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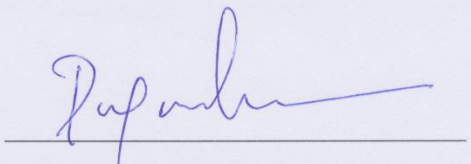
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in this thesis.

This thesis has also not been submitted for any degree in any university previously.



Priyanka Balaganapathy

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I dedicate this thesis to my advisor and mentor,
A/Prof Thiruma Arumugam

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ABSTRACT

Stroke is the world's second leading cause of mortality, with a high incidence of morbidity. Oxidative stress, excitotoxicity, inflammation and apoptosis are cellular events regulating post-ischemic neuronal cell death, although the underlying molecular mechanisms are incompletely understood. Numerous neuronal membrane receptors are activated by endogenous ligands and may contribute to infarct development. Notch is a well-characterized membrane receptor involved in cell differentiation and proliferation and now shown to play a pivotal role in cell death during ischemic stroke. Blockade of Notch signaling by inhibition of γ-secretase, an enzyme that generates the active form of Notch (Notch intracellular domain, NICD), is neuroprotective following stroke. Many pieces of evidence of Notch-controlled cell death are in association with crucial hypoxic state eliciting transcription factors including Nuclear factor-κB (NF-κB) and Hypoxia inducible factor-1α (HIF-1α). Interestingly, Pin1, a peptidyl-prolyl isomerase that regulates p53 transactivation under stress, has been identified to promote the pathogenesis of ischemic stroke via Notch signaling. Also, transcription factors, NF-κB and HIF-1α are p53 activation promoting entities widespread in many cellular models. Moreover, Notch in early neural progenitor cells selectively induces apoptosis by elevating levels of nuclear p53 and transactivation. The current study has investigated the interplay between Notch and p53 under ischemic stroke conditions. Using pharmacological inhibitors, it has been demonstrated that a NICD/p53 interaction is involved in transcriptional regulation of genes downstream of p53 and NICD to modify stroke severity. Furthermore, p53 expression is maintained at a minimal level by mouse double minute 2 homolog (MDM2)-

mediated ubiquitination and proteasomal degradation under normal conditions and can be stabilized by various cellular stresses. This study has demonstrated that the NICD/p53 interaction confers stability to p53 by rescuing it from MDM2-mediated ubiquitination. Here we provide the first evidence that Notch and p53 interact to activate apoptotic and neurodegenerative pathways during ischemic brain injury, and that suppression of Notch/p53 signaling ameliorates the disease process of ischemic stroke in a mouse model. Together, this study indicates Notch contribution to the pathogenesis of ischemic stroke by promoting p53 stability and signaling, and inhibition of the Notch/p53 complex is a novel approach for treating ischemic stroke.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ADP	Adenosine Diphosphate
AIF	Apoptosis inducing factor
AIM-2	Absent in melanoma 2
AIP1	Apoptosis inducing protein-1
AKT	Protein kinase B (PKB)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANK	Seven ankyrin/cdc10 repeats (ANK)
ANOVA	Analysis of variance
AP sites	Apurinic/apyrimidinic sites
Apaf-1	Apoptotic protease activating factor 1
ASC	Apoptosis-associated speck-like protein containing a CARD
ASIC	Acid-sensing ion channels
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine Triphosphate
AVM	Arteriovenous malformation
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BBB	Blood-brain barrier
BCL2	B-cell lymphoma 2

Bcl-xL	B-cell lymphoma-extra large
Bcl-XS	B-cell lymphoma-short isoform
BH	B-cell lymphoma 2 homology
BID	BH3 interacting-domain death agonist
BSA	Bovine serum albumin
BNIP3	Bcl-2/adenovirus E1B 19 kDa-interacting protein3
Ca^{2+}	Calcium ion
CARD	Caspase recruitment domain
cbEGF	Calcium binding epidermal growth factor
CBF / RBPJ	Centromere Binding Factor / Recombining binding protein suppressor of hairless
CBP	CREB-binding protein
CCA	Common carotid artery
CD95	Cluster of differentiation 95
CED	Cell death protein
CHX	Cycloheximide
CICR	Calcium-induced calcium release
CNS	Central nervous system
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
CSL	CBF1, Suppressor of Hairless, Lag-1
Cyt-C	Cytochrome-C
Cyp-D	Cyclophilin-D
DAMP	Damage associated molecular patterns
DAPI	4’6-diamidino-2-phenylindol

DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DBD	DNA binding domain
DISC	Death-inducing signaling complex
DJL	Delta-like/Jagged ligands
DLL	Delta-like homologous
DMEM	Dulbecco modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
DSL	Delta/Serrate ligands
ECA	External carotid artery
ECL	Chemiluminescent enhancer reagent
ECM	Extracellular matrix
EGF	Epidermal growth factors
EndoG	EndonucleaseG
ETC	Electron transport chain
FADD	Fas-associated death domain
FASL	Fas Ligand
FasR	Fas receptor
FSG	Fish skin gelatin
GFP	Green fluorescence protein
GSI	Gamma-secretase inhibitor
H2O2	Hydrogen peroxide
HAT	Histone acetyl transferase

HD	Hetero-dimerization
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Herp	Hes-related protein
HIF-1α	Hypoxia inducing factor-1α
HIS	Histidine
HNE	4-hydroxynonenal
HRP	Horse radish peroxidase
IAPs	Inhibitors of apoptosis
iASPP	Inhibitor of apoptosis-stimulating protein of p53
ICA	Internal carotid artery
ICAM-1	Intercellular adhesion molecule-1
IGF	Insulin-Like Growth Factor
IGF-BP3	Insulin-Like Growth Factor-binding protein-3
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAG	Jagged
JNK	c-Jun-N-terminal kinase
LAG-1	Lymphocyte-activation gene-1
LDH	Lactate dehydrogenase
LLOMe	L-leucyl-L-leucine methyl ester
LMN	Lysosomal-mediated necrosis
LNR	Lin-12-Notch
MAC	Membrane attack complex

MAML	Mastermind-like proteins
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MCU	Mitochondrial calcium uniporter
MCUR1	Mitochondrial calcium uniporter regulator 1
MDM2	Mouse double minute 2 homolog
MICU1	Mitochondrial calcium uptake 1
MMP	Matrix metalloproteinases
MnSOD	Manganese superoxide dismutase
MPTP	Mitochondrial permeability transition pore
mTOR	Mechanistic target of rapamycin
MTP	Mitochondrial transition pore
Na^+	Sodium ion
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAS	Notch antisense
NES	Nuclear export signal
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NGFR	Nerve growth factor receptor
NICD	Notch intracellular domain

NLRP	NBD – nucleotide-binding domain (NACHT), leucine-rich repeat (LRR) and pyrin domain (PYD) domains-containing protein
NLS	Nuclear localization signal
NLRC	Nucleotide-binding domain and leucine-rich repeat containing CARD
NMDA	N-methyl-d-aspartic acid
NO	Nitric oxide
NRR	Negative regulatory region
NTR	Neurotrophin receptor
O_2^-	Superoxide
OH^-	Hydroxyl anionic-radical
$ONOO^-$	Peroxynitrite
OGD	Oxygen glucose deprivation
OPA	Polyglutamine tract
PAG608	p53-activated gene 608
PAR	Poly (ADP-ribose)
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PEST	Proline-, glutamic acid-, serine- and threonine-rich
PFA	Paraformaldehyde
PFT	Pifithrin-α
PIG	p53-inducible gene (PIG)
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
PKC	Protein kinase C

PLA2	Phospholipase A2
PPIase	Peptidyl-prolyl isomerases
Pro	Proline rich domain
PRR	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog
PUMA	p53-upregulated modulator of apoptosis
RAGE	Receptor for advanced glycation end products
RAIDD	RIP-associated ICH1/CED3-homologous protein with death domain (RAIDD)
RAM	RBP-J κ-associated module (RAM)
RD	Regulation domain
RIP	Receptor-interacting protein
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SH-SY5Y	Thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH which was established in 1970 from a metastatic bone tumor
SIVA	Zinc-containing intracellular ligand of the CD4 receptor that promotes HIV-1 envelope-induced apoptosis in T-lymphoid cells
SOD	Superoxide dismutase
TD	Tetramerization domain
TAD	Transcriptional transactivation domain

TBST	Tris-buffered saline-Tween
Thr	Threonine
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRP	Transient receptor potential channels
TTC	2,3,5-triphenyltetrazolium chloride
TWEAK	TNF-related weak inducer of apoptosis
UPP	Ubiquitin-Proteasome Pathway
VCAM	Vascular cell adhesion molecule

CHAPTER 1

INTRODUCTION

1.1 Stroke

Before the Common Era, this condition was referred by the medical professionals as Apoplexy which originated with Hippocrates circa 400BC. The term ‘Apoplexy’ was used to refer the symptoms of paralysis and convulsions; an outcome of stroke occurrence. Upon the understanding the causes of stroke, the term ‘apoplexy’ faded way and the term ‘stroke’ was later followed in the medical setting and also described as Central Nervous System Infarction (Nilsen, 2010; Sacco et al., 2013). Stroke is the second leading cause of mortality globally resulting in 6 million deaths each year approximately and a leading cause of long-term paralysis. A 12% increase in mortality is recorded in 2012 since the beginning of the 21st century (Reeve, 2009; Organization, 2014; Walter Johnson, 2016).

Stroke is a condition encompassing an acute decrease in blood flow to the brain tissue affecting the cellular physiology and neurological impairment. Typical characteristics of stroke is a sudden depletion of oxygen and glucose which are the energy driving essentials in cells and maintains the metabolic activity and physiological function of the brain. Stroke is clinically classified into two categories namely, hemorrhagic stroke and ischemic stroke according to the nature of an insult to the cerebral blood vessel network (Ahmad and Graham, 2010; Marti and Kunze, 2016).

1.1.1 Hemorrhagic stroke

Hemorrhagic stroke accounts for 13-15% of the total stroke incidences and results from an impaired vessel rupturing and bleeding into the brain. The accumulated blood compresses the surrounding brain tissue. The condition is either presented as an intracerebral hemorrhage or a subarachnoid hemorrhage. Hemorrhagic stroke caused by impaired blood vessels are presented as the formation of the balloon at weak spots of blood vessels which ruptures to bleed into the brain known as an aneurysm or formation of an abnormal cluster of blood vessels known as arteriovenous malformation (AVM) (Gilgun-Sherki et al., 2002; Duan et al., 2016).

1.1.2 Ischemic stroke

Ischemic stroke contributes for 85-87% of all stroke cases. It is initiated by obstruction due to a blood clot at the arterial circuit supplying the brain tissues. The blood clot deprives the brain tissue from the supply of oxygen and nutrients, causing damage to the brain tissue (Bacigaluppi et al., 2010; Zhang et al., 2017). The obstruction will be instigated either by an embolic or a thrombotic occlusion of the cerebral artery. An embolic stroke occurs when a blood clot that forms elsewhere (embolus) due to cardiovascular condition breaks loose to travel and lodge in the arterial circuit of the brain causing a stroke whereas, a thrombotic stroke is a result of a blood clot (thrombus) developed at the clogged region of an arterial vessel already suffered from plaque deposits. Ischemic stroke is further classified as global ischemia and

focal ischemia (Bottiger et al., 1999). Global ischemia is characterized by severe obstruction of blood flow to either the entire brain organ or an extensive tissue portion of the brain. This condition is commonly observed in occlusion of coronary arteries associated with myocardial Infarction. On the contrary, focal ischemia is displayed with a specific brain region deprived of blood flow depending on the type of cerebral artery occluded either by an embolus or thrombus. The occlusion is observed either transiently or permanently and thus determines the extent of the damage. Focal ischemic stroke is by far, the most prevalent stroke condition reported (Lindblom et al., 2016; Sakai et al., 2016).

There are various risk factors contributing to ischemic stroke and the direct risks include 1) Hypertension: A predicament which has 35-40% of cases at the disposition of suffering ischemic stroke making it the leading cause (Johansson, 1999; Kupferman et al., 2016; Smith et al., 2016), 2) Carotid artery stenosis: A condition in which the carotid arteries supplying blood to the brain suffers fatty plaque deposits in the interior walls, thus hardening and narrowing the blood vessels (Alagoz et al., 2016; Reznik et al., 2017), 3) High cholesterol: Presented with high intake of food with low density lipoprotein; the high levels of cholesterol leads to an increased risk of severe fatty plaque deposits in the arteries (Xing et al., 2016), 4) Diabetes mellitus: High glucose levels is reported as an independent risk factor and when presented with high blood cholesterol, overweight or high blood pressure increases stroke incidence (Yu et al., 2016; Arthur et al., 2017) and 5) Other indirect risk factors include existing cardiovascular disease, obesity and environmental habits such as, physical

inactivity, diet intake involving high saturated fat content, tobacco smoking and alcohol consumption (Jena et al., 2016; Laroche et al., 2016).

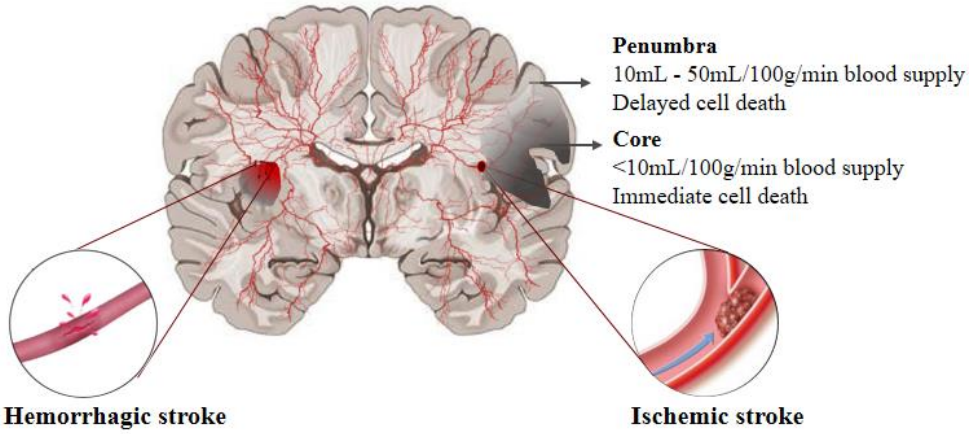


Figure 1: Ischemic and hemorrhagic stroke brain

There are two types of stroke, Hemorrhagic stroke and Ischemic stroke. The left hemisphere of the brain in the image shows a ruptured blood vessel bleeding into the tissues and resulting in a severe infarct. The right hemisphere of the brain in the image shows an ischemic clot occurring in the arteries supplying blood to the tissue. This blockade of blood flow will result in 2 distinct infarct region, the core receiving <10mL/100g/min of blood flow where the cells die immediately; the second region where there is still a collateral blood supply 10mL-50mL/100g/min, this region experience delayed cell death and is the salvageable area. (Adapted : bbc.com images)

1.2 Ischemic stroke pathophysiology

Brain tissue suffered an ischemic stroke damage encompasses an ischemic territory due to the formation of two regions, an ischemic core in the center and a surrounded ischemic penumbra also referred as peri-infarct region due to inadequate blood supply. The ischemic core will be found separated from the healthy tissues by the penumbra layer. The sizable extent of core and penumbra region will depend on the duration and severity of the cerebral blood flow obstruction. Following the blockage, the level of cerebral blood continuing

to perfuse the severed region regulates the advancement of ischemic core and penumbra size (Kumar et al., 2010; Ernst et al., 2015). To carry out a normal physiological function such as glycolysis and oxidative phosphorylation by utilizing oxygen and glucose supplied by an adequate blood flow, the rate of flow must sustain at 50mL/100g/minute. When the rate of flow drops to <10mL/100g/minute, an ischemic core develops making the core region suffer irreversible necrotic cell death and thus rendering the tissue metabolically, electrically and functionally inactive (Bisdas et al., 2004). The penumbra region develops due to the incomplete cessation of blood supply, which is an outcome of the intact collateral blood supply. The hypo-perfusion observed here will have a flow rate between 10mL and 50mL/100g/minute resulting in the tissue electrically and functionally affected yet metabolically active (Astrup et al., 1981; Mehta et al., 2007). Unlike the cellular death observed in the ischemic core, the minimal oxygen and glucose availability drives a slow paced apoptotic cellular death to be manifested in ischemic penumbra making it the target for the therapeutic rescue of the severed tissue (Dharmasaroja et al., 2015; Ginsberg, 2016).

Cerebral ischemia comprises of complex molecular and cellular mechanisms interconnected to trigger an apoptotic or necrotic cell death. The inadequate supply of glucose and oxygen during hypo-perfusion, an initial event in cerebral ischemia induces bio-energetic failure due to loss of ATP production (Vanlangenakker et al., 2008). Loss of ATP production impairs the ATP-dependent proton pumps, voltage-gated ion channels, Na^+/K^+ ATPase pumps rendering the neurons and glial cells susceptible to an imbalance of ionic

gradient. This ionic imbalance results in extensive neuronal and glial membrane depolarization. The loss of ATP production further instigates the production and accumulation of lactate through anaerobic glycolysis (Padosch et al., 2001; Gusev and Skvortsova, 2002). The accumulated lactate leads to the acidic extracellular environment which regulates calcium permeable acid-sensing ion channels (ASICs) providing a path for predominantly Na^+ and also low Ca^{2+} ions permeability. The influx of Ca^{2+} thereby initiates the widespread cascade of events along with anoxic depolarization, excitotoxicity, oxidative stress and inflammation (Lust et al., 2002; Xiang et al., 2004; Xiong et al., 2004).

1.2.1 Neuronal depolarization and Excitotoxicity

At homeostatic neuronal physiology, the synaptic transmission is either electric or chemical mode. The chemical mode of synaptic signaling is through amino acid neurotransmitters. Glutamate is a predominant neurotransmitter known to elicit action potential by activating the glutamate receptors such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-d-aspartic acid) at the post-synaptic terminal of the neuron (Gusev and Skvortsova, 2002; Brassai et al., 2015). These receptors are cationic channels, which allow Na^+ and Ca^{2+} ions influx in the postsynaptic neurons triggering depolarization followed by an action potential. At basal physiology, the glutamate molecules found in the synaptic cleft is regulated through glutamate-glutamine cycle controlled by the astrocytes population. During ischemic insult, glutamate at the synaptic cleft is not converted to glutamine as a consequence of impaired homeostasis in astrocytes. The postsynaptic neurons

become susceptible to glutaminergic overstimulation, extensively activating the glutamate receptors and thereby enormous intra-cellular Ca^{2+} influx occurs. The depolarized neuron further amplifies the uptake of Ca^{2+} ions and release of glutamate (Sattler and Tymianski, 2000; Hinzman et al., 2015). If the ATP supply is not restored, Ca^{2+} ion influx regulated together by the activation of ASICs, glutamate receptors, impaired Na^{+}/Ca^{2+} exchanger and Ca^{2+}-ATPase pump will result in a cascade of nuclear and cytoplasmic events leading to an adversity known as excitotoxicity (Kristian and Siesjo, 1998). In addition, the influx of Na^{+} ions into neurons develops an osmotic movement of water from extracellular matrix through aquaporin, resulting in swelling of the cell and edema in brain tissue (Iijima, 1998; Jarvis et al., 2001; Anderson et al., 2005).

1.2.2 Oxidative stress

Disrupted calcium homeostasis initiates to multiple mechanisms to damage the neurons and glial cells. Ca^{2+} ions are a powerful activator of Mitochondria-mediated oxidative stress. Mitochondria are the mastermind of metabolism and mitochondrial glycolysis. The mitochondrial inner membrane is the platform of oxidative phosphorylation through electron transport chain (ETC) with the use of ATP synthase (Sims and Muyderman, 2010; Chehaibi et al., 2016). During cerebral ischemia, the excess Ca^{2+} ion influx is up taken by the calcium uniporter, MCU/MCUR1 of the mitochondrial matrix. The abnormal mitochondrial accumulation of Ca^{2+} ions will form the calcium precipitates affecting the transmembrane potential and perturbing the electron transport chain (Kristian and Siesjo, 1998; Ying and Padanilam, 2016).

Perturbed electron transport chain results in a premature electron leakage to oxygen to produce superoxide (O_2^-), an anionic free radical (Sims and Muyderman, 2010; Murphy, 2016; Ying and Padanilam, 2016). Numerous Ca^{2+} mediated path contribute to the production of superoxide (O_2^-). Ca^{2+} mediated protein Kinase C (PKC) activation generates O_2^-, through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Perez-Pinzon et al., 2005). Production of prostaglandins and leukotrienes through Ca^{2+} induced Phospholipase A2 (PLA2), activation generates O_2^- as well. O_2^- along with other reactive oxygen species (ROS) such as hydroxyl anionic-radical (OH^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) induces oxidative stress (Kristian and Siesjo, 1998; Ste-Marie et al., 2000; Lushchak, 2014; Pisoschi and Pop, 2015). Oxidative stress is a predominant cause of tissue damage by targeting the essential cellular organelles. Cellular damage is elicited by the generated ROS stealing electrons and resulting in oxidation of the cellular components nucleic acids, proteins and lipids (Zitnanova et al., 2016). Consequently, a multitude of pathway cascade including nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs) and c-Jun-N-terminal kinase (JNK) known to modulate caspase-mediated apoptosis are activated (Manzanero et al., 2013). ROS damages the rich calcium storage site, Endoplasmic reticulum mediating excessive calcium release in response to an amplified initial calcium insult a process termed calcium-induced-calcium-released (CICR). In turn, the cumulative calcium insult perturbs mitochondria facilitating the release of apoptosis-mediating factors (Kristian and Siesjo, 1998; Love, 1999).

In addition to triggering reactive oxygen species, calcium influx also activates the catabolic enzymes, endonuclease, and calpain. Endonuclease is a nuclear protease cleaving DNA upon activation generating DNA breaks, which can trigger an apoptotic cascade (Hayashi et al., 1999; Cui et al., 2000; Saki and Prakash, 2016). Calpains are calcium activated non-lysosomal neutral proteases, located in cytoplasm performing hydrolysis of proteins to an inactive state. These calpains-inactivated functional proteins include cytoskeletal proteins such as actin, tubulin, and spectrin; membrane proteins including glutamate receptors and ryanodine receptors; anti-apoptotic B-cell lymphoma 2 (Bcl-2) family of proteins such as B-cell lymphoma-extra large (Bcl-XL) and Bcl-2 (B-cell lymphoma 2); pro-apoptotic Bcl-2 family of protein apoptosis-inducing-factor (AIF). Unlike other functional proteins, hydrolysis of AIF renders it active in its truncated form. Consecutively, calpains can trigger the cathepsin-mediated proteolysis of cellular proteins and together with calpains mediated proteolysis contributes to cellular demise through necrosis (Vanlangenakker et al., 2008; Potz et al., 2016; Quillinan et al., 2016).

1.2.3 Inflammation

Inflammation is an innate immune reaction to damage and infection known to revive the host from cellular insult. However, inflammation is a characteristic event in the ischemic reperfusion injury as the inflammatory pathway in ischemic brain damage contributes to the exacerbation of the injury. As a result of the ischemic damage, dissociated cellular debris from the necrotic cell death of ischemic core region functions as ligands known as damage

associated molecular patterns (DAMPs) which can trigger danger signal by activating cellular pattern recognition receptors (PRRs). Such PRRs include cell surface receptors including toll-like receptors (TLRs) TLR2 and TLR4; receptor for advanced glycation end products (RAGE); c-type lectin Mincle receptor; IL-1 receptor1 (IL-1R1). Together these receptors will elicit an inflammatory signaling pathway upon specific DAMP-interaction in neurons, microglia, astrocytes and endothelial cells (Ishikawa et al., 2005; Kigerl et al., 2014; Li et al., 2014; Okun et al., 2014).

These activated cells secrete the resultant inflammatory functional proteins known as pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α); interleukins (IL)-1β, IL-16 and IL-18. The predominant signaling pathways, mitogen-activated protein kinase (MAPK) and nuclear factor kappa beta (NF-κB) mediated intracellular multi protein complexes formation termed inflammasomes, promotes the pro-inflammatory cytokines release (Allan and Rothwell, 2001; Fann et al., 2013[a;] Arumugam et al., 2016). The released pro-inflammatory cytokines promote immune cells infiltration by inducing various cell adhesion proteins in endothelial cells, platelets and leukocytes. The expression of cell adhesion proteins including, intercellular adhesion molecule-1 (ICAM-1), selectins, integrins and vascular cell adhesion molecule (VCAM) are regulated by the pro-inflammatory cytokines which have a vital role in facilitating immune cells infiltration including monocytes/macrophages and neutrophils at the site of ischemic territory during reperfusion (Arumugam et al., 2004[a;] Yilmaz and Granger, 2008).

Consecutively, in addition to pro-inflammatory cytokines released by neurons and glial cells, monocyte chemo-attractant protein-1 (MCP-1) is secreted initiating the leukocytes migration to the ischemic territory, a process called chemotaxis. The infiltrated immune cells additionally contributes to the ischemic reperfusion injury by releasing various cytotoxic components such as, nitric oxide (NO) from inducible nitric oxide synthase (iNOS), NADPH oxidase-derived ROS, pro-inflammatory cytokines, matrix metalloproteinases (MMPs) which particularly affect the blood brain barrier (BBB) and extracellular matrix exacerbating brain damage and edema promoting more neuronal and glial cell demise in addition to the cell death caused by initial ischemic event (Dimitrijevic et al., 2006; Yang et al., 2007; Liu and Mu, 2014). Further, leukocyte recruitment is mediated by the complement system, an inevitable event involved in ischemic reperfusion injury. A characteristic event is the formation of membrane attack complex (MAC) in neurons and glial cells, mediated by anaphylatoxins, including C1, C3a and C5a. This complement cascade causes cell lysis and results in damage to tissue as well (Arumugam et al., 2004[b;] Pavlovski et al., 2012; Alawieh et al., 2015).

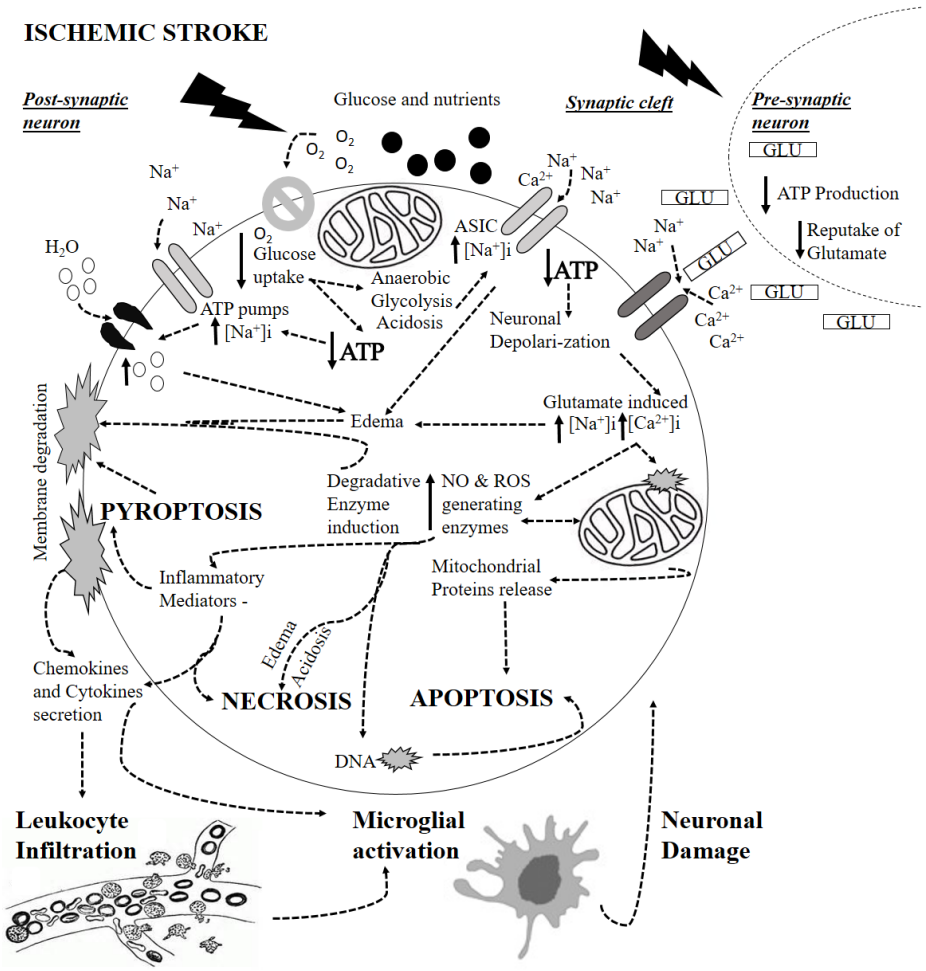


Figure 2: Cellular events of ischemic stroke pathophysiology

Ischemic stroke initiates major mechanisms affecting the neurons. The initial depletion of blood flow restricts the availability of glucose and oxygen leading to decreased ATP production through glycolysis and oxidative phosphorylation. Alternatively, anaerobic glycolysis starts accumulating lactic acid resulting in acidotoxicity mediated Na^+ influx through ASICs and ATP-Na^+ pumps resulting in edema due to osmotic movement of water which leads to necrosis. The failing of ATP-pumps causes anoxic depolarisation and the voltage-gated Ca^{2+} channels allow the influx of ions inducing uncontrolled glutamate release. The glutamate transporters are impaired leaving the accumulated glutamate at synaptic cleft to continue to sensitize the Ca^{2+} channels resulting in excitotoxicity. Up-regulated Ca^{2+} ions mediate mitochondrial proteins release to execute apoptosis and in parallel triggers degradative enzymes and production of ROS which degrades key cellular proteins such as, membrane proteins, cytoskeletal proteins; lipids and nucleic acids contributing to necrosis and inflammatory release. The released inflammatory mediators recruit immune cells and activate glial cells at the site of entry and as a positive feedback cycle, ROS and cytokines released by glial cells damage the other neighboring neurons. (Adapted : Dirnagl et al., 1999)

1.3 Cellular death mechanisms

The cellular events of pathophysiology including neuronal depolarization mediated excitotoxicity, oxidative stress and inflammation together trigger the different mechanism of cell death. Programmed cell death where the cellular bodies are highly maneuvered for an organized disposal and a non-programmed cell death culminated by the release of cellular content into the extracellular matrix without organized packaging. The cell death mechanism is further categorized as a caspases-dependent mechanism and caspases-independent mechanism. Caspases are protease enzymes cysteine-dependent aspartate-directed playing a vital role in cleaving cellular proteins at c-terminal after aspartate residue during the programmed cell death pathway. Study of embryonic development in *Caenorhabditis elegans* revealed the amino acid sequence of CED-3 sharing the functional similarity with a mammalian version of protease known for mature IL-1β conversion to its active form and was named 'caspase-1' leading to the subsequent discovery of a family of these proteases. Caspases exist in the cytosol as inactive pro-forms and are activated by other caspases by proteolytic cleavage. Caspases are broadly categorized either as apoptotic caspases including Caspase-2, -3, -6, -7, -8, -9 and -10 or inflammatory caspases such as, Caspase-1, -4, -5, -13 and -14; murine homolog -11 for -4 and -5; murine homolog -12 for -13 and -14 (Nicotera, 2002; Fink and Cookson, 2005; Crowley et al., 2016).

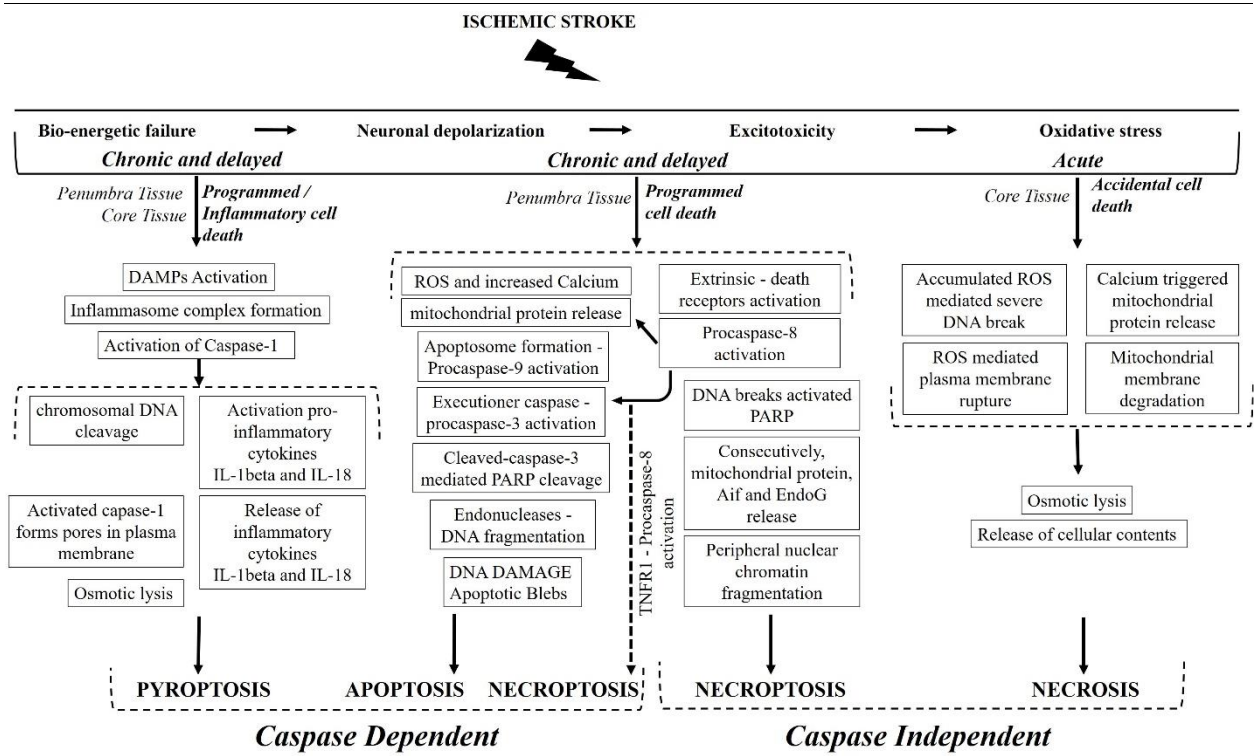


Figure 3: Distinct types of cellular death in ischemic injury

Ischemic stroke cellular events mediate three distinct types of cell death depending on the severity of the injury and the proximity of the tissue to the artery obstructed in blood flow. The cellular events including bio-energetic failure, excitotoxicity and oxidative stress when acute, core tissue is formed characterized by necrotic cell death and partially pyroptosis mediating inflammatory cascade. Chronic effects of these cellular mechanisms leads to penumbra characterized predominantly with apoptosis and later by pyroptosis during reperfusion.

1.3.1 Necrosis

Necrosis is a non-programmed, accidental cell death resulting from environmental perturbation, which is morphologically and mechanistically differentiated from the highly programmed apoptotic pathway by its characterized presence of dead tissues or cells unlike the bulging of apoptotic vesicles. Necrosis is also termed oncosis derived from ‘oncos’ meaning swelling. Necrotic cell death is characterized by acute calcium influx elicited excitotoxicity, the release of excitotoxins, stored cellular energy depletion, oxidative stress mediated chromosomal DNA breaks and disintegration of membrane pumps (Nicotera and Lipton, 1999; Snider et al., 1999; Lossi et al., 2015). Necrosis involves process demanding uncontrolled cellular ATM consumption resulting in energy depletion in response to severe DNA energy. DNA strand breaks activate nuclear enzyme such as, Poly (ADP-ribose) polymerase (PARP) which during a moderate DNA damage facilitates DNA repair through the addition of poly (ADP-ribose) to a variety of nuclear proteins. During ischemic stroke, chromosomal DNA experiences acute insult, nicotinamide adenine dinucleotide (NAD) coenzyme dependent PARP catalysis depletes the coenzyme reserve promoting ATP-dependent excess NAD synthesis (Murphy, 1999; Pieper et al., 1999; Walisser and Thies, 1999; Cho, 2014). In addition, necrotic cell death also include cyclophilin-D (Cyp-D) forming mitochondrial permeability transition pore (MPTP) to mediate necrosis, calpains-mediated Cathepsins C and D triggered lysosome-destabilizing agent L-leucyl-L-leucine methyl ester (LLOMe) drives proteolytic cascade in lysosomal-mediated necrosis (LMN) (Galluzzi et al., 2014; Brojatsch

et al., 2015; Ying and Padanilam, 2016). Tumor necrosis factor-α (TNF-α) along with its cognate receptor TNFR1 mediates RIP1 kinase mechanism of cell death which is similar to the cell death lead mitochondrial protein, AIF effected chromatinolysis. Cumulatively, TNF-α mediated and AIF regulated unique cell death pattern is recently defined 'necroptosis' describing the coalescence of apoptosis and necrosis or rather an orderly regulation of necrosis (Majno and Joris, 1995; Murphy, 1999; Zaremba, 2000; Hallenbeck, 2002; Lossi et al., 2015; Hanson, 2016).

1.3.2 Pyroptosis

Pyroptosis is a morphologically distinct form of programmed and a highly inflammatory mode of cell death, a response that occurs due to pathogen associated molecular patterns (PAMPs) and DAMPs interacting PRRs signaling mechanism. The term 'pyroptosis' is of greek origin 'pyro' and 'ptosis' meaning fire and falling respectively denoting its characteristic feature in releasing inflammatory mediator and triggering a chain of the inflammatory process in the neighboring cellular population (Fink and Cookson, 2005). In an ischemic stroke, this process is characterized by inflammation triggered through DAMPs from tissue injury resulting in a release of inflammatory mediators in the extracellular region. Pyroptosis is predominantly a caspase-1 dependent mechanism which has been described in neurons, monocytes, macrophages and dendritic cells. Caspase1 activation involves the formation of inflammasome complex of proteins including NLRP-1, NLRP-3, NLRC4 and AIM-2 as well as ASC leading ultimately to the functional outcome of the pyroptotic pathway

involving the release of pro-inflammatory cytokines such as IL-1beta and IL-18. Initial activation of Caspase-1 leads to the removal of CARD domain by cleavage at the specific site resulting in the p10 and p20 catalytic domain fragments separation. (Fink and Cookson, 2005; Miao et al., 2011; Chang et al., 2013; Fann et al., 2013[a;] Fann et al., 2013[b;] Adamczak et al., 2014; Sangiuliano et al., 2014).

Pyroptosis is characterized by the release of pro-inflammatory mediators in the extracellular environment due to pores formed on the plasma membrane and rapid rupture mediated by cleaved capase-1 through an unknown mechanism. The pores are of diameter 1.1–2.4 nm which eventually disintegrates the membrane ionic gradient pumps, allowing an excessive influx of cationic ions facilitating the osmotic movement of water through aquaporins resulting in swelling of the cell. The swelling results in large spherical protrusions in the membrane making it detach from the cytoskeleton structure (Fink and Cookson, 2006). Cleaved caspase-1 mediated pyroptosis is also characterized by chromosomal DNA breaks cleaved by caspase-1 dependent endonucleases (Walsh et al., 2011). Cleaved caspase-1 has been described to inactivate enzymes including fructose-bisphosphate aldolase, α-enolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase of the glycolysis pathway thus leading to bioenergetic failure (Barrington et al., 2016; Yuan et al., 2016). Pyroptosis executes the cell death by osmotic lysis in the acute scenario, chromosomal DNA cleavage by a chronic or delayed stress which will consecutively lead to an apoptotic cell death, another form of programmed caspase-dependent cell death. Moreover, cleaved caspase-1 was

found to activate both executioner caspase-3 and caspase-7, and Bid by cleaving to its truncated form (Bergsbaken et al., 2009; Fann et al., 2013[a;] Shi et al., 2016).

In addition to the caspase-1 promoted canonical pyroptotic pathway, caspase-11 is involved in the non-canonical pathway of pyroptosis although the mechanism behind remains to be well-understood. Caspase-11 is the murine homolog for human caspase-4 and caspase-5 and its cleaved form has been described to activate executioner caspase-3 and caspase-7 to induce apoptotic cellular death. Hence, these events together cause lysis of the affected neuronal cell in ischemic stroke either through the canonical caspase-1 pathway or through caspase-11 (Bergsbaken et al., 2009; Vigano and Mortellaro, 2013; Shi et al., 2016).

1.3.3 Apoptosis

Apoptosis is an instructive energy-dependent mechanism, genetically embedded and involves execution of a cascade containing a series of components and when activated elicits exclusion of cells through fragmented membrane-bound cellular bodies otherwise termed ‘apoptotic blebs’. The term ‘apoptosis’ is of Greek origin denoting ‘falling off’ which connotes the morphological characteristic feature observed during atrophy of tissue and organ development paradigm to dispose of redundant cells with the purpose to promote healthy cell turnover; hence, perceptibly a deregulation in apoptosis initiation will be associated with deformities and abnormalities. The cellular

morphology associated with this phenomenon involves dismantling of cell organelle by nuclear and cytoplasmic condensation from within the cell packaged into apoptotic bodies in an organized way and unlike other modes of cell death, minimizes damage and disruption to neighboring cells. An early process of apoptosis is cell shrinkage characterized with condensed cytoplasm and tightly packed organelles and pyknosis as a result of chromatin condensation, the landmark feature in apoptosis (Kerr et al., 1972; Hacker, 2000). In the later steps, packaged apoptotic bodies are phagocytized by neighboring cells through endocytosis mechanism following distinct steps involving, invagination of the plasma membrane, engulfment of phagocyte vesicle, internalization and fusion of the phagocyte vesicle with lysosomes forming phagosomes inside which the apoptotic bodies are degraded. Besides maintaining a healthy homeostasis, apoptosis is an inevitable process during immune reactions, noxious agents or tissue injury and cellular impairment situation (Hiramine, 1997; Kerr, 2002). During ischemic stroke, penumbra region undergoes apoptotic death few hours and days after the blood flow obstruction, unlike the core region, which is predominantly featured with acute necrotic death. Hence, penumbra befits the salvageable region and can be a therapeutic option for rescue. The situation leading to apoptotic death remarkably explains the cells inability to meet the demand of nutrients for the cellular machinery of metabolism and likely tipped the cellular balance towards death (Porn-Ares et al., 1998; Charriaut-Marlangue, 1999). The important family of proteases executing this phenomenon is caspases existing in an inactive ‘pro’ state. As mentioned earlier, the apoptotic caspases are Caspase-2, -3, -6, -7, -8, -9 and -10 which could be further classified as initiator caspases

(caspase-2, -8, -9 and -10) having long domains dimerize through the death effector domain or caspase recruitment domain motifs; executioner caspases (caspase-3, -6 and -7) containing relatively shorter domains which executes the cell death process by cleaving the cellular substrates. Apoptosis regulators although not successful has emerged as the targets for therapeutic methods to modulate the cellular death decision (Hengartner and Horvitz, 1994; Kumar, 1995; Whyte and Evan, 1995)

Apoptosis is subdivided into 2 pathways including, intrinsic mechanism carried out through calcium and ROS generated from mitochondrial oxidative stress; and extrinsic mechanism executed by ligand-receptor mediated both directly and through mitochondrial downstream signaling respectively (Mattson et al., 2000; Charriaut-Marlangue, 2004; Fischer and Schulze-Osthoff, 2005).

1.3.3a Intrinsic pathway of apoptosis

ROS generation and effect on mitochondrial activity

ROS contributes majorly for tissue injury after cerebral ischemia. As explained earlier, the reactive oxygen species are O_2^-, H_2O_2, OH^-. The production of the ROS is controlled by xanthine oxidase, mitochondrial electron transport chain, arachidonic acid, NADPH oxidase, endogenous antioxidants such as superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD), glutathione peroxidase, glutathione and catalase. Perturbed electron transport chain is a major event giving rise to ROS. Events like excitotoxicity (glutamate), and Ca^{2+} overload after cerebral ischemia and during reperfusion are

the triggering mechanism to develop ROS accumulation (Sugawara and Chan, 2003; Perez-Pinzon et al., 2005; Loh et al., 2006; Miller et al., 2006; Sims and Muyderman, 2010; Murphy, 2016; Ying and Padanilam, 2016). O_2^-, OH^-, H2O2 and highly toxic molecule, peroxynitrite ($ONOO^-$) generated from interaction of O_2^- with NO causes tissue damage during cerebral ischemia, either destroying cellular proteins, lipids and DNA by direct contact or indirectly disrupting the normal cellular signaling and gene regulation. Collectively, ROS can trigger either the mitochondrial caspase-dependent or mitochondrial and non-mitochondrial caspase-independent cascade of apoptosis characterized by altered Bcl-2 family proteins (Kristian and Siesjo, 1998; Morita-Fujimura et al., 2001; Yagami et al., 2002; Love, 2003; Lushchak, 2014; Pisoschi and Pop, 2015).

Calcium entry

Ca^{2+} entry regulated intrinsic mechanism includes the events which are described earlier, such as excitotoxicity and oxidative stress. As mentioned before, the onset of ischemic stroke limits the delivery of oxygen and glucose due to blood supply obstruction impairing the bioenergetics required to maintain homeostasis. The rapid imbalance of bioenergetics can lead to membrane potential loss and depolarization of the cells causing excitotoxicity. These mechanisms are carried out by the presence of voltage–gated channels present at the cell surface (Dirnagl et al., 1999; Mergenthaler et al., 2004; Jeanneret et al., 2016; Radak et al., 2016). Various voltage-gated channels are activated upon the binding of glutamate amino acid, released by the pre-synaptic cells into the

synaptic cleft of the extracellular space. The most-widely studied channels are the receptors opened by accumulated glutamate to operate the cationic inflow into the post-synaptic junction neuron. Ionotropic receptors like NMDA and AMPA gate the entry of Ca^{2+} ions under glutamate influence increasing the intracellular Ca^{2+} concentration. In addition, acid-sensing ion channels (ASICs) and transient receptor potential channels (TRP) triggered by acidosis can act as a major contributor of neuronal apoptosis through mediating Na^{+} ions permeability and to a lesser degree of Ca^{2+} ions. The two subunits of ASICs, ASIC1a and ASIC2a has a contributory role in cerebral ischemia. The cationic channels mediated Na^{+} and Ca^{2+} ions influx in the postsynaptic neurons triggers depolarization and together with NMDA and AMPA elicits an action potential. The depolarized neurons further amplify the uptake of Ca^{2+} ions and release of glutamate resulting in a cascade of nuclear and cytoplasmic events leading to an excitotoxic adversity. (Kristian and Siesjo, 1998; Verkhratsky and Petersen, 1998; Horn et al., 2001; Liu et al., 2007; Simard et al., 2007; Miller et al., 2014).

Effects of increased calcium on Mitochondrial activity

As an organelle responsible for maintaining the Ca^{2+} homeostasis mitochondria functions as a buffering and a storage system of the intracellular calcium and in regulating physiological stimulation upon Ca^{2+} signals. Mitochondrial sensing of the increased cytoplasmic Ca^{2+} takes place by the negative membrane potential developed across the inner mitochondrial membrane. In addition to Ca^{2+} clearance, the inner membrane also participates in the Ca^{2+} ions elevation when required to regulate Ca^{2+} mediated function in

the cytoplasm. The mitochondrial proteins involved in the regulation of the calcium uptake include a complex of proteins forming channels called the uniporter along with the assistance of voltage present across the inner membrane due to the protons generated by the respiratory chain. Excessive cytoplasmic Ca^{2+} is up-taken by the ion-conducting pore forming calcium uniporter, mitochondrial calcium uniporter (MCU) in the presence of mitochondrial calcium uniporter regulator 1 (MCUR1) interaction at the inner mitochondrial matrix. Consequently, mitochondrial protein MICU1 is identified as an integral part of MCU/MCUR1 complex regulating the mitochondrial Ca^{2+} threshold for intracellular Ca^{2+} uptake; functioning as a gatekeeper ensuring the MCU-mediated Ca^{2+} uptake kinetic to be intact; and prevents mitochondrial Ca^{2+} overload associated stress. The imbalance in the mitochondrial kinetics is associated with the release of ROS (Verkhratsky and Petersen, 1998; Mallilankaraman et al., 2012b; Mallilankaraman et al., 2012a; Manzanero et al., 2013; Kaufman and Malhotra, 2014).

In cerebral ischemia, several events occur in mitochondria unfavorable for the survival of neurons and centrally regulates the neuronal apoptosis through release of various pro-apoptotic proteins. The glutamate receptors sensitization results in a large volume of Ca^{2+} influx triggering endoplasmic mediated Ca^{2+}-induced Ca^{2+} release (CICR). Following an increase in Ca^{2+} levels activation of Ca^{2+} dependent protease, calpains occur which hydrolyze specific proteins including cytoskeletal, Membrane, and several Bcl-2 family of proteins located within cytoplasm and mitochondria. Calpains inactivate anti-apoptotic mechanism by truncating proteins including Bcl-2 and Bcl-XL and on

the contrary, Calpains activate pro-apoptotic mechanism by truncating Bid and AIF. Bid, predominantly prevailing in the cytoplasm when activated to tBid translocates to mitochondria targeting the outer membrane and simultaneously induces a conformational change in proteins such as Bak, Bax, Bad, and Bcl-XS activating its proapoptotic property. AIF, originally present in the inner mitochondrial membrane upon truncation to tAIF can translocate to nucleus along with endonucleaseG (EndoG) to potentiate chromatinolysis (Folbergrova et al., 1992; Verkhratsky and Petersen, 1998; Plesnila et al., 2001; Plesnila et al., 2002; Sugawara et al., 2004; Culmsee et al., 2005; Manzanero et al., 2013; Curcio et al., 2016; Potz et al., 2016).

Bad attains its activated state through dephosphorylation and translocates to mitochondria from the cytosol forming dimers with Bcl-XL, an anti-apoptotic protein in regard to inhibiting its anti-apoptotic role. The inhibition of Bcl-XL and Bcl-2 is also carried out by Puma, a cytosolic pro-apoptotic protein. Subsequently, pro-apoptotic Bax which is inhibited by anti-apoptotic proteins Bcl-XL and Bcl-2 gets released following Bad and Puma interaction with both the inhibitory proteins. Following release from inhibition, Bax translocates to the outer mitochondrial matrix in association with Bak can form pores facilitating the release of cytochrome-C (Cty-C) from the mitochondrial intermembrane space (Fujimura et al., 1999; Antonsson et al., 2000; Kirkland et al., 2002; Saito et al., 2003; Webster et al., 2006).

Caspase cascade dependent apoptosis

Caspase cascade is regulated by the release of pro-apoptotic proteins from the intermembrane space of the mitochondria through the transition pores. Cyt-C, Smac/DIABLO and Omi/HtrA2 are the prominent pro-apoptotic agents released to activate the caspase cascade. The released Cyt-C in the cytoplasm is bound to form the apoptosome complex by clustering with procaspase-9 and apoptotic protease activating factor 1 (Apaf-1). The formation of apoptosome is the triggering event of procaspase-9 activation by cleavage mechanism making caspase-9 the initiator of Cyt-C-dependent caspase signaling. Meanwhile, the enhanced apoptosome formation is facilitated by the mitochondrial release of Smac/DIABLO and Omi/HtrA2 which binds to the inhibitors of apoptosis (IAPs) (Jayanthi et al., 2004). Enhanced apoptosome formation is the characteristic apoptotic feature shortly after the onset of ischemic stroke and a limiting step for procaspase-3 activation. Various neuroprotective compounds have been studies targeting this complex of proteins to rescue the cells experiencing ischemic stress. The activated procaspase-3 in a truncated form known as an executioner caspase, cleaved-caspase-3 and so far identified as a key mediator of apoptosis. An up-regulated activation of caspase-3 is observed shortly after the initiation of stroke extending during reperfusion duration as well (Asahi et al., 1997; Namura et al., 1998; Fujimura et al., 1999; Rami et al., 2003; Cao et al., 2004).

Major cellular events which take place by the executioner, caspase-3 are cleavage of substrate proteins. PARP, a nuclear enzyme activated by DNA

strand breaks during damage facilitates DNA repair in an ATP-dependent process. Caspase-3 mediated inactivation of PARP by cleavage preserves cellular ATP for apoptosis requirement. Absence of PARP leads to unrepairable DNA damage promoting ATP-dependent programmed compartmentalization of the cellular organelles into apoptotic blebs (Endres et al., 1997; Murphy, 1999; Pieper et al., 1999; Walisser and Thies, 1999; Le et al., 2002; Baxter et al., 2014; Cho, 2014).

Caspase cascade independent apoptosis

Programmed cell death independent of caspase is initiated by a collective group of proapoptotic proteins released from the mitochondrial transition pores, into the cytoplasm. These proteins include and Bcl-2/adenovirus E1B 19 kDa-interacting protein (BNIP3), apoptosis inducing factor (AIF) and endonuclease G. AIF-induced cell death is independent of functional caspases serving as an alternative death pathway. Depletion of cellular energy which is an unfavorable event for caspase activation can still stimulate AIF-mediated cell death (Cregan et al., 2002). When AIF is truncated by calpains and released from the mitochondria to translocate in the nucleus causing DNA fragmentation and peripheral nuclear chromatin condensation, which is distinct from caspase-dependent chromatin condensation and DNA fragmentation. AIF release from mitochondria is mediated by the presence of PAR polymers generated by PARP-1 and tBID. Activated AIF inhibits protein translation by its association with the translation inhibition factor, eIF3g and its translocation to the nucleus is mediated by cytoplasmic peptidyl-prolyl

isomerase enzyme, cyclophilinA. Recently, this mechanism is termed, programmed necrosis or necroptosis, but whereas it's widely recognized as caspase-independent apoptosis (Daugas et al., 2000; Cregan et al., 2004; Culmsee et al., 2005; Cho and Toledo-Pereyra, 2008; Baxter et al., 2014).

Endonuclease G nuclear translocation occurs alongside AIF translocation and is also associated with fragmenting DNA during cell death after ischemic stroke. A proapoptotic member of the Bcl-2 family, BNIP3 contributes to dysfunction of mitochondria and hence mediates apoptosis independent of functional caspases and AIF pathway in neurons following stroke. These proteins form the non-canonical pathway different from Cty-C mediated caspase-dependent intrinsic apoptotic pathway (Lee et al., 2005; Zhang et al., 2007; Delavallee et al., 2011).

1.3.3b Extrinsic pathway of apoptosis

Extrinsic pathway as the name suggests is instigated from the signals originated outside the cell. This pathway includes the engagement of the death receptors present in the plasma membrane, and thus also referred to as the death receptor pathway. The best-characterized death receptors such as, Fas receptor (FasR) also termed as cluster of differentiation 95 (CD95) and TNFR-1 also referred as p55 or CD120a possesses an important characteristic cytoplasmic domain of 80 amino acids long termed 'death domain' and extracellular cysteine-rich domain. The death receptors sharing these features are grouped

together as a family of tumor necrosis factor receptors (TNFR). Besides Fas and TNFR-1, the other death receptors of the TNFR family includes, death receptor 3 (DR3), DR4, DR5 and p75 neurotrophin receptor (p75NTR) also known as nerve growth factor receptor (NGFR) (Kenchappa et al., 2010). Characterized ligands activating the death receptors belong to the TNF gene superfamily and share structurally similar molecules in a homo trimerizing pattern with its respective receptors such as, Fas ligand (FasL) activates Fas receptor, tumor necrosis factor-α (TNF-α)activates TNFR-1, Apo3 ligand (Apo3L) or TWEAK binds DR3, Apo2 ligand (Apo2L) or TRAIL activates DR4 and DR5. The receptors when stimulated are conjugated with cytosolic adapter proteins, including Fas-associated death domain (FADD), TNFR-associated death domain (TRADD), TNFR-associated factor (TRAF), receptor interacting protein (RIP) and RIP-associated ICH1/CED3-homologous protein with death domain (RAIDD) or CASP2 and RIPK1 domain containing adaptor with death domain (CRADD) (Ashkenazi and Dixit, 1998; Sugawara et al., 2004; Fink and Cookson, 2005; Underwood and Coulson, 2008; Broughton et al., 2009; Kraemer et al., 2014).

Mechanism of death receptors

The mechanism involved in the signaling of Fas and TNFR-1 receptor follow a similar pattern. In Fas-mediated apoptosis mechanism, the ligand binding to the receptor marks the initiation of the pathway, triggering a cascade in the cytoplasm by recruiting adapter protein, Fas-associated death domain protein (FADD or called Mort-1). Death effector domain is a classified region

of homophilic interaction also termed caspase recruitment domain (CARD). Located at the N-terminal of an adapter protein, CARD is the site of initiator caspase, procaspase-8 interaction. Together the complex of FasL (Ligand)-Fas (death receptor)-FADD **(**adapter protein**)**-procaspase-8 (initiator caspase) is a death-inducing signaling complex (DISC) and a the state at which procaspase-8 drives its activation through self-cleavage resulting in cleaved-caspase-8 (Boldin et al., 1995; Chinnaiyan et al., 1995; Chinnaiyan et al., 1996; Hofmann et al., 1997; Vincenz and Dixit, 1997; Muzio et al., 1998).

Procaspase-8 activation mediates cleavage of Bid to genetate truncated form of Bid (tBid). tBid integrates with different death pathways at the mitochondrial apoptosis checkpoint or activates caspase-3 directly by cleaving into its cleaved form. At the mitochondrial membrane tBid interacts with pro-apoptotic, Bax to dimerize and facilitate the release of Cyt-C, which execute conversion of procaspase-9 to its active state, caspase-9 in combination with apaf-1. This complex of proteins triggers procaspase-3 dependent PARP inactivation and cell death. Alternatively, cleaved-caspase-8 can directly cleave procaspase-3 surpassing the mitochondrial route (Vier et al., 1999; Plesnila et al., 2001) (Earnshaw, 1995; Le et al., 2002).

Consequently, TNFR-1 mechanism induces apoptosis following ligation with the tumor necrosis factor (TNF) ligand. Well established pathways by the TNF engaging TNFR1 includes pro-inflammatory signaling mediated by NF-κB and AP-1 (Roulston et al., 1998). The trimerization of the ligand with the receptor induces association of the death domains with the adapter protein

TRADD, a platform adaptor enabling the attachment of several molecules forming a complex with the death domains of the receptor. The sequential molecules include TRAF-2 and RIP triggering the serine-threonine kinase enzymatic activity based NF-κB and AP-1 pathway; and FADD recruitment to the TNFR death domain complex triggers the apoptosis pathway similar to the Fas-mediated apoptosis. TNFR1 is also a site of recruitment for another adapter protein called RAIDD/CRADD which binds to the death domain of RIP. These adapter proteins in turn interact with the CARD motif of death effector caspase-2, thereby inducing apoptosis. (Smith et al., 1994; Gruss and Dower, 1995; Hsu et al., 1995; Rothe et al., 1995; Chinnaiyan et al., 1996; Ting et al., 1996; Ahmad et al., 1997; Duan and Dixit, 1997). DR3 is a receptor mimicking TNFR1 similar mechanism, in response to the Apo3 ligand. DR3 initiates the same order of TNFR death domain recruitment of proteins including, TRADD-RIP/TRAF2 and TRADD-FADD mediated apoptosis. DR4 and DR5 receptors are activated by Apo2L or TRAIL resembling the FasL mediating adapter recruitment for apoptosis. Although the p75NTR mechanism is not well studied, the self-association of its receptor death domain is one of the cell death mechanism initiating step (Qu et al., 2013).

Death receptors in cerebral ischemia

In stroke, Fas, TNFR-1, p75NTR, DR5 play role in apoptosis in neurons activating the downstream executioner caspase, procaspase-3. The expression of DR5 death receptors is observed within 12 hours after cerebral ischemic formation and is observed to have a sustained activation until 48 hours

contributing to the apoptotic death in neuronal cells. A knockout can show a 93% reduction in the infarct size rescuing the penumbral tissue from demise (Rubin et al., 1994).

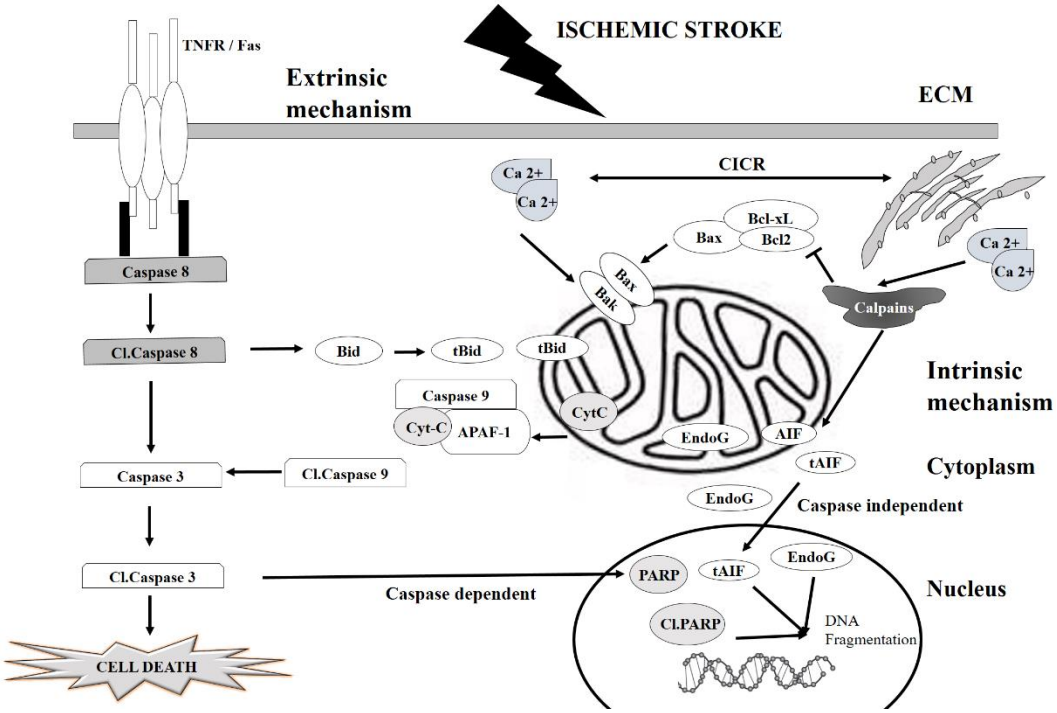


Figure 4: Extrinsic and Intrinsic apoptotic pathway

Ischemic stroke elicits an acute Ca^{2+} increase affecting endoplasmic reticulum, which releases Ca^{2+} through a calcium-induced calcium release (CICR) mechanism. Accumulated Ca^{2+} triggers the pro-apoptotic proteins, Bax and Bak and permeabilizes mitochondria and mediates release of Cyt-C to form the apoptosome complex with Apaf-1 and procaspase-9. This complex activates an initiator caspase of intrinsic pathway, procaspase-9 to its cleaved form which in turn along with cleaved caspase-8 from the extrinsic pathway cleaves the procaspase-3, the executioner caspase to mediate DNA fragmentation. The other mitochondrial protein mediating DNA fragmentation is EndoG and AIF in its truncated form, tAIF. The truncation of AIF and its release is mediated by Ca^{2+} activated calpains. The mitochondrial permeabilization is also mediated by the extrinsic pathway through death receptors mediated activation of Bid to its truncated form (t-Bid). Bid activation is regulated by the activated procaspase8, an initiator caspase of the extrinsic pathway. Together extrinsic and the intrinsic pathway cleave functional proteins and DNA to be assembled into apoptotic blebs. (Adapted : Molkentin Lab-Howard Hughes Medical Institute images)

In sympathetic neurons, P75NTR has an NGF ligand-dependent and oxidative stress-mediated mechanism also involving 4-hydroxynonenal (HNE), a lipid peroxidation product generated naturally during oxidative stress. Proteolysis of p75 (NTR) by the metalloprotease TNFα-converting enzyme and γ-secretase is found necessary for p75(NTR)-mediated apoptotic signaling (Martin-Villalba et al., 1999; Harrison et al., 2000; Rosenbaum et al., 2000; Sugawara et al., 2004; Angelo et al., 2009; Cui et al., 2010; Kraemer et al., 2014).

1.4 DNA damage and p53 mediated apoptosis in neurons

1.4.1 p53 and post-translational activation

p53 mediated apoptosis is an evident mechanism in cell death resulting from collective events of cellular insults. The acutest feature integrating various neurodegenerative stimuli is oxidative stress and DNA damage which remains as the stimulus for p53 transcriptional and non-transcriptional activity (Amundson et al., 1998; Lane, 1998). p53 known as a transcription factor for decades regulates cell cycle and is an enabled tumor suppressor protein. p53 transcriptionally regulates genes of p53- regulating control, target genes of cell cycle regulation, involved in angiogenesis controlling genes, genes involved in cellular stress response, DNA repair and apoptosis (Amundson et al., 1998; Tokino and Nakamura, 2000; Rahman-Roblick et al., 2007). The structure of this protein has conserved regions with specialized functions including 1) TAD domain, required for transcriptional transactivation at the N-terminal domain 2) DNA binding domain which binds to its sequence-specific motif 3) a tetramerization domain and regulatory domain near the C-terminal end.

Together the C-terminal domain interacts directly with single-stranded DNA facilitating transcription (Balagurumoorthy et al., 1995; Joerger and Fersht, 2010; Saha et al., 2015).

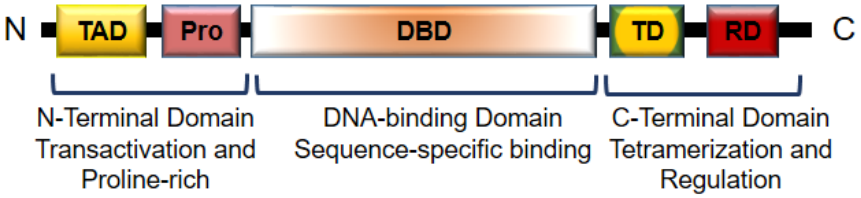


Figure 5: p53 protein domains

The p53 protein structure briefly divided as N-Terminal domain consisting of Transactivation (TAD) and Proline-rich (Pro) domain where the post-translational modification including phosphorylation occurs to activate the protein; N-Terminal is followed by the DNA binding domain specific to its binding motif on target genes; C-Terminal domain is the sequence which is involved in gene regulation based on the acetylated residues. It consists of tetramerization domain (TD) and Regulation domain (RD).

The several stress signals activating p53 are hypoxia, DNA damage, radiation or chemotherapeutic drugs. Under ischemic conditions, while the cell suffers a collective description of the earlier explained events of cellular damage, the resultant DNA breaks, trigger the p53 regulated genes expression resulting in functional death proteins (Norbury and Zhivotovsky, 2004). Oxidative stress related free radical development and particularly generation of highly toxic $O2^{-}$, OH^{-} $ONOO^{-}$ are the characteristic features of oxidative damage mediated DNA breaks. Such DNA breaks are the resultant of highly specialised lesions in DNA bases, at the apurinic/apyrimidinic sites (AP sites) formed by the hydroxyl radical (OH^{-}) generation (Liu et al., 1996; Lan et al., 2003; Sugawara and Chan, 2003; Singh and Englander, 2012). Such radicals

mediated degradation of proteins involved in cell cycle regulation and DNA repair further accumulates excessive DNA breaks which are a characteristic feature of ischemic injury and a well-documented stimulus for p53 activation shifting the paradigm of the suffering cell towards an apoptotic decision.

Multiple post-translational modifications of p53 are mandatory events for defining its function. The orchestrated modifications including phosphorylation predominantly at N-terminal residues, poly (ADP) ribosylation or acetylation of residues in the C-terminal region either promotes stability of the protein or enhances its DNA-binding affinity. These modifications are controlled by various kinases emerging in its role by sensing the DNA damage and initiate the post-translational modification at site-specific regions largely involving serine, threonine, lysine, arginine residues (Malanga et al., 1998; Coultas and Strasser, 2000; Appella and Anderson, 2001; Brooks and Gu, 2003). Important kinase executing the initial activation of p53 is the ataxia telangiectasia mutated (ATM) kinase mediating phosphorylation of p53 at serine-15 and serine-20 by acting upstream of p53 upon genotoxic stress (Saito et al., 2002). ATM-deficient neurons were partially rescued from excitotoxicity-mediated neuronal damage illustrating the significance of ATM for p53 activation (Waterman et al., 1998; Keramaris et al., 2003). Such excitotoxin-induced apoptosis presented with DNA damage is also controlled by the major kinases including mitogen-activated protein kinase (MAPK) family members, Jun N-terminal kinase (JNK), and exclusively, the p38 kinase after hypoxia downregulates E3 ubiquitin-protein ligase MDM2, a negative regulator of p53,

resulting the neuronal accumulation of phosphorylated p53 (Yang et al., 1997; Mielke and Herdegen, 2000; Tournier et al., 2000; Zhu et al., 2002).

MDM2 executes p53 ubiquitination through interaction at the p53 N-terminal transactivation domain and enhancing p53 nuclear export to be degraded by the cytoplasmic proteasome complex. In favor of p53 activity as a transcriptional regulator, the inhibition of ubiquitin-dependent degradation of the p53 protein is vital. The ubiquitination of p53 is interestingly regulated by itself since MDM2 is a p53 transcriptional target. This p53- regulated MDM2 expression prevails in either a positive or negative feedback loop controlling the cellular paradigm towards survival or demise (Lane, 1998; Prives, 1998; Lev Bar-Or et al., 2000; Feng et al., 2004). Hypoxia inducible factor-1α (HIF-1α) signaling is predominant in early phases of mild hypoxia conditions supporting cell survival, however, HIF-1α promotes enhanced p53 transcriptional activity towards neuronal death under conditions of sustained hypoxia. HIF-1α is found to interact with MDM2 during cellular stress and may indirectly influence activation of p53 by inhibiting MDM2 negative regulation over p53. Moreover de-phosphorylated HIF-1α stabilizes p53 via interaction to promote cell death during hypoxia (An et al., 1998).

Further p53 and HIF-1α shares the same co-activator, p300 and by competing with HIF-1α, p53 attracts the co-activators while the cellular paradigm has to be shifted towards an apoptotic mechanism. Also, p53 interaction with HIF-1α is enhanced by de-phosphorylation of HIF-1α executed during cellular stress and the interaction facilitates the apoptosis pathway. (Halterman and Federoff, 1999; Chen et al., 2003a; Greijer and van der Wall,

2004; Schmid et al., 2004). These factors primarily lead to phosphorylation of p53 at various sites resulting in conformational instability. An offset of the conformational instability is governed by a peptidyl-prolyl cis-trans isomerase (PPIase), Pin-1 which binds to motifs of phospho-Serine/Threonine-Proline. During the phosphorylation of p53, the sites containing these motifs are immediately recruited with Pin-1 upon phosphate addition. Such sites include Ser 46, Ser 33, Ser 315 and Thr 81 (Zacchi et al., 2002; Sorrentino et al., 2013). These regulate the acetylation of p53 by exposing its lysine residues to various HATs following the removal of iASPP, an apoptosis inhibitor. The acetylation of the protein elicits its transactivation (Mantovani et al., 2007).

Some of the immediate response to DNA damage in neurons are elicited by PARP-1 mediating DNA repair mechanism and before the apoptotic signaling incites. PARP-1 repairs DNA break in an NAD^+ dependent manner which is derived by utilizing the ATP stores of the cell, resulting as one of the factors for bioenergetic failure (Hong et al., 2004). In addition, PARP-1 plays a role in poly (ADP) ribosylating p53 protein and promoting its stability by an unknown mechanism in neurons undergoing apoptosis. However, some study demonstrates loss of binding of poly (ADP) ribosylated p53 to DNA which is essential for transactivation of target genes. PARP-1 promoting apoptotic signaling is yet to be elaborated in neurons (Malanga et al., 1998).

1.4.2 p53-mediated transactivation of apoptotic factors

After the onset of ischemia, p53 transactivation is instigated to regulate target genes including genes regulating its control, target genes involved in cellular stress response, DNA repair and apoptosis (Tokino and Nakamura, 2000; Menendez et al., 2009). The proteins resulting from the p53 triggered apoptotic gene regulation widely belong to the Bcl-2 family member consisting of the various BH domains such as, BH1, BH2, BH3 or BH4 domain. Regulated pro-apoptotic protein expression includes the multidomain Bcl-2 family member Bax, BH3-only members such as p53-upregulated modulator of apoptosis (PUMA), Noxa, and Bid plays a key role in specific cases of neuronal apoptosis (Rahman-Roblick et al., 2007; Menendez et al., 2009).

Proapoptotic protein, Bax is a key transcriptional target for p53 induced by DNA breaks during ischemia and exists as a central mediator of p53-induced apoptosis pathway. Triggers during the ischemia such as glutamate and oxidative stress are associated with Bax translocation to mitochondrial membrane (Xiang et al., 1998; Raghupathi, 2004). At basal condition, Bax is inhibited by an anti-apoptotic Bcl-2 family of proteins. The role of Bax in the mitochondrial intrinsic apoptotic pathway is the limiting step of mitochondrial Cyt-C secretion through the disrupted MTP into the cytoplasm. The release of Cyt-C signifies the irreversible apoptotic trigger with the traceable amount of apoptosome complex formation consisting Apaf-1, cytochrome-C and procaspase-9. As mentioned earlier apoptosome complex indicates the caspase cascade initiation to promote apoptosis (Kirkland et al., 2002; Love, 2003). The

proapoptotic BH3-only proteins such as Puma and Noxa are also the target genes of p53 transcription factor and contributes to cellular infarction by functioning as inhibitors of the anti-apoptotic Bcl-2 family proteins by located at the mitochondria thus making the pro-apoptotic Bax free to trigger the Cyt-C release. (Vousden and Lu, 2002; Jeffers et al., 2003; Reimertz et al., 2003; Webster et al., 2006).

The other target genes of p53 involved in cellular death pathway including Insulin-Like Growth Factor binding protein-3 (IGF-BP3), p53-activated gene 608 (PAG608), DR5/KILLER, FAS1 and FASL are found to play a role in ischemic conditions. IGF-BP3 and PAG608 are observed to be up-regulated during ischemia in humans, however, the signaling mechanism is not elaborate (Tomasevic et al., 1999; Hermann et al., 2001; Johnsen et al., 2005). Genes such as DR5, FAS1 and FASL coding for the proteins involved in apoptosis, are associated with the extrinsic pathway. Dr5 and Fas are well-characterized receptors widely found in ischemic stroke conditions and FasL controls the activation of Fas receptor (Owen-Schaub et al., 1995; Geske et al., 2001; Reimertz et al., 2003; Culmsee and Mattson, 2005; Webster et al., 2006) Other proteins expressed by p53 gene regulation include p53-regulated apoptosis inducing protein-1 (p53AIP1), p53-inducible gene (PIG) which are related to mitochondrial dysfunction leading to ROS accumulation; protein SIVA and its role in ischemia establishing direct interaction with TNFR family proteins as well as anti-apoptotic Bcl-2 family proteins is indispensable (Prasad et al., 1997; Oda et al., 2000; Matsuda et al., 2002; Xue et al., 2002; Fortin et al., 2004).

1.4.3 p53-mediated transcription-independent apoptosis

p53 dependent apoptosis in the absence of transcriptional activation of p53-target genes is an inevitable mechanism and evidently, occurs in many disease models. This mechanism bounds the parallel events during a conventional apoptotic pathway. Mitochondrial translocation of p53 is a characteristic non-transcriptional apoptotic function resulting in mitochondrial dysfunction and ROS production in response to excitotoxicity, oxidative damage of DNA. p53 acquires a non-transcriptional apoptotic role in the cytoplasm by carrying out interaction with the anti-apoptotic proteins, Bcl-XL and suppressing its effect on mitochondrial membrane stability (Caelles et al., 1994; Moll and Zaika, 2001; Gilman et al., 2003; Petros et al., 2004; Endo et al., 2006). In addition to anti-apoptotic inhibition process, p53 can mediate mitochondrial permeabilization for apoptotic factors through promoting pro-apoptotic Bcl-2 family members Bax and Bak activity through interaction (Chipuk et al., 2004; Leu et al., 2004; Petros et al., 2004).

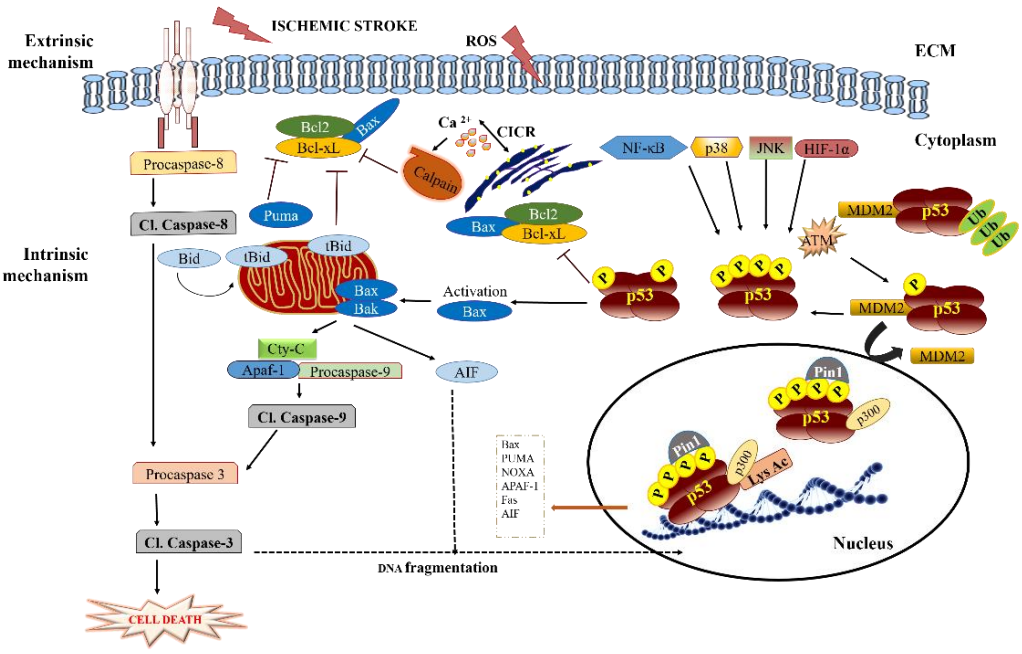


Figure 6: p53 regulated apoptosis pathway

At the resting state, p53 is inhibited by MDM2 protein facilitating its ubiquitination. During ischemic stroke, the accumulated ROS and Ca^{2+} ions trigger a cascade of events including DNA breaks. DNA breaks activate kinases particularly, ATM to phosphorylate p53 resulting in a structural change to detach from MDM2. Following, the other residues serine, threonine of p53 gets phosphorylated by many cellular pathways including NF-κB, p38, JNK and various kinases which are found to be up-regulated by upstream mechanisms independent of p53. The different regions of phosphorylation destabilize p53, however, the interaction of peptidyl-prolyl isomerase, Pin-1 interaction increases p53 stability making it available for HIF-1α regulated stability and recruited of co-activator, p300. In addition, the transactivation role of p53 is gained by acetylation at the lysine residues found in the C-terminus of the protein. The genes transcribed by p53 assumes its role in the apoptosis pathway through the extrinsic and intrinsic mechanism. Additionally, p53 has a transcription-independent role by inhibiting the anti-apoptotic protein, Bcl-XL by interaction, making Bax free for activation and dimerization at the mitochondrial transition pores.

1.5 Cell surface receptors in pathogenesis

Cell surface receptors are integral proteins activated by external signals such as ligand binding which gets transduced into an intracellular signaling mediated by various proteins to carry out a specialized biological role. These transmembrane proteins regulates a wide range of pathogenesis including reactivity to immune response, cancer, injury, inflammation and cell death (Okun et al., 2009; Okun et al., 2011; Pei et al., 2015; Coutaz et al., 2016; Lv et al., 2016; Mayer et al., 2016; North, 2016). Widely studied brain ischemic injury regulating transmembrane receptors include Pattern recognition receptor (PRRs) such as Toll- like receptors, C-type lectin myeloid receptor- Mincle and RAGE which promote sterile inflammation following its response to endogenous stimuli referred to as "damage associated molecular patterns" (DAMPs) (Okun et al., 2010; Kigerl et al., 2014; Okun et al., 2014; Lok et al., 2015; Arumugam et al., 2016). The other trans-membrane receptors associated with stroke are and Notch receptors (Arumugam et al., 2006; Arboleda-Velasquez et al., 2008; Meng et al., 2015; Zhang et al., 2015; Li et al., 2016).

1.5.1 Notch receptors

Notch receptor signaling defines one of the many fundamental cellular communication. Notch receptor is a highly conserved trans-membrane protein activated through cell-cell interaction to govern the cell fate across various species from metazoans to mammalian origin (Kimble et al., 1998; Hurlbut et al., 2007). The Notch receptor has diverse function in determining cell fate. The

resultant mechanism of Notch is diverse and pleiotropic as both its activation and inhibition can regulate cellular events from the developmental stages. Notch is involved in the development of the nervous system, including neuronal differentiation and glial determination, brain development, stem-cell differentiation and termination, cellular proliferation, angiogenesis, cancer progression and cell death mechanism. A variety of signaling proteins are involved in performing the biological and physiological outcome destined to be obtained (Artavanis-Tsakonas et al., 1999; Radtke and Raj, 2003; Bray, 2006). The different types of Notch receptor paralogues are identified as Notch1, Notch2, Notch3 and Notch4. The biological function of each of these receptors have a small degree of overlapping but vary with non-identical expression patterns depending on the cellular context and organ type (Hofmann and Iruela-Arispe, 2007; Greenwald and Kovall, 2013).

1.5.2 Mechanism of Notch Receptor Structure

The Notch receptor is a single pass transmembrane protein with distinct 2 regions and 3 cleavable sites. The extracellular domain, the transmembrane domain and the intracellular domain with the cleavable sites S1 and S2 at the extracellular domain and the S3 cleavable site at the trans-membrane domain (Kopan, 2009). During the maturation of the protein, one of the post-translational modification, O-fucosylation carried out by furin-like convertase process the receptor protein by cleaving at the S1 cleavage site in the trans-Golgi to produce the final structure of the non-covalently associated

heterodimer and prepares it to be launched at the cell surface (Sanchez-Irizarry et al., 2004; Gordon et al., 2009).

The Extra cellular domain structure contains a 36 epidermal growth factors (EGF)-like repeat domain, with few domains containing calcium binding sites. Subsequent to the EGF-like repeat is the negative regulatory region (NRR) (Rebay et al., 1991). The NRR regions contain the three Lin-12-Notch (LNR) repeats lying close to the EGF-like repeats and a hydrophobic hetero-dimerization (HD) domain lying adjacent to the cell membrane. NRR region conceals the S2 cleavage site to prevent the ligand-independent activation. S2 at the NRR gets unfolded and exposed during ligand interaction (Schroeter et al., 1998; Sanchez-Irizarry et al., 2004).

The next, S3 cleavage site is present at the trans-membrane segment and susceptible to be cleaved upon the slicing of the extracellular domain. The cleavage at the S3 site liberates the intracellular domain (NICD) into the cytoplasm. NICD consists 4 to 5 domains depending on the type of paralogue. RAM, ANK-containing seven ankyrin repeats, TAD which is the trans-activation domain, OPA and PEST are the 5 types of domains (Tamura et al., 1995; Kelly et al., 2007). TAD domain is present only in Notch1 and Notch2 paralogues but not in Notch3 and Notch4.

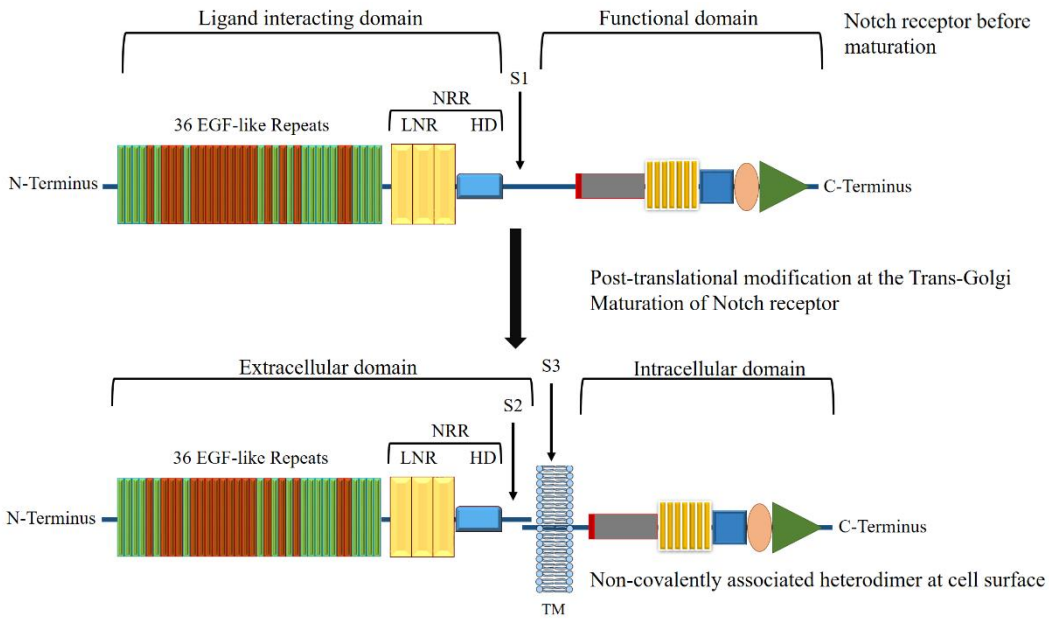


Figure 7: Notch Receptor structure before and after maturation

The structure of Notch receptor before its maturation and after maturation through post-translational modification at the trans-Golgi. The ligand interacting domain contains 36-EGF like repeats region with cbEGf (brown) and non-cbEGF (green) followed by LNR (Lin-12-Notch) repeats lying between the EGF repeats and hydrophobic HD (Hetero-dimerization) domain. The LNR and HD domain together form the NRR region. The S1 cleavage site lies between the ligand-interacting domain and the functional transactivation domain. After translation of Notch receptor protein, it undergoes post-translational modification at the trans-Golgi through furin-like convertase cleaving it at the S1 site and making a non-covalently associated heterodimer protein before getting launched at the cell surface. After maturation, NRR region of the extracellular domain conceals the S1 cleavage site from being susceptible to ligand-independent activation. The trans-membrane (TM) consists of the S3 site which upon cleavage generates the active intracellular Notch domain (NICD). (Adapted : Sanchez-Irizarry et al., 2004)

Each domain of NICD contributes to a unique functionality (Gordon et al., 2008). RAM and ANK are involved in its interaction with the CSL transcription factors to initiate transactivation (Zweifel et al., 2003), whereas PEST domain is identified to regulate NICD proteolytic degradation (Weng et al., 2004). Studies have supported this identification, by proving deletion of this domain promotes faster ubiquitination of NICD (Baik et al., 2015). ANK fold

comprises a pair of antiparallel helices, which participates in protein-protein interactions (Zweifel et al., 2003). Notch transcription function includes the interaction of NICD to the transcription factors CSL and the co-activator MAML. NICD and CSL interaction predominantly takes place at the RAM domain (Lubman et al., 2007) and the co-activator, MAML binding to NICD is completely ANK domain dependent (Nam et al., 2006).

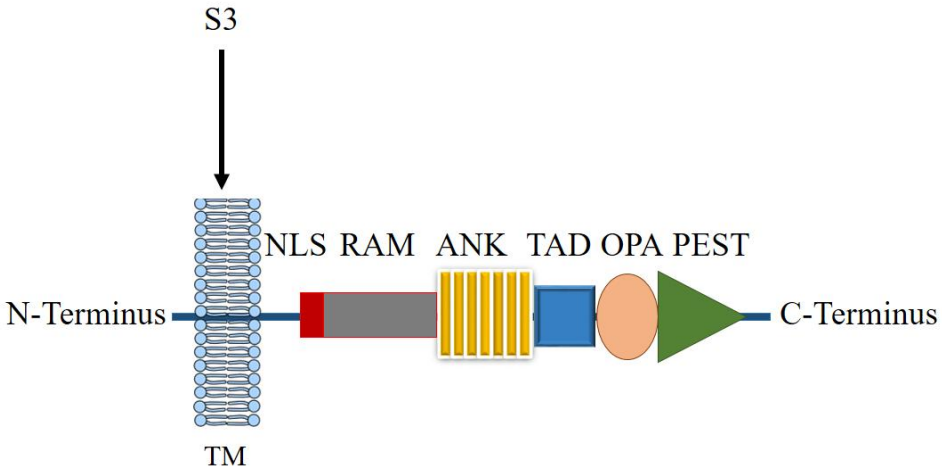


Figure 8: Structure of Transmembrane and Notch Intracellular domain

The structure of the trans-membrane encompasses S3 cleavage site which embarks the Notch activation step from being catalyzed by γ-secretase enzyme complex. Following the trans-membrane region is the 5 domain region of the intracellular portion of the receptor (NICD). RAM containing the nuclear localization signal helps in its nuclear translocation, followed by 7-Ankyrin repeat domain, ANK, TAD which is the transactivation region, OPA and PEST identified for its role in inhibiting ubiquitination of NICD.

Notch signaling

Ligand-mediated signaling

Notch signaling is canonically ligand-dependent. Notch ligands alike Notch receptors are single-pass transmembrane which belong to the Delta/Serrate ligands (DSL) family of proteins identified in *Drosophila*

corresponding with Delta-like/Jagged ligands (DJL) family of proteins in the mammalian forms that consists of six mammalian notch ligands including, delta-like homologous (DLL)-1, DLL-2, DLL-3, DLL-4 and serrate-like homologous JAG-1 and JAG-2 (Artavanis-Tsakonas S, 1995; Love, 2003; Kopan, 2009). The ligand-dependent signaling is initiated when the opposing cell expressing Notch ligand is in close proximity to potentiate the ligand interaction with heterodimer Notch receptor (de Celis and Bray, 1997). Interaction of the ligand to the EGF-like repeats of the extracellular domain triggers a structural change in the extracellular domain leading to the NRR domain to expose the S2 cleavage site for catalyzes by the metalloprotease ADAM enzyme (Vardar et al., 2003). The cleaved Notch-ligand complex undergoes endocytosis at the membrane surface of the cell expressing the ligand (Weinmaster, 2011; Musse et al., 2012). The remaining tethered fragment of the heterodomain receptor at the transmembrane region is now susceptible to S3 cleavage by the γ-secretase complex to liberate the NICD, an active version of Notch receptor and hence γ-secretase complex activity embarks the activated stated of Notch receptor.

NICD translocates to the nucleus to facilitate the formation of transcription enabling CSL-MAML-NICD complex interaction. This organized transcription enabling complex is initially designed to rescue the target genes from a complex of repressor proteins, in order to be transcribed (Schroeter et al., 1998; De Strooper, 1999; Tanigaki, 2010; Borggrefe, 2012; De Strooper et al., 2012). In addition, the recruitment of Histone acetyltransferase (HAT) and chromatin remodeling complexes leads to transcription of target genes such as

Hes-1, Hes-5 which are identified with CSL binding sites (Kurooka, 2000; Wallberg, 2002).

Alternatively, ligands expressed on the same cell surface as the receptor can lead to cis-inhibition to circumvent the ligand-receptor trans-interaction (Micchelli et al., 1997). The molecular mechanism of the cis-inhibitory process is unknown, yet studies reveal the existence of cis-interaction in the similar regions on the cell surface where the receptor is also involved in trans-interaction (de Celis and Bray, 2000). Calcium binding sites containing EGF repeats (cbEGF) of a receptor is studied to be rigid near-linear in architecture. However, since Notch extracellular domain consists of the N-terminal region and C-terminal region without calcium binding sites and rich with EGF-EGF repeats along with few cbEGF repeats and thus may make the structure more compact and flexible (Morgan et al., 1999; Hambleton et al., 2004). Without cis-inhibition mechanism, the facilitation of the cis-interaction of receptor-ligand may arise from the EGF–EGF tandem domain's flexible non-linear orientation of structure and may assume a bent position (Chillakuri et al., 2012).

Ligand-independent signalling

The ligand-mediated Notch signaling is an extensively studied mechanism while the other mechanisms of Notch activation does not have a widely collected mammalian evidence. The endogenous cellular system is under a constant state of Notch receptor production and degradation making it attain a constant state of turnover. Various studies from *Drosophila*, have highlighted

the mechanisms through which ligand-independent Notch activation is accomplished. Endocytosis and intracellular trafficking play a vital role. The drosophila, E3 ubiquitin ligase, Deltex promotes Notch internalization and activates Notch by interacting with its ankyrin domain. Deltex facilitates the internalization into the late endosome to execute Notch activation (Diederich et al., 1994; Hori et al., 2004).

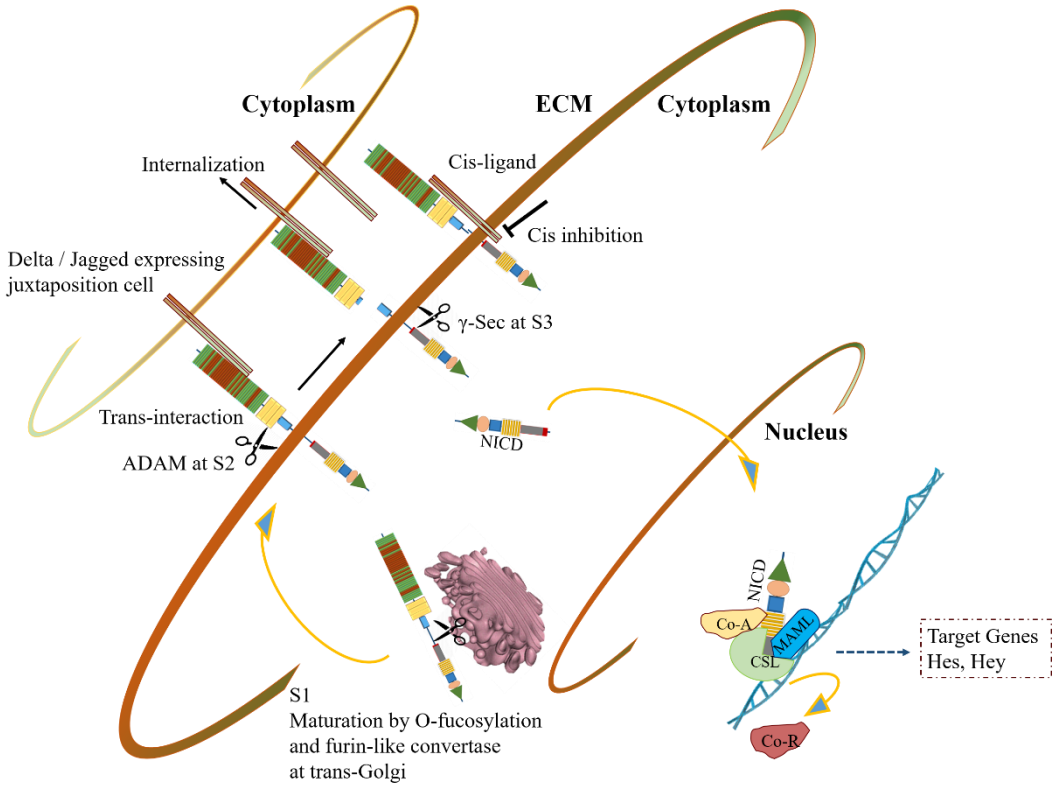


Figure 9: Notch ligand-mediated signaling

The receptor is post-transcriptionally modified at the trans-Golgi by O-fucosylation priming the ligand interacting domain and through furin-like convertase acting at the S1 site producing a non-covalently bonded heterodimer to be launched at the cell surface. The ligand-receptor trans-interaction initiates the S2 cleavage by ADAM metalloprotease followed by the S3 cleavage by γ-secretase liberating NICD to translocate into the nucleus. The extracellular domain remains intact with ligand and gets internalized into the ligand-expressing cell for ubiquitination. In the nucleus NICD associates with CSL and MAML, meanwhile, the transcription complex formation dissociates the co-repressors from the gene targets. The NICD/CSL/MAML complex recruits other chromatin modeling proteins to carry out the transcription of Hes and Hey genes. The cis-interaction between the receptor-ligand of the same cell acts as cis-inhibition making the receptor not available for trans-interaction signaling.

Ligand-independent Notch activation is also buffered by DSL ligands. As explained earlier cis-ligand represses the canonical Notch signaling through making the receptor not available for trans-interaction (Chillakuri et al., 2012). An absence of both cis- and trans-ligands have found to increase ligand-independent Notch activation activated cell-autonomously. In addition, the *Drosophila* Hif-1α homolog, sima has endosomal Notch activation role through interaction in development (Palmer et al., 2014). ADAM protease, an S2 cleaving protease is found to be active independent of the ligand when present in abundance. NRR region of the extracellular domain which conceals the S2 site from the ADAM protease activity is perturbed by the calcium chelation due to the LNR repeats stability is dependent on the Ca^{2+} ions for bringing stability to the NRR region through calcium bridging (Rand et al., 2000; Delwig and Rand, 2008). In mammals, the ligand-independent activation is observed in the cancer stem cells which is found to regulate the sphingosine-1 phosphate receptor (Hirata et al., 2014). These mechanisms are yet to be explored in ischemic stroke conditions although the physiological events of ischemic onset lead to the calcium chelation in the extracellular region which could be a crucial step in mediating Notch activation. Further, the S3 cleavage site in Notch is executed by γ-secretase, presenilin containing the enzyme and the function of the γ-secretase enzyme is actively dependent on the intracellular calcium sensor protein, calsenilin. In an ischemic stroke, the increased level of intracellular calcium regulates the Notch activity by promoting calsenilin and presenilin interaction (Park et al., 2013).

1.5.3 Notch in ischemic stroke pathogenesis

Notch role is observed in all phases of brain development and in other cellular events in the adult, however, there is growing evidence that Notch may also play a contributing role in pathological events including cortical post-mitotic neurons, dendritic branching and in neural plasticity (Redmond et al., 2000).

Notch paralogues are associated with ischemic stroke. Focal cerebral ischemia in rodents has shown Notch1 receptor activation. Various studies have employed γ-secretase inhibitor, DAPT to explore the biological outcome of Notch inhibition. During cerebral ischemia-reperfusion in adult mice and rats, DAPT has effected a reduced infarct size and better neurological function outcome. Interestingly these studies have observed decreased apoptosis in the ischemic region of rodent brain subjected to stroke (Li et al., 2016). Moreover, ischemic stroke has been associated with Notch2 paralogue. Rac1, a GTPase protein found up-regulated during ischemic stroke in mice IR injury was identified to regulate Notch2 receptor. An inhibition of Rac1 GTPase decreased the Notch2 activation and resulted in the reduced generation of NICD2. The protection caused in mice brain neurological function from Rac1 GTPase has been reproduced in DAPT studies as well (Meng et al., 2015). Coherently, hippocampal neurons have shown to express Notch2 activity after the onset of ischemic stroke (Alberi et al., 2010). Additionally, Notch3 and Notch4 types of receptors have been recently reported to contribute to ischemic stroke injury in rodent through unknown mechanisms (Zhang et al., 2015).

By far the widely studied paralogue of Notch receptors is the Notch1 receptor (Notch). Extensive mechanistic studies have elaborated the intracellular pathways associated with Notch activation. Along with pharmacological inhibitor studies using DAPT, Notch reduction at the genetic level by employing a Notch antisense mice, the similar protective effect was able to be obtained in mice subjected to stroke. Further, an improved neurological score in conjunction with reduced infarct size was obtained (Arumugam et al., 2006). Notch activation has been observed to increase in neurons and microglial population during ischemic stroke. And the main mechanism shared by both the cell populations is the Nuclear factor-κB (NF-κB) pathway and serves as one of the factors for mediating cellular death mechanism in neuronal cells shortly after ischemic condition and an early microglial inflammatory response mediated leukocyte infiltration followed by a delayed cellular death in microglial population (Wei et al., 2011; Li et al., 2012; Marumo et al., 2013; Yuan et al., 2015). In neuronal cells, NF-κB pathway endangers neurons through Bcl-2-interacting mediator (Bim) protein. Additively, modified NICD with the nuclear export signal (NES) has proven to promote more cell death rate compared to the NICD with nuclear localization signal (NLS) depicting neuronal death elicited by NICD may also be transactivation-independent.

Similarly, a role for Notch/HIF-1α interaction in neuronal cell death has also been documented in ischemic stroke (Cheng et al., 2014[a]). Cell death was potentiated by combined overexpression of Notch intracellular domain (NICD)

and HIF-1α compared to that seen with expression of either protein alone. The early inhibition of HIF-1α reduced neurological deficits and brain infarct volume, and additional protection was obtained by combined inhibition of Notch-activating γ-secretase and HIF-1α. Notch signaling can modulate mitogen-activated protein kinase (MAPK) pathways and regulate inflammation, cell death, and cell proliferation (Pallavi et al., 2012; Yamashita et al., 2013; Cheng et al., 2014[b]). Additional molecular entities likely to play an interacting role with Notch following stroke is the c-Jun N-terminal kinases (JNK). Studies have also demonstrated that the peptidyl-prolyl cis/trans isomerase, Pin1, promotes mechanisms associated with Notch-dependent cell death following stroke and was found to interact with NICD to increase its stability by circumventing FBW7-induced polyubiquitination (Baik et al., 2015). Pin1 is a peptidyl-prolyl isomerase family of enzymes and it catalyzes cis/trans isomerization of proline bonds preceded by phosphorylated serine or threonine residues (pSer/Thr-Pro). The inhibition of Pin1 independently reduces neuronal cell death during oxidative stress indicating the pathogenic role of Pin1 (Shen, 1998; Lu, 1999; Verdecia, 2000; Kesavapany, 2007).

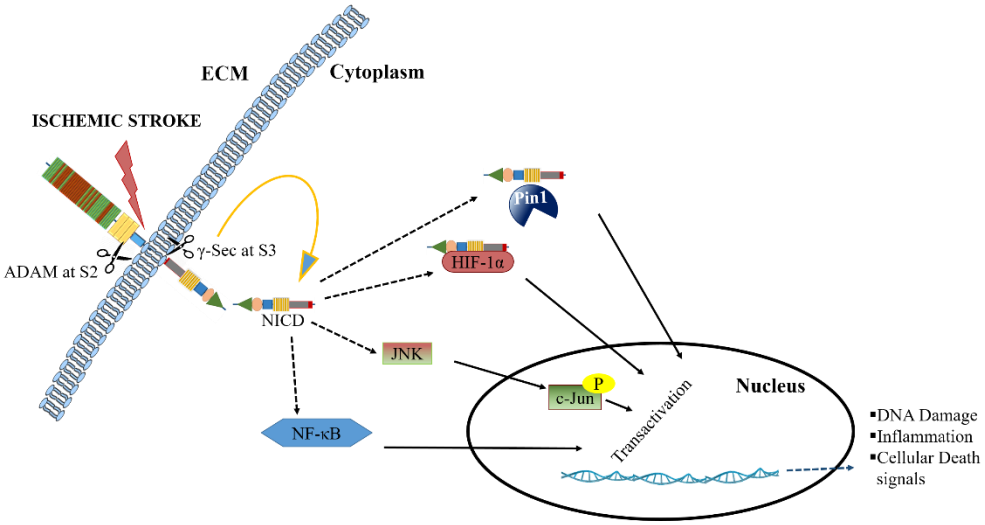


Figure 10: Notch-mediated cell death pathway in neurons

The ligand-receptor trans-interaction initiates the S2 cleavage by ADAM metalloprotease followed by the S3 cleavage by γ-secretase liberating NICD to translocate inside the cell. The figure illustrates the various pathways by which NICD is identified to regulate cell death. NICD interaction with HIF-1α and Pin is found to enhance its molecular capacity to endanger neurons. NICD is found to upregulate NF-κB and JNK-cJun signaling. Together NICD modulates DNA damage, inflammation and cell death by regulating these pathways.

1.5.4 Crosstalk of Notch downstream with p53

A surplus of evidence suggests that Notch could promote cell death under stroke conditions. Role for Notch in the regulation of NF-κB-mediated cell death and inflammatory signaling pathways (Arumugam et al., 2011; Wei et al., 2011), Notch/HIF-1α interaction in neuronal cell death (Cheng et al., 2014[a]) and additionally molecular entities including c-Jun N-terminal kinases (JNK) and mitogen-activated protein kinase (MAPK) pathways to mediate inflammation, cell death, and cell proliferation (Pallavi et al., 2012; Cheng et al., 2014[b]) are well-established. These pathways have an elaborated role in activating apoptosis by promoting the phosphorylation of p53 in various other

disease conditions (Buschmann et al., 2001; Chen et al., 2003a; Choi et al., 2011). Collaborative roles of transcription factors such as nuclear factor-κB (NF-κB), hypoxia-inducible factor-1α (HIF-1α) with p53 have been demonstrated in multiple tissues under different conditions (Halterman and Federoff, 1999; Ryan et al., 2000). The peptidyl-prolyl cis/trans isomerase, Pin1, mechanisms associated with Notch-stability (Baik et al., 2015) is also a stabilizing candidate for post-translationally modified p53 under genotoxic stress (Zacchi, 2002; Zheng, 2002). Yet, Notch association with p53 in positively regulating cellular death pathway is yet to be established. However, it was shown that conditional expression of a constitutively active form of Notch in early neural progenitor cells selectively induces extensive apoptosis by elevating levels of nuclear p53 and up-regulating transcription of target pro-apoptotic genes, Bax and Noxa (Yang et al., 2004), the mechanism behind the collaborative apoptotic role of Notch and p53 was not elaborated. Hence understanding the collaborative role of NICD and p53 in promoting neuronal cell death after the onset of ischemia and the mechanisms via which this complex can execute ischemic stroke pathophysiology will broaden the prospects to the therapeutic field for ischemia.

1.6 Hypothesis

The current study is hypothesized that activated Notch receptor is a key entity in regulating apoptosis by assisting p53 cellular mechanism. As widely studied, p53 stabilization by different proteins has found to be a prerequisite for activating p53-mediated various cellular functions. This study proposes a mechanism of NICD regulated protein level alteration in p53 to drive its transcriptional function of genes involved in apoptosis during the ischemic cerebral stroke. Additionally, this study also postulates the established stability of NICD conferred through Pin1 interaction will be an indispensable molecular mechanism to establish NICD/p53 interaction for stabilizing and activating p53 mediated transcription to result in ischemic neuronal death. A perturbation in this mechanism will render both the protein functionally impaired and fail to promote neuronal cell death after the onset of cerebral ischemia.

Aims

- The severity of apoptotic pathway in cerebral ischemia.

  This was achieved by establishing *in vitro* ischemic conditions at various duration to examine the neuronal adversity in ischemia.

- Modulation of Notch activation to determine the effect of Notch on p53 activation and its transcriptional steadiness.

  This study was achieved through exogenous Notch expression and pharmacological inhibition of Notch mechanism during basal and ischemic conditions to investigate the p53-mediated neuronal death mechanism.

- Hampering p53 cell death pathway to investigate Notch regulation.

  Using pharmacological inhibition of the active sites in p53, the mechanism of Notch activation and transcriptional regulation was investigated for variation.

- Molecular elaboration of protein-protein interaction.

  The interaction was probed by endogenous pull-down of the complex in basal and ischemic condition. Through hindering the endogenous cellular ubiquitination and translation mechanism interaction mediated utility was explored.

- The synergistic mechanism through inhibitory approaches.

  Established *in vitro* and *in vivo* ischemic model were examined for the synergistic function of both proteins and the effect on functional outcome and infarct area was estimated with the combined inhibition of both proteins during ischemia.

CHAPTER 2

MATERIALS & METHODS

2.1 Primary mice cortical neuronal cultures and cell lines

Dissociated neuron-enriched cell cultures of cerebral cortex were established from day 16 C57BL/6NTac mouse embryos. Experiments were performed in 7-9 days-old cultures. SH-SY5Y and HEK293T were cultured in Dulbecco modified Eagle medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (HyClone Laboratories, GE Healthcare Life Sciences) and 1% penicillin/streptomycin (Life Technologies, Thermo Fischer Scientific). For transfection experiments, cells were seeded at a density of $2x10^5$/well onto 6-well plates on the day before transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and then incubated with complete medium for 24 h. Cells were transfected with mouse human Myc-NICD, HA-p53, HA-p53/ΔN, HA-p53/DBD and HA-p53/ΔC domain mutant cDNAs vector for ubiquitination and immunoprecipitation studies.

2.2 Oxygen-Glucose Deprivation (OGD)

Cultured neurons were incubated in glucose-free Locke's buffer containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM HEPES, pH 7.2, supplemented with gentamicin (5 mg/L) in an oxygen-free chamber for various time points. γ-Secretase inhibitor (GSI), N-

[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), (Calbiochem-Merck Millipore) and the p53 inhibitor pifithrin-α (Calbiochem-Merck Millipore) were dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich) and used at final concentrations as indicated in the results section. To observe the effect of GSI (DAPT) or p53 inhibitor (PFTα), DMSO-dissolved drugs were added to cultures during OGD with the glucose-free Locke's buffer. Control conditions included exposure to vehicle of the volume used for the drug treatment.

Figure 11: Oxygen-glucose Deprivation chamber

The chamber employed in ischemic simulation. The two vents is used as inlet and outlet for replacing O_2 in the chamber with 95% N_2+5% Co_2. It consists of an air-tight lock to avoid gaseous exchange.

2.3 Immunoblot analysis

Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis using a Tris-SDS-glycine running buffer. Gels were then electro-blotted using a transfer apparatus (Bio-Rad) in

transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 20 % (v/v) methanol for 1.5 h at 500 mA onto a nitrocellulose membrane (Bio-Rad). The membrane was then incubated for 1 h at RT in blocking agent (1 % Fish Skin Gelatin – FSG) dissolved in 1x TBST (Tris-buffered saline-T) constituted of 20 mM Tris-HCl, pH 7.5, 137 mM NaCl and 0.2 % Tween-20. The membrane was then incubated overnight at 4 °C with primary antibodies including those that selectively bind NICD1 (Abcam), NICD-Myc (Roche), P-p53 (Thr81) (Bioss), Total p53 (Cell Signaling Technology), Bax (Abcam), Puma (Abcam), Bcl-2 (Cell Signaling Technology), P-Pin-1 (Abcam), Pin-1 (Abcam), Hes1 (Abcam), cleaved caspase-3 (Cell Signaling Technology), Caspase-3 (Cell Signaling Technology) and β-actin (Sigma-Aldrich) in 1x TBST. Subsequently, the membrane was incubated with secondary antibodies tagged with horseradish peroxidase (HRP) against the primary antibody for 1 h at room temperature (RT). The membrane was washed with in 1x TBST and incubated in Clarity Western ECL (Chemiluminescent enhancer reagent) before scanning using Chemidoc Plus (Bio-Rad). Quantification of protein levels was achieved by densitometry analysis using Image J software.

2.4 Immunocytochemistry and immunohistochemistry

Coverslips containing cortical neurons subjected to either control or OGD conditions were fixed in 4 % buffered paraformaldehyde (PFA) in PBS. Fixed cells were permeabilized using 0.1 % Triton-X in PBS at RT for 15 min and incubated in blocking solution (1 % BSA and 0.1 % Triton-X in PBS) at RT for 1 h before overnight incubation at 4 °C with microtubule-associated

protein 2 antibody (MAP2, Millipore) along with primary antibodies that selectively bind NICD (Abcam), P-p53 Thr81 (Bioss) diluted in blocking solution. Following incubation with primary antibodies, cells were incubated with the appropriate Alexa Fluor conjugated secondary antibodies (In-vitrogen, Life Technologies, Thermo Fisher Scientific) for 1 h at RT. The nuclei were counterstained with DAPI (AbD Serotec) for 10 min at RT. Following secondary antibody incubation, coverslips were sealed with Vectashield Fluorescent Mounting Medium (Vector Laboratories) on glass slides. For immunohistochemistry, frozen cryostat brain sections were obtained from animals subjected to either sham surgery or cerebral ischemia-reperfusion (I/R) followed by trans-cardiac perfusion with 4% paraformaldehyde. The brain sections were processed using 20% sucrose overnight at 4 °C and 30% sucrose overnight at 4 °C. The processed sections were mounted using OCT using a mold and sectioned as 20μm thickness using cryostat and immunostained with primary antibodies against microtubule-associated protein 2 antibody (MAP2, Millipore) or primary antibodies that selectively bind NICD (Abcam), P-p53 Thr81 (Bioss). Images were acquired using an Olympus FluoView FV1000 confocal laser-scanning inverted microscope (Olympus, Tokyo, Japan) with a 60X and 100X oil immersion objective.

2.5 Immunoprecipitation and *In Vitro* Ubiquitination

SH-SY5Y cells were transfected with NICD-Myc and HA-p53 (WT, ΔN, DBD, ΔC). Transfected cells were lysed in PBS (0.1 % Tween-20) after 24 h and total lysates were centrifuged at 10,000 x g for 10 min at 4 °C. For

ubiquitination, SH-SY5Y cells were transiently transfected with NICD-Myc or HIS-ubiquitin, and HA-p53 and incubated for 24 h. Subsequently, cells were treated with MG132 (10 µg/mL) for 9 h. Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) (200 mM NaCl, 50 mM Tris-cl, 1 % tritonX-100, 0.1 % SDS) and centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was incubated with Protein A/G Sepharose (GE Healthcare) beads overnight at 4 °C. The mixture was centrifuged at 2,000 rpm for 3 s and then washed 3 times with 1 ml of radioimmunoprecipitation assay buffer. The supernatant was discarded, and remaining beads were supplemented with 15 µL of 2x sample buffer and then boiled at 95 °C for 10 min. Subsequently, immunoprecipitated materials were subjected to immunoblotting with anti-HA, anti-Myc, and anti-p53 antibodies.

2.6 p53 Stability

SH-SY5Y cells were transfected with Myc-tagged NICD and p53 or HA-tagged p53 (WT, ΔN, DBD, ΔC) for 24 h and then treated with cycloheximide (CHX; 40 µg/mL) at various time points. The cells were harvested at the indicated times, followed by an immunoblot analysis with anti-Myc, anti-p53 and anti-HA antibodies.

2.7 Focal middle cerebral artery I/R stroke model

Experiments were carried out on wild-type (WT) C57BL/6NTac mice obtained from In Vivos (Singapore). Three-month-old C57BL/6NTac male mice were

subjected to transient middle cerebral artery I/R. Briefly, after making a midline incision in the neck, the left external carotid and pterygopalatine arteries were isolated and ligated with 6-0 silk thread. The peripheral site of the bifurcation of the internal carotid artery (ICA) was occluded with a small clip and the common carotid artery (CCA) was ligated with 6-0 silk thread. The external carotid artery (ECA) was cut, and a 6-0 nylon monofilament with a tip that was blunted (0.20–0.22 mm) with a coagulator was inserted into the ECA. After the clip at the ICA was removed, the nylon thread was advanced to the origin of the middle cerebral artery until light resistance was evident. The nylon thread and the CCA ligature were removed after 1 h to initiate reperfusion. In the sham group, surgery was performed until the arteries were visualized. Mice were administered with either 40 mg/kg of a gamma-secretase (γ-sec) inhibitor (GSI, Calbiochem-Merck Millipore), 2 mg/kg of a p53 inhibitor Pifithrin-α (Santa Cruz) or vehicle (DMSO) by infusion into the femoral vein 1 h after the start of reperfusion. Experimental doses were determined based on previously published work by our group and others. The mice were euthanized with isoflurane after either 6 h (for protein analysis) or 24 h (for infarct analysis and immunostaining) reperfusion. The animals were included in the study if they underwent successful MCA occlusion, defined by an 80 % or greater drop in cerebral blood flow seen with laser Doppler flowmetry. The animals were excluded if insertion of the thread resulted in perforation of the vessel wall determined by the presence of sub-arachnoid blood at the scheduled time of euthanasia. A total of 81 mice were employed for the animal study including 30 mice for protein analysis study with each of the 5 groups having N=6 mice; 45 mice for the Neurological deficit and infarct area study with each of the 5 groups

having N=9 mice; 6 mice for immunostaining study with each of the 2 groups having N=3 mice.

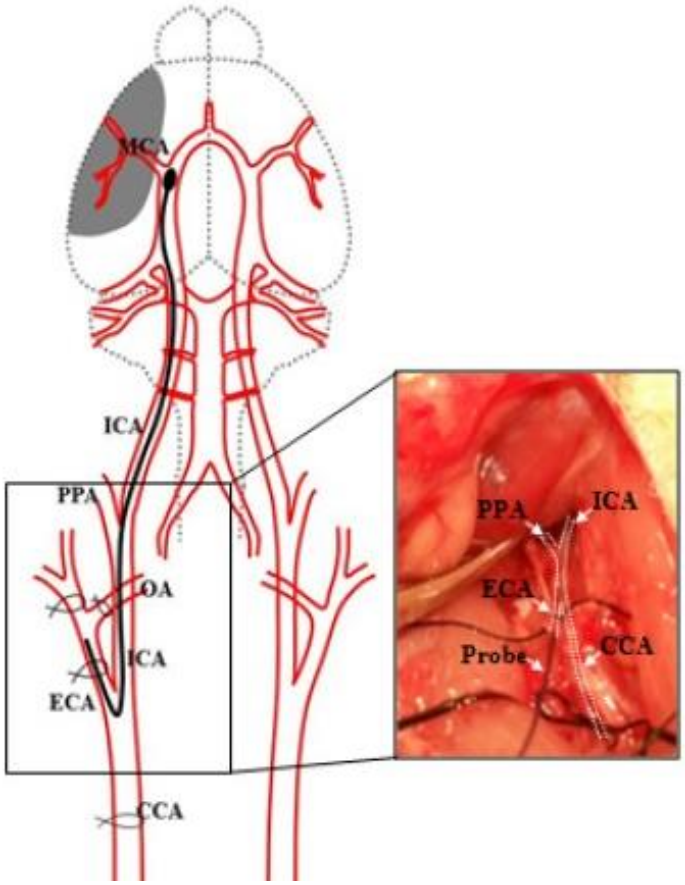


Figure 12: Middle cerebral artery occlusion method

Structural diagram of mice Middle Cerebral Artery Occlusion microsurgery (Lee et al., 2014). The figues explains the filament insertion path through the carotid arteries and into the cerebral artery. The filament marked in black, is introduced inside the left external carotid after an incision through the left internal carotid artery until reaching the middle cerebral artery region and sutured for until 1hr to subject the mice to ischemia.

2.8 Neurological assessment and infarct size determination

The functional consequences of I/R injury were evaluated using a five-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2,

circling to the right; 3, falling to the right; and 4, unable to walk spontaneously) and were assessed in a blinded fashion. Brains were immediately removed and placed into phosphate buffered saline (PBS, Sigma-Aldrich) at 4 °C for 15 min, and four 2-mm coronal sections were made from the olfactory bulb to the cerebellum. The brain sections were stained with 2 % 2,3,5-triphenyltetrazolium chloride (TTC) in PBS at 37 °C for 15 min. The stained sections were photographed and the digitized images used for analysis. Borders of the infarct in the image of each brain slice were outlined and the area quantified (Image J software). To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. The infarct volume was determined by calculating the percentage of infarcted area in each brain slice, and then integrating the infarct areas for all slices of each brain.

2.9 Statistical Analysis

Statistical analysis of all data except the behavioral score data were performed using a one-way ANOVA followed by a Bonferroni post hoc test to determine between-group differences. Statistical difference was accepted if $P<0.05$. Neurological behavior scores were analyzed using a non-parametric Kruskal–Wallis test and Dunn's multiple comparison test. Statistical analyses were performed using GraphPad Prism.

CHAPTER 3

RESULTS

3.1 Neuronal cell death regulated by notch receptor and p53 during Ischemic conditions

3.1.1 Neuronal cell death mediated by caspase cascade pathway

To investigate the cell death mechanism initiated by ischemic condition, the cell death executing protease, procaspase-3 activation mediated either by a mitochondrial damage triggered apoptosome formation or alternatively by extrinsic apoptotic signaling by cell surface receptors was observed (Culmsee and Mattson, 2005). The activation of procaspase-3 to produce the active form of cleaved caspase-3 (Cl.Cas3) signifies a quantitative neuronal death occurring during ischemic condition. The level of Cl.Cas3 elevates gradually over a different period of ischemic condition having a significant increase from 3hrs of OGD until the prolonged duration of 12hrs OGD (Fig. 13 A and B). Obtained significance mimics the neuronal cell population suffering the loss of combat against the ischemic-like insult occurring over the indicated time periods.

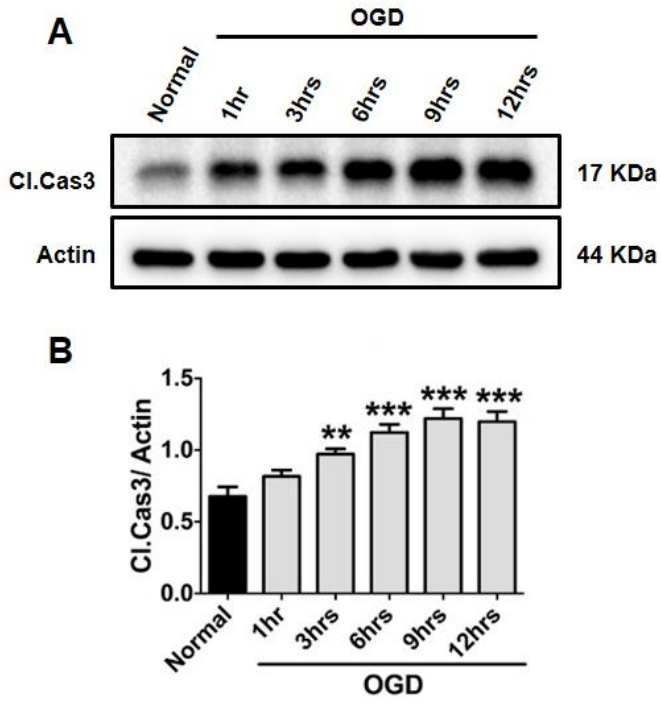


Figure 13: Apoptotic cell death during *in vitro* ischemia

In vitro ischemia increases cell death mediated by a caspase-cascade pathway in primary cortical neurons. Neurons were subjected to OGD and analyzed for apoptotic cell death marker, cleaved-caspase-3. A and B, Representative immunoblot and analysis of cleaved-caspase-3 at the indicated time points during OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=5 cultures. **P<0.005 and ***P<0.001 compared with normal cell culture.

3.1.2 Ischemic conditions activate cell surface notch receptor

The activation profile of Notch receptor was examined in ischemic conditions to determine the contribution of the receptor during ischemic cell death. The biological activity of Notch receptor can be triggered within the vista of its cleavage by the gamma-secretase enzyme complex (Arumugam et al., 2006). The elevated cleaving of Notch receptor to produce the notch intracellular domain (NICD), its active form signifies the transcriptional function of NICD in association with co-activators such as CBF, MAML, CSL.

Established evidence of NICD association with other transcription factors like HIF-1α and NF-κB plays a role in regulating the expression of target genes (Artavanis-Tsakonas et al., 1983; Lathia et al., 2008). Here, the activation of Notch receptor is significant in the early time points of OGD hours (Fig. 14 Aand B). Activation of Notch receptor is observed with significance in the initial hours of ischemic condition depicting the supporting role of NICD in neuronal death.

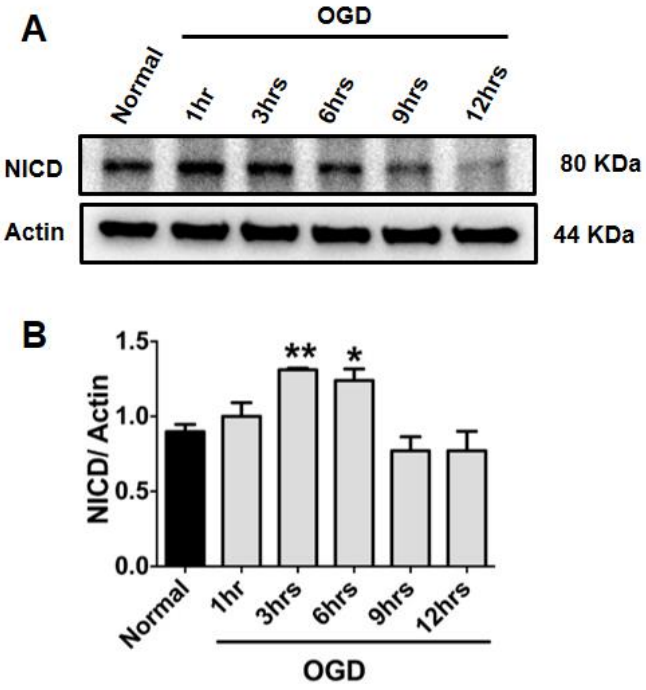


Figure 14: Notch receptor activation during ischemic conditions

In vitro ischemia increases notch receptor activation in primary cortical neurons. Neurons were subjected to OGD and analyzed for notch intracellular domain to determine the activation of the receptor. A and B, Representative immunoblot and analysis of NICD at the indicated time points during OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=5 cultures. *$P<0.05$ and **P<0.005 compared with normal cell culture.

3.1.3 Apoptosis initiator protein is activated shortly after the onset of ischemia

The cellular diversity of p53 protein comprises a major responsibility as an apoptosis initiator by transcribing the target genes responsible for the pathway after being activated (Hong et al., 2010). The activation involves major sites of phosphorylation and acetylation. One of the major sites of phosphorylation where Threonine-81 (Thr-81) residue lies, enhances the stability of the protein to be available for co-activators recruitment and acquire gene transcription ability (Buschmann et al., 2001). The elevation of the phosphorylated p53 occurs in the early time points of ischemia with a significance observed in 3hrs and 6hrs of OGD (Fig 15-A and B).

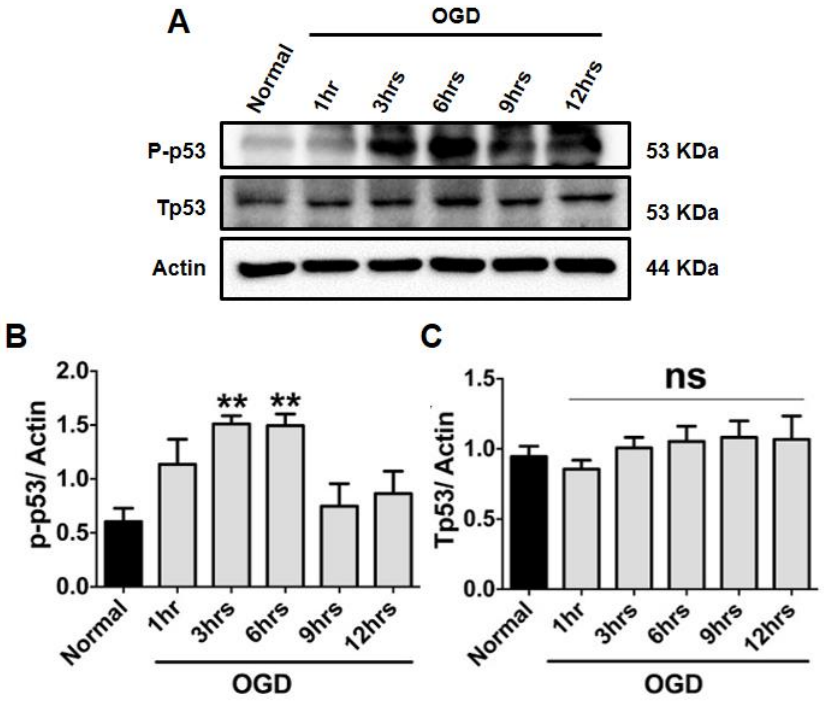


Figure 15: Apoptotic initiator, p53 activation during ischemic condition

In vitro ischemia increases p53 activation in primary cortical neurons. Neurons were subjected to OGD and analyzed for phosphorylated levels of p53 to determine the transcriptional activation of the protein. A-C, Representative immunoblots and analysis of Tp53 and P-p53 at the indicated time points during

OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=5 cultures. **P<0.005 compared with normal cell culture.

3.1.4 p53 mediated apoptosis and mitochondrial permeability

p53 mediated apoptosis involves important protein up-regulation including, Bax and Puma. Both the proteins regulate the release of apoptotic factors such as Cyt-C, AIF from the mitochondrial inter-membrane space. Permeabilization of mitochondria is controlled by Bax dimerization (Eskes et al., 1998; Gross et al., 1998) which, is promoted by Puma interacting with anti-apoptotic proteins, that are otherwise found to inhibit the action of Bax via interaction (Yee and Vousden, 2008). Investigation in ischemic condition mediated death pathway reveals the significant up-regulation of Bax and Puma (Fig. 16 A-C) in the early time points of OGD. Bax is also found to show a sustained increase. The up-regulation of these proteins signifies intrinsic apoptotic signaling being triggered following Notch and p53 activation in the neurons subjected to *in vitro* ischemia.

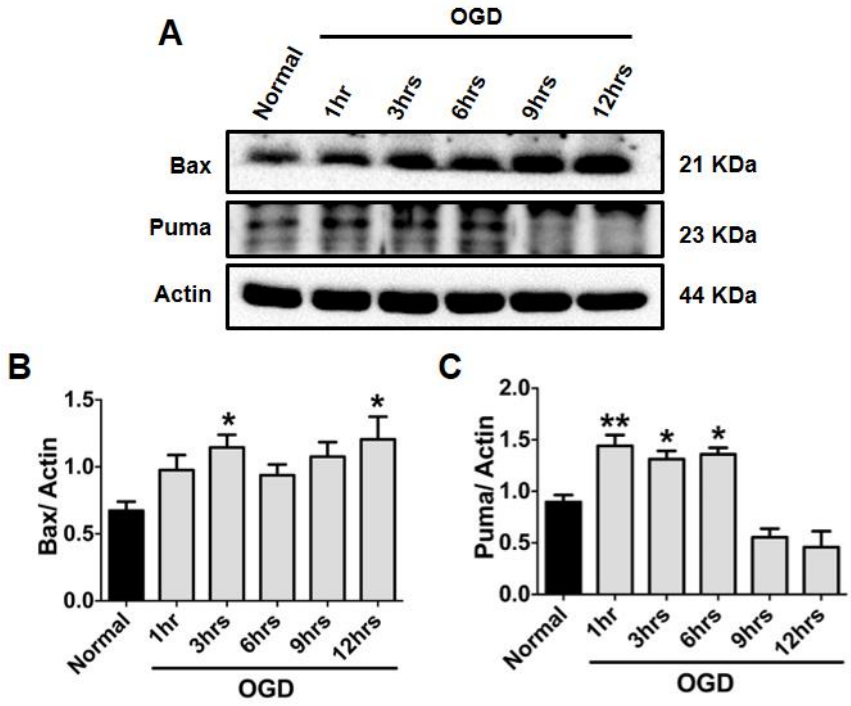


Figure 16: p53 mediated intrinsic apoptotic pathway during ischemic condition.

In vitro ischemia activates the intrinsic apoptotic proteins in primary cortical neurons. Neurons were subjected to OGD and analyzed for apoptotic proteins Bax and Puma to determine the cell death pathway. A-C, Representative immunoblots and analysis of Bax and Puma at the indicated time points during OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=5 cultures. *P<0.05 and **P<0.005 compared with normal cell culture.

The other mechanism of apoptosis is caspase-independent mediated by apoptosis inducing factor (AIF) fragmenting DNA. AIF is subjected to the positive gene regulation controlled by p53 to mediate a caspase-independent cell sensitization and is up-regulated significantly during the ischemic conditions to initiate a large-scale DNA fragmentation (Culmsee et al., 2005; Stambolsky et al., 2006; Cho and Toledo-Pereyra, 2008). Ischemic condition executes p53 mediated cell death also through AIF up-regulation (Fig 17, A and B).

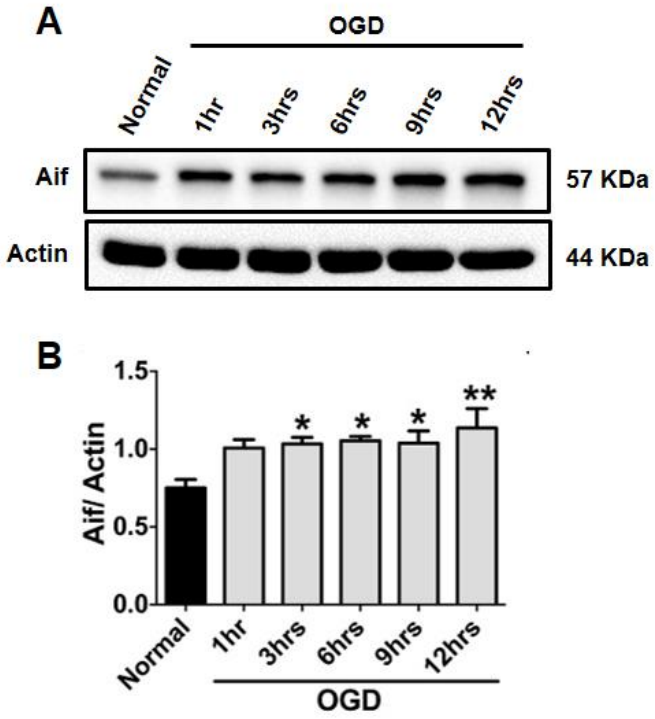


Figure 17: AIF regulated during ischemia

In vitro ischemia triggers AIF-mediated mitochondrial permeabilization of apoptotic proteins in primary cortical neurons. Neurons were subjected to OGD and analyzed for AIF to determine mitochondrial permeability. A and B, Representative immunoblot and analysis of AIF at the indicated time points during OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=5 cultures. **P<0.005 compared with normal cell culture.

3.2 NICD and p53 translocate to the nucleus for transcriptional activity

Both NICD and p53 are transcription factors regulating specific target genes. The presence of these proteins in the cytoplasm does not contribute to its transcriptional role rather translocation of the proteins in the nucleus determines its transcriptional role. After the onset of ischemic condition the translocation of P-p53 and NICD can be observed. The rise in the NICD levels and translocation was investigated in primary cortical neurons (Fig. 18 A) and neuroblastoma SH-SY5Y (Fig. 18 B). Similarly, the activated transcription factor, P-p53 levels and translocation was also investigated in primary cortical neurons (Fig. 19 A) and SH-SY5Y cells (Fig. 19 B).

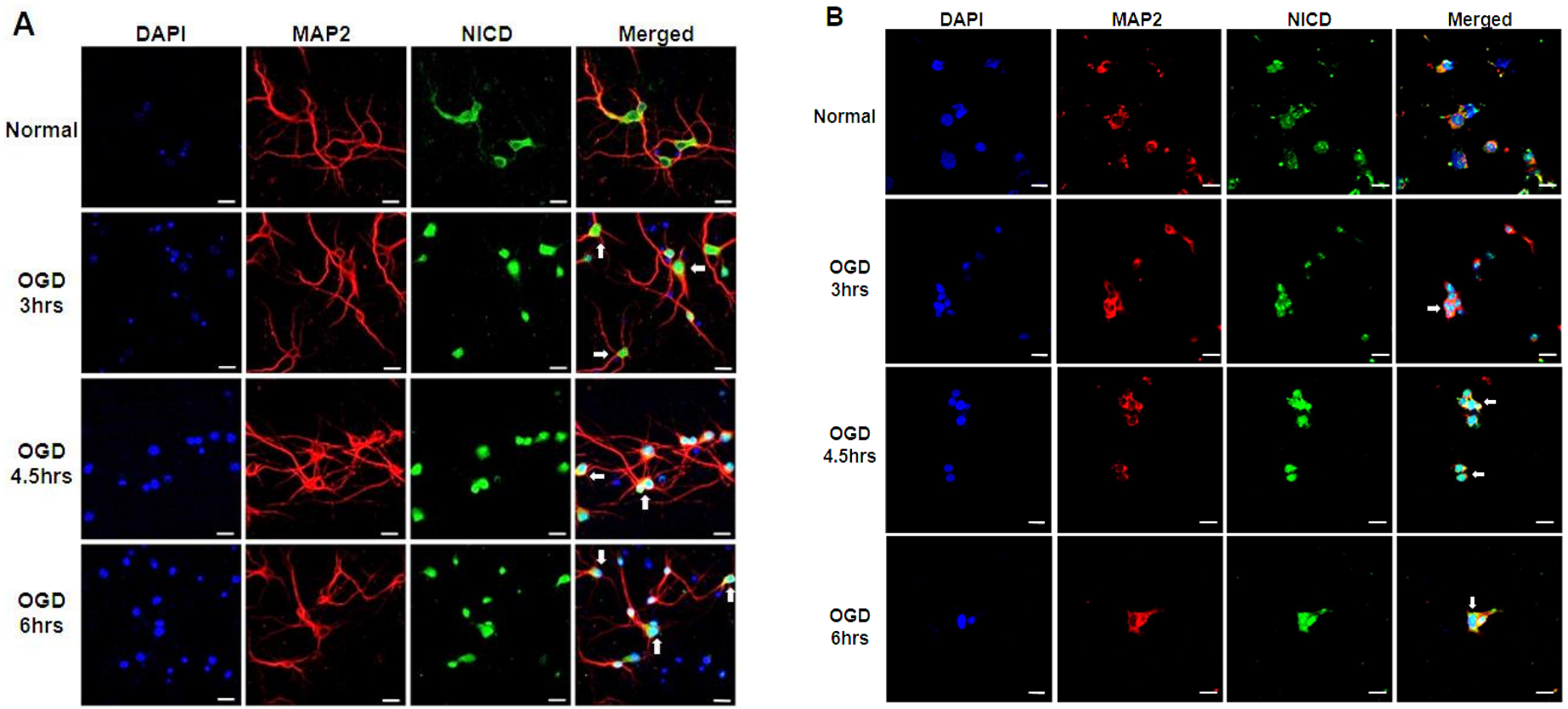


Figure 18: Nuclear translocation of NICD following ischemic onset

I*n vitro* ischemia triggers the translocation of NICD in primary cortical neurons and SH-SY5Y neuroblastoma secondary cell lines. Cells were subjected to OGD at the indicated time points and stained for NICD, MAP2. A and B, Immunofluorescence staining of primary cortical neurons and SH-SY5Y respectively, showing confocal images of NICD (green), the neuronal marker MAP2 (red) and the nuclear marker 4'6-diamidino-2-phenylindol (DAPI) (blue). Both P-p53 staining showed an increase and nuclear translocation during OGD. Scale bar: 20 µm.

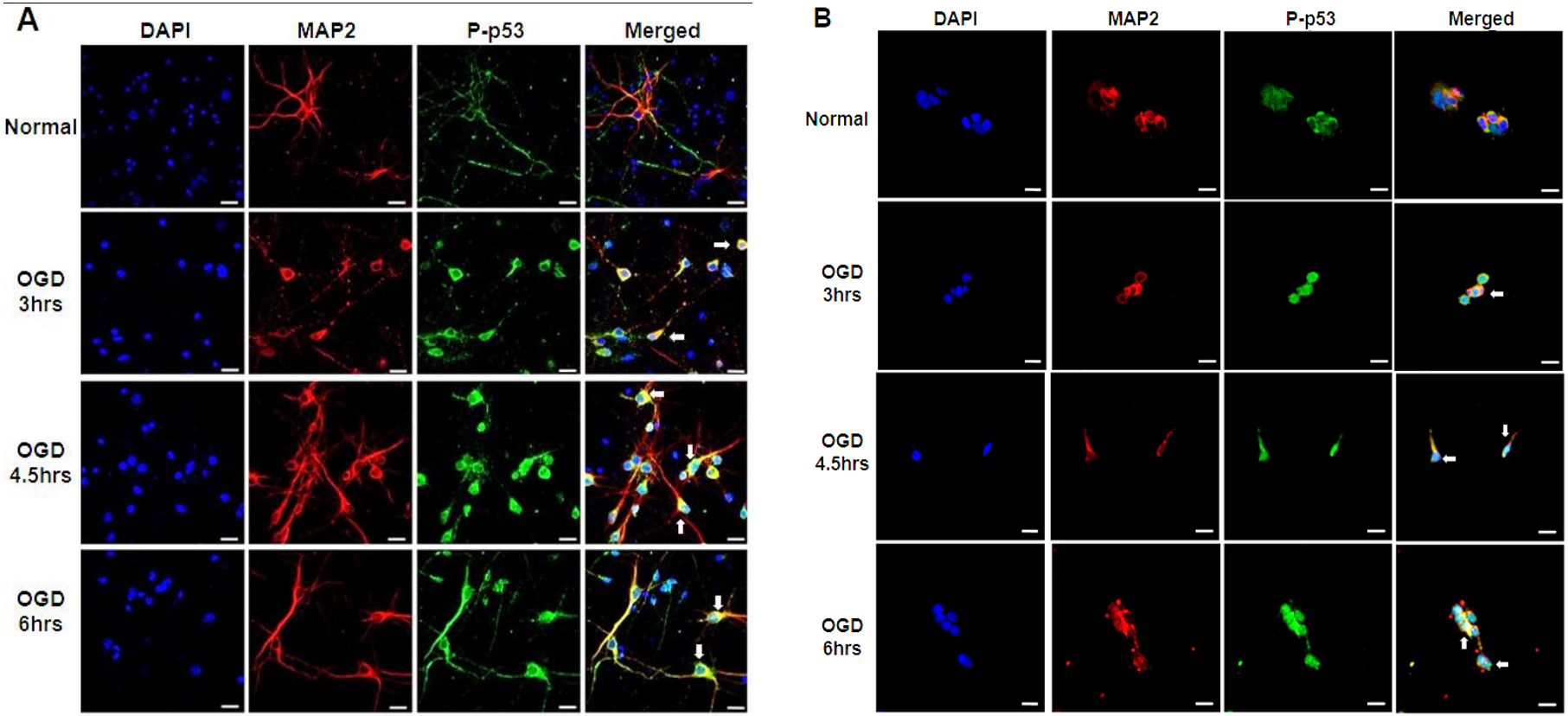


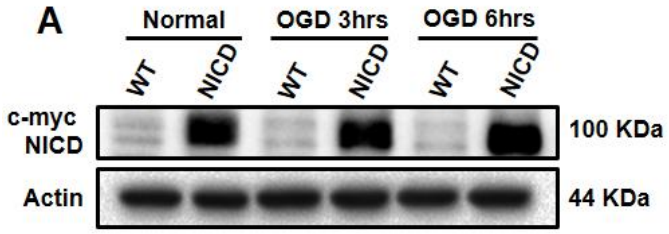
Figure 19: Nuclear translocation of activated p53 following ischemic onset

I*n vitro* ischemia triggers the translocation of p53 in primary cortical neurons and SH-SY5Y neuroblastoma secondary cell lines. Cells were subjected to OGD at the indicated time points and stained for P-p53, MAP2. A and B, Immunofluorescence staining of primary cortical neurons and SH-SY5Y showing confocal images of P-p53 (green), the neuronal marker MAP2 (red) and the nuclear marker 4'6-diamidino-2-phenylindol (DAPI) (blue). Both P-p53 staining showed an increase and nuclear translocation during OGD. Scale bar: 20 µm

3.3 Notch receptor activation regulates p53 mediated cell death pathway

3.3.1 Exogenous NICD expression mediates endogenous p53 activation and regulates apoptosis

To determine the mechanism of activated Notch receptor in cell death pathway NICD over-expressed model was selected. p53 mediated pathway in the exogenous NICD expressing groups at basal condition and ischemic condition was demonstrated. The over-expression of myc tagged NICD (myc-NICD) levels were probed in basal and i*n vitro* ischemic conditions (Fig. 20 A). The p53 activation levels significantly raised in the myc-NICD expressing groups at basal and ischemic condition (Fig. 20 B and C). Consistently, P-p53 mediated expression of Bax and Puma elevates in the myc-NICD groups compared to the endogenous NICD group during basal and ischemic condition. A significant rise of Bax and Puma observed in later time of the OGD treatment (Fig. 20 B, D and E) signifying the elevated NICD mediates an apoptotic signal by regulating p53 activation.



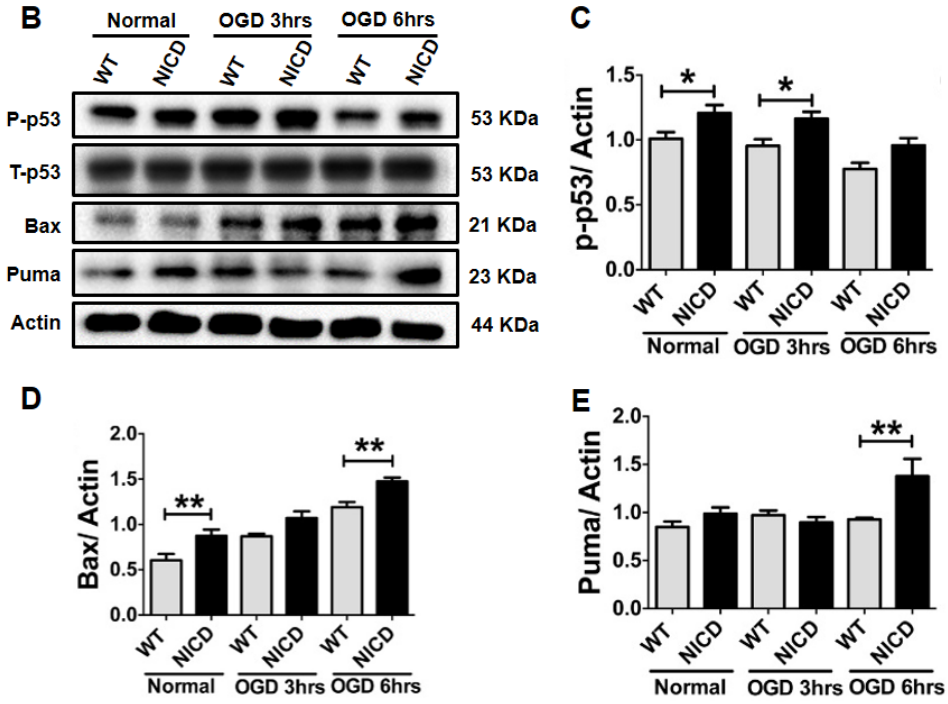


Figure 20: Exogenous NICD mediates ischemic apoptosis

NICD overexpression triggers p53-mediated apoptotic cell signaling. HEK 293T cells were subjected to OGD at the indicated time points and analyzed for NICD and p-53 signaling proteins. A, Representative immunoblot of exogenous myc tagged NICD over-expressed protein in HEK 293T cells at the indicated time points during OGD. B-E, Representative immunoblots and analysis of P-p53, Bax and Puma in HEK 293T myc tagged NICD-overexpressing cells at the indicated time points during OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05 and **P<0.005 compared with wild-type control.

3.3.2 Exogenous NICD expression affects cellular anti-apoptotic mechanism

Bcl-2 is a protein with the anti-apoptotic role. It executes its mechanism by interacting with the apoptotic protein, Bax to inhibit the promotion of mitochondrial permeabilization. The Bcl-2 profile is repressed by p53 upon cellular insult, liberating the apoptotic protein from Bcl-2 inhibition. Interestingly, the myc-NICD expressing population of cells has decreased Bcl-

2 protein levels at basal and ischemic conditions (Fig. 21 A and B) with a significant decrease during the time of OGD 6hrs. This observation correlates with the rise of apoptotic proteins observed (Fig. 20 B, D and E) at the same time point in the myc-NICD expressing groups denoting the positive control of NICD over p53 transactivation.

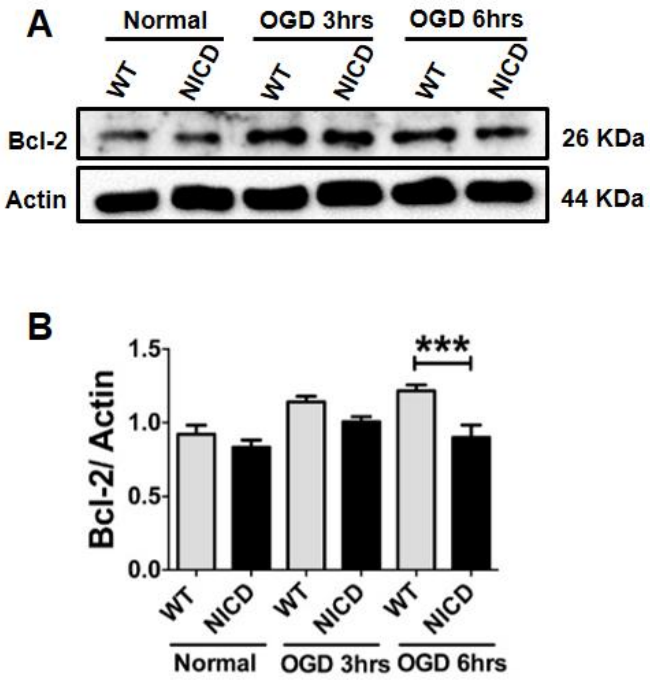


Figure 21: Exogenous NICD expression decreases cellular antiapoptotic role during ischemia

NICD overexpression decrease anti-apoptotic protein levels. HEK 293T cells were subjected to OGD at the indicated time points and analyzed for anti-apoptotic protein, Bcl-2. A and B, Representative immunoblots and analysis of Bcl-2 in HEK 293T myc tagged NICD-overexpressing cells at the indicated time points during OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. ***P<0.001 compared with wild-type control.

3.4 Inhibiting Notch receptor activation rescues cell from p53 signaling-mediated demise

3.4.1 Notch activation regulates γ-secretase enzyme inhibition demonstrated in non-neuronal cells for mechanistic study

3.4.1a γ–secretase enzyme inhibition decreases apoptotic signaling following notch receptor cleavage

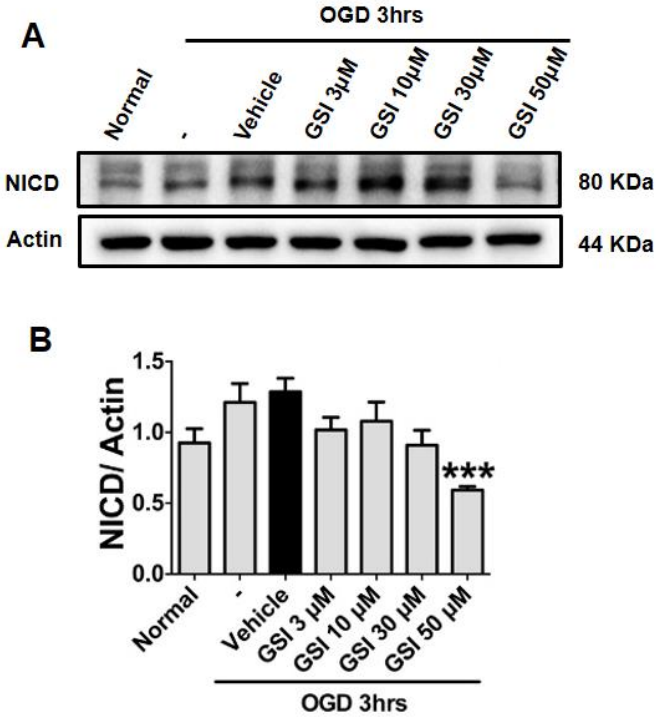


Figure 22: γ–secretase inhibition (GSI) induced decrease Notch receptor activation during ischemia

NICD generation decreases during γ–secretase inhibition (GSI). Cells were subjected to OGD at the indicated time point and analyzed for NICD. A and B, Representative immunoblot and analysis of NICD in HEK 293T cells treated with different concentrations of GSI or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. ***P<0.001 compared with vehicle-treated control.

γ-secretase enzyme executes the activation of Notch receptor and the inhibition of its enzyme property limits the cleavage of Notch. Using WT HEK 293T cell lines p53 mediated activity was determined during the inhibition of

the Notch-activating enzyme using Gamma-secretase inhibitor, GSI – DAPT (3-50 μM) under *in vitro* ischemic conditions. As expected, 3hrs of OGD induced NICD was reduced significantly by GSI – DAPT at 50 μM concentration (Fig. 22 A and B). Intriguingly, the OGD-induced increase in P-p53 was also attenuated by GSI treatment (Fig. 23 A and B). Coherently, p53 mediated apoptotic proteins Bax and Puma expression in OGD condition were significantly down-regulated by GSI treatment (Fig. 23 A, C and D).

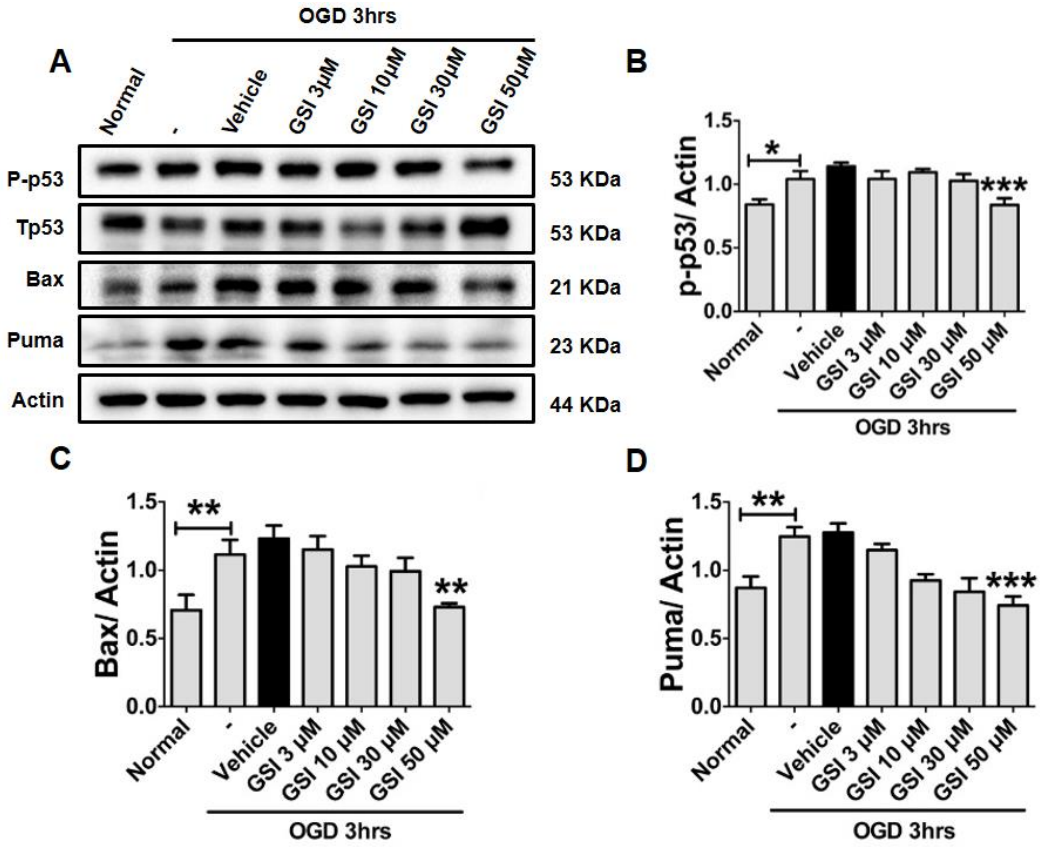


Figure 23: Notch inhibition suppresses ischemia triggered p53 activation and transcription

p53-mediated apoptotic cell signaling, suppressed by Notch inhibition. Cells were subjected to OGD at the indicated time point and analyzed for p53 signaling proteins. A-D, Representative immunoblots and analysis of P-p53, Bax, Puma, in HEK 293T cells treated with different concentrations of GSI or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

3.4.1b γ–secretase enzyme inhibition positively regulates the anti-apoptotic signal

Using WT HEK 293T cell lines p53 mediated activity was determined during the inhibition of the Notch-activating enzyme using GSI – DAPT (3-50 μM) under *in vitro* ischemic conditions. The anti-apoptotic protein negatively regulated by p53 during 3hrs of OGD was found to increase with GSI treatment at 50 μM (Fig. 24 A and B).

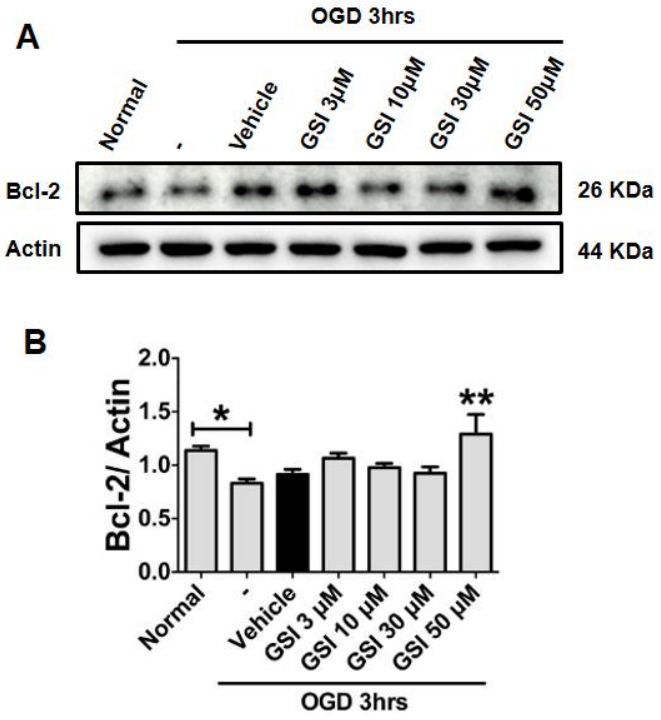
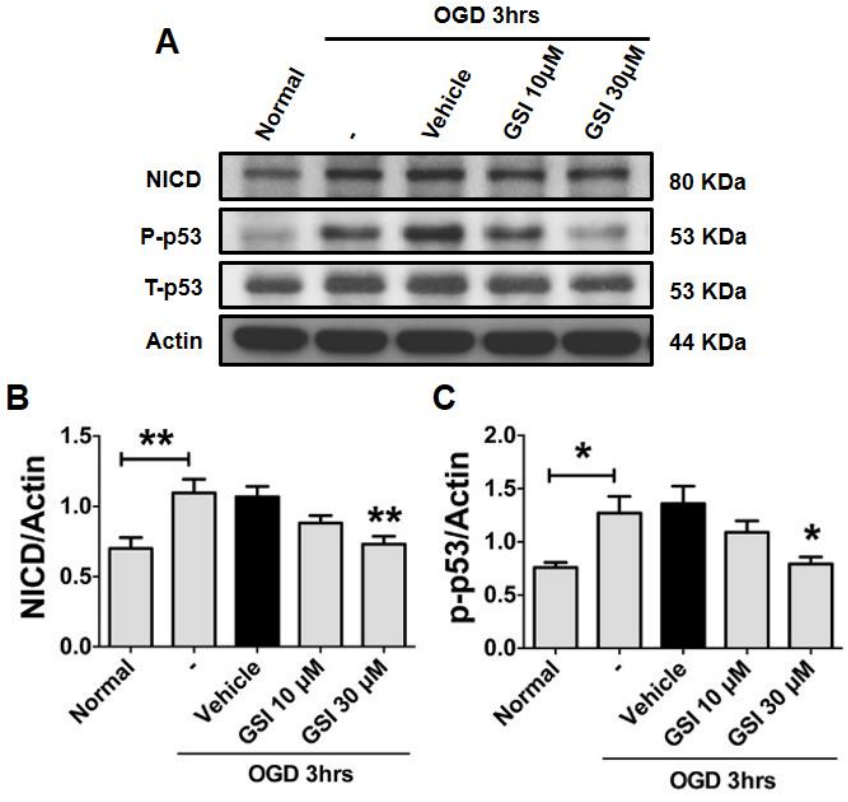


Figure 24: Notch inactivation benefits cellular antiapoptotic role during ischemic condition

Suppression of Notch signaling positively regulates anti-apoptotic property of the cell. Cells were subjected to OGD at the indicated time point and analyzed for anti-apoptotic protein, Bcl-2. A and B, Representative immunoblots and analysis of Bcl-2, in HEK 293T cells treated with different concentrations of GSI or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05 and **P<0.005 compared with vehicle-treated control.

3.4.2 Notch activation controlling γ-secretase enzyme inhibition demonstrated in SH-SY5Y neuroblastoma cells

γ-secretase enzyme executes the activation of Notch receptor and the inhibition of its enzyme property limits the cleavage of Notch. Using neuroblastoma SH-SY5Y cell lines p53 mediated activity was determined during the inhibition of the Notch-activating enzyme using Gamma-secretase inhibitor, GSI – DAPT (10 and 30 μM) under i*n vitro* ischemic conditions. As expected, NICD and P-p53 was reduced significantly by GSI – DAPT at 30 μM concentration during 3hrs of OGD (Fig. 25 A-C) and 6hrs of OGD (Fig. 25 D-F) signifying the effect of Notch receptor signaling in p53 activation.



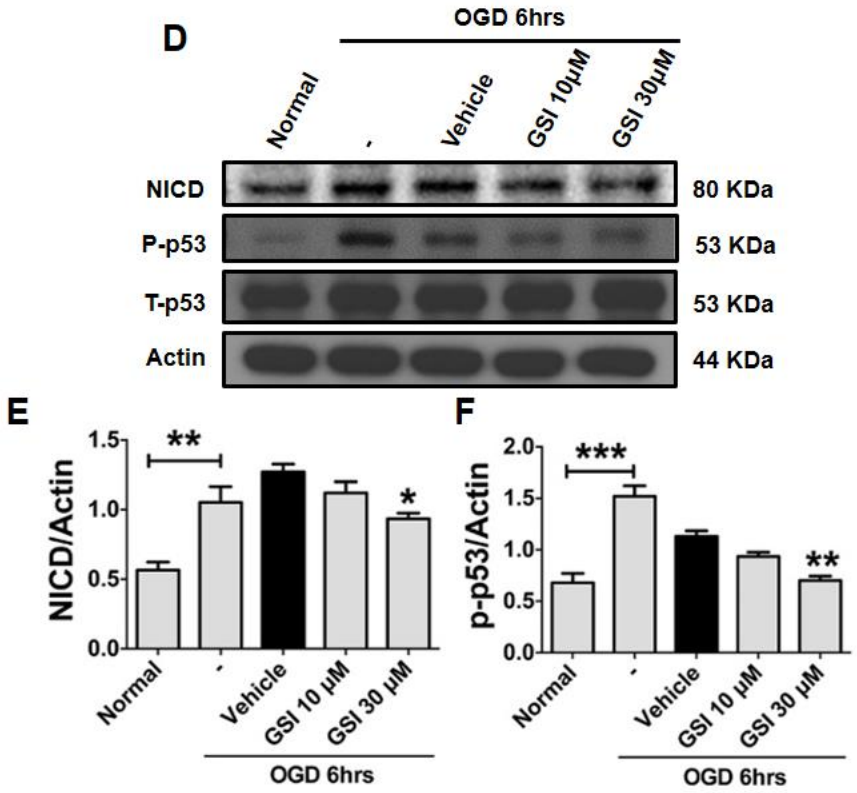


Figure 25: GSI induced Notch inhibition negatively regulates p53 activation during ischemia in neuronal secondary cells

Notch receptor controls the p53 death signaling in secondary cells of neuronal origin. The cells were subjected to OGD at the indicated time points and analyzed Notch and p53. A-F, Representative immunoblots and analysis of NICD and P-p53 in SH-SY5Y culture treated with different concentrations of GSI or vehicle during 3hrs (A-C) and 6hrs (D-F) of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

3.4.3 Notch activation inhibition demonstrated in primary cortical neurons

To confirm the consistency in the observed findings, p53 mediated cell death was determined during the inhibition by Gamma-secretase inhibitor, GSI – DAPT (3-30 μM) under i*n vitro* ischemic condition using primary cortical neuron cultures. OGD induced cleaved form of executioner caspase-3, as

expected, was reduced significantly by GSI – DAPT treatment (Fig. 26 A and B) signifying the cellular death promoted by notch activation.

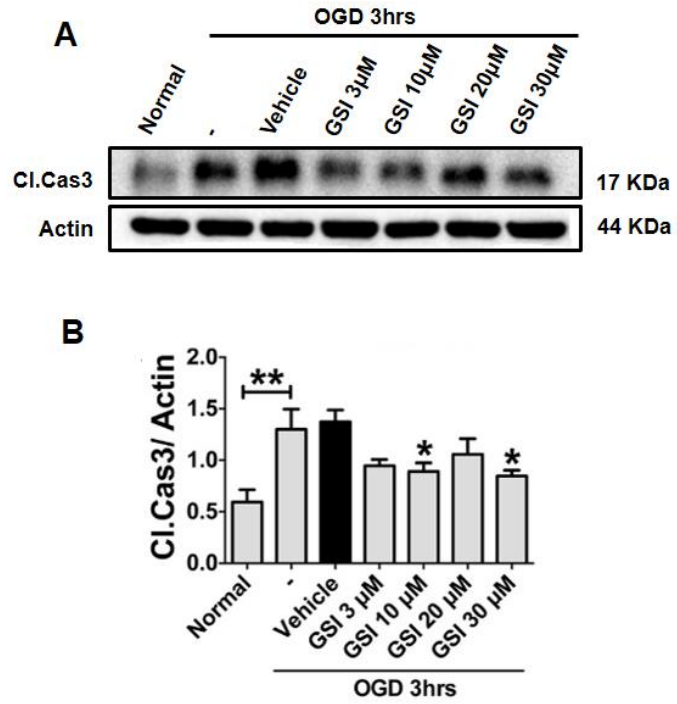


Figure 26: Inhibition of executioner caspase activation following ischemia by notch inactivation

Rescue of neuronal cell death during ischemia attained by notch inhibition. The cells were subjected to OGD at the indicated time point and analyzed for apoptotic-marker. A and B, Representative immunoblot and analysis of cleaved-caspase-3 in primary cortical neuron culture treated with different concentrations of GSI or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05 and **P<0.005 compared with vehicle-treated control.

In addition to demonstrated cell death protein study Notch and p53 activity was investigated. As expected, the OGD induced increase in Notch activation was attenuated by GSI treatment. OGD mediated NICD increase was attenuated during 3hrs of OGD treated with GSI treatment (Fig. 27 A and B).

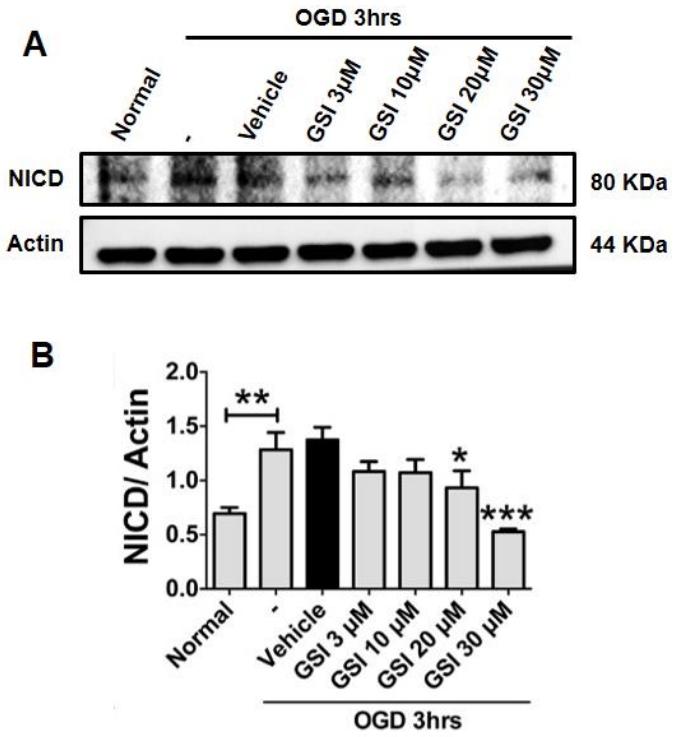


Figure 27: GSI induced decrease of Notch activation during ischemia in primary neuronal cells

GSI decreases the generation of activated notch upon ischemia. The cells were subjected to OGD at the indicated time point and analyzed for Notch activation. A and B, Representative immunoblot and analysis of NICD in primary cortical neuron culture treated with different concentrations of GSI or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

Consistently, a coherent inhibition of p53 activity with GSI treatment in primary cortical neurons was observed. OGD induced increase in P-p53 was attenuated by GSI treatment (Fig. 28 A and B). p53 regulated apoptotic protein Bax expression in 3hrs of OGD condition was also decreased by GSI treatment (Fig. 28 A and D).

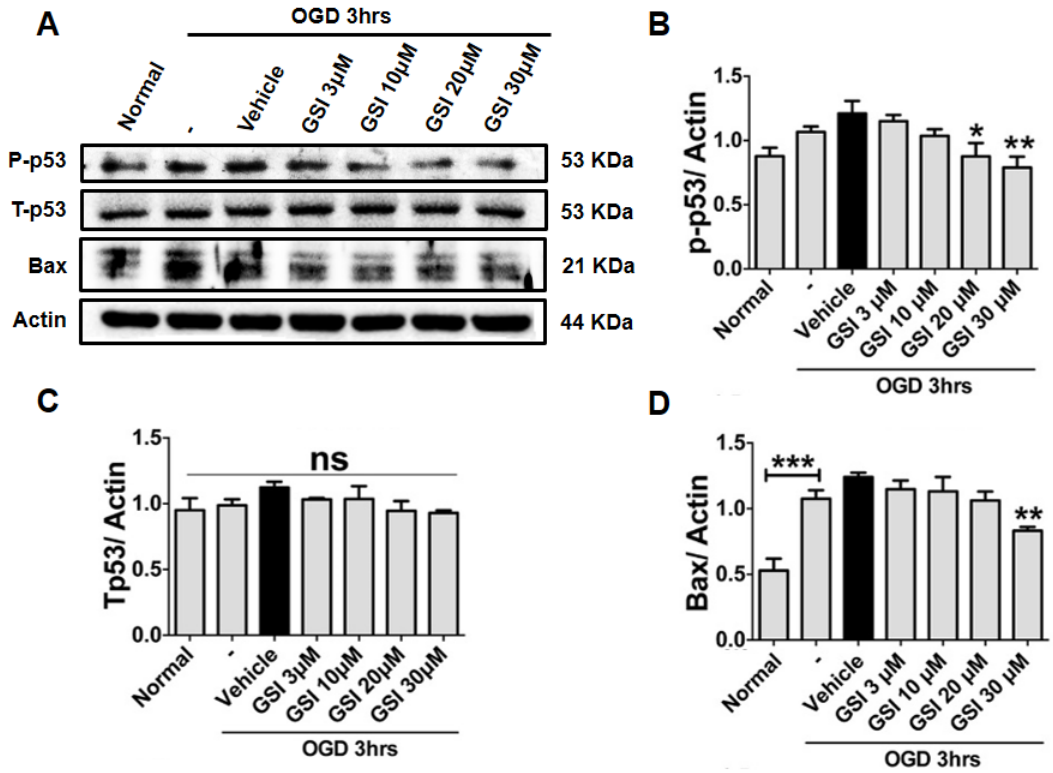


Figure 28: Notch inactivation negatively regulates p53 activation and transcriptional role

Transcriptional role of p53 is regulated by Notch. The cells were subjected to OGD at the indicated time point and analyzed for p53 apoptotic signaling proteins. A-D, Representative immunoblots and analysis of P-p53, Tp53 and Bax in primary cortical neuron culture treated with different concentrations of GSI or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

3.5 p53 regulates the activation of NICD and its transcriptional role

3.5.1 p53 inhibiting compound toxicity screening

Subsequently, the effect of p53 on Notch activation was investigated by analyzing the expression of NICD in primary cortical neurons during OGD in combination with p53 inhibition. Thus the p53 inhibitor Pifithrin-α (PFT) was tested initially for toxicity at a concentration ranging from 300 nM - 20 μM.

Lactate dehydrogenase (LDH) release from primary cortical neurons subjected to *in vitro* ischemia for 6hrs was found to be reduced significantly with the PFT treatment (Fig. 29 A) denoting minimal cellular damage. In consistency to minimized cellular insult, 3hrs of OGD induced cell death marker, cleaved-caspase-3 was reduced with PFT treatment at 20 μM (Fig. 29 B and C).

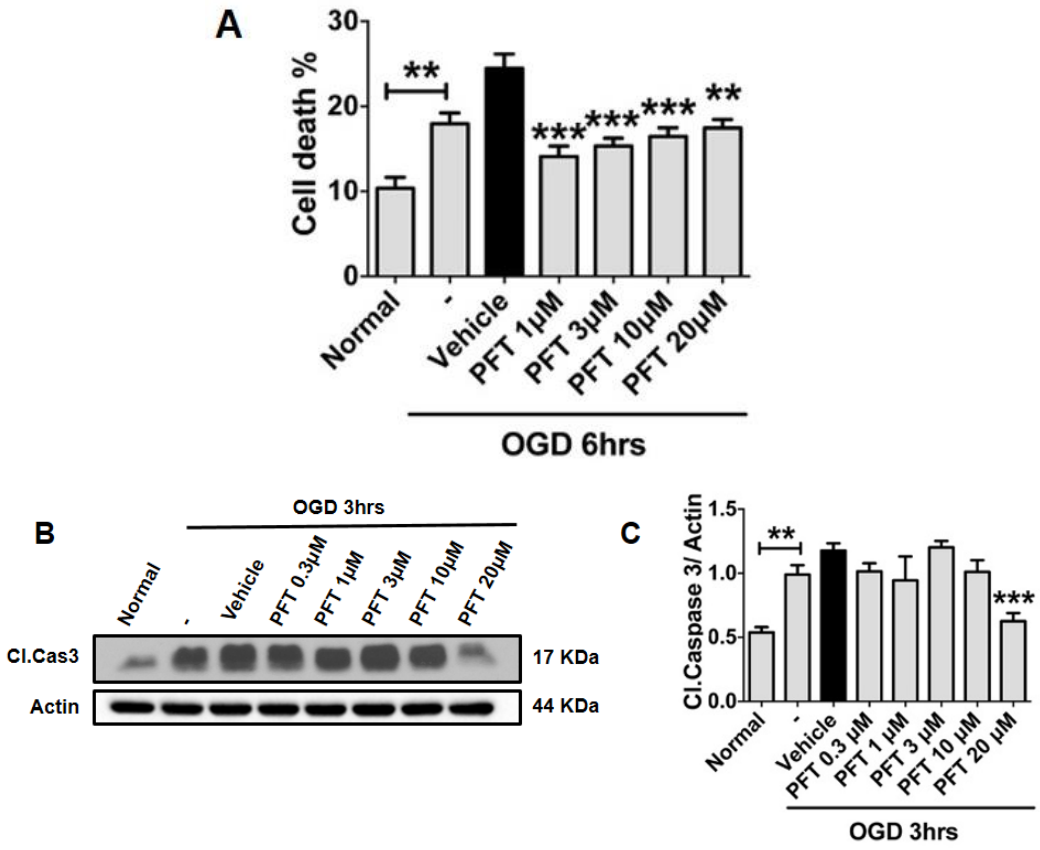


Figure 29: p53 inhibiting compound, Pifithrin-α (PFT) decreases cell toxicity during ischemic condition

Pifithrin-α (PFT) decreases cell toxicity caused by ischemic treatment by decreasing p53 activation. The cells were subjected to OGD at the indicated time point and analyzed for apoptotic marker and LDH release. A, cell death analyzed by detecting LDH released by primary cortical neuron cultures treated with different concentration of PFT or vehicle during 6hrs of OGD. B and C, Representative immunoblot and analysis of apoptotic marker, cleaved-Caspase-3 in primary cortical neuronal cultures treated with different concentration of PFT or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. **P<0.005 and ***P<0.001 compared with vehicle-treated control.

3.5.2 Inhibition of p53 activation negatively regulates notch activation and its transcriptional role

Effect of p53 inhibition on its activation was established in primary cortical neurons during 3hrs of OGD. The p53 inhibitor PFT was treated in cultures subjected to OGD and the data indicated the decrease of induced P-p53 levels during ischemic condition (Fig. 30 A and B). Surprisingly, the Tp53 levels were found to be decreased as well (Fig. 30 A and C).

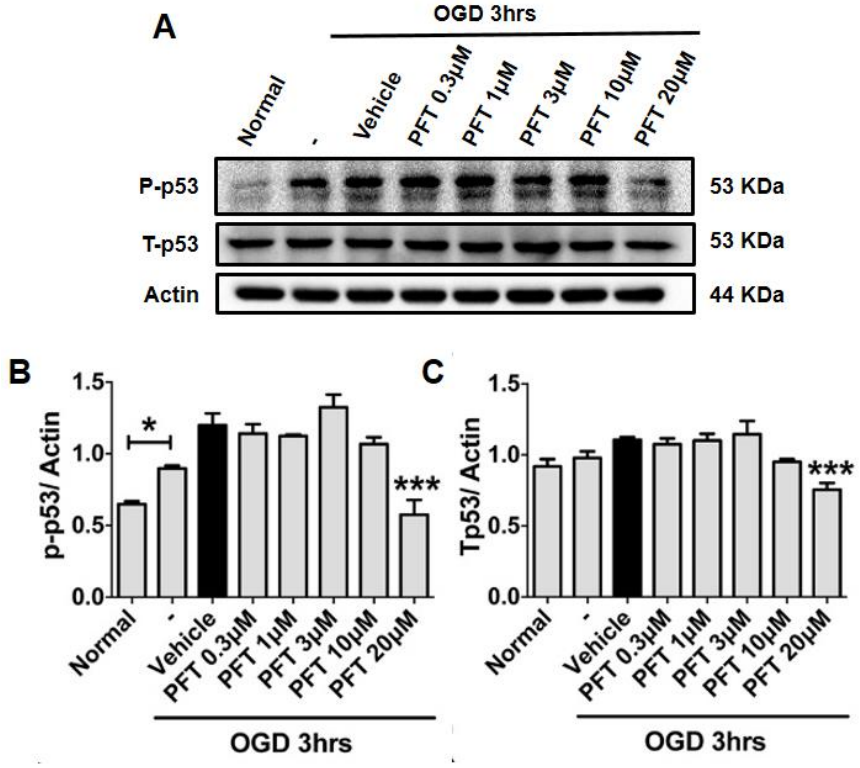


Figure 30: Pifithrin-α (PFT) controls p53 expression and activation

Pifithrin-α (PFT) decreases p53 activation mediated by ischemic treatment. The cells were subjected to OGD at the indicated time point and analyzed for p53 protein. A-C, Representative immunoblots and analysis of P-p53 and Tp53 in primary cortical neuronal cultures treated with different concentration of PFT or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05 and ***P<0.001 compared with vehicle-treated control.

The effect of p53 inhibition indicated that p53 regulates Notch receptor activation and thus its transcriptional function. Cultures subjected to OGD and PFT treatment indicated the OGD induced Notch activation was attenuated with p53 inhibition (Fig. 31 A and B). Coherently, the NICD target, Hes-1 was decreased in PFT treatment during 3hrs of OGD condition (Fig. 31 A and C).

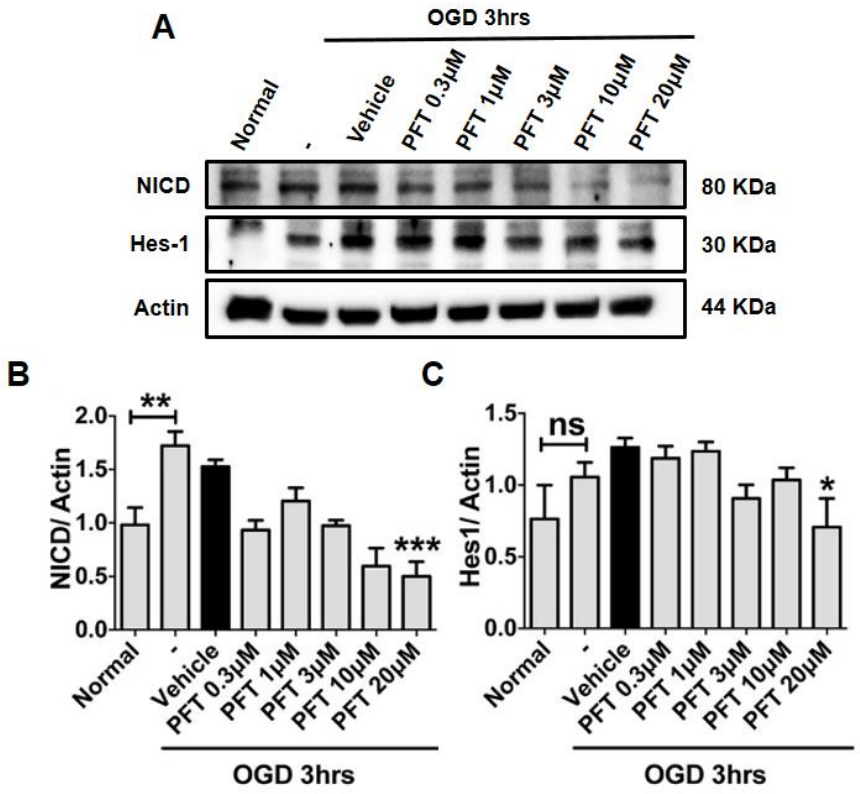


Figure 31: p53 inactivation decreases notch receptor activity consequently its transcriptional role

p53 mediates notch activation during ischemic treatment. The cells were subjected to OGD at the indicated time point and analyzed for notch pathway. A-C, Representative immunoblots and analysis of NICD and Hes-1 in primary cortical neuronal cultures treated with different concentration of PFT or vehicle during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

3.6 Interaction of NICD and P-p53 and neuronal nuclear co-localization occurs during cellular ischemia

To investigate Notch and p53 collaboration at the nuclear level, Immunostaining and co-immunoprecipitation were performed in different cultures. Immunostaining of NICD (Fig. 32 A, red; Fig. 33 A, green) and P-p53 (Fig. 32 A, green; Fig. 33 A, red) in cells subjected to OGD confirmed increased levels and co-localization of NICD and p53 in the nucleus of neuroblastoma SHSY5Y cell lines (Fig. 32 A) and Primary cortical neurons (Fig. 33 A) in response to ischemic condition. Co-immunoprecipitation in neuronal cells demonstrated NICD and p53 physically interact together in primary cortical neurons (Fig. 32 B and C) and in SH-SY5Y cells (Fig. 33 B) under both normal and OGD conditions.

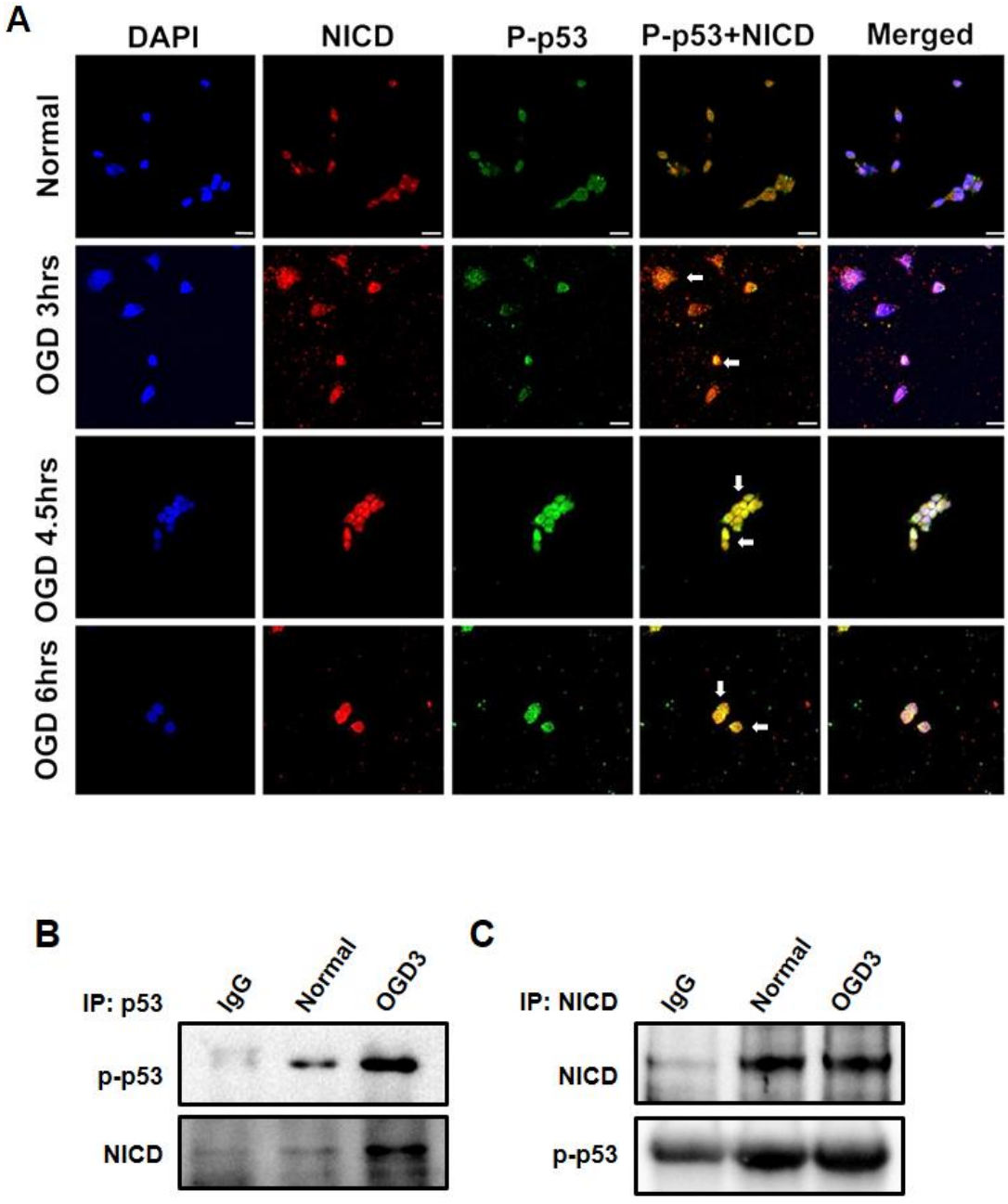


Figure 32: Nuclear co-localization and interaction of transcription factors NICD and P-p53 in SH-SY5Y neuroblastoma cells

In vitro ischemia promotes interaction and neuronal nuclear co-localization of both transcription factors. The cells were subjected to OGD at the indicated time points for immunoprecipitating NICD/p53 complex and for staining NICD and P-p53. A, Immunofluorescence staining of SH-SY5Y cultures showing confocal images of NICD (Red), P-p53 (Green), and nuclear marker 4'6-diamidino-2-phenylindol (DAPI) (blue). Both NICD and P-p53 staining are increased and co-localized at the indicated OGD time points. Scale bar: 20 µm. B and C, Representative immunoblots of NICD and p53 co-immunoprecipitation in SH-SY5Y cultures following 3hrs of OGD.

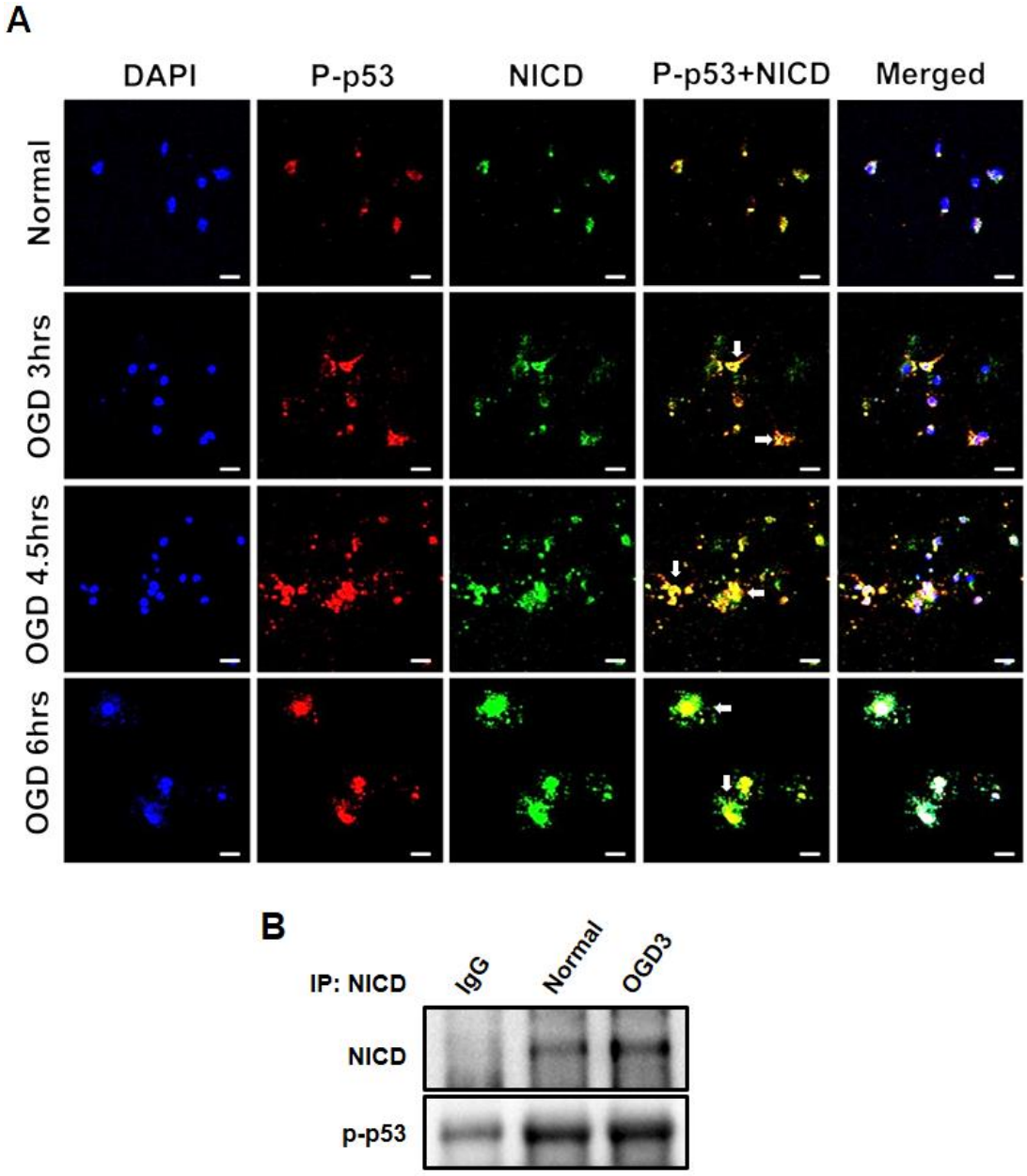


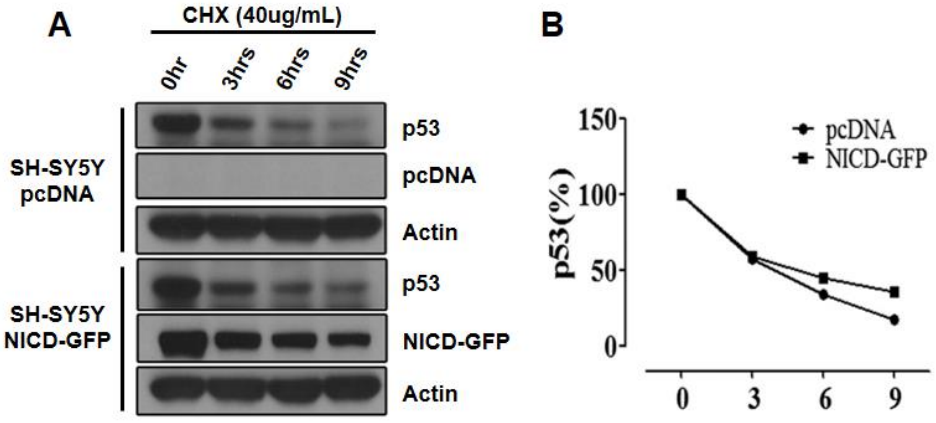
Figure 33: Nuclear co-localization and interaction of transcription factors NICD and P-p53 in primary cortical neurons

A, Immunofluorescence staining of primary cortical neuron cultures showing confocal images of P-p53 (Red), NICD (Green) and nuclear marker 4'6-diamidino-2-phenylindol (DAPI) (blue). Both NICD and P-p53 staining are increased and co-localized at the indicated OGD time points. Scale bar: 20 µm. B and C, Representative immunoblots of NICD and p53 co-immunoprecipitation in primary cortical neuron cultures following 3hrs of OGD.

3.7 NICD promotes stability of p53

3.7.1 Exogenous NICD regulates stability of the WT-p53

To test whether NICD regulates p53 stability, a GFP-tagged NICD-overexpressed SH-SY5Y cell line was employed, and p53 stability was assessed using cycloheximide (CHX) chases. The data indicated NICD overexpression increased endogenous p53 stability in comparison to pcDNA-transfected cells over 9 h in the presence of CHX chases (Fig. 34 A and B). Subsequently, exogenous p53 turnover was demonstrated in SH-SY5Y cells transiently transfected with NICD-MYC or pcDNA and WTp53-HA. NICD overexpression increased exogenous WT p53 stability over 9 h in the presence of CHX chases (Fig. 34 C and D).



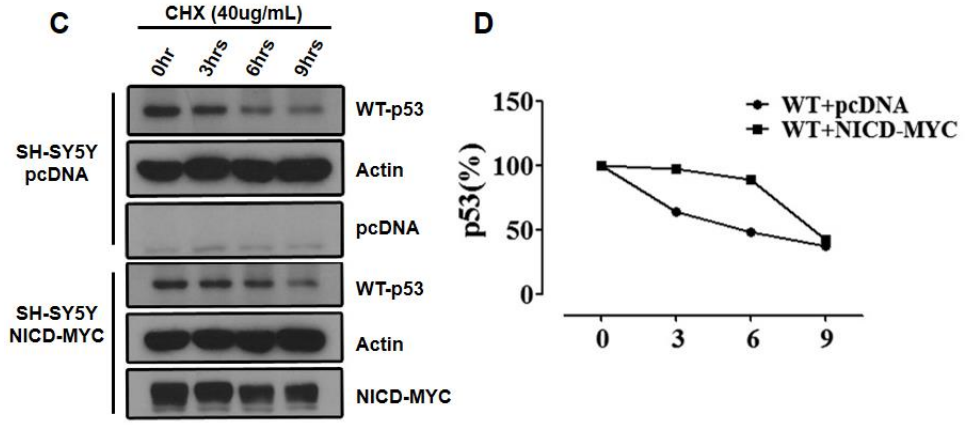


Figure 34: Stability of WTp53 increases in the presence of exogenous NICD

NICD increases the stability of WT-p53. pcDNA and NICD-GFP were transiently transfected in SH-SY5Y cells and after 24hrs, the cells were treated with 40 ug cycloheximide (CHX) during the indicated time points. A and B, Representative immunoblots and analysis showing p53 stabilization in NICD-GFP overexpressing cells. WTp53-HA and pcDNA or NICD-GFP were transiently transfected in SH-SY5Y cells and after 24hrs, the cells were treated with 40 ug cycloheximide (CHX) during the indicated time points. C and D, Representative immunoblots and analysis showing exogenous WTp53-HA stabilization in NICD-MYC overexpressing cells. Statistical analysis were not done due to insufficient N.

3.7.2 NICD fails to improve stability of p53 mutants due to loss of interaction

To understand the mechanism involved in NICD regulation of p53 stability, exogenous mutated p53 turnover was demonstrated in SH-SY5Y cells transiently transfected with NICD-MYC or pcDNA and N-terminal deleted p53 (ΔN)-p53-HA or DNA binding domain (DBD)-p53-HA or C-terminal deleted (ΔC)-p53-HA in the presence of CHX chases. The data indicated that stability of the exogenous HA-tagged (ΔN)-p53 (Fig. 35 A and B), (DBD)-p53 (Fig. 35 C and D) and (ΔC)-p53 (Fig. 35 E and F) were unrestored by NICD overexpression, a quality observed in WTp53 stability (Fig. 34 C and D) over 9 h in the presence of CHX chases.

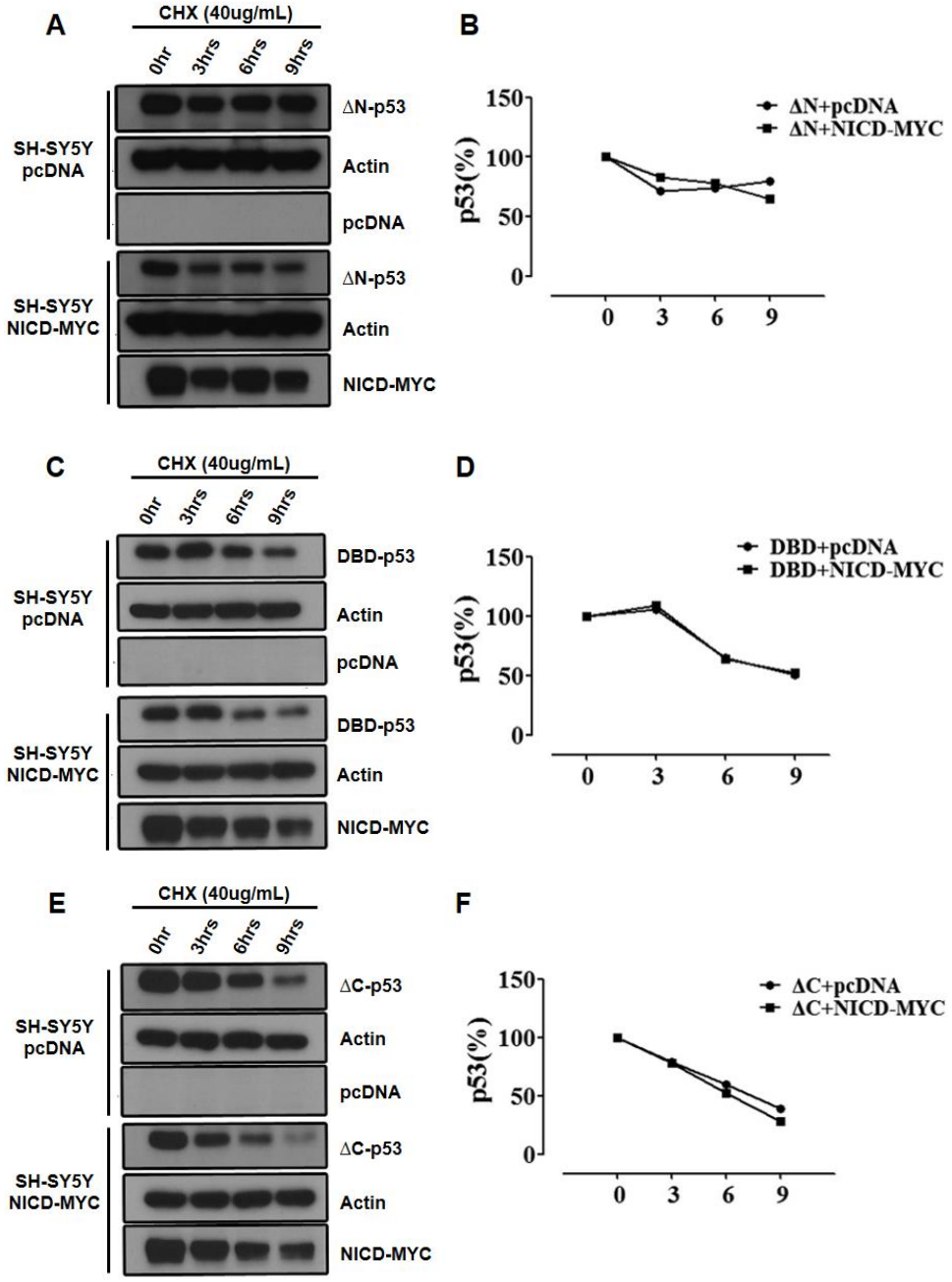


Figure 35: Mutated exogenous p53 stability remains unchanged during NICD presence

NICD fails to improve the stability of exogenous mutated p53-HA. SH-SY5Y cells were transiently transfected with ΔNp53-HA, DBDp53-HA, ΔCp53-HA and NICD-MYC or pcDNA and after 24hrs, the cells were treated with 40 ug cycloheximide (CHX) during the indicated time points. A-F, Representative immunoblots and analysis showing the stability of p53 deletion mutants following overexpression of NICD-MYC. Statistical analysis were not done due to insufficient N.

NICD promotes WTp53 stability, whereas fails to restore mutant p53 stability, hence, NICD association with the exogenous WT and mutant p53 was demonstrated. Co-immunoprecipitation was performed in SH-SY5Y cells transiently transfected with NICD-MYC and WTp53-HA or ΔNp53-HA or DBDp53-HA or ΔCp53-HA after 24hrs of transfection. Co-immunoprecipitation with an anti-HA antibody indicated that NICD interaction decreased with ΔNp53-HA, DBD-p53 and ΔCp53-HA groups (Fig. 6A and B) signifying the interaction is crucial for p53 stability.

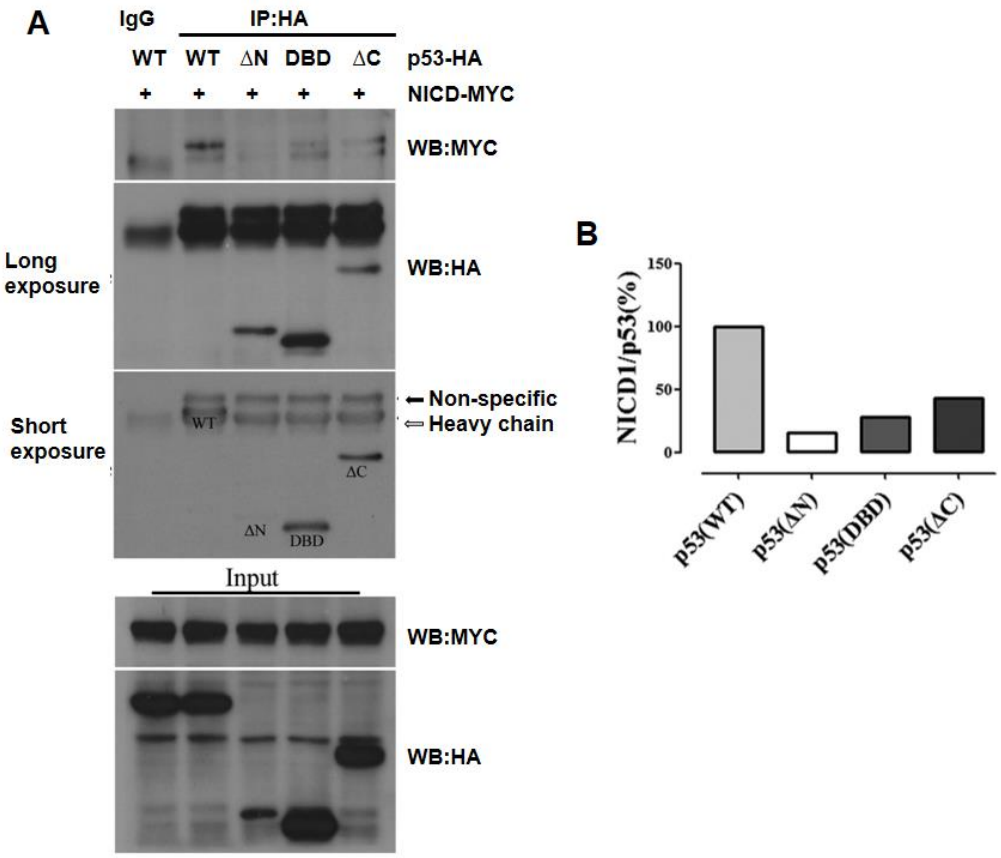


Figure 36: NICD interaction with WT-p53 and mutants

NICD interaction with p53 is facilitated by all p53 domains. SH-SY5Y cells were transiently transfected with WTp53-HA, ΔNp53-HA, DBDp53-HA, ΔCp53-HA and NICD-MYC or pcDNA and Immunoprecipitation was carried out with anti-HA. A and B, Representative immunoblot and analysis showing NICD binding with p53 is decreased in ΔNp53-HA, DBD-p53 and ΔCp53-HA domain-deleted mutants compared to WTp53-HA following overexpression of NICD-MYC. Statistical analysis were not done due to insufficient N.

3.8 NICD/p53 complex reduces p53 ubiquitination

p53 protein stability is regulated by MDM2 mediation of the ubiquitin-proteasome pathway (UPP) (Levine, 1997; Wu and Levine, 1997). As NICD was also found to regulate p53 stability, the effects of NICD on p53 ubiquitination was demonstrated. SH-SY5Y cells were transfected with HIS-tagged ubiquitin (Ub-HIS), MYC-NICD, or HA-p53, and then treated with 26S proteasome activity blocker, MG132 for 12hrs. Co-immunoprecipitation using an anti-HA antibody showed that polyubiquitinated p53 was markedly reduced by NICD overexpression. In addition, interaction with E3 ubiquitin-protein ligase MDM2 was markedly reduced by NICD overexpression (Fig. 37).

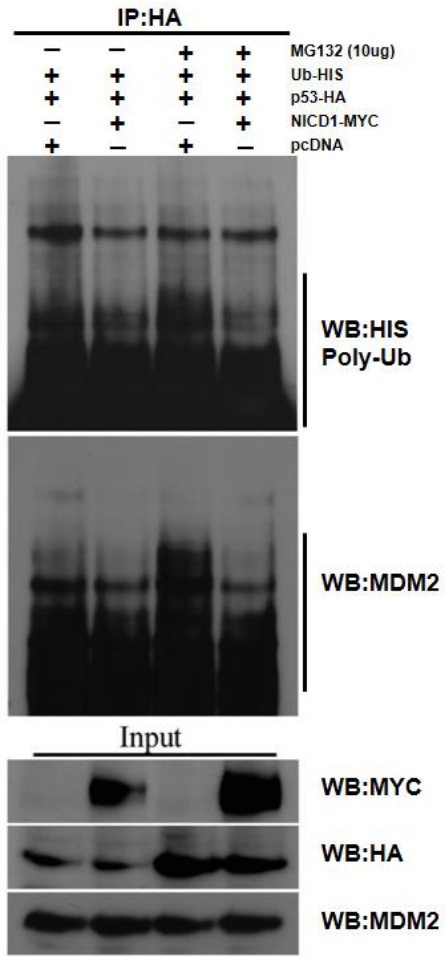


Figure 37: NICD/p53 complex decreases the binding of p53 with its negative regulator MDM2 mediating reduced ubiquitination of p53

Interaction of NICD-p53 reduces p53 ubiquitination mediated by its negative regulator, MDM2. pcDNA, NICD-Myc, Ub-HIS and p53-HA were transiently transfected into SH-SY5Y cells and after 24hrs, the cells were treated with proteasomal inhibitor MG132 (10 μg) for 9hrs. A co-immunoprecipitation representative blot shows reduced p53-polyubiquitination and a reduced interaction between p53 and MDM2 upon overexpression of NICD.

3.9 p53 associates with NICD at the N-Terminus ANKYRIN (ANK) domain

Notch intracellular domains include RAM, ANK, TAD, OPA and PEST. Using deletion mutants of NICD, regions p53 interaction was sought to be identified. HEK293T cell lines stably expressing Myc-tagged WT NICD or individual domain-deleted NICD mutants (WT, ΔRAM, ΔANK, ΔTAD, ΔOPA, or ΔPEST) were established. NICD mutant cells were co-immunoprecipitated using anti-Myc antibodies. A reduction in the interaction between NICD and p53 was observed in the ANK domain-deleted mutant (Fig. 38).

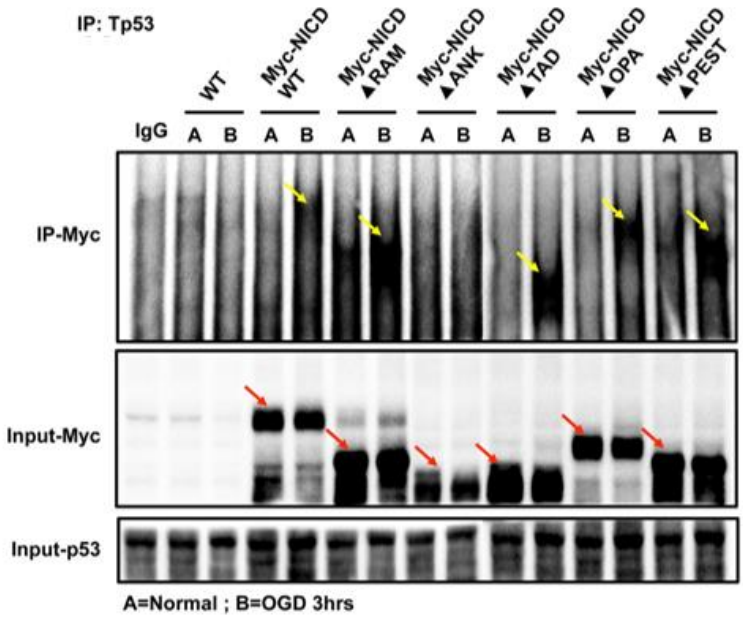


Figure 38: p53 associates at the N-Terminus ANKYRIN (ANK) domain of NICD

NICD associates with p53 at the N-Terminus ANKYRIN (ANK) domain. HEK 293T stable over-expressing NICDWT-Myc, NICDΔRAM-Myc, NICDΔANK-Myc, NICDΔTAD-Myc, NICDΔOPA-Myc and NICDΔPEST-Myc were subjected to 3hrs of OGD for immunoprecipitation of NICD/p53 complex. Representative immunoblot of p53 immunoprecipitation in WT or deletion mutants of stable Myc-tagged NICD-overexpressing HEK 293T cells.

3.10 p53/Notch synergistically activates apoptosis

3.10.1 Ischemia-mediated cellular death rescue during combined p53/Notch inactivation

To determine whether blockade of Notch activation or p53 individually can protect against cell death by reducing the NICD-p53 interaction, γ-secretase inhibitor (GSI) DAPT and PFT (p53 inhibitor) was employed. The data indicated that primary cortical neurons cells treated with either DAPT or PFT had lower levels of cell death marker, cleaved-caspase-3, compared to vehicle-treated cells following 3hrs of OGD (Fig. 39 A and B).

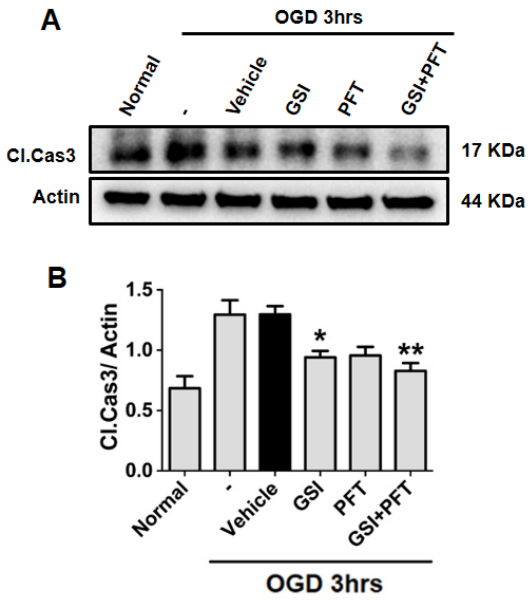


Figure 39: Inhibition of executioner caspase activation following ischemia by combined notch and p53 inhibition

Combined inhibition of p53 and Notch synergistically decreases the apoptosis execution through caspase-3 during ischemia. Cells were subjected to OGD at the indicated time point and analyzed for apoptotic marker protein. A and B, Representative immunoblot of cleaved-Caspase-3 in primary cortical neurons treated with GSI (30μM) and/or PFT (20μM) compared with vehicle treatment during 3 h of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05 and **P<0.005 compared with vehicle-treated control.

3.10.2 p53/Notch initiated cellular death suppressed effectively in combined inhibition treatment

The combined inhibition treatment was found to decrease the Notch and p53 activated apoptotic pathway. The data indicated that primary cortical neurons cultures treated with either DAPT or PFT had lower Notch activation compared to vehicle-treated cells following 3hrs of OGD (Fig. 40 A and B).

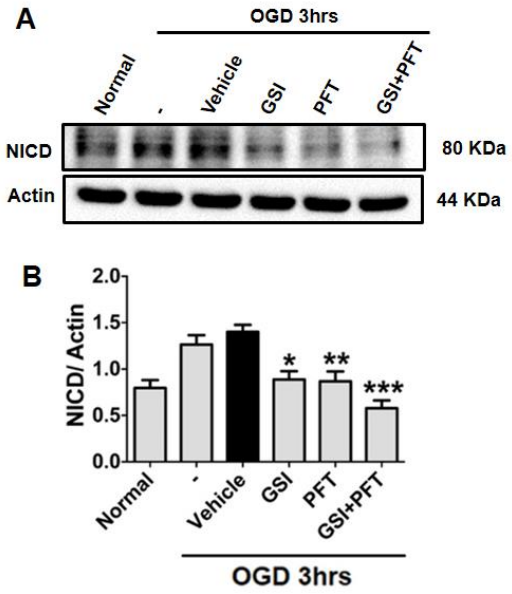


Figure 40: Combined notch and p53 inhibition furthermore decreases NICD generation following ischemia

Combined inhibition of p53 and Notch synergistically inhibits Notch activation further effectively during ischemic stroke. Cells were subjected to OGD at the indicated time point and analyzed for Notch activation. A and B, Representative immunoblots of activated NICD in primary cortical neurons treated with GSI (30μM) and/or PFT (20μM) compared with vehicle treatment during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared vehicle-treated control.

The combined inhibition treatment was found to decrease p53 activated apoptotic pathway. The data indicated that primary cortical neurons cultures treated with either DAPT or PFT had lower P-p53 compared to vehicle-treated cells following 3hrs of OGD (Fig. 41 A and B). Apoptosis-mediating downstream target of activated p53 including Bax, and Puma expression was lower in either DAPT or PFT treatment compared to vehicle-treated cells following 3hrs of OGD (Fig. 41 A, C and D).

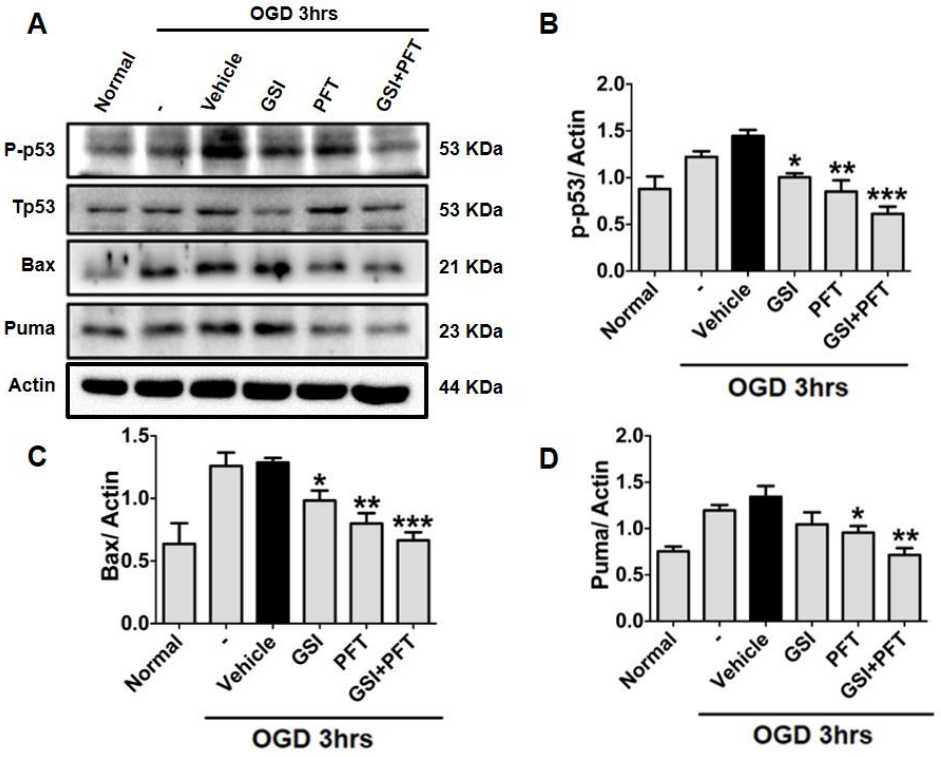


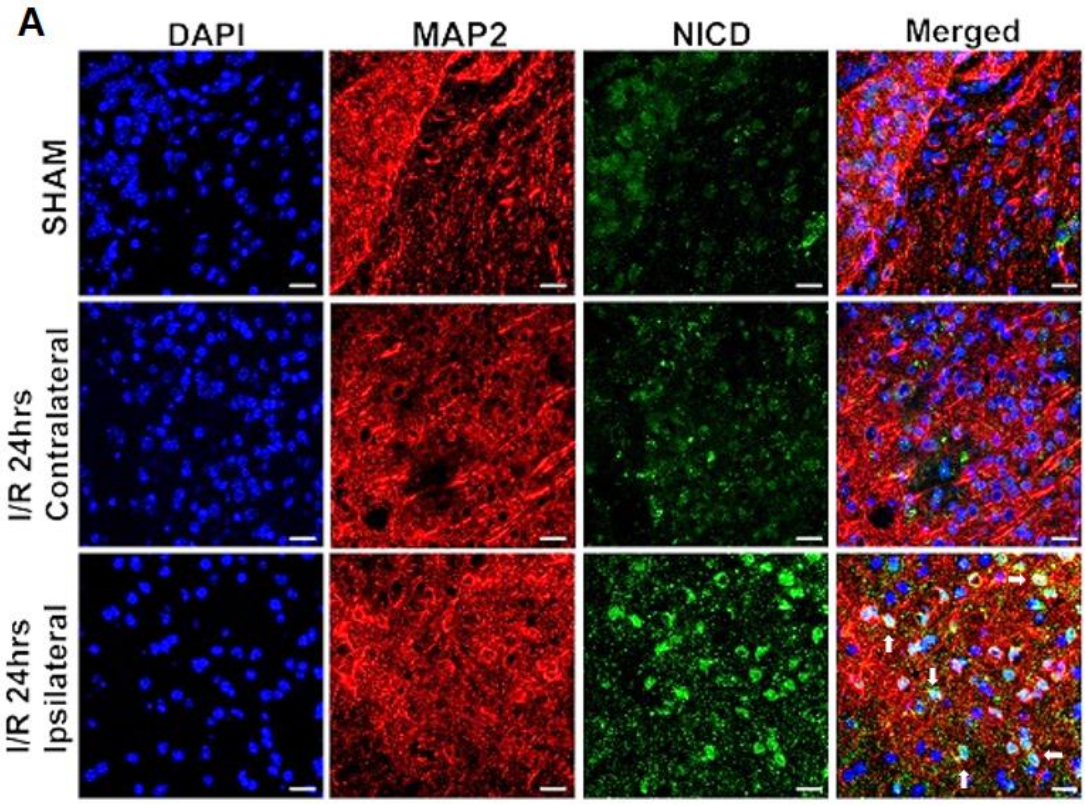
Figure 41: Combined notch and p53 inhibition effectively decreases the apoptotic pathway than independent inhibition

Combined inhibition of p53 and Notch synergistically inhibits p53-mediated downstream apoptotic signaling and rescues cells from ischemic death. A-D, Representative immunoblots of P-p53, Bax, Puma in primary cortical neurons treated with GSI (30μM) and/or PFT (20μM) compared with vehicle treatment during 3hrs of OGD. β-actin was used as the loading control. Data are represented as mean ± S.E.M. n=3-5 cultures. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

3.11 NICD and p53 shows neuronal co-localization in ischemic brain hemisphere in vivo

3.11.1 Following stroke the mice ischemic brain hemisphere has elevated NICD and p53 expression

The expression patterns of NICD and p53 were confirmed *in vivo* following 1hr ischemia and 24hrs reperfusion. In the cerebral cortex of sham-operated control mice, very little immunoreactivity with NICD and p53 antibodies was observed, and both NICD and p53 appeared restricted to the neuronal cytoplasm (Fig. 42 A and B). After 24hrs of post-ischemic reperfusion, neurons in the ischemic cortex (ipsilateral) exhibited robust NICD and p53 immunoreactivity in both nucleus and cytoplasm (Fig. 42 A and B).



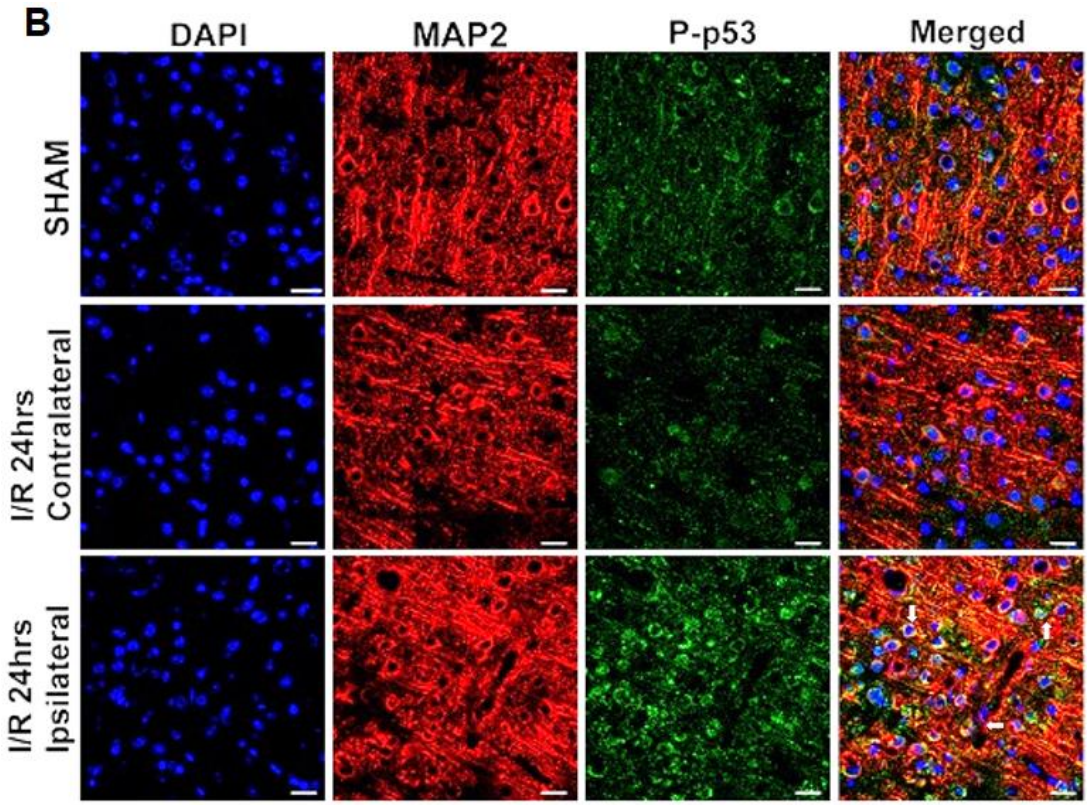


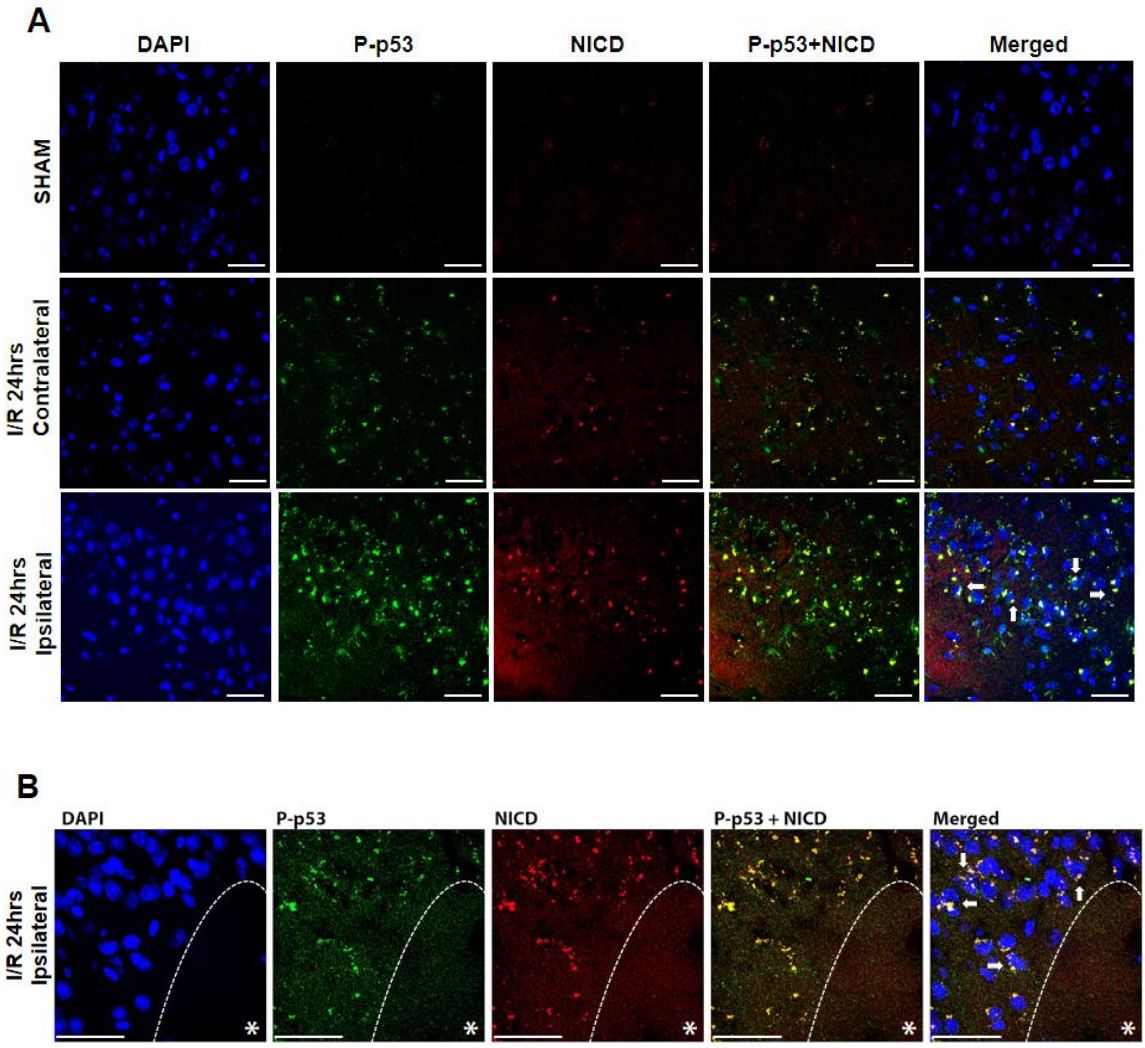
Figure 42: NICD and p53 expression in ipsilateral region of ischemic brain

NICD and p53 expression elevate in ischemic brain hemisphere of mice following ischemic stroke. Mice underwent 1hr MCAO and 24hrs reperfusion (I/R). Obtained brain sections were stained. A and B, Immunofluorescence staining of ischemic (ipsilateral and contralateral) and sham (ipsilateral) brain sections showing confocal images of NICD (A, green), P-p53 (B, green), the neuronal marker MAP2 (red), and the nuclear marker 4'6-diamidino-2-phenylindol (DAPI) (blue) in the cortical neurons of mice brain. Scale bar: 20 µm.

3.11.2 NICD/p53-Pin1 complex presence and NICD and p53 nuclear co-localization in the ischemic brain

The co-localization of NICD and p53 were confirmed *in vivo* following 1hr ischemia and 24hrs reperfusion. In the cerebral cortex of sham-operated control mice, very little immunoreactivity with NICD and p53 antibodies was observed, and both NICD and p53 shows no localization in the nucleus of

neurons in the ischemic cortex (Fig. 43 A). After 24hrs of post-ischemic reperfusion, neurons in the ischemic cortex (ipsilateral) exhibited robust NICD and p53 expression and neuronal nucleus co-localization (Fig. 43 A and B). Furthermore, co-immunoprecipitation was performed in the ipsilateral brain tissue from mice subjected to focal ischemic stroke and the findings indicate NICD and p53 are a complex (Fig. 43 C and D). A peptidyl-prolyl isomerase, Pin1 proven to confer stability to NICD and p53 separately (Zheng, 2002; Sorrentino et al., 2013; Baik et al., 2015) was also co-immunoprecipitated with NICD/p53 complex (Fig. 43 C; Fig. 43 D) in post-ischemic brain tissue of mice.



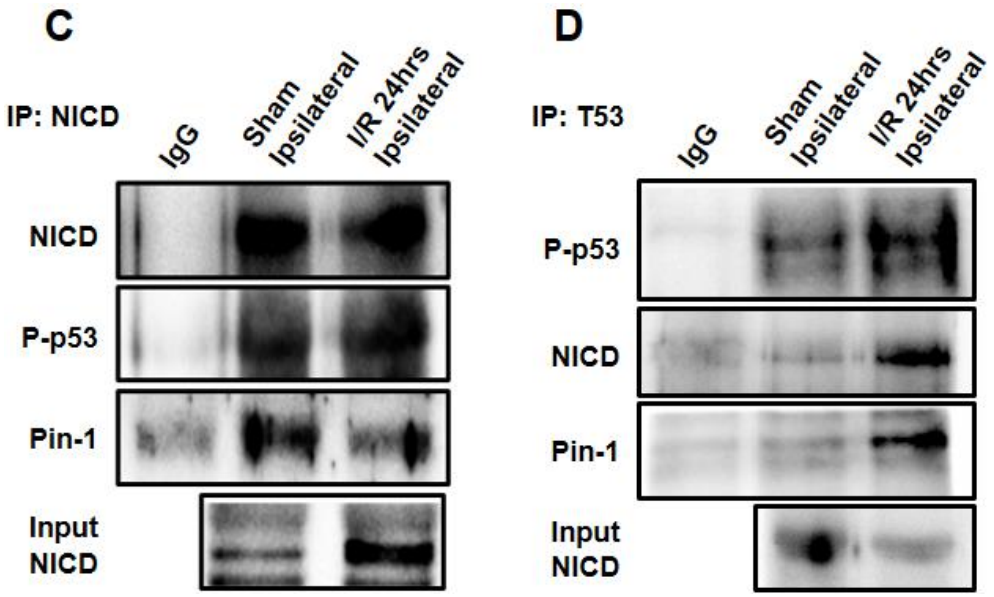


Figure 43: NICD and p53 nucleus co-localization and NICD/p53-Pin-1 complex formation in ipsilateral region of ischemic brain

NICD and p53 nuclear co-localization and NICD/p53-Pin-1 complex interaction in ischemic brain hemisphere of mice following ischemic stroke. Mice underwent 1hr MCAO and 24hrs reperfusion (I/R). Obtained brain was sectioned and stained or lysed for immunoprecipitation. A and B, Immunofluorescence staining of ischemic (ipsilateral and contralateral) and sham (ipsilateral) brain sections showing confocal images of NICD (red), P-p53 (green) and the nuclear marker 4'6-diamidino-2-phenylindol (DAPI) (blue) in the cortical neurons of mice brain. Scale bar: 20 µm; *core region. C and D, Representative immunoblots of NICD, p53 and Pin-1 co-immunoprecipitation in the ischemic brain of mice following 24hrs reperfusion.

3.12 Inhibition of NICD and P-p53 may reduce ischemic brain injury in vivo.

3.12.1 Caspase-mediated cell death may decrease during p53 and Notch inhibition in MCAO mice

Finally, the effect of either combined inhibition or individual inhibition of γ-secretase (using DAPT) and p53 (using PFT) was studied on the degree of stroke-induced brain injury *in vivo*. Intravenous infusion of either DAPT or PFT was administered immediately after ischemia for 6hrs. The elevated protein levels of cell death marker, cleaved-caspase-3 appears to be reduced by both DAPT and PFT treatment (Fig 44 A and B).

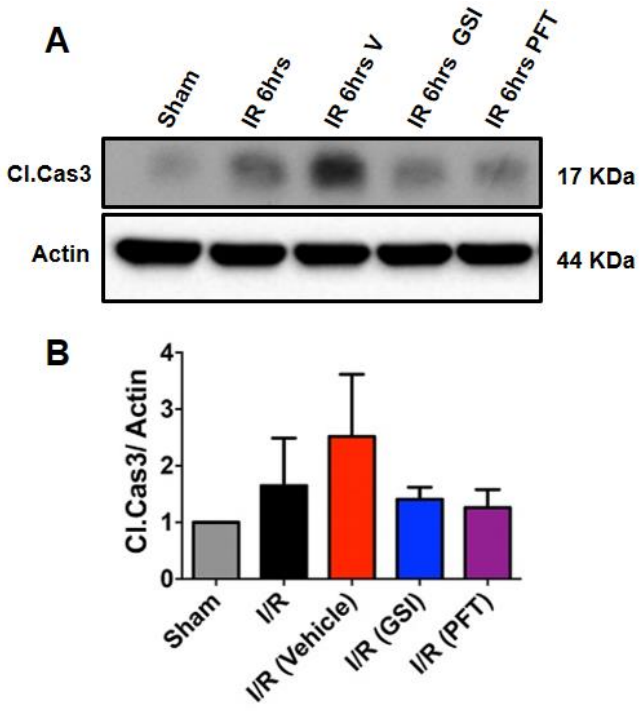


Figure 44: Notch/p53 inhibition could decrease cell death in ischemic mice

Caspase-activated cellular death is suppressed in the ischemic hemisphere of stroke mice during the treated with separate inhibition of p53/NICD proteins. Mice underwent 1hr of MCAO and 6hrs reperfusion (I/R) and were analyzed for cell death marker. A-B, Representative and quantification of cleaved-caspase-3 in the ischemic hemisphere of stroke mice treated with either GSI (40mg/Kg) or PFT (2mg/Kg). β-actin was used as the loading control. n=6 in each group.

3.12.2 Ischemia-mediated apoptotic pathway could be affected during inhibition of p53 and Notch activation *in vivo*

The effect of either combined inhibition or either individual inhibition of γ-secretase (using DAPT) or p53 (using PFT) was studied on the degree of stroke-induced Notch and p53 activation *in vivo*. NICD and p53 in the ischemic hemisphere at 6hrs of reperfusion indicated that both NICD and P-p53 were elevated after stroke and moderately reduced by either DAPT or PFT (Fig. 45 A and B; Fig. 46 A and B), confirming that DAPT and PFT may exert protection very early following cerebral ischemia. In addition, while we found no significant effect of DAPT or PFT on the expression of Hes1 (Fig. 45 A and C), Bax, and Puma (Fig. 46 A, C and D) at 6hrs, there were notable reductions in these proteins.

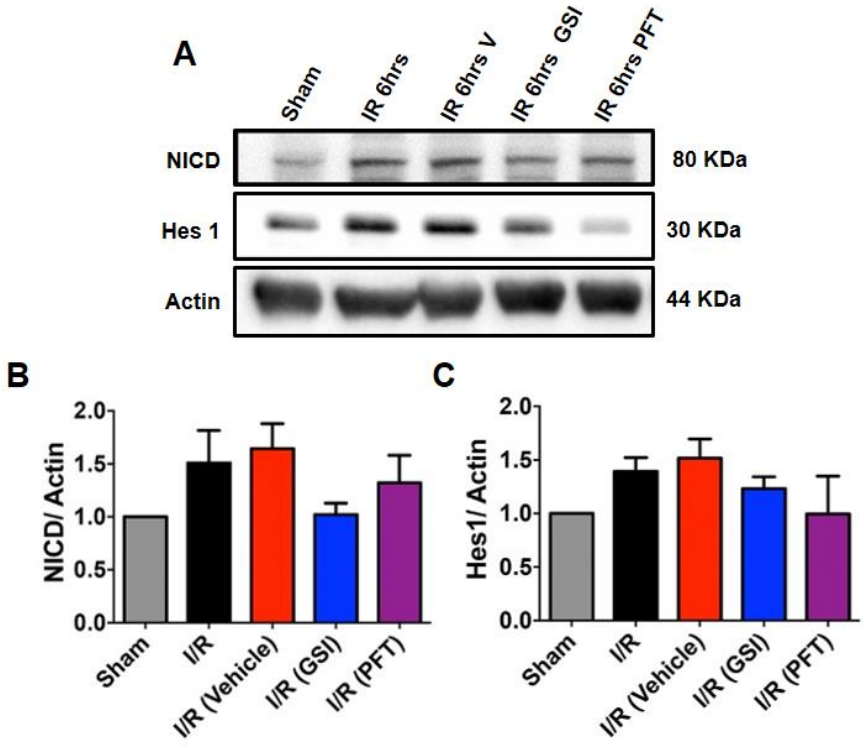


Figure 45: Notch and p53 inhibition affects NICD activity in ischemic mice

Notch activation is suppressed consistent with apoptotic marker in the ischemic hemisphere of stroke mice during the treated with separate inhibition of p53/NICD proteins. Mice underwent 1hr of MCAO and 6hrs reperfusion (I/R) and were analyzed for Notch activation. A-C, Representative and quantification of NICD and Hes1 in the ischemic hemisphere of stroke mice treated with either GSI (40mg/Kg) or PFT (2mg/Kg). β-actin was used as the loading control. n=6 in each group.

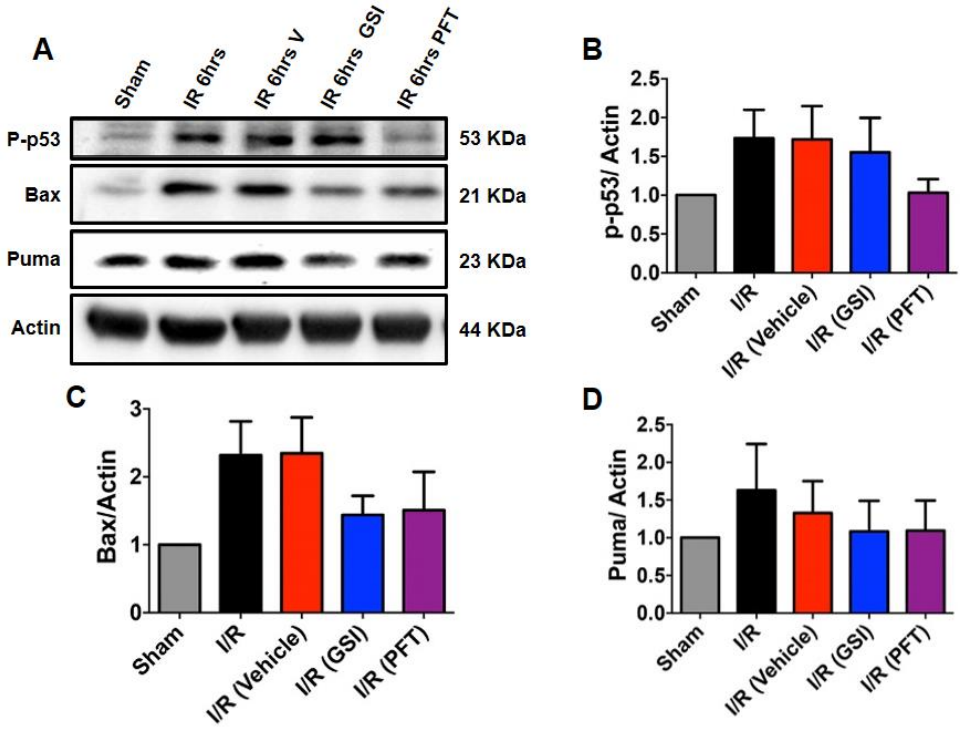
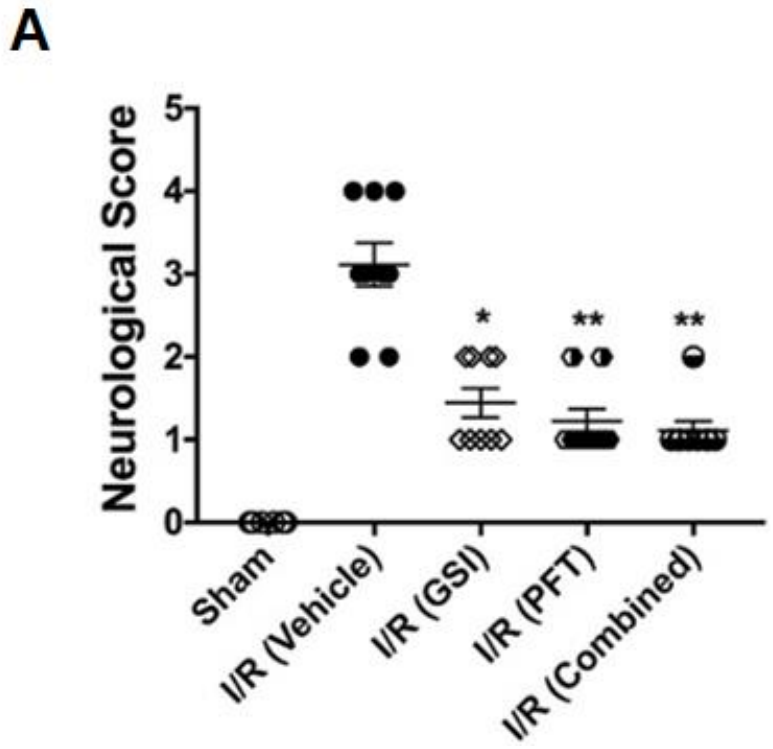


Figure 46: Notch and p53 inhibition modulates the apoptosis mediating proteins in ischemic mice

Ischemia-mediated apoptotic cellular death is suppressed in the ischemic hemisphere of stroke mice during the treated with separate inhibition of p53/NICD proteins. Mice underwent 1hr of MCAO and 6hrs reperfusion (I/R) and were analyzed for proteins. A-D, Representative and quantification of P-p53, Bax, Puma in the ischemic hemisphere of stroke mice treated with either GSI (40mg/Kg) or PFT (2mg/Kg). β-actin was used as the loading control. n=6 in each group.

3.13 Inhibition of NICD/P-p53 reduces ischemic brain injury and improves the functional score in vivo

Finally, the effect of either combined inhibition or either individual inhibition of γ-secretase (using DAPT) or p53 (using PFT) was studied on the degree of stroke-induced brain injury *in vivo*. Intravenous infusion of either DAPT or PFT alone immediately after ischemia reduced the extent of neurological deficit and brain damage compared to vehicle treatment as assessed after 24hrs (Fig. 47 A and B). Interestingly, combined DAPT and PFT treatment did not provide greater protection than did administration of the individual drugs (Fig. 47 A and B).



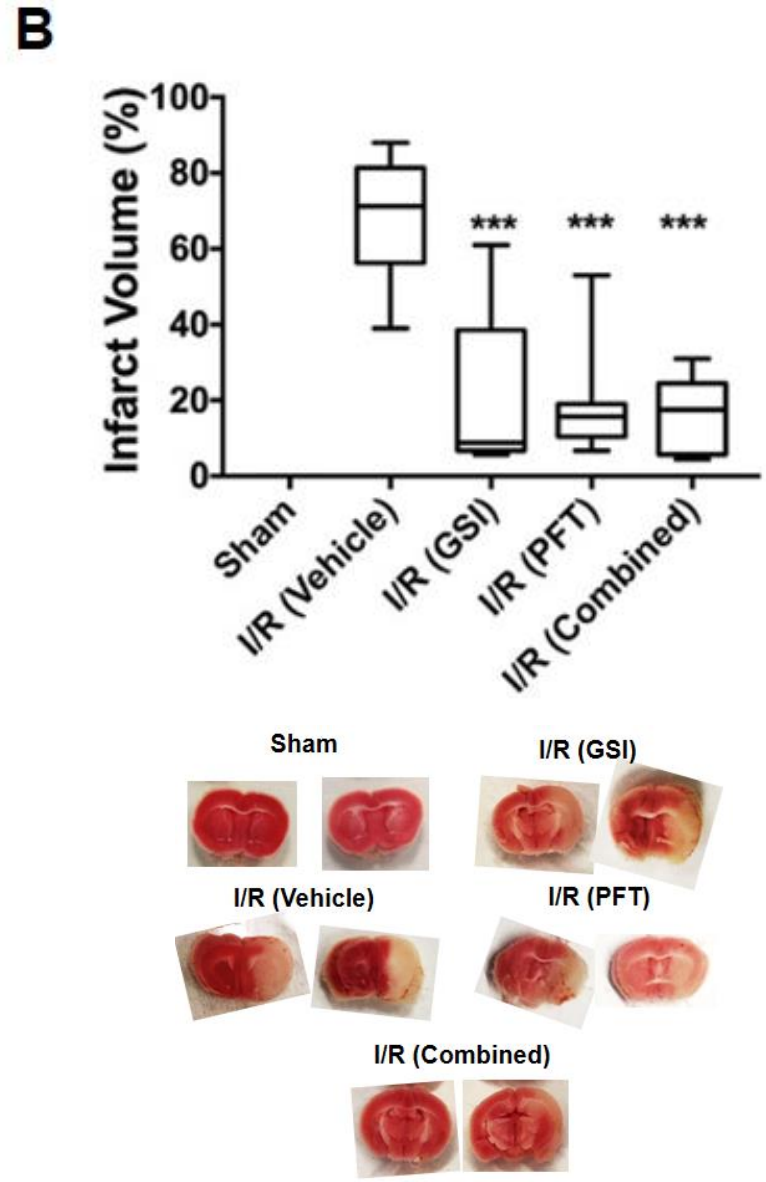


Figure 47: Improved Neurological function and reduced infarct volume of the drug treated ischemic mice

Improved neurological function and reduced infarct volume in the ischemic mice treated with separate or combined inhibition of p53/NICD proteins. Mice underwent 1hr of MCAO and 24hrs reperfusion (I/R) and were analyzed for the neurological deficit and infarct volume. Mice treated with GSI (40mg/Kg) (n=9) and PFT (2mg/Kg) (n=9), alone or in combination (n=9), exhibited improved neurological function and reduced brain injury after stroke, compared to vehicle-treated (n=9) mice. Neurological score and infarct volume were measured at 24hrs following reperfusion. Data are represented as mean ± SEM n=9 mice. *P<0.05, **P<0.005 and ***P<0.001 compared with vehicle-treated control.

CHAPTER 4

DISCUSSION

The findings of this study majorly point out the novel attribute of the Notch receptor in endangering neurons by p53 mediated apoptosis. Notch interaction with p53 is a critical molecular basis for an apoptotic signaling leading to compromised neuronal viability after ischemic stroke. The functional role of NICD-p53 interaction in modifying disease outcome the following stroke involves the stabilization of p53 and the transcriptional regulation of p53 and NICD target genes which can execute the cellular demise through an extremely methodical-apoptosis pathway. In addition, p53 inhibition study in vitro has revealed that p53 may positively regulate NICD expression at the receptor cleaving level. Importantly, this study has demonstrated NICD conferred stability to p53 through substantial interaction and arbitrates the rescue of p53 from ubiquitination positively regulated by MDM2.

Various cell surface receptors including Toll-like receptors, RAGE, Mincle having an established role in stroke pathophysiology such as inflammation, cytokines secretion and release of pathological cell mediators constitutes a pattern of profound effects in neuronal cells similar to their function observed with other pathophysiological conditions involving different cell types, glial cells and immune cells are to name a few (Lok et al., 2015; Arumugam et al., 2016). The peculiarity of Notch receptor signaling is its diversified biological role in an organ specific and cell specific manner

executing mechanisms like differentiation and proliferation during developmental stages and adulthood (Artavanis-Tsakonas S, 1995; Artavanis-Tsakonas et al., 1999). During development of CNS, Notch signaling is involved in the maintenance of neural progenitors in an undifferentiated state, in part by inhibition of neurogenesis (Redmond et al., 2000). Also, Notch receptor influences synaptic plasticity, learning, and memory in the adult brain (Lathia et al., 2008; Tan et al., 2014). The activation of the receptor leads to NICD nuclear translocation where it attains functions in conjunction with co-activators such as CBF-1/Su(H)/LAG-1 (CSL) family of DNA-binding proteins by recruiting general transcription factors, including CBP/p300 (Schroeter et al., 1998; Brou et al., 2000; Miele, 2006). NICD-mediated transcription induces expression of genes such as Hairy-Enhancer of Split (Hes) and Hes-related protein (Herp) (Borggrefe and Oswald, 2009; Guruharsha et al., 2012). Notch-mediated neuronal cell fate determination or other events could be influenced not only by its target genes but also by NICD interaction with other proteins, such as Nuclear factor- Kappa B (NF-κB) (Cheng et al., 2001; Arumugam et al., 2011), HIF-1α (Mukherjee et al., 2011; Cheng et al., 2014[a]) and c-Jun (Cheng et al., 2014[b]; Chen et al., 2016).

Interestingly, a cellular death mechanism of Notch in focal ischemic stroke was supported by studies showing that mice overexpressing Notch1 antisense (NAS) and normal mice treated with inhibitors of the Notch-activating enzyme, γ–secretase, exhibit reduced damage to brain cells and improved functional outcome (Arumugam et al., 2006; Arumugam et al., 2011; Cheng et al., 2014[a]). Consequently, the mechanisms underlying Notch-mediated neuronal

death in ischemic stroke were further investigated. Notch was shown to bind to P-p65 and to prolong NF-κB-mediated proinflammatory responses in immune cells (Shin et al., 2006). Notch signaling was also shown to interact with NF-κB and to endanger neurons after ischemic stroke by modulating NF-κB activity (Arumugam et al., 2011; Wei et al., 2011; Li et al., 2012). Accordingly, inhibition of γ-secretase protects against ischemic neuronal cell death by targeting NF-κB and the pro-death BH3-only protein, Bcl-2-interacting mediator of cell death (Bim) (Arumugam et al., 2011; Li et al., 2012). Furthermore, inhibitors of γ-secretase reduce activation of NF-κB and expression of proinflammatory mediators, resulting in marked attenuation of neurotoxic actions of microglia (Wei et al., 2011; Li et al., 2012).

It seemed plausible that other death-promoting transcription factors may also interact with NICD and contribute to brain injury following stroke, and previously shown is that NICD and HIF-1α bind directly to each other to form part of a multiprotein transcriptional complex that may include NF-κB and other NICD binding partners (Cheng et al., 2014[a]). Levels of NF-κB, p-p65, and cell death marker, cleaved Caspase-3 were reduced by inhibition of either γ-secretase/Notch or HIF-1α and combined inhibition during hypoxic conditions (Cheng et al., 2014[a]). Interestingly, cell death was effectively reduced by combined inhibition of γ-secretase/Notch and HIF-1α than separately. Transfection with NICD, HIF-1α, or both, increased the level of the phosphorylated (p-p65) and non-phosphorylated (p65) forms of the 65 kDa subunit of NF-κB under both normal and hypoxic/ischemic conditions (Cheng et al., 2014[a]). Likewise, HIF-1α inhibition in neuronal cells transfected with

either NICD, HIF-1α, or both, resulted in decreased levels of total and P-p65 under both normal and hypoxic conditions (Cheng et al., 2014[a]). Independent of NICD, an interaction between HIF-1α and NF-κB was also demonstrated to exist in normal and other disease conditions (Wang et al., 2001; Shin et al., 2006; Arumugam et al., 2011). Collectively, the evidence described above suggests that NICD, HIF-1α, and NF-κB signaling pathways communicate with each other.

Nuclear factor-Kappa B (NF-κB) is a diversely triggered protein and studies have demonstrated its role in inflammation mediating pathway (Yao et al., 2013; Simmons et al., 2016) and in mediating survival mechanism in Hodgkin's lymphoma cells rescuing them from undergoing apoptosis under stress conditions (Bargou et al., 1997). Moreover, NF-κB has been proven to protect against TNF-α stimulated cell death in rodent fibroblasts (Beg and Baltimore, 1996). Nevertheless, contrasting studies have elaborated NF-κB role in apoptosis (Ryan et al., 2000). Focal cerebral ischemic damage triggered NF-κB activation promotes cell death in rodent models, whereas, the NF-κB knockout or inhibition of NF-κB activation studies resulted in minimal cell injury in neurons (Hickenbottom et al., 1999; Schneider et al., 1999; Gu et al., 2012). In addition, negatively regulating NF-κB activation in neonates decreases hypoxia-mediated p53 neuronal and mitochondrial accumulation in neurons (Nakai et al., 1999; Nijboer et al., 2008).

Neuronal death mechanisms in ischemia also include documented NICD regulation of MAP kinase-mediated JNK-cJun pathway. The γ-secretase

inhibition was previously shown to decrease the phosphorylated JNK and cJun proteins leading to minimized cellular stress (Cheng et al., 2014[b]). c-Jun N-terminal kinase (JNK) is a kinase widely phosphorylating many proteins including cJun, a transcriptional factor contributing to cell death (Chen et al., 2003b). Moreover, JNK targeted phosphorylation of p53 is an essential mechanism for p53 stability in the transcriptional role (Buschmann et al., 2001; Chen et al., 2003b; Choi et al., 2011; Shi et al., 2014). Active MAPK signaling is observed in cells when lacking oxygen and is a core regulatory mechanism for p53 phosphorylation in primary cortical neurons subjected to hypoxia (Huang et al., 1999; Sanchez-Prieto et al., 2000; Zhu et al., 2002). Although, it seems possible for Notch-mediated MAPK signaling to regulate cell death through p53, the direct evidence of Notch in accordance with p53 to contribute to cell death under ischemic stroke conditions has not been established. Hence the current study about the interplay of two proteins p53 and NICD regulating cellular apoptosis is essential. The substantial findings of this study represent the first evidence that interactions between NICD and p53 are vital for p53 stabilization and to promote cell death pathway in the brain following ischemic stroke.

The Notch-p53 association in apoptosis was previously established in early neural progenitor cells by Yang and colleagues (2004), who showed that conditional expression of a constitutively active form of Notch1 selectively induces extensive apoptosis by elevating levels of nuclear p53 and up-regulating transcription of target pro-apoptotic genes, such as Bax and Noxa (Yang et al., 2004). p53 is normally maintained at a low level by MDM2-mediated

ubiquitination and proteasomal degradation, and it can be stabilized by various cellular stresses (Prives, 1998; Lev Bar-Or et al., 2000). The p53 protein is also involved in the cellular response to ischemia. The severity and the duration of hypoxia/ischemia exert very different influences on the activity, level, and apoptotic function of p53 (Hubert et al., 2006; Sano et al., 2007). Early studies reported that the total absence of p53 was marginally protective in ischemic stroke (Crumrine et al., 1994) Another study found that a majority of p53-deficient mice displayed no signs of cell damage following subcutaneous injection of kainic acid compared to the extensive hippocampal cell loss in wild-type animals (Morrison et al., 1996). Furthermore, it was shown that pifithrin-α protected neurons against apoptosis induced by the DNA-damaging agents, amyloid beta-peptide and glutamate (Culmsee et al., 2001). Animals treated with pifithrin-α displayed less cell death, decreased expression of the p53 target gene BAX (Culmsee et al., 2001) and dose-dependently increased the number and size of new neurospheres formed following ischemic stroke (Luo et al., 2009).

In the present study, a comparable dose of pifithrin–α was employed and as expected a similar degree of neuroprotection in ischemic stroke was observed. The apoptotic pathway initiated by p53 either regulated extrinsically or intrinsically activates procaspase-3 by cleaving at the CARD domain. In the current study, ischemic condition results in elevated cleaved-caspase 3 and AIF levels defining the caspase-dependent cell death pathway and caspase-independent DNA fragmentation occurring during i*n vitro* ischemia (Cregan et al., 2002). Expectedly, activated p53 regulating apoptotic pathway was also

high. p53 executes cell death by mediating the transcription of apoptotic genes including Bax, Puma and Noxa. As a transcription factor p53 can be abundantly found in the nucleus in parallel to its degree of presence in the cytoplasm as well (Caelles et al., 1994; Chipuk et al., 2004). Hence localization studies can define the role of p53 elaborately in conjunction with the protein expression study. This study has identified the localization of p53 in SH-SY5Y cultures and primary cortical neurons *in vitro* and in neurons of tissue sections from the ipsilateral hemisphere of the ischemic stroke mice *in vivo* where the neurons were identified with MAP2 protein, a marker of neuronal cells (Caceres et al., 1984; Ingvar et al., 1994). Here, the translocation of P-p53 from the cytoplasm to nucleus was evidently traced in the neuronal cultures and the immunostained tissues after the onset of ischemic stroke followed by 24hrs reperfusion, signifying the p53 transcriptional activity is initiated to maneuver programmed cell death during ischemia conditions. Following this study Pifithrin-α treatment was employed to determine Notch activity at doses showing decreased p53 activation reduced cleaved-caspase-3. Surprisingly, Notch receptor activity was effectively reduced by Pifithrin-α treatment in primary cortical neurons *in vitro.* Due to the lessening in NICD levels, its target, Hes-1 showed minimal expression benefitting the outcome of stroke. Hes-1 has been proven to be involved in microglial inflammatory mediated pathway exhibiting detrimental effects in rat ischemic stroke brain and Hes-1 has been associated with apoptosis by regulating Hes-1-PTEN-AKT-mTOR signaling, observed as a novel mechanism of cell death (Yuan et al., 2015; Zeng et al., 2016). In the current study p53 inactivation has negatively regulated the Hes-1 mediated possible detrimental mechanism in neurons.

Simultaneously, Notch receptor, the pivotal entity of this study is a well-established co-activator controlling the activity of transcriptional proteins to express target genes and has revealed a synchronized molecular emplacement with p53. As a transcriptional co-activator, the translocation of Notch-activated receptor domain, NICD from the plasma membrane to the nucleus remains indispensable. In accordance with the obtained P-p53 results, localization studies of NICD have revealed the translocation of the protein to the nucleus upon ischemic stress in SH-SY5Y and primary cortical neuron culture *in vitro* as well as in the tissue sections from an ischemic hemisphere of stroke mice *in vivo.* The intriguing section of the evidence is the observed co-localization of NICD along with P-p53 in the neuronal nucleus after the onset of stroke. This noted co-localization has further been elaborated by immunoprecipitating either of the proteins only to confirm the interaction between NICD and p53 in normal as well as ischemic conditions. Likewise, the γ–secretase inhibition exhibited the similar pattern of reduction in apoptotic pathway proteins, Bax and Puma and resulted in a decrease of Notch activation induced P-p53 levels. γ–secretase inhibition was demonstrated in various cellular models which ultimately displays the reduction of phosphorylation of p53. Importantly, γ–secretase inhibition treatment also decreases the cell death by causing less cleaved-caspase-3 levels. These results demonstrate that Notch and p53 activation are interdependent following ischemia and either of the activation control could modulate the survival attribute of stroked neurons. We also observed an additive protective effect by combined Notch/p53 inhibition in a cell culture model of i*n vitro* ischemic stroke, but such an additive effect was not replicated in the degree

of attenuation of infarct size and neurological deficit score *in vivo*. It is possible that this was due to the severity of our ischemic model such that the maximum possible protective effect could be obtained with just a single treatment targeting either Notch or p53.

Severe hypoxia, induce p53 in HIF-1α-dependent mechanism in different cell models. The direct association of HIF-1α with p53 is found to be p53 stabilizing prerequisite (An et al., 1998). Further, the post-translational modification of HIF-1α determines its role in providing p53 stability, where the hypoxia induced HIF-1α de-phosphorylation favors the stability and accumulation of p53 through interaction to initiate apoptosis, on the contrary, the phosphorylated form of HIF-1α switches the cellular paradigm to survival in hypoxic glioma cells, thus acting as a double-edged sword. (Suzuki et al., 2001). Concurrently, in similar cellular models, Notch activity is shown to be dependent on HIF-1α (Qiang et al., 2012) and the interaction between HIF-1α and NICD confers stability for the dynamic activity of NICD (Qiang et al., 2012; Hu et al., 2014) and these evidence are in uniformity with the interaction of HIF-1α/NICD observed in promoting neuronal cell death during *in vitro* ischemia (Cheng et al., 2014[a]). Also, additive studies offer the supportive evidence for HIF-1α-mediated p53 stabilization and HIF-1α/p53 synergistic regulation of apoptosis during hypoxia (Halterman et al., 1999; Robertson et al., 2014; Liu et al., 2016). In the current study, it is evidently proven that the stability of p53 in overexpression of exogenous NICD is observed to increase the stability of endogenous p53 and exogenous WTp53 in comparison to pcDNA-transfected cells. Whereas the exogenous mutant p53 including

deletion of N-terminal, deletion of C-terminal and presence of the only DBD abolished the WTp53 stability resulting from exogenous NICD overexpression. This reduction in stability is due to the loss of interaction of the mutant p53 with NICD. The interaction between NICD and WTp53 was not consistent with p53 mutants including the DBD only domain, N-terminal domain deletion or C-terminal domain deletion mutants.

MDM2 which is a negative regulator of p53 (Lev Bar-Or et al., 2000) is found in association with HIF-1α. HIF-1α binding of MDM2 may be the possible mechanism of HIF-1α mediated stabilization and activation of p53, signifying the rescue of p53 from ubiquitination (An et al., 1998; Robertson et al., 2014; Liu et al., 2016). Interestingly in this study, it was observed the interaction between the E3 ubiquitin-protein ligase MDM2 and p53 was also noticeably reduced by NICD overexpression in neuronal cells following conditions of i*n vitro* ischemia. This observation was coherent with the markedly reduced polyubiquitinated p53 with the presence of NICD overexpression in neuronal cells following conditions of i*n vitro* ischemia. This body of new evidence strongly suggests that the NICD-p53 interplay plays pivotal role in determining pro-apoptotic signaling in the brain after ischemic stroke. In addition to the NICD overexpression-mediated p53 ubiquitination rescue, the non-neural cells stably expressing high NICD, experienced an increase in an apoptotic pathway mediated cell death. At basal and i*n vitro* ischemic conditions, NICD overexpressing cells is found to have high p53 activation and hence results in increased levels of Bax and Puma promoting the mitochondria-mediated caspase activation.

Recently report demonstrated an increased NICD ubiquitination in Pin1 knockout neuronal cells (Baik et al., 2015). Pin1 is a peptidyl-prolyl isomerase (PPIase) with an established role to convene stability to post-translationally modified protein by catalyzes of the protein isomerization when phosphorylated at the peptide bond between pSer/Thr-Pro. Pin1 is associated with the pathogenesis of Alzheimer's disease (Shen et al., 1998; Butterfield et al., 2006; Sultana et al., 2007). In addition, Pin1 also plays a crucial role in neuronal death following ischemic stroke (Becker and Bonni, 2007; Baik et al., 2015). Specifically, the Pin1-FBW7-Notch1 axis compromises neuronal vulnerability after ischemic stroke, and neuronal injury can be ameliorated *in vitro* and *in vivo* by either a Pin1 inhibitor or in Pin1 deficiency (Baik et al., 2015). Pin1 interacts with NICD in the brain to increase its stability after ischemic stroke by inhibiting FBW7-mediated polyubiquitination, resulting in facilitation of NICD-induced neuronal death. Notably, a decreased stability of the NICD mutants due to the loss of Pin1 interaction is observed along with NICD mutant mediated cell death at minimal in comparison with WT NICD expressing population of cells. Thus, the coordinated interaction between Pin1/FBW7 and NICD, and the well-established function of Notch1 in ischemia-induced neuronal damage provides strong evidence that Pin1 regulates neuronal death by stabilizing NICD following ischemic stroke (Baik et al., 2015). Concurrently, earlier established work has shown that the phosphorylated p53 at the pSer/Thr-Pro that is induced by stress must form a complex with Pin1 and then undergo a conformational change in order to fulfill its biological roles associated with apoptotic gene regulation and cellular events through

mitochondrial cell death (Buschmann et al., 2001; Zheng et al., 2002; Zheng, 2002; Becker and Bonni, 2007; Sorrentino et al., 2013). In the current study, the provided evidence displays Pin1 interaction with both NICD and p53 following ischemic stroke, indicating that Pin1-mediated NICD stability and/or p53 phosphorylation play a vital role in the pro-apoptotic function of the p53-NICD complex. Furthermore, this study indicates that Pin-1 is pull down as a complex with p53 and NICD in the ischemic hemisphere of stroke mice brain. Pin1 interaction was lost with the deletion of NICD domains, especially presenting with a decreased binding of Pin1 to the ANK domain deleted NICD (Baik et al., 2015). This finding had brought an attention to explore the domain of NICD involved with p53 association. Investigated finding shows p53 interaction with NICD occurs via the ANK domain under i*n vitro* ischemic condition. This finding is noteworthy, as we have previously reported ANK-domain mutant cells to exhibit profound protection under ischemic conditions compared to that by other NICD domains such as RAM, TAD, OPA, and PEST deleted or in wild-type NICD expressing cells (Baik et al., 2015). Current and previous observations imply that the coordinated interactions between Pin1-NICD, NICD-p53, NICD-HIF1α and NICD-NFκ-B may each impact the stability of the other protein interactions to promote ischemia-induced neuronal cell death. The NICD mediated MAP kinase signaling up-regulation is the additive factors in regulating the interaction of NICD/p53 complex signaling mechanism. Thus together these proteins as a complex may have added biological roles and hence could enumerate novel cellular mechanisms underlying the pathogenesis of the neuronal disease.

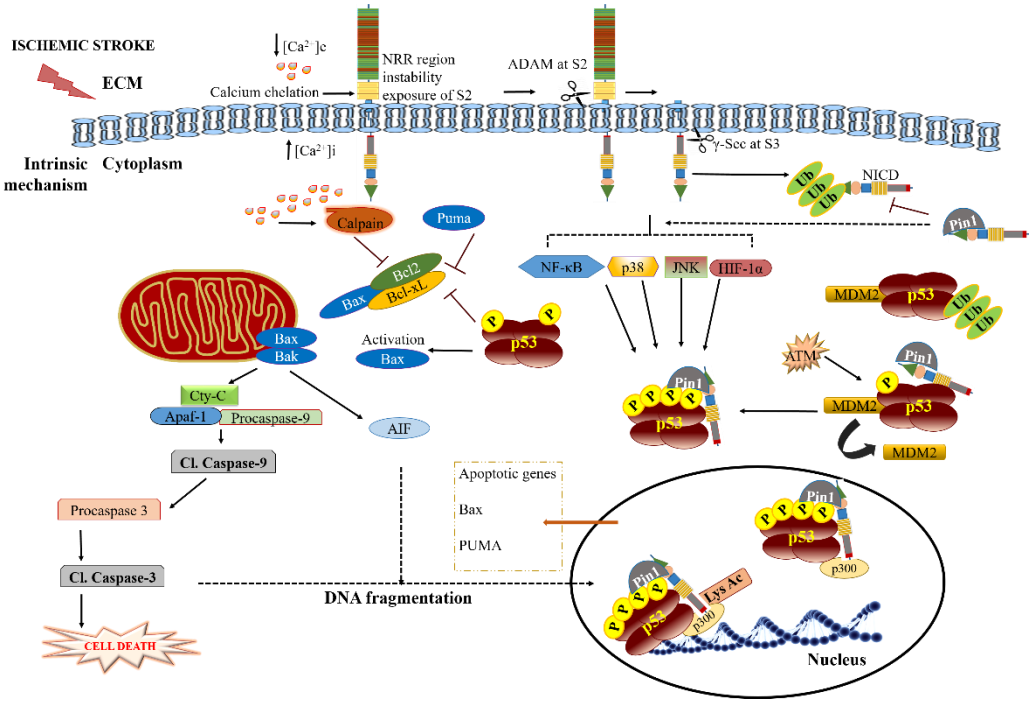


Figure 48: Proposed mechanism of ischemic insult executed by Notch

Ischemic stroke increases neuronal calcium influx from the synaptic cleft resulting in calcium chelation. The Ca^{2+} ions dependent LNR repeats of extracellular domain of Notch destabilizes allowing ADAM protease activity at the S2 site. The cleavage of S2 actively triggers the spontaneous γ-sec S3 cleavage liberating NICD in the cytosol. Individually, NICD is ubiquitinated, however, Pin1 interaction stabilizes and inhibits degradation of NICD. Simultaneously, MDM2 mediated p53 ubiquitination is inhibited by a combined mechanism of ATM kinase-added phosphate group and NICD/Pin1 complex interaction. At this molecular state, p53 is susceptible to various sites of phosphorylation by kinases, some of which are found to be mediated by NICD. The phosphorylated form of p53 recruits Pin1 at its Ser/Thr-proline-motifs to gain a stable conformation, which mobilizes p300 interaction to acquire transactivation role following lysine acetylation. As a complex p53/NICD up-regulate apoptotic genes expression which results in proteins involved in intrinsic apoptotic pathway. Whether NICD plays a role in p53 transcription-independent mechanism is yet to be explored.

CHAPTER 5

LIMITATIONS OF THE STUDY

In the present study, the individual inhibition of the protein activation has a reduced effect on the apoptotic proteins and a combined inhibition effectively carried out in the in vitro primary cortical mice neurons were not reproducible at the similar degree of consistency in the combined drug-treated stroke mice. Although a higher number of animals can make a significant difference in the effect observed; alternatively, it can be possible that this was due to the severity of our ischemic model such that the maximum possible protective effect could be obtained with just a single treatment targeting either Notch or p53.

Although the interaction studies of the NICD/Pin1-p53 complex of proteins revealed the existence of this complex in basal and ischemic conditions, the role of its interaction in the basal state has not been addressed in the study. However, since p53 transcription ranges across the self-regulating gene, cell cycle control gene regulation, cellular stress, DNA breaks and apoptosis, the exhibited interaction in the basal state may be a regulatory mechanism for stabilizing the activated p53 to carry out its other biological roles. Importantly, the factors that determine whether the interplay of NICD-p53 switches to be detrimental for neurons following ischemic stroke need further investigation. Moreover, the interaction study was performed within a whole cellular protein cocktail. The cellular compartment based interaction study will be an evidence to elaborate the intricate mechanism contributed by this complex of proteins to

the apoptotic pathway in detail. Since p53 is also involved in a transcription-independent mechanism of apoptosis, effect on this functional aspect of p53 by the established NICD/Pin1-p53 complex role remains to be investigated. Additionally, the mechanism involved in p53 inhibition-mediated decreased NICD generation is yet to be elaborated. As the activation of Notch receptor is executed by the γ-secretase enzyme (Kimble et al., 1998), inhibition of p53 may regulate Notch activation either by acting at the level of γ-secretase enzyme complex or via an unknown mechanism mediated to suppress Notch activation.

Notch-1 signaling is associated with various pathogenesis including UV radiation, leukemia and various other cancers (Ellisen et al., 1991; Kimble et al., 1998; Kim et al., 2007). The mechanism of notch signaling in this pathogenesis are proliferative and contains cancer-promoting feature. On the contrary, Notch induces p21 expression to cause cell cycle arrest and triggers terminal differentiation of mouse keratinocytes (Rangarajan et al., 2001). This multi-signaling role of Notch receptor is cell-specific and carries out its destined biological role in specific pathways distinct to each organ. Interestingly some studies have elaborated the mechanism of cancer progression involving NICD interaction promoted inhibition of p53 transactivation (Kim et al., 2007; El Khatib et al., 2013) which is a reversal effect of the finding from this current study. Nevertheless, tumor suppressor role of Notch is also evident in certain studies without elaborate mechanism (Shou et al., 2001; Nicolas et al., 2003). Due to such non-similarity in Notch signaling towards pathogenesis, the mechanism reported from this study is unique and predominant in the neuronal population of ischemic brain.

FUTURE DIRECTION

As discussed earlier from the current study and previously existing observations, the coordinated interaction between Pin1-NICD, and NICD-p53, NICD-HIF1α and NICD- NFκ-B may each impact the stability of the other protein interactions to promote ischemia-induced neuronal cell death. MAP kinase signaling up-regulation mediated by NICD are the additive factors in regulating the interaction of NICD/p53 complex signaling mechanism. It is likely that the NICD/p53 complex plays a key functional role by associating with the other protein including Pin1, HIF-1α which are proven to associate with NICD.

It is important for the current study to be reproduced *in vivo* and elaborate the mechanisms required to address the limitations. Nevertheless, it is plausible for the complex of these multiprotein to transcriptionally regulate genes associated with death signaling which ultimately causes apoptosis in the brain following ischemic stroke. These observation when well characterized in the upcoming projects, can pave a platform for multiple target for treatment and ameliorative post-ischemic damage. An identification of such process can be of great importance in understanding the molecular mechanisms of stroke pathophysiology and a novel approach for therapeutic development.

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APPENDICES

Appendix I

Tissue culture media, buffers and reagents

1) Media for Neuroblastoma SH-SY5Y cell line culture

For preparing 500 ml

Reagent	Volume	v/v (%)
DMEM/F12 media (Gibco)	450 mL	
Heat inactivated FBS (Biowest)	50 mL	10%

2) Media for HEK 293T cell line culture

For preparing 500 ml

Reagent	Volume	v/v (%)
DMEM (Gibco)	450 mL	
Heat inactivated FBS (Biowest)	50 mL	10%

3) Media for Primary cell culture

For preparing 500 ml

Reagent	Volume
Neurobasal medium (Gibco)	450 mL
B-27 supplement	10 mL
HEPES	5 mM
L-glutamine	0.5 mM

4) Locke's Buffer For preparing 1 L

Reagent	Final concentration
NaCl	154 mM
KCl	5.6 mM
Na_2HCO_3	3.6 mM
$CaCl_2$	2.3 mM
$MgCl_2$	1 mM
HEPES	5 mM
HCl	To adjust pH to 7.2
Gentamicin	5 mg/L
ddH_2O	Top up to 1 L

5) 1x PBST For preparing 1 L

Reagent	Quantity
10x PBS	100 mL
Tween-20	500 µL
ddH_2O	Top up to 1 L

6) 10x TBST For preparing 1 L

Reagent	Quantity	Final concentration
NaCl	80 g	1.5 M
Tris	24.2 g	200 mM
HCl	To adjust pH to 7.5	
Tween-20	10 mL	
ddH_2O	Top up to 1 L	

7) 4% PFA For preparing 1 L

Reagent	Quantity
PFA	40g
ddH_2O	500 mL
1M NAOH	10 mL
10XPBS	100 mL
HCl	To adjust pH to 7.4
ddH_2O	Top up to 1 L

8) RIPA Buffer For preparing 1 mL

Reagent	Quantity
RIPA (Thermo Scientific)	1000 µL
Phosphatase Inhibitors	10 µL
Protease Inhibitors (Thermo Scientific)	10 µL

9) 2× Laemmli sample buffer For preparing 1 mL

Reagent	Quantity
2xLaemmli (Bio-rad)	950 µL
β-mercaptoethanol	50 µL

10) 10X Towbin-Electro-Blotting buffer (Transfer buffer)

For preparing 1 L

Reagent	Quantity
Tris	30.1 g
Glycine	144 g
ddH_2O	1000 mL
1XTransfer Buffer	
ddH_2O	700 mL
Methanol	200 mL
10X Transfer Buffer	100 mL

11) 10X SDS-PAGE Running buffer

For preparing 1 L

Reagent	Quantity
Tris	30.27 g
Glycine	138.9 g
SDS	10 g
ddH_2O	1000 mL
1XRunning Buffer	
ddH_2O	900 mL
10X Transfer Buffer	100 mL

12) Lower Tris Buffer For making 250 mL

Reagent	Quantity
Tris	45.25 g
ddH_2O	200 mL
HCl	To adjust pH to 8.8
20% SDS Sol in ddH_2O	5 mL
ddH_2O	Top up to 250 mL

13) Upper Tris For making 100 mL

Reagent	Quantity
Tris	6.6 g
ddH_2O	75 mL
HCl	To adjust pH to 6.8
20% SDS Sol in ddH_2O	3.75 mL
ddH_2O	Top up to 100 mL

14) Resolving gel (12%) For preparing 4 Gels

Reagent	Quantity
ddH_2O	17.5 mL
Lower Tris-HCl, pH 8.8	10 mL
40% Acrylamide/bis	12.5 mL
10% Ammonium persulfate	375 µL
TEMED	32.5 µL

15) Stacking gel For preparing 4 Gels

Reagent	**Quantity**
ddH_2O	11.5 mL
Upper Tris-HCl, pH 6.8	2 mL
40% Acrylamide/bis	1.5 mL
10% Ammonium Persulfate	154 µL
TEMED	15.4 µL

Appendix II

Manuscript submitted

Priyanka Balaganapathy, Sang-Ha Baik, Karthik Mallilankaraman, Christopher G. Sobey, Dong-Gyu Jo*, Thiruma V. Arumugam* (2017) Interplay Between the Notch and p53 Promotes Neuronal Cell Death in Ischemic Stroke. *Journal of Cerebral Blood Flow & Metabolism.*

Local and international conference presentations

1) **Balaganapathy, P.** and Arumugam, TV. The role of Notch in p53 mediated neuronal cell death in cerebral ischemia, 7th Models of Physiology and disease symposium, Singapore, September 2015, NUS, Singapore (Poster presentation).

2) **Balaganapathy, P.** and Arumugam, TV. Interplay between Notch and p53 enhances neuronal cell death during ischemic stroke, *NUS Yong Loo Lin School of Medicine 6th Annual Graduate Scientific Congress, January 2016, NUS, Singapore* (Oral presentation).

3) **Balaganapathy, P.** and Arumugam, TV. Interplay Between the Notch and p53 Promotes Neuronal Cell Death in Ischemic Stroke, *9th International Symposium on NEUROPROTECTION and NEUROREPAIR, April 2016, Leipzig, Germany* (Oral presentation).

4) **Balaganapathy, P.** and Arumugam, TV. Interplay Between the Notch and p53 Promotes Neuronal Cell Death in Ischemic Stroke, *CUSAT-NUS Joint International Conference on Biotechnology and Neuroscience (CUSBAN-2016), December 2016, Kochi, India* (Oral presentation)