BH3-only proteins. Although these may not induce apoptosis efficiently in certain cell types, given a longer time period they may fulfill their apoptotic function. Whatever the reason for the abundance of targets, it will be important to identify these in order to fully understand the mechanism by which p53 induces apoptosis.

The importance of p53-mediated transcription-independent mechanisms of neuronal cell death

In non-neuronal cells, it has been observed that p53 can induce apoptosis through mechanisms other than transcriptional activation of target genes. These include transcriptional repression and extranuclear roles at the mitochondria. Do these have a role in neuronal cell death?

p53-mediated neuronal cell death could require both the transcriptional upregulation of proapoptotic genes as well as the transcriptional repression of survival genes. Our research, however, clearly establishes a role for transcriptional activation. Transcriptional repression is not well documented in neurons. There is evidence suggesting that p53 can block the activity of the transcription factor NF-κB following induction of apoptosis in several *in* vivo and *in* vitro models of neuronal injury (Bhakar, Tannis et al. 2002; Culmsee, Siewe et al. 2003; Kassed, Butler et al. 2004). It is believed that p53 functions by out-competing NF-κB for limiting amounts of p300 thereby disrupting NF-κB transcriptional activity (Wadgaonkar, Phelps et al. 1999; Culmsee, Siewe et al. 2003). NF-κB promotes survival in neurons by inducing the expression of anti-apoptotic members of the Bcl-2 family and IAPs (Mattson, Culmsee et al. 2000). Thus, p53 can indirectly repress survival factors which may be required for p53-mediated neuronal cell death (Culmsee, Siewe et al. 2003). More research is needed to fully understand a role for p53 transcriptional repression in neuronal apoptosis.

The extranuclear role of p53 at the mitochondria is very much more controversial. Could p53 function at the mitochondrial level in neurons? p53 is believed to regulate apoptosis at the mitochondrial level in two ways: 1) either as an inhibitor of the anti-apoptotic members of the Bcl-2 family; or, 2) as an activator of the multi-domain proapoptotic member Bax. Our research has failed to co-localize p53 with the mitochondria during DNA damage induced neuronal apoptosis. p53 was exclusively located within the nucleus (Cregan, Arbour et al. 2004). Following oxidative stress, p53 was detected at the mitochondria in cultured glial cells and astrocytes (Bonini, Cicconi et al. 2004). No evidence, however, exists of its occurrence in neurons. This would suggest that the mitochondrial function of p53 may be cell type specific and, that, in neurons, p53 functions primarily in the nucleus. Thus far, an extranuclear role at the mitochondria appears to be nonessential for apoptotic activity in injury-induced neuronal cell death.

P53 and E2F1 transactivate common proapoptotic genes

p53 and E2F-1 can both independently regulate neuronal demise. E2F1 has been shown to mediate apoptosis independently of p53 in models of neuronal cell death involving potassium-deprivation (O'Hare, Hou et al. 2000) and expression of β-amyloid (Giovanni, Keramaris et al. 2000). They have been shown, however, to co-operate in certain models of neuronal cell death. For example, deficiency in p53 or E2F-1 has been shown to decrease the size of the infarct following ischemic damage (Crumrine, Thomas et al. 1994; MacManus, Koch et al. 1999). The link between the p53 pathway and the pRB/E2F-1 pathway was always believed to involve the transactivation of the E2F-1

target gene, $p19^{ARF}$. $P19^{ARF}$ stabilizes p53 expression by inhibiting the negative regulator of p53, MDM2. Current research, however, has discovered that E2F-1 can promote p53-dependent apoptosis through transcriptional activation of several cofactors of p53 including ASPP1, ASPP2, JMY and p53DINP1 (Hershko, Chaussepied et al. 2005). These cofactors positively influence transactivation of proapoptotic p53 target genes. The ASPPs can enhance p53 binding to promoters of proapoptotic genes (Samuels-Lev, O'Connor et al. 2001). Likewise, JMY, a p300 interactor, can direct p53 to proapoptotic targets (Shikama, Lee et al. 1999). p53DINP1 mediates phosphorylation of p53 on ser46, a modification important for the induction of the proapoptotic target p53AIP (Okamura, Arakawa et al. 2001). Thus, it is clear that the influence of the pRB/E2F-1 pathway on the p53 pathway is much more complex than previously thought. Interestingly, many p53 proapoptotic genes have also been described as direct targets for E2F-1. In chapter 3, I describe SIVA as a direct target for both p53 and E2F-1. Apaf1, the focus of chapter 2, was also identified as a direct target for E2F-1. Other proapoptotic p53 target genes known to be transcriptionally regulated by E2F-1 include the BH3-only proteins, PUMA and Noxa, and as mentioned above p53DINP1. The question now becomes: Does the pRB/E2F-1 pathway co-operate with p53 to induce neuronal cell death by promoting p53 accumulation and proapoptotic gene transcription in an additive fashion or do they synergize on promoters of proapoptotic target genes to multiply the response? These questions await further studies.

In search of neuroprotective strategies

The evidence suggests that p53 is an important mediator of apoptosis in many acute neuronal injury models and possibly in neurodegenerative disorders such as Parkinson's disease and Huntington's disease. It is, thus, crucial to study the mechanisms by which p53 mediates neuronal demise if we are to fully understand its mode of action. Basic scientific research is expected to lead to the identification of specific targets or to the development of unique strategies for neuroprotective intervention, potentially lessening the damaging effects of injury.

A clinically relevant model could be ischemic injury such as that induced by stroke. There are an estimated 40,000-50,000 strokes suffered every year in Canada. With 16,000 deaths per year caused by strokes, it ranks as the fourth leading cause of death. As many as 300,000 Canadians are stroke survivors living with the effects of stroke, which can be negligible or can involve severe paralysis (www.canadianstrokenetwork.ca). Following ischemic injury, neurons of the core region die a necrotic death and are lost. The neurons of the surrounding penumbral region, however, succumb to a delayed apoptotic death usually within 2-5 days. It is the death of these neurons which determine the severity of the effects for stroke survivors. Understanding the mechanisms leading to the loss of these neurons will allow us to establish effective approaches to minimize brain damage after stroke.

One approach could be the generation of blocking peptides for p53 targets such as PUMA. PUMA has been identified as a key target in the regulation of apoptosis in various cell types including neurons (Cregan, Arbour et al. 2004) A peptide which could inhibit the interaction of PUMA with multi-Bcl-2 homology domain family members could abrogate its apoptotic activity, perhaps minimizing the loss of neurons in the

penumbral region. Even prolonging the survival of these neurons will provide a larger window of opportunity for therapeutic intervention. Although the role of PUMA in E2F1-mediated neuronal cell death is not clear, genes targeted for neuroprotective strategies encompassing more than one pathway such as PUMA could be favourable over those targeting p53 alone. It is clear that neuronal cell death following ischemic injury is not a simple process and an effective treatment will have to target many genes. p53 does, however, play a prominent role and will factor highly in establishing of an efficient neuroprotective strategy.

In summary, tumour suppressor p53 is a key regulator of neuronal cell death following injury. Our studies reveal that this is accomplished through transcriptional activation of target genes. In a search for proapoptotic genes directly regulated by p53, I have identified two new genes, Apaf1 and SIVA, which are induced in *in vitro* and *in vivo* models of neuronal injury. Although the role of Apaf1 in apoptosis has been well established and is crucial for caspase activation in neurons, the mechanism by which SIVA mediates cell death is less clearly defined and will require further research. The characterization of these new targets has expanded our knowledge regarding the mechanism by which p53 induces neuronal cell death. More work, however, is needed if we are to exploit this complex gene and its targets to develop novel approaches for the treatment of acute neuronal injury.

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Career History

Sudbury Countertop Distributors May 1993 – August 1999

Collins Barrow Maheu Noiseux September 1988 – April 1993

Experience

My experience has engendered strong scientific research skills along with sound knowledge of accounting principles.

University of Ottawa September 1999 - Present

PhD Student

- Collaboratively worked with Principal Investigator (Dr. Ruth Slack) to ensure that PhD research goals were met.
- Designed, organized and performed PhD experiments.
- Obtained first class honours in all courses taken.
- Presented monthly reports on my research.
- Performed critical review of internal scientific manuscripts prior to submission.
- Reviewed external manuscripts for scientific journals.
- Participated in writing and critiquing of grant proposals.
- Presented research at national and international conferences.
- Trained incoming graduate students.
- Supervised summer students.
- Followed animal care training and maintained a mouse colony.

Sudbury Countertop Distributors May 1993 – August 1999

Office Administrator (full-time/part-time)

- Lead and managed all aspects of accounting including receivables, payables, payroll, and inventory.
- Prepared weekly and monthly cash flow analysis and bank reconciliations.
- Prepared year-end review of financials and drafted financial statements for tax purposes.
- Prepared cost-benefit/risk analysis and advised management on selection and pricing of new products.
- Conducted business analysis of current customers and competitors.
- Implemented Customer Relationship Management to ensure customer satisfaction.
- Oversaw all aspects of IM/IT/IS.

Collins Barrow Maheu Noiseux September 1988-April 1993

Accounting student/technician

- Worked as an integral part of a team to complete interim and year-end audits of large corporate clients.
- Prepared year end review of financials, drafted financial statements and prepared tax returns for smaller corporate clients and businesses.
- Compiled financial information and prepared personal tax returns for individual clients.
- Communicated verbally as well as in writing tax reports to Managers / Partners and then Clients.

Awards & Scholarships

2004 – Present	**Canada Graduate Scholarship, Canadian Institute of Health Research** ($30,000/yr + $5,000/yr research allowance). Special award recognizing exceptionally high potential for future research success.
2004	**Ontario Graduate Scholarship**, Ontario Government ($15,000/yr) (declined).
2004	**Heart & Stroke Foundation of Canada-Focus on Stroke** ($20,000/yr + $1000/yr travel allowance) (declined).
2003	**Award – Poster Presentation – Canadian Stroke Network,** Annual General Meeting ($500).
2002	**Brain Star Award, Institute of Neurosciences, Mental Health and Addiction** ($1,000).
2001 – Present	**Ottawa Academic Excellence Scholarship**, University of Ottawa. (tuition fees waived, ~ $5,500/yr).
1999 – 2001	**Ontario Graduate Scholarship Science & Technology, Ontario Government** ($15,000/yr).
1999	**Admissions Scholarship**, University of Ottawa (value of tuition, ~ $5,000).
1999	**Award of Excellence – Science, Association of Laurentian University Part-time Students** ($ 500).
1998 – 1999	**Part-time Student Bursary, Laurentian University Alumni Association** ($500/yr).

Peer-Reviewed Publications

Sean P. Cregan, Nicole A. Arbour, Jason G. MacLaurin, Steven M. Callaghan, **Andre Fortin**, Eric C. C. Cheung, Alexander E. Mackenzie, David S. Park and Ruth S. Slack (2004) p53 activation domain 1 is essential for PUMA up-regulation and p53-mediated neuronal cell death **J. Neurosci.** 24(44) : 10003-12.

Andre Fortin, Jason G. MacLaurin, Nicole Arbour , Sean P. Cregan, Neena Kushwaha, Steven M. Callaghan, David S. Park, Paul R. Albert and Ruth S. Slack (2004) The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1 **J. Biol. Chem.** 279(27) : 28706-14.

Cregan S.P., **A. Fortin**, J. G. MacLaurin, S.M. Callaghan, F. Cecconi, S. Yu, T.M. Dawson, D.S. Park, G. Kroemer, and R.S. Slack (2002) Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death **J. Cell Biol.** 158(3) : 507-517.

Fortin A., S.P. Cregan, J.G. MacLaurin, N. Kushwaha, E.S. Hickman, C.S. Thompson, A. Hakim, P.R. Albert, F. Cecconi, K. Helin, D.S. Park and R.S. Slack (2001) Apaf-1 is a key transcriptional target for p53 in the regulation of neuronal cell death **J. Cell Biol.** 155(2) : 207-216.

Submitted Manuscripts

Jahani, A., Cheung, E.C., MacLaurin, J.G., **Fortin, A.**, Park, DS, McBride, H., and Slack, RS. (2006) Activation of mitochondrial fusion protects neurons against injury-induced cell death.

Jacqueline L. Vanderluit, Crystal Wylie, **Andre Fortin,** Steve Callaghan, Jason G. MacLaurin, David S. Park, and Ruth S. Slack. (2006) P107, the cell cycle regulator, impacts the mode of neural precursor division and differentiation by repressing Hes1.

Abstracts

A. Fortin, J. G. MacLaurin, S. M. Callaghan, D. S. Park and R. S. Slack. (2005) Mechanisms of Siva-mediated neuronal cell death. Programmed Cell Death, Cold Spring Harbor, NY

A. Fortin, J. G. MacLaurin, S. M. Callaghan, D. S. Park and R. S. Slack. (2005) Mechanisms of Siva-mediated neuronal cell death. Cellular Senescence and Cell Death, Keystone Symposia, Keystone, CO

A. Fortin, J. G. MacLaurin, S. M. Callaghan, D. S. Park and R. S. Slack. (2004) Mechanisms of Siva-mediated neuronal cell death. Society for Neuroscience, San Diego, CA

Andre Fortin, Sean P. Cregan, Neena Kushwaha, Steve M. Callaghan, Charlie Thompson, David S. Park, Paul R. Albert and Ruth R. Slack. (2003) Siva is upregulated in response to

p53-mediated neuronal cell death. Society for Neuroscience, Annual Meeting, New Orleans, LA

Andre Fortin, Sean P. Cregan, Neena Kushwaha, Steve M. Callaghan, Charlie Thompson, David S. Park, Paul R. Albert and Ruth R. Slack. (2003) Siva is upregulated in response to p53-mediated neuronal cell death. Canadian Stroke Network, Annual Meeting, St-Andrews-by-the-Sea, NB

Andre Fortin, Sean P. Cregan, Neena Kushwaha, Steve M. Callaghan, Charlie Thompson, David S. Park, Paul R. Albert and Ruth R. Slack. (2003) Siva is upregulated in response to p53-mediated neuronal cell death. Keystone Symposia, Apoptosis, Banff, AB

S.P. Cregan, **A. Fortin**, J.G. MacLaurin, S.M. Callaghan, F. Cecconi, S.W. Yu, T.M. Dawson, V.L. Dawson, D.S. Park, G. Kroemer, and R.S. Slack. (2002) The Involvement of AIF and Apaf-1 in the regulation of p53-mediated neuronal cell death. Cancer Genetics and Tumor Suppressor Genes, Cold Spring Harbor, New York

S.P. Cregan, **A. Fortin**, J.G. MacLaurin, S.M. Callaghan, F. Cecconi, D.S. Park, G. Kroemer, and R.S. Slack. (2002) Apaf-1 and AIF regulate distinct pathways in p53-mediated neuronal cell death. Keystone Symposia, Mitochondria and Pathogenesis, Copper Mountain, CO

Fortin A., S.P. Cregan, J.G. MacLaurin, D.S. Park, F. Cecconi, P.Gruss, K. Helin and R.S. Slack (2001) Apaf1 is a downstream effector of p53-induced apoptosis in neurons. Society for Neuroscience, Annual Meeting, San Diego, CA

S.P. Cregan, **A.J. Fortin**, J.G. MacLaurin, S.M. Callaghan, D.T. McBride, D.S. Park, F. Cecconi, P. Gruss, G. Kroemer, and R.S. Slack. (2001) P53 induces neuronal cell death through parallel pathways. Society for Neuroscience, Annual Meeting, San Diego, CA

Fortin A., S.P. Cregan, J. MacLaurin, J. Tauskela, P. Morley, A. Hakim, D.S. Park and R.S. Slack (2000) Involvement of p53 signaling pathway in cell death of neurons. Society for Neuroscience, Annual Meeting, New Orleans, LA

Community Involvement

Running Room Running Club

- An active member of the Running Room Running Club Bank Street location.

Sudbury Fitness Challenge (SFC)

Treasurer 1997-1999

- Responsible for all financial aspects of the SFC including receivables, payables payroll and financial statements. Searched for funding opportunities for summer student projects.
- Worked closely with the rest of the SFC team to organise and run the five yearly sporting events of the SFC.

Other Interest and Hobbies

Triathlons, swimming, running, skiing (downhill and cross country), cycling, the arts, cooking, reading suspense thrillers, antique collecting, travelling, and listening to jazz music. I hope to compete in the 2007 world triathlon championship as an elite age grouper.

References

References available upon request.