Regulatory activities of topoisomeraseIIβ in DNA repair, HIV-1 transcription and senescence

A thesis submitted for the degree of

Doctor of Philosophy

By

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the **Department of Biotechnology and Bioinformatics**, School of Lifesciences, University of Hyderabad, under the supervision of **Prof.Anand K. Kondapi**. I further declare that this has not been submitted before for the award of degree or diploma from any Institute or University.

Date:

Place: **Chekuri Anil Kumar**

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CERTIFICATE

This is to certify that thesis entitled "**Regulatory activities of topoisomeraseIIβ in DNA repair, HIV-1 transcription and senescence"** submitted to the University of Hyderabad by **Mr. Chekuri Anil Kumar** for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. This work has not been submitted before for the award of degree or diploma from any University or Institution.

Prof. Anand K. Kondapi
Supervisor

Head, Department of Biotechnology and Bioinformatics

Dean, School of Life Science

Date:

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Anil

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Abbreviations

Topoisomerase IIβ	:TopoIIβ
mRNA	: Messenger RNA
DNA	:Deoxy Ribo Nucleic acid
HIV	: Human immunodeficiency Virus
NHEJ	: Non homologous end joining
DSB	: Double strand breaks
His tag	: Histidine tag
Tat	: Transactivator of transcription
MTT	: 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide
siRNA	: Small interfering RNA
A2M	: Alpha-2-Macroglobulin
GNA14	: Guanine nucleotide binding protein, alpha 14
GRIA1	: Glutamate receptor 1
MASP1	: Mannan-binding lectin serine protease 1
NPY	: Nueropeptide Y
SLIT2	: Slit homolog 2 protein
CGNs	: Cerebellar granule neurons
XRCC1	: X-ray repair cross-complementing protein 1
XRCC4	: X-ray repair cross-complementing protein 4

µg	: Microgram
µl	: Microlitre
µM	: Micromolar
AD	: Alzheimer's disease
ATP	: Adenosine triphosphate
BER	: Base excision repair
BSA	: Bovine Serum Albumin
bp	: Base pairs
CNS	: Central Nervous System
CS	: Cockayne's Syndrome
cDNA	: Complementary DNA
CO2	: Carbon dioxide
DAPI	: 4',6-diamidino-2-phenylindole
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxynucleotidetriphosphates
dsDNA	: Double-stranded DNA
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra acetic acid
FBS	: Fetal Bovine Serum
FACS	: Fluorescence-activated cell sorting
GFAP	: Glial cell fibrilary acidic protein
HRP	: Horse radish peroxidase
IgG	: Immunoglobulin G

IR	: Ionizing radiation
kDa	: Kilodaltons
LIG	: Ligase
mg	: Milligram
mM	: Millimolar
NAD	: Nicotinamide adenine dinucleotide
NER	: Nucleotide excision repair
NSE	: Neuron specific enolase
PAGE	: Polyacrylamide gel electrophoresis
PARP-1	: Poly ADP- ribose polymerase 1
PD	: Parkinon’s disease
PDL	: Poly-D-lysine
PMSF	: Phenylmethyl sulfonyl flouride
POLβ	: DNA polymerase β
PBS	: Phosphate buffer saline
SSBs	: Single strand breaks
TBS	: Tris buffered saline
TCA	: Trichloroacetic acid
TCR	: Transcription coupled repair

Chapter 1

Introduction

Topoisomerases

The higher order chromatin organization of DNA becomes a constraint for various processes in nucleic acid metabolism. Chromosomes must be folded and compacted, should be confined to the cellular boundaries. The processes involving nucleic acid metabolism such as transcription, replication and repair requires access to nucleotide sequence information that necessitates duplex-melting events involving overwinding and supercoiling of nucleic acid segments (Clancy, 2008). Furthermore, DNA repair and replication generate entanglements between chromosomal regions that, if left unresolved, can lead to potentially mutagenic or cytotoxic DNA strand breaks. (Arsuaga et al, 2002).

Enzymes known as topoisomerases manage the topological transactions of DNA. Endowed with an ability to cut, shuffle and religate DNA strands, topoisomerases can regulate the DNA supercoiling and disentangle snarled DNA segments (Wang , 2009, CSHL 2009).

All topoisomerases can be assigned into two primal classes, type I and type II, depending on the cleavage reaction. For the type I enzymes, the DNA strands are transiently broken one at a time; for the type II enzymes, by contrast, a pair of strands in a DNA double helix are transiently broken in concert by a dimeric enzyme molecule.

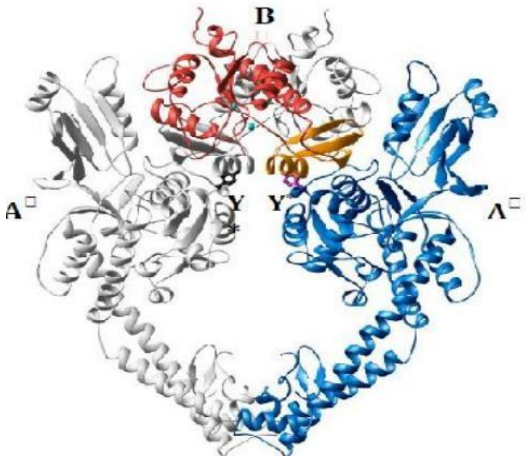
Type I Topoisomerases can effect the topological state of the DNA through a 'strand passage' mechanism (Wang, 1996), where a single DNA strand is cleaved and physically opened, and a second DNA strand is navigated through the gap; following passage of the second DNA segment, the broken strand is resealed. Although type-1 enzymes are dispensible for cell survival they are known to be

playing important roles in replication (Rampakakis, 2009) and transcription (Ju et.al, 2008).

Type II Topoisomerases employ an active strand passage mechanism for effecting topological changes in DNA. However, type IIA enzymes differ in that they cleave both strands of a DNA duplex and pass a second intact duplex using ATP through the transient break (Roca,1994).Type II topoisomerases are involved in diverse nuclear processes including mitotic and meiotic Chromosome segregation, structural maintenance and condensation of the chromosome, thus proving its indispensability in cellular functions.

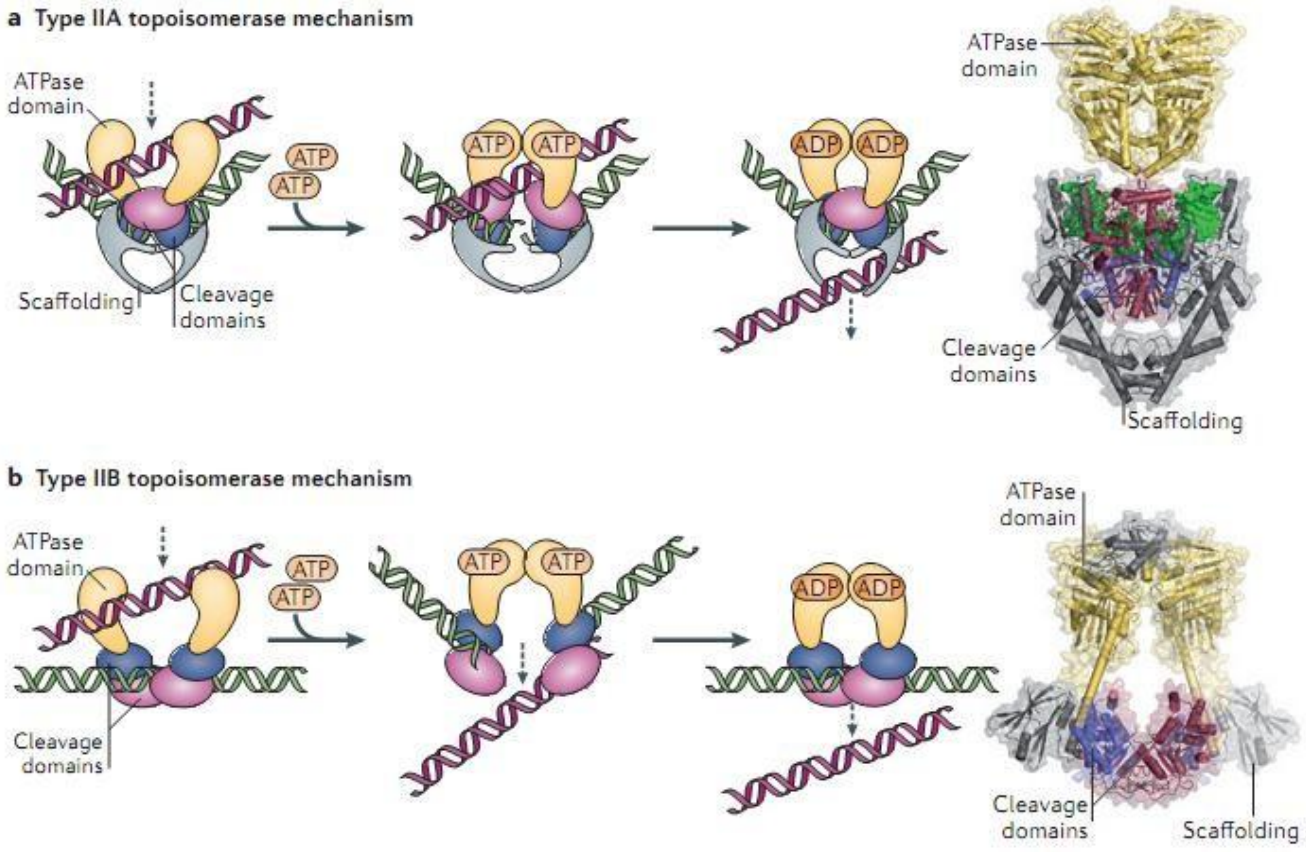
Studies on topoisomerase II from vertebrates, including human (Drake et al.1987, 1989), mouse (Adachi et al., 1992), rat (Tsutsui et al., 2001), have revealed the existence of two isoforms α and β having molecular weight of 170 kda and 180 kda respectively. They are highly similar but genetically, immunologically, and biochemically distinct and display different cellular localization and cell cycle dependent expression patterns. The studies *invivo* have revealed that topo IIα is expressed in developing tissues such as thymus and testis, while topo IIβ is expressed in somatic tissues (Holden et al.,1990; Capranico et al., 1992).Topo IIα gene has been mapped to chromosome 17q21-22, while topo IIβ gene resides on chromosome 3p24. There is evidence to suggest that human topo IIα and topo IIβ might perform nonoverlapping functions in vivo (Meyer et al., 1997; Grue et al., 1998). Homozygous topoIIβ mutant exhibited neonatal death phenotype due to neuromuscular dysfunction (Yang et al., 2000).

Structure and catalysis of Topoisomerase II The crystal structure of TopoII was worked out by Berger et al., (1996). The study shows that TopoII in its active form is a heart shaped homodimer with a large central hole. The monomer is a flat crescent shaped fragment, which can be divided into three discrete domains. The first is the ATP binding domain in the N-terminal region (B' region). It has a consensus sequence for ATP binding and has the capacity to hydrolyze ATP. This domain dimerizes with the other monomer upon binding to ATP and imposes a conformational change all over the enzyme, required for catalytic activity. The second is the DNA binding domain or the DNA breakage/reunion domain, present in the A' region. The active site containing tyrosine, which associate with the broken ends of DNA during the catalytic cycle, is present in this domain. The third is the primary dimer interface in the C-terminal region, which forms the dimer interface of the enzyme by associating with the other monomer. Apart from forming the dimer interface, this region is also implicated in regulation of enzyme activity and nuclear localization.



(From *Berger et al 1996)*

Type IIα cleave both strands of a duplex DNA and pass another duplex DNA (pink) through the transient break in a reaction that is coupled to ATP turnover. The cleaved strands are religated, and the products of the reaction are released from the enzyme. Type IIβ use a duplex strand passage mechanism similar to that of type IIα enzymes and have the same ATPase and cleavage domains but differ in overall tertiary structure.



James C. Wang **Cellular roles of DNA topoisomerases: a molecular Nature Reviews Molecular perspective** Cell Biology 3, 430-44.

Functions of topoisomerase II

Yeasts and Drosophila possess a single molecular species of type II DNA topoisomerase, whereas in higher eukaryotes there are two closely related isoforms, DNA topoisomerase IIα and IIβ. Although the two isoforms possess very similar catalytic properties in vitro, they exhibit high spatio- temporal variations in their expression (Tsutsui 2001). The IIα isoform is expressed in proliferating cells, such as undifferentiated culture cells, whereas expression of the IIβ isoform is very low or negligible in such cells (Heck 1988; Drake et al., 1987; Woessner et al., 1991; Austin & Marsh, 1998). It has been shown that DNA topoisomerase IIα is essential in the process of chromosome segregation after DNA synthesis (Wang, 1996). Inhibition and/or knock down of functional topoisomerase IIβ depressed the induction of a subset of neuronal genes during neuronal cell differentiation (Tiwari, 2012). The enzymatic activity of the IIβ isoform is involved in an early stage of induced expression of neuronal genes, probably through decondensation of the chromatin structure of the gene containing region. The nucleoplasmic DNA topoisomerase IIβ is actively involved in its catalytic reaction. The IIβ enzyme, localized in nucleoli, is inaccessible to chromatin DNA, although it is still active on naked DNA.

Diverse biological functions of Topollβ

The major clues about the diverse biological roles of TopoIIβ in various cellular processes came from its expression pattern throughout the cell cycle unlike that of α isoform (Wang, 1996). This expression of β isoform in non-replicative phases of cell cycle lead to the idea that it exhibits diverse functions in various cellular processes. It has been shown that TopoIIβ is specifically required in certain neuronal cells, and its requirement for transcription of some genes lead to unknown

biological roles for TopoIIβ. TopoIIβ has also been proposed to play roles in DNA repair (Kingma and Osheroff,1998; Yamane et al.,2002), especially in itsability to repair DNA lesions such as abasic sites to generate enzyme mediated DNA damage. A better understanding of the protein complexes that include TopoIIβ may unleash its more possible non –replicative functions. As with other proteomic studies, identification of the relevant protein complexes is only the first step in understanding the relevant biological processes.

Role of DNA topoisomerase II in brain:

The development of the central nervous system with ongoing neuronal differentiation is strictly controlled in a spatio-temporal manner (Vellis and Carpenter,1999).This is clearly illustrated in the cerebellum because of a highly specific and uniform laminar arrangement of cells in the cerebellar cortex. Purkinje cells and granule cells, the two major neurons in the cerebellar cortex, express the topoisomerase II isoforms with similar timing during development (i.e. the α isoform in proliferating cells and the β isoform in post-mitotic cells), although these cells are generated and differentiated at different times and at different sites in the developing cerebellum (Capranico et al., 1992).

Oxidative DNA damage in brain:

The nervous system is also very rich in polyunsaturated fatty acids (PUFAs) and has a high content of transition metals and ascorbate levels, which together act as potent oxygen radical-generating system. On the other hand, it possesses a relative paucity of antioxidant systems compared with other organs, which makes the nervous system highly vulnerable to oxidative stress. Neurons are particular in being terminally differentiated, postmitotic cells, and also being extremely

metabolically active. They, in fact, display high rates of transcription and translation, which are associated with high metabolic rate and mitochondrial activity, and thus with a high rate of oxygen consumption. Excessive levels of ROS can be therefore be generated and constantly attack DNA, producing several lesions (Evans et al., 1996, Dizdaroglu,1992, Breen et al.,1995)

Free radicals are produced in cells by cellular metabolism and by exogenous agents. These species react with biomolecules in cells and one of the important targets is DNA. This kind of damage, often referred to as oxidative DNA damage, has consequences in various organs and particularly in brain, due to its high metabolic activity and oxygen consumption. The consequences include mutagenesis of various kinds, ranging from simple oxidation of bases to large deletions through single and double strand breaks. In brain, because of its post-mitotic nature, oxidative damage to DNA is seen more often at the level of bases. A major route for repairing oxidative damage to bases is base excision repair (BER). It is increasingly becoming apparent that defects in repairing oxidative DNA damage can lead to a number of neurological disorders like Alzheimer and Parkinson (Rolig and Mckinnon, 2000).

Neuronal DNA repair remains one of the most exciting areas for future DNA repair investigations, particularly as a means to compare the DNA repair response in mitotic (cancer) versus post-mitotic (neuronal) cells. In addition, the role of DNA repair in neuronal cell survival and response to aging and environmental insults is of particular interest.

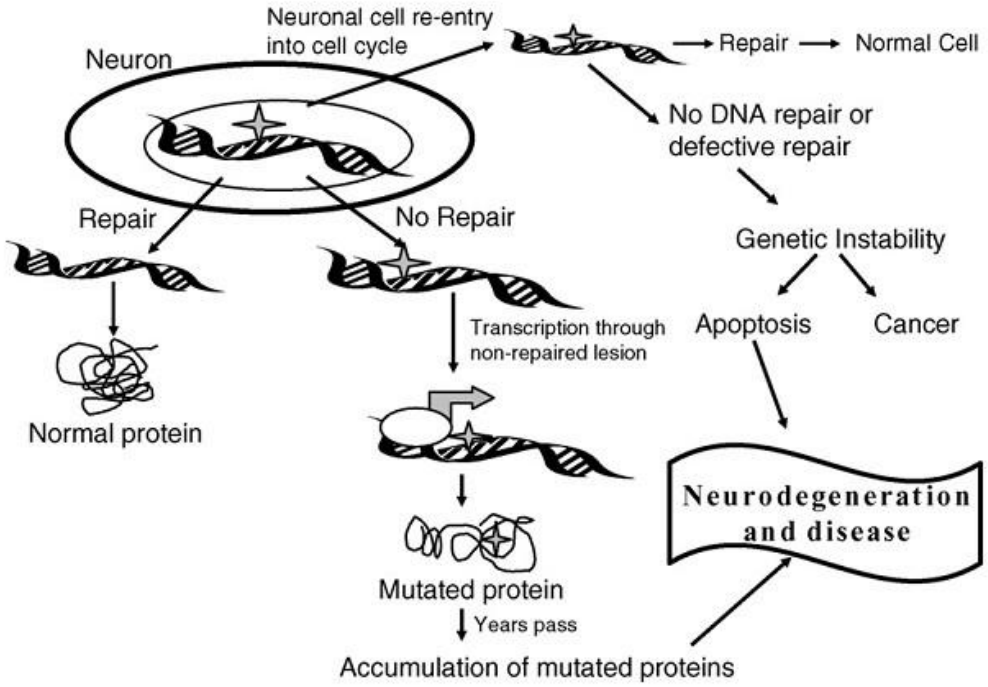
Free radical induced DNA damage:

Free radicals, most notably •OH, react with organic compounds by addition and abstraction. Hydroxyl radical adds to double bonds of heterocyclic DNA bases and

abstracts an H atom from the methyl group of thymine and from each of the C–H bonds of 2-deoxyribose (Evans et al.,1997). Hydroxyl radical reacts with the sugar moiety of DNA by abstracting an H-atom from each of the carbon atoms. Further reactions of thus formed carbon-centered sugar radicals generate various sugar products, DNA strand breaks and base-free sites by a variety of mechanisms (Vieira and Steenken,1990).

In the CNS, excessive production of reactive oxygen and nitrogen species (ROS/RNS) has been invoked in neurodegeneration associated with various insults to neurons, such as hypoxia and hypoglycemia (Halliwell et al., 1992; Friberg et al., 2002), as well as with the neurodegeneration seen in AD (Zhu et al., 2004b), PD (Fahn, 1992) and ALS (Carri et al., 2003). Oxidative damage is caused by an imbalance between overproduction of ROS and/or RNS and the enzymatic or non-enzymatic detoxification of these highly reactive species. The highly reactive ROS and RNS, when over-produced or under-detoxified due to factors such as aging and disease, are detrimental to cells since they chemically modify lipids, proteins, and nucleic acids. Such oxidative modification of macromolecules may be an initiating event in the causation of neuronal injury. As mentioned above, brain neurons respond to such stresses differently. While most brain neurons can tolerate oxidative damage well, neurons in certain parts of the brain, such as those in the hippocampal CA1 region and cerebellar granule cell layer, are particularly vulnerable to oxidative damage (Wang et al., 2005, 2007, 2009). The vulnerability of the cerebellar granule neurons to oxidative stress might play an important role in their significant loss in aged individuals (Andersen et al., 2003). As Oxidative stress appears to be a common underlying factor in the various adverse conditions characterized by Selective Neuronal Vulnerability

(Sayre et al., 2001; Valko et al., 2007), the study of Selective Neuronal Vulnerability to Oxidative stress might improve our understanding of how this particular form of cell stress causes selective neuronal losses in brain.



Potential consequences of a neuronal cell repairing a DNA lesion. Alternative results could be obtained in a non-dividing neuronal cells vs.one that has re-entered the cell cycle(Melissa L. Fishel, Michael R. Vasko b, Mark R. Kelley **(DNA repair in neurons: So if they don't divide what's to repair?** Mutation Research 614 (2007) 24–36.)

Types of DNA damage in neurons:

Accumulation of DNA damage in neurons has long been suggested to be one of the major forms of damage involved in brain ageing and neurodegeneration (Hamilton et al., 2001). The predominant class of oxidative DNA lesions in the aging brain are single base modifications, such as 8-oxoguanine, which increase with aging in rodent models and humans. More than 100 different types of DNA lesions have been reported, including base modifications (for instance, 8- oxo-7, 8-dihydro-2`-deoxyguanosine [8-oxo-dG], thymidine glycol, and 8-

hydroxycytosine), single- and double-strand breaks, and interstrand cross-links (Cooke et al., 2003). The oxidation of nucleotides in the DNA leads to attack of the sugar-phosphate backbone by hydroxyl radical, which can generate a SSB. If two reactions of that type occur in close vicinity (clusters), a DSB formation is possible (Mladenov and Iliakis, 2011) . Breaks of both DNA strands could also occur after conversion of labile lesion to SSB or after enzymatic processing of base damage (Mladenov and Iliakis, 2011). Nevertheless, if left unrepaired, or repaired incorrectly, DNA lesions may result in massive loss of genetic information, genomic rearrangements, or cell death. Therefore, the cell has evolved a number of pathways to repair DNA damage.

DNA Repair pathways

As a major defense against the environmental damage to cells, DNA-repair is present in all the organisms studied to date, including bacteria, yeast, fish, amphibians, rodents and humans. The DNA repair process would minimize cell killing, mutations, persistent DNA damage and errors in replication. The role of DNA repair in neuronal cell survival and response to aging and environmental insults is of particular interest.

The four major pathways for repairing damage to DNA are

1. Mismatch repair (MMR),
2. Nucleotide excision repair (NER),
3. Base excision repair (BER), and
4. Double-strand break repair (DSBR).

DNA mismatch repair (MMR) is a highly conserved pathway that removes base-base mismatches and insertion-deletion loops that arise during DNA replication and recombination (Jiricny, 2006). The nucleotide excision repair (NER) is a

complex DNA repair system that recognizes bulky, helix-distorting lesions, such as pyrimidine dimers and 6–4 photoproducts, intrastrand crosslinks (Niedernhofer et al., 2004), and some DNA adducts caused by oxidative damage (Nouspikel, 2008). NER involves the excision of a single-stranded lesion-containing oligonucleotide fragment, thus creating a single-strand gap in the DNA. This gap is subsequently filled during repair synthesis by a DNA polymerase using the undamaged strand as a template .There is, however, an alternative NER pathway that is coupled to active transcription and is termed transcription-coupled repair. BER involves the removal of one nucleotide (short-patch BER, SP-BER) or 2–13 nucleotides (long-patch BER, LP-BER) by a glycosylase action (i.e., 8-oxoguanine DNA glycosylase, OGG1), in which the other strand is used as a template to repair the specific lesion (Wilson.,1997)

NHEJ in the Nervous System

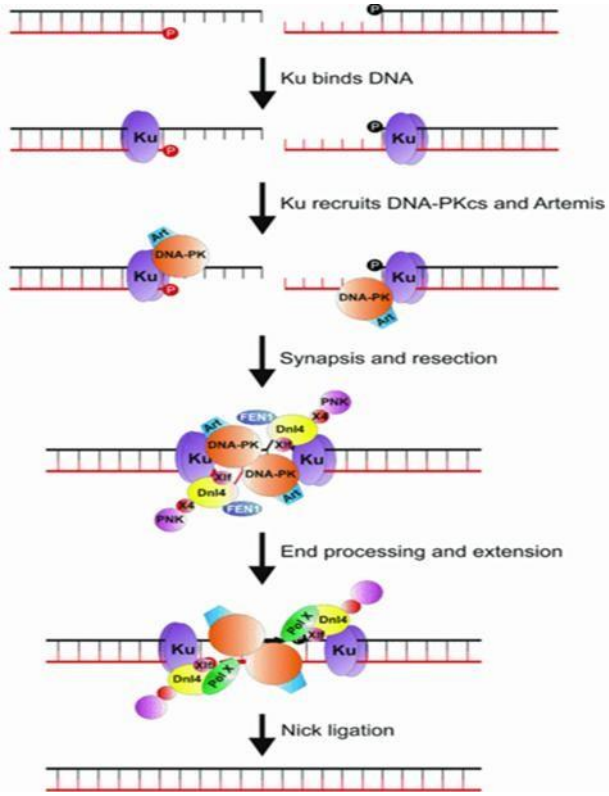
Since the mature nervous system is largely postmitotic, NHEJ is the main DNA DSB repair pathway in the brain (Orii et al., 2006, McKinnon, 2009.). In fact, several observations in the mouse knockout models of NHEJ factors, and different analyses of NHEJ activity in mature rat brain (OrtRen K et al.,2002) point towards the importance of NHEJ in neuronal function and homeostasis. Among them DSB, although being less frequent, is one of the most toxic and mutagenic lesions (Fishel et al., `2007). Furthermore, defective DNA repair systems in neurons can lead to a high accumulation of DNA damage, such as chromosomal breaks. In particular, during neural development, defects in NHEJ can result in neuropathology (i.e.neurodegeneration and microcephaly), suggesting that responding to DNA DSBs is essential for neural homeostasis (McKinnon ,2009).

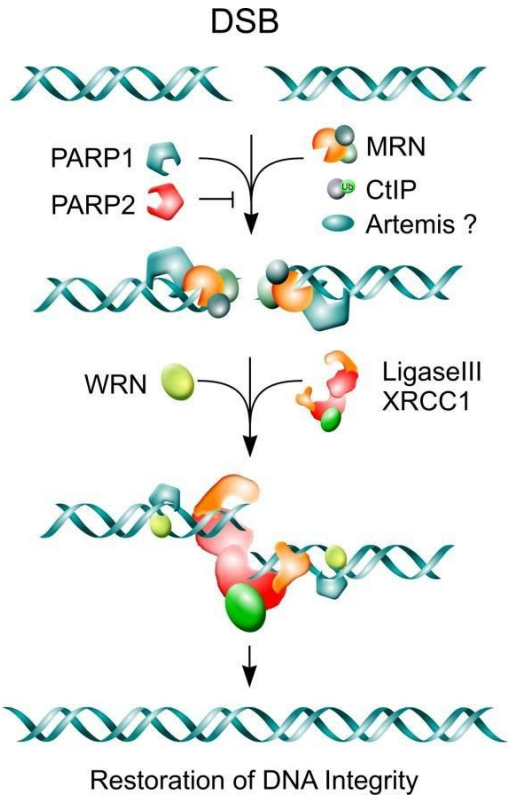
Furthermore, in mature brain, the inability to respond to DNA DSBs may lead to neurodegenerative disorders. In particular, it has been reported that genes involved in signaling pathway which coordinates cell cycle arrest after DNA DSB, such as the ataxia telangiectasia mutated gene (ATM), becomes defective in neurodegenerative disorders (ataxia-telangiectasia and ataxia-telangiectasia-linked disorder, AT and ATLD (Dar et al.,2006). Moreover, the high oxidative DNA damage and the decreased DNA repair observed in Alzheimer's patients(Coppedè ,2009) have been correlated to defects in the NHEJ repair process, although a precise locus or gene(s) affected in this pathway is not yet been identified (Kanungo et al.,2013).

Mechanism of NHEJ pathway

"Clean" DSBs with complementary overhangs, 5′ phosphates and 3′ hydroxyl groups, such as those produced by nucleases, can be precisely repaired by NHEJ. In yeast and mammalian cells, 25-50% of nuclease DSBs are repaired by precise NHEJ (Clikeman et al., 2001, Lin et al., 1999). When ends cannot be precisely rejoined, NHEJ typically involves alignment of one or a few complementary bases ("microhomology") to direct repair, leading to small deletions and sometimes small insertions. In mammalian cells NHEJ proceeds in a stepwise manner beginning with limited end-processing by the MRE11/RAD50/NBS1 (MRN) complex and perhaps other factors, end-binding by Ku comprising the Ku70 and Ku80 subunits, and recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), forming the trimeric DNA-PK holoenzyme. Once bound to broken ends, DNA-PK is activated and it phosphorylates itself and other targets including RPA, WRN, and Artemis; in cells lacking ATM, DNA-PK can also phosphorylate histone H2AX, termed γ-H2AX (Burma et al., 2001). In the final step, DNA ligase IV, with its binding partners XRCC4 and XLF

(also called as Cernunnos), seals the break. The nuclease Artemis helps repair a subset of IR- induced DSBs by NHEJ, and is important for opening hairpins formed during V(D)J recombination .Terminal deoxynucleotidyl transferase (TdT) and two members of the polymerase X family (pol μ and pol λ) can modify NHEJ outcomes by non-templated addition of nucleotides to ends or by extending a 3′ single-stranded DNA (ssDNA) tail that can transiently pair via microhomology on the other broken end (Nick et al,2005). In 1996, Boulton and Jackson provided the first evidences for the existence of an alternative DNA end-joining pathway. This pathway was about 20-fold less efficient than NHEJ and repair junctions displayed both nucleotide deletions and overlapping microhomologies of 3–16 nucleotides (Boulton and Jackson, 1996). Although it was known at that time that short microhomologous regions of up to five nucleotides were commonly recovered at





NHEJ repair junctions of mammalian cells (Roth et al., 1985), this DNA repair pathway was clearly able to operate in a NHEJ-deficient background (Decottignies et al., 2013). XRCC1,LigaseIII and PARP-1 are known to be involved in back NHEJ pathway, wherein new function of PARP-1 for synapsis of DNA ends that is uncoupled from the subsequent ligation step dependent on XRCC1/DNA ligase III (XL). Finally, DNA ligase III reseals the break. Further characterization of the DNA-protein complexes would be necessary in order to know if two XL complexes associate with a PARP-1 homodimer.

Role of topoisomerase IIβ in DNA repair

Predominance of topoisomerase IIβ in non-proliferating cells, namely neurons, suggests its role in non-replicating functions of DNA (Tsutsui et al. 2001, Kondapi et al. 2004). The decreasing activity of topo IIβ with aging (Kondapi et al. 2004) points out its possible role in DNA repair activity in neurons during aging. It has been reported that several DNA damaging agents and Topo II poisons can enhance the activity of DNA repair enzymes. Emmons et al studied activities of TopoII α and β in repair of melphalan-induced cross-links in the cellular DNA of K562 Leukemia and U937 histocytic lymphoma cells, the results could correlate TopoIIβ levels with the repair of cross-links. TopoIIβ may be activated through signaling proteins expressed during DNA repair (Nitiss etal., 1998,Yamane et al ,2002). Repair process mediated by TopoIIβ may also promote repair of DSBs formed due to TopoIIα mediated DNA damage, through Non-homologous end-joining (NHEJ) repair pathway (Mandraju et al 2008). It has been shown that TopoIIβ is involved in regulation of transcription of proteins involved in DNA repair (Robb et al., 2012). Moreover the functional activity of TopoIIβ may occur

through an association with DNA rearrangements during branch migration and resolution of crossover intermediates. This facilitates the release of torsional stress in order to maintain the topological integrity in DNA. In the absence of such an activity of TopoIIβ, the migration and forward movement of the repairing DNA ends will be inhibited, thereby blocking the progression of DNA repair.

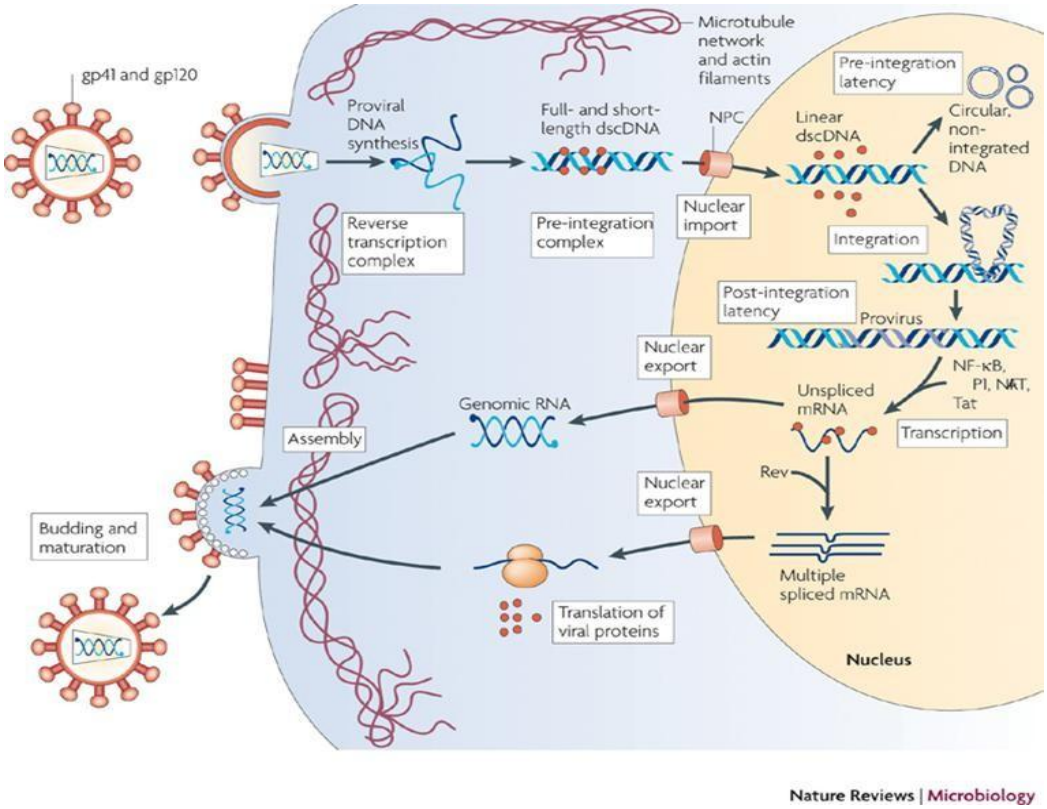
Human Immuno Defeciency Virus type 1:

Human Immunodeficiency Virus type 1 (HIV-1) is the cause of the acquired immunodeficiency syndrome (AIDS) and the responsible of a devastating pandemic that affects around 34 million people worldwide. Untreated HIV-1 infection is characterized by continuous viral replication that drives CD4+ T cell loss and predicts disease progression. HIV-1 belongs to the family "Retroviridae". The evolutionary success of this family is in contrast to its deceptive simplicity. HIV‑1 can persistently infect humans by subverting the innate and adaptive immune systems, despite encoding only 15 mature proteins. Viral replication at the cellular level proceeds through a series of steps that starts when a virus productively engages cell surface receptors and ends when nascent particles mature into infectious virions. During this process, HIV‑1 exploits a myriad of cellular factors to replicate, whereas host restriction factors fight to suppress this replication.

HIV-1 life cycle:

Viral fusion and entry requires the binding of glycoprotein gp120 to CD4 receptors at the cell surface as well as to CC chemokine receptor type 5 (CCR5) or CXC chemokine receptor type 4 (CXCR4). The viral nucleocapsid enters the cytoplasm and uses cytoplasmic dynein to move toward the nuclear pore complex (NPC). The viral RNA is retrotranscribed into proviral double-stranded cDNA (dscDNA),

which can stay in the cytosol, where it is highly unstable and exists in a transient, reversible pre-integration latent state, or can form a pre-integration complex consisting of ds cDNA, viral proteins and some host cell proteins. When ATP



Understanding HIV-1 latency provides clues for the eradication of long-term reservoirs. Mayte Coiras, María Rosa López-Huertas, Mayte Pérez-Olmeda & José Alcamí **Nature Reviews Microbiology** 7, 798-812 (2009).

Viral fusion and entry requires the binding of glycoprotein gp120 to CD4 receptors at the cell surface as well as to CC chemokine receptors. The viral nucleocapsid enters the cytoplasm moves toward the nuclear pore complex (NPC). The viral RNA is retrotranscribed into proviral double-stranded cDNA (dscDNA), which can stay in the cytosol or form a pre-integration complex consisting of dscDNA. The pre-integration complex is transported into the nucleus through the NPC, and the dscDNA either circularizes as one or two long terminal repeat-containing circles or is integrated into a host cell chromosome. After integration, the provirus remains quiescent or undergoes activation. On activation, the viral genome is transcribed by the synergic interaction of cellular transcription factors (nuclear factor-kappaB (NF-kappaB),

nuclear factor of activated T cells (NFAT) and specificity protein 1 (SP1)) and the viral transactivator, Tat. Rev, a viral protein, regulates the splicing and cytosolic transport of some of the viral mRNAs, which are translated into regulatory and structural viral proteins. New virions assemble and bud through the cell membrane, maturing through the activity of the viral protease.

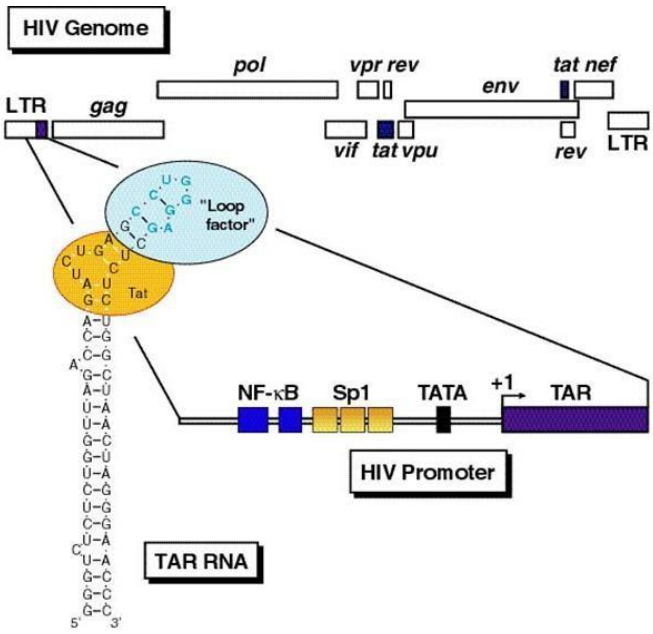
levels are adequate, the pre-integration complex is transported into the nucleus through the NPC, and the dscDNA either circularizes as one or two long terminal repeat-containing circles or is integrated into a host cell chromosome. After integration, the provirus remains quiescent, existing in a permanent post-integration latent state. On activation, the viral genome is transcribed by the synergic interaction of cellular transcription factors (nuclear factor-kappaB (NF-kappaB), nuclear factor of activated T cells (NFAT) and specificity protein 1 (SP1) and the viral transactivator, Tat. Rev, a viral protein, regulates the splicing and cytosolic transport of some of the viral mRNAs, which are translated into regulatory and structural viral proteins. New virions assemble and bud through the cell membrane, maturing through the activity of the viral protease.

Organisation of HIV-1 genome:

The HIV-1 genome encodes nine open reading frames. Three of these encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins common to all retroviruses. The four Gag proteins, MA (matrix), CA (capsid), NC (nucleocapsid), and p6, and the two Env proteins, SU(surface or gp120) and TM (transmembrane or gp41), are structural components that make up the core of the virion and outer membrane envelope. The three Pol proteins, PR (protease), RT (reverse transcriptase), and IN (integrase), provide essential enzymatic functions and are also encapsulated within the particle. HIV-1 encodes six additional proteins, often called accessory proteins, three of which (Vif, Vpr, and Nef) are found in the viral particle. Two other accessory proteins,

Tat and Rev, provide essential gene regulatory functions, and the last protein, Vpu, indirectly assists in assembly of the virion.

The retroviral genome is encoded by an 9-kb RNA, and two genomic-length RNA molecules are also packaged in the particle. Tat and rev are regulatory proteins that accumulate within the nucleus and bind to defined regions of the viral RNA: TAR (transactivation-response elements) found in the LTR; and RRE (rev response elements) found in the env gene, respectively.



Structure of the HIV genome. Karn, J. (1999). Tackling Tat. J. Mol. Biol. 293, 235-254.

The Tat gene is encoded by two exons that are highlighted on the HIV genome map. The viral promoter has a structure typical of promoters activated by RNA polymerase II. Immediately upstream of the TATA box are two tandem NF-kB binding sites and three tandem SP-1 binding sites. Immediately downstream of the start of transcription is the transactivation response region (TAR). TAR encodes an RNA that can fold into the stem-loop structure shown at left. Tat is able to bind directly to the highlighted region near to the apex of the stem containing a UCU bulge. Critical residues for Tat binding are shown in bold. In addition to acting as the binding site for

Tat, TAR acts as the recognition signal for a cellular co-factor interacting with its apical loop sequence (From (Karn, 1999)).

HIV-1 transcription

After its entry into the cell, the HIV -1 RNA is reverse transcribed to form DNA that moves to the nucleus via the preintegration complex and becomes integrated into the cellular chromatin (Bukrinsky and Haffar,1999). These complexes are either activated by cytokines, by chemokines, by various cellular events, or can be recruited by the viral Tat protein after its production (Copeland, 2005; Pumfery et al., 2003).Tat assists the recruitment of the components of a competent Pre Integration Complex and cellular factors. Tat acetylated in K28 by PCAF binds strongly to CycT-CDK9, and the complex has increased affinity for TAR RNA. Acetylation of Tat by p300/CBP and hGCN5 at K50 and K51 releases the complex from TAR, which contributes to the recruitment of TBP, TFIIB, PCAF, RNAPII, and SWI/SNF at the initiation site. Active progression of the transcription complex occurs in the presence of Tat-P-TEFb. Cellular factors recruited by Tat to the HIV-1 promoter form an active PIC. The complex initiates the transcription of 15-nt RNA. The phosphorylation of the RNAPII CTD by CDK9 triggers polymerase departure and effective transcription elongation. The phosphorylation of the RNAPII CTD, NELF, and DSIF by CDK9 is maintained throughout the elongation process. Transcriptional elongation is inhibited by the activity of NELF and by the DSIF that prevents the phosphorylation of the RNAPII CTD which terminates the transcription.

Role of cellular host factors in HIV-1 transcription:

As with other lentiviruses, HIV-1 must be integrated into host cell chromosomes to acquire permanent residence as a provirus. Once integrated, HIV-1 provirus can

subsequently be considered as a host-inducible gene that requires the concerted action of different transcription factors, as well as the RNA polymerase II (RNAPII) and associated transcription machinery, to produce full-length viral mRNA that will be translated into viral proteins.

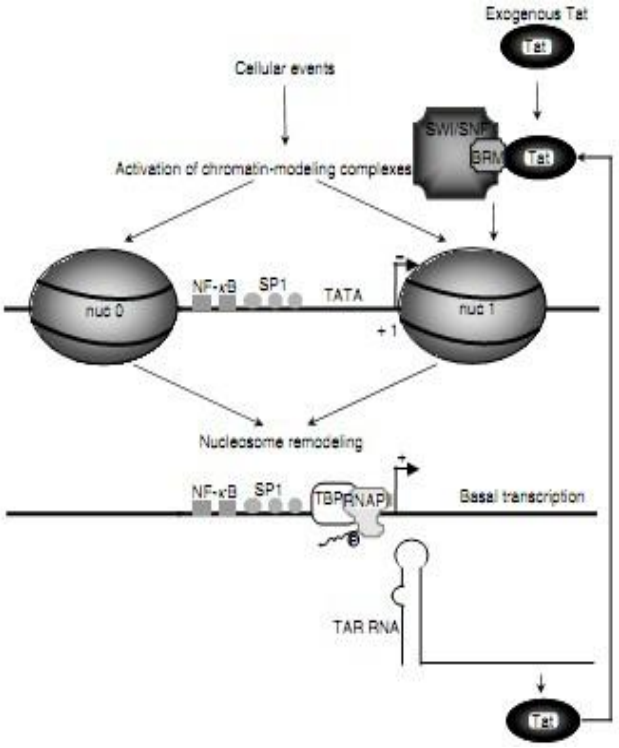
The site of proviral integration is critical to promote efficient transcription. Integration is a highly restrictive process that occurs preferentially into chromosomal regions where viral transcription may be assured. After the provirus integration into the cellular chromosome, HIV-1 replication begins when the cellular transcription machinery can be recruited to the LTR once the open chromatin structure allows access to the proviral DNA.Once the chromatin around 5'LTR is in a permissive state, several host transcriptional factors, such as NFκB, specific protein 1 (Sp1), activated protein 1 (AP-1), and nuclear factor of activated T-cells (NF-AT), are recruited to their consensus sites, promoting a dynamic viral transcription. Many of the putative host transcription factors aiding viral replication were yet to be identified.

Molecular regulation of HIV-1 transcription is a multifaceted process dictated in part by the abundance of cellular transcription factors that induce or repress HIV-1 promoter activity and by the viral Tat protein. The 5'LTR functions as the HIV-1 promoter and contains DNA binding sites for several ubiquitously expressed cellular transcription factors, such as Sp1 and TFIID, and inducible transcription factors, including NF-kappaB, NFAT and AP-1.

Chromatin modifications during HIV-1 expression:

The integrated HIV-1 LTR is transcriptionally inactive due to the formation of nucleosomes as shown in the proximal promoter. Nucleosome unfolding is a prerequisite to any transcriptional initiation and this mechanism is accomplished

by chromatin remodeling complexes that modify histone–DNA interactions. The integration of the HIV-1DNA in human chromatin is not site specific, although it integrates preferentially within active transcription units, but the chromosomal environment influences the level of basal transcriptional activity (Jordan et al., 2001; Lewinski et al., 2005; Schroder et al., 2002).



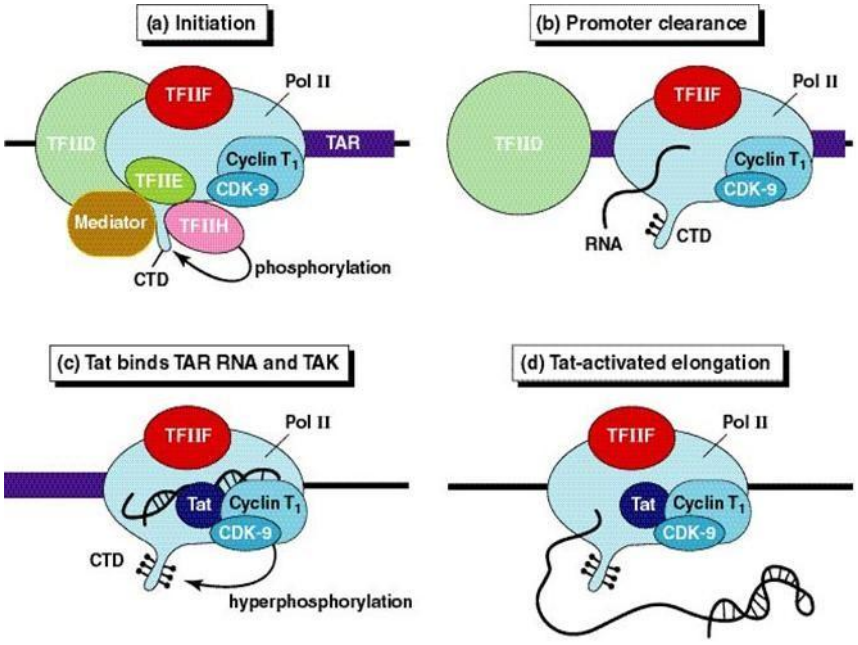
Transcription of HIV: Tat and Cellular Chromatin. Anne Gatignol, Advances in Pharmacology, Volume 55,2007.

Cellular events for the initial transcription, or newly synthesized Tat, or exogenous Tat from other infected cells induce the recruitment of chromatin-modeling complexes that will disrupt the nucleosome structure and allow basal transcription.

The role of chromatin-modifying complexes is to disrupt the nucleosomal structure so that the DNA becomes more accessible to interacting proteins. These complexes belong to two main groups: the ATP-dependent remodeling complexes that alter histone–DNA interactions and proteins that regulate histone acetylation and histone deacetylases which act as acts as an activation/repression switch in transcription by regulating DNA accessibility to regulatory proteins. Chromatin-remodeling complexes act as a first step on the integrated provirus to destabilize the nucleosomal structure and allow basal transcription. After a threshold amount of RNA is produced, Tat is synthesized and will recruit more chromatin-remodeling factors.

HIV-1Tat and viral transcription:

Tat is the viral trans-activator of transcription that binds the transactivation response RNA element TAR at the 5'-end of all viral transcripts to promote transcriptional elongation (Karn, J 1999.). Tat is a promiscuous viral protein that has been shown to associate to a number of host factors. The RNA polymerase II



Model for the activation of RNA polymerase II by Tat and cellular co-factors. (a) The RNA polymerase II holoenzyme is recruited to the HIV LTR through its interactions with TFIID and other components of the basal transcription apparatus. (b) The nascent RNA chain corresponding to the TAR RNA transcript folds into its characteristic stem-loop structure and binds the RNA polymerase. (c) Tat is recruited to the transcription complex because of its ability to bind to the bulge sequence found near the apex of the TAR RNA structure and forms a ternary complex with TAK.(d) The activated transcription complex is able to transcribe the remainder of the HIV genome. (From Karn, 1999).

holoenzyme is recruited to the HIV LTR through its interactions with TFIID and other components of the basal transcription apparatus (Keen et al.,1997). The CTD domain of the RNA polymerase is phosphorylated by the CDK-7 kinase found in TFIIH and the modified polymerase clears the promoter and begins transcription of TAR. (b) The nascent RNA chain corresponding to the TAR RNA transcript folds into its characteristic stem-loop structure and binds the RNA polymerase. (c) Tat is recruited to the transcription complex because of its ability to bind to the bulge sequence found near the apex of the TAR RNA structure and forms a ternary complex with TAK. The activated TAK kinase catalyses phosphorylation of the CTD and TAR is displaced from the polymerase. (d) The activated transcription complex is able to transcribe the remainder of the HIV genome (Karn,1999)

The pattern of transcription from the HIV LTR suggested that Tat does not stimulate initiation. Initial transcription from HIV LTR characteristically produce two populations of transcripts, a set of short non-polyadenylated RNAs that terminate near the promoter, and a set of full-length polyadenylated mRNAs (Kao et al., 1987). In the absence of Tat, the short transcripts predominate, whereas in the presence of Tat there is a dramatic increase in the levels of long transcripts

(Kao et al., 1987; Kessler & Mathews, 1992; Ratnasabapathy et al., 1990; Toohe& Jones, 1989). Thus, Tat participates in a positive feedback mechanism that ensures high levels of HIV transcription following the activation of cells carrying HIV proviruses. Furthermore, the shift from short to long transcripts observed in these experiments strongly implies that Tat actives transcription elongation.

Role of topoisomerases in Transcription

Until recently, the role of topoisomerase II in transcriptional regulation was restricted to resolving topological problems regarding the passage of RNA polymerase II, as it transcribes genes into messenger RNA. Remarkably, Ju et al. (2006) report that regulated gene transcription involves the unexpected recruitment and unique enzymatic function of TopoIIβ. This enzyme produces a transient nucleosome-specific DNA double-strand break that is required for transcriptional activation. They further demonstrate that the presence of the transient double-strand break is mechanistically due to the action of the component of a complex, which also includes two enzymes that sense DNA damage, poly(ADP-ribose) polymerase–1 (PARP-1) and Ku70

Role of Topoisomerases in viral replication

The absence of a virus-encoded type II topoisomerase creates a problem, because the complex structures formed during genome replication, presumably have to be resolved into monomers, condensed, and packaged into developing particles. The telomere resolution reaction and cleavage of branched DNA structures could be catalyzed by a viral Holliday junction resolvase, but neither this enzyme nor the viral topoisomerase is well suited for unknotting the complex structures that are likely formed during virus replication and recombination. One solution could be that many viruses might use cellular type II topoisomerases to disentangle duplex

DNAs. Topoisomerases move from cytoplasm to nucleus over the course of the cell cycle and thus might be accessible to viruses replicating in the cytoplasm of infected cells. This hypothesis is supported by the observation that replication of many viruses including vaccinia virus, herpex simplex virus, SV40 and Epstein barr virus replication is inhibited by the topoisomerase II inhibitors (Purushothaman 2012, Hammersten.,1996, Michiko Kawanishi 1993, Benson and Huang 1998, James Lin 2008.)

The involvement of topoisomerase II in the viral DNA Replication has been hypothesized by considering the following evidence:

(i) Topoisomerase II influences many aspects of DNA metabolism (Wang, 1996) including recombination (Christman et al., 1988; Kim & Wang, 1989)

(ii) Integration sites in the host DNA are found within regions of chromatin that are preferentially accessible to DNase I (Vijaya et al., 1986)and that are transcriptionally active

(iii) A strong topoII binding and cleavage cluster was found within the local chromatin into which an HIV genome had integrated (Howard & Griffth, 1993)

(iv) HIV infection leads to phosphorylation of topo II, which stimulates its activity (Matthes et al., 1990).

Topoisomerase II may influence the chromatin structure, keeping it in an open configuration that could favor viral transcription .The enzyme may play a role in modulating the topology of the chromatin fiber before and/or after transcriptional

induction. From this stand point, topo II may influence retroviral transcription by facilitating the open state of the chromatin fiber.

Ageing

All multi-cellular organisms undergo change with time. Conception heralds the onset of growth and development, leading to reproductive competence and propagation of the species with time organisms age, leading to death as a final end-point. Ageing is the process of accumulative changes to molecular and cellular structure that disrupts metabolism with the passage of time, resulting in deterioration and eventually bringing about death. Oxidative damage to various biomolecules like proteins, lipids and DNA increases with age (Bokov et al., 2004). These changes progresses inevitably over time. However, the rate of the progression of such damage can be very different from person to person.

Theories of Ageing

Explanations on aging mechanisms have now become unexpectedly complicated. However, it is gradually accepted that 'senescence is a collective consequence of both inheritance and environment'

Important theories of aging at the whole animal level are:

Wear and tear theory

Error catastrophe theory

Stress damage theory

Autointoxicationtheory

Major theories of aging at the organ level are:

Endocrine theory

Immuno-biological theory

Key aging theories at the cellular level are:

The cell membrane theory

Somatic mutation theory

The mitochondrial theory

The mitochondrial-lysosomal axis theory

Limited cell proliferation theory(programmed aging theory).

Important aging theories at the molecular level are:

Accumulation of DNA alterations

Trace element theory

Free radical theory (Harman, 1956,2003)

Crosslinkage theory

Oxidative stress theory

Non-enzymatic glycosylation theory

Carbonyl toxification theory

Garbage catastrophe theory

But, the most widely accepted are structural damage theory and the one theory that encompasses all of the previous theories - is the Free Radical Theory of aging.

Based on radiation-related free radical studies, Dr Harman formally put forward the free radical theory of aging in 1956 (Harman, 1956, 2003). Harman suggested that free radicals produced during respiration cause cumulative oxidative damage, resulting in aging and death. Free radicals are highly reactive molecules or atoms that have an unpaired electron in an outer orbital that does not contribute to molecular bonding (Halliwell et al., 1989). Atoms or small molecules that are free radicals tend to be the most unstable, because larger molecules can have the capacity to form resonance structures. Under normal physiological O_2 levels, 1-2% of the O_2 consumed is converted to reactive oxygen species (ROS). For biological systems, oxygen free radicals are the most important, in particular superoxide ($.O_2$ -), nitric oxide (.NO) and the hydroxyl radical (.OH).

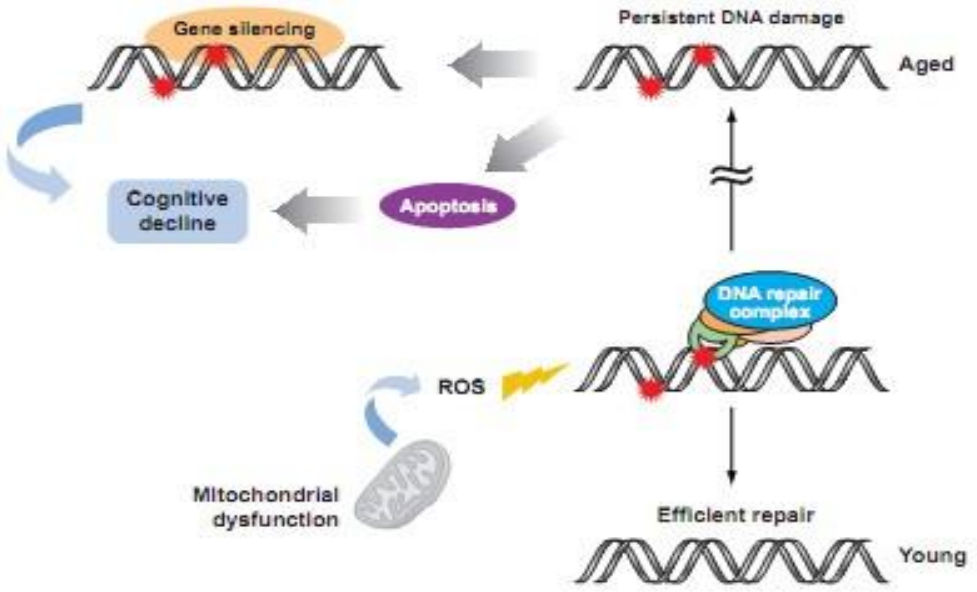
DNA damage and repair theory

The first theoryto suggest that DNA damage per se, apart from its role in inducing mutations, may be a primary cause of ageing. This theory postulates that the DNA damage, which is bound to occur in the body of an organism, is repaired efficiently up to a certain age of an organism, but thereafter is compromised in a predetermined manner. Thus, from some point of lifespan, DNA repair capacity decreases, therefore DNA damage accumulates. This accumulation of DNA damage leads to the breakdown of all the vital processes in the cell finally leading to death. In mammals, long-lived neurons, differentiated muscle cells and other differentiated cell types that do not divide or divide slowly, accumulate DNA damage with age. These cells are likely candidates to govern the rate of mammalian ageing. In brain, the level of DNA repair is low, endogenous damages

accumulate with age, mRNA synthesis declines and protein synthesis is reduced. Furthermore, cell loss, tissue function decline and functional impairments directly related to the central processes of ageing. Thus, for the brain, there appears to be a direct relationship between the accumulation of DNA damage and ageing.

Brain Ageing

In theory, the CNS should be particularly susceptible to damage by ROS because of its high demand for oxygen, the abundance of redox-active metals (iron and copper), the high levels of brain PUFAs and the fact that mature neurons are postmitotic. In practice, increased oxidative damage is readily detectable in the ageing brain, which is paralleled by an increase in oxidative stress response gene expression with ageing in Brain. The characteristic symptomology of brain ageing include decrease in global gene expression due to promoter destruction as a result of DNA damage. The progressive loss of post-mitotic tissue during aging may be due to the death of damaged post-mitotic cells which cannot be replaced, or possibly their malfunction. The damage may derive from either intrinsic or extrinsic sources, and may consist of damage to proteins as well as DNA. The loss of neuronal cells is particularly problematic, since they are least readily replaced. There are increasing evidences, that neurodegeneration and cognitive decline may be associated with specific changes in gene expression. Neurodegenerative diseases share common predisposing factors like loss of neuronal circuit, memory and synaptic plasticity during brain aging as in the case of Alzheimer's and Parkinson's diseases.

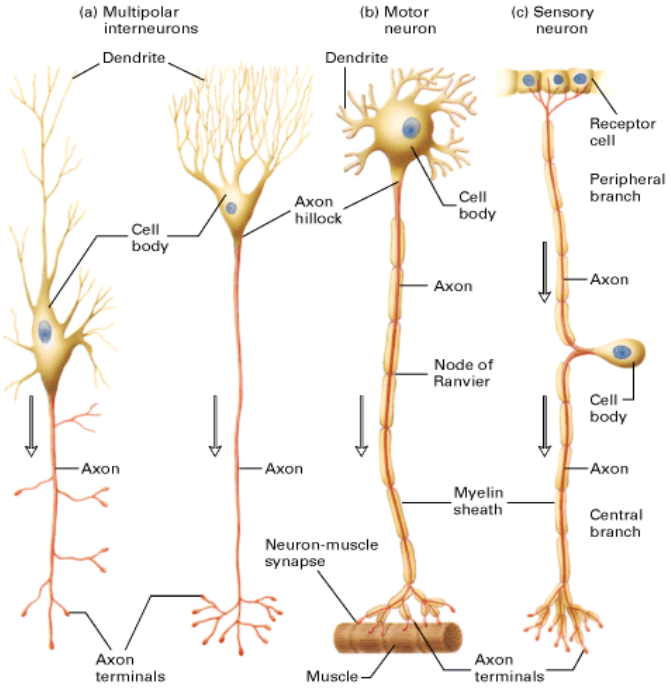


Types of neurons

Neurons are the information-processing cells of the nervous system. They are categorized as receptors, interneurons, or effectors, depending on their function. The dendrites of a neuron provide an extended receptive surface for the cell, increasing greatly the number of synaptic inputs. Many dendrites have dendritic spines at their more distant synapses.

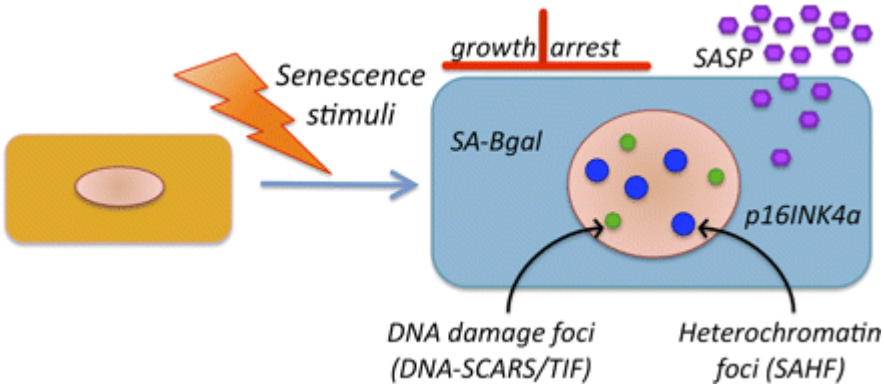
1. Motor neurons or Multipolar neurons carry signals from the CNS to muscles and glands. These neurons have many processes originating from the cell body. Motor neurons account for 9% of all neurons. (Examples are spinal motor neurons, pyramidal neurons and Purkinje cells.)

2. Interneurons or Pseudopolar cells form all the neural wiring within the CNS. These have two axons (instead of an axon and a dendrite). One axon communicates with the spinal cord and the other communicates with either the skin or muscle.



Cell culture as a model for ageing

Given the duration of ageing in humans, cell culture studies are a promising approach to the study of human ageing. It is reasonable to assume that human ageing has, at least partly, a cellular origin. Cellular senescence was first described by Hayflick and Moorfield in 1961 who observed that cultures of normal human fibroblasts had a limited replicative potential and eventually became irreversibly arrested (Hayflick and Moorhead, 1961). The majority of senescent cells undergo, a large number of changes in gene expression, protein processing and chromatin organization (Gonos et al., 1998). Senescent cells maintain metabolic activity and can remain viable essentially indefinitely (Matsumura et al., 1979 and Pignolo et al., 1994). An important component of this stability in culture may be the capacity of senescent cells to resist apoptosis (Marcotte et al., 2004 and Hampel et al., 2005).



Four faces of cellular senescence Francis Rodier and Judith Campisi. (2011) JCB;192,547-556.

The key features of senescent cells are

(a) The senescence mediated growth arrest is essentially permanent and cannot be reversed by known physiological stimuli.

(b) Senescent cells increase in size, sometimes enlarging more than twofold relative to the size of non senescent counterparts (Hayflick, 1965).

(c) Senescent cells express a senescence-associated β-galactosidase (SA-Bgal; Dimri et al., 1995), which partly reflects the increase in lysosomal mass (Lee et al., 2006).

(d) Most senescent cells express p16INK4a, which is not commonly expressed by quiescent or terminally differentiated cells (Alcorta et al., 1996)

Neuronal Senescence

Ageing of neuronal cells in vitro was not described until the end of the twentieth century (Lesuisse and Martin, 2002; Toescu and Verkhratsky, 2000). Providing the optimal conditions over long period of time was the biggest obstacle. Hippocampal

and cortical neurons have been able to survive up to 60 days in culture (Lesuisse and Martin, 2002).

The predominant ageing mechanism of post-mitotic cells (i.e. neurons, myoblasts and osteocytes) is often thought to be due to the accumulation of damage to DNA, protein and lipids caused by reactive oxygen species (ROS). Since these cells do not divide in tissues and can potentially persist for a lifetime, they are more likely to accumulate damage which may subsequently result in an age-related phenotype. Also, since mitotic cells are mostly found in a non-dividing state in vivo known as quiescence, with very little cell turnover, they could also in some regard be treated similar to post-mitotic cells. However, the question remains, whether investigation of replicative senescence will shed insight into the senescence of postmitotic differentiated cells (Cristofalo, 1996; Rubin, 1997). Several investigators suggested that the ‘‘stationary phase’’ cell cultures (Rubin, 1997) might be a more advisable model to study mechanisms of ageing of post mitotic differentiated cells.

CGNs as ageing model

Granule cells of the cerebellum are the smallest neurons in the brain. (The term granule cell is used for several unrelated types of small neurons in various parts of the brain). Cerebellar granule cells are the most numerous neurons in the brain: in humans, estimates of their total number average around 50 billion, which means that they constitute about 3/4 of the brain's neurons (Linnas, 2004). These cells constitute the most abundant neuronal population in the mammalian brain. When cultured from early postnatal rats, cerebellar granule cells differentiate, acquiring several morphological, biochemical, and electrophysiological characteristics of mature neurons (Lasher et al., 1972, Hockberger et al.,1987). CGNs of the cerebellum are vital for co-ordination and control. Associated disorders and symptoms of cerebelluminclude tremors, nystagmus (involuntary movement of the

eye) and ataxia (lack of co-ordination). Previously, long-term cultures of CGNs were shown to survive in vitro for a maximum of 17 days (Ishitani et al., 1996), while, CGNs could be maintained for 23 DIV (days in vitro) in studies on the Ca^{2+} homeostasis related to ageing (Toescu and Verkhratsky, 2000). CGNs were chosen for this study because primary cultures of postnatal rat cerebellum make an excellent model system for molecular and cell biological studies of neuronal development and function.

Our present study completely focusses on cerebellar granule neurons as a cell culture model of ageing.

Gene expression during neuronal senescence

A number of microarray studies has been conducted to monitor genome-wide changes in gene expression associated with aging, particularly in the brain.

Two primary themes arose from comparison of the aging process in different organisms.

1. Specific biological pathways are being altered as a result of the aging process, as opposed to a genome-wide dysregulation of transcription.

2. Although the underlying cause has not been identified, there is an age-associated induction of stress response genes and a significant reduction in the expression of mitochondrial, DNA repair genes suggesting that mitochondrial dysfunction and DNA damage may be the sources of increased stress.

Role of topoisomerase IIβ in Ageing

Concomitant decrease in the level of topoisomerase IIβ during *invitro* ageing model along the other repair proteins and senescence associated genes clearly hypothesized the role of topoisomerase IIβ in ageing process. Along with the expression profile of topoisomerase IIβ, its role in SSB (Gupta et al 2013) and DSB repair pathways (Mandraju et al 2011) also added strength to the hypothesis. The correlation of expression profile of topoisomerase IIβ with age *invivo* (kondapi et al 2004) also clearly established its importance in ageing *invitro*.

Rationale

TopoIIβ is predominantly present in non-proliferating cells namely neurons suggesting its role in non-replicating functions of DNA (Tsutsui et al., 2001; Kondapi et al., 2004). The decreasing activity of TopoIIβ with ageing (Kondapi et al., 2004) points out to its possible role in DNA repair activity in neurons during aging. Cell cultures are widely used as models to study the molecular mechanisms of aging. CGNs were chosen for this study because primary cultures of postnatal rat cerebellum make an excellent model system for molecular and cell biological studies of neuronal development and function and moreover TopoIIβ was known to be decreased in *invitro* ageing of CGNs along with other repair proteins gave clues about its regulation in DNA repair.

Transcriptome analysis of Cerebellar granule neurons in culture was done by (Gupta 2012, PhD Thesis) micro array analysis of 1-4 week cultures. The study characterized the expression pattern of genes during *invitro* ageing, with an aim of identification of novel markers such as – A2M, GNA14, GRIA1, MASP1, NPY and SLIT2 are upregulated during ageing of CGNs *invitro*. The expression pattern of topoisomerase IIβ during *invitro* ageing has shown to be down regulated in an

age dependent manner, thus establishing the relationship between TopoIIβ and neuronal senescence. Investigation of the effect of down regulation of potential genes which are known to be upregulated during *invitro* ageing on neuronal survival will through light on their importance in neuronal ageing. Expression of TopoIIβ along with some repair genes during *invitro* ageing also gave clues in its involvement in intricate transcriptional regulation of some genes during neuronal senescence, but the mechanism by which TopoIIβ is involved in the process is not clearly understood.

As the expression of TopoIIβ is correlated with other repair genes during *invitro* ageing, whether TopoIIβ interacts with some repair genes to form a complex to function in DNA repair, and whether such a complex is the major cause or consequence of ageing is to be addressed. Since TopoIIβ associated complex has already been reported during transcriptional regulation of genes, similar kind of complexes are involved in TopoIIβ associated gene expression during various stress conditions such as DNA damage, HIV-1 transcription involving host transcriptional factors has to be studied. This regulation of gene expression would be a model to study the molecular association of TopoIIβ associated proteins involved in transcriptional induction of HIV-1 genes.

Objectives

Based on the above observations and findings we framed the objectives as

a. Correlation of TopoIIβ and DNA repair genes in neuronal senescence.

b. Analysis of association of TopoIIβ in DSB repair in cerebellar granule neurons.

c. Regulatory activities of TopoIIβ in HIV-1 transcription.

Chapter 2

Materials and Methods

Materials

Reagents

Rosewell memorial institute medium 1640, Eagle Essential medium with Earal`s salts (EMEM), non-essential amino acids, sodium pyruvate (Invitrogen, Carlsbad, CA), Fetal Bovine Serum (FBS) (Lifetech). Oligonucleotides, Taq DNA polymerase, dNTPs (Integrated DNA Technologies (IDT, USA). PMSF, BSA, Leupeptin, Pepstatin, Aprotinin , Triton X 100, Trizol (TRI-REAGENT), DTT, MTT, ICRF-193,and Merbarone, PVDF, Nitrocellulose membrane (NC) (PALL Life Sciences, USA), all other chemicals were from Sigma and SRL unless specified.

Kits:

First Strand Synthesis Kit (Superscript III,SIGMA)

HIV-1p24 antigen capture kit (ABI, USA)

Animals:

Wistar rats (rattus novergicus), provided by University Animal house facility (UOH, Hyd, INDIA). All experiments were performed in accordance with University animal ethics committee and guidelines were followed to minimize pain and discomfort.

Age group:

Post natal 6-8 day rat pups.

Cell lines

SK-N-SH (neuroblstoma) and HEK 293T cell lines were obtained from NCCS, Pune, India .

SupT1, TZM-bl obtained from NIH AIDS Reference Reagent Programme, USA.

Virus

HIV-$1_{93IN101}$ is biotype-NSI(X5) (Dr. R. Bollinger), isolated from a seropositive individual in India.

Antibodies

Mouse monoclonal Rat anti- Ku70 and PARP-1 and Mouse anti- Topoisomerase IIβ were from Pharmingen group of Becton–Dickinson biosciences. Mouse monoclonal anti HIV-1 Tat is from NIH AIDS Reference reagent programme, Mouse monoclonal anti- Glial fibrilar acidic protein (GFAP) and Rabbit polyclonal anti-Neurofilament-M chain (NF) were from Sigma. Rabbit polyclonal Poly ADP Ribose antibody is from calbiochem, USA, Rabbit polyclonal anti- Topoisomerase II β and Rabbit polyclonal γ-H2AX antibody were from ABCAM, USA. Mouse monoclonal GFP is from Clonetech, USA. The secondary antibodies were purchased from UPSTATE, USA.

Plamids and constructs:

pMC-TopoIIβ GFP is a generous gift by Christian Mielke, Heinrich-Heine-University, Medical School, Düsseldorf, Germany. Pc-Tat and pHIV LTR-Luc were kindly gifted by Prof. Debhashis mitra NCCS Pune, India. pNL4-3 molecular clone was obtained from NIH AIDS reference reagent program. All transfections

were performed using lipofectamine (Invitrogen) and X-treme GENE HP DNA Transfection Reagent (Roche).

Primary neurons

Primary cerebellar granule neuronal cultures were prepared from post natal 6 – 8 day old rat pups cerebellum

Methods:

2.1 Isolation and culture of cerebellar granule neurons

The preparation of cerebellar granule cells was based on a previously described method. Anesthetized D7 rat pups were decapitated. Isolated cerebella were collected and washed with phosphate-buffered saline (PBS) at 40C. Cerebella were minced into 500 µm or smaller sections with a no. 11 scalpel blade. Minced tissue was incubated at room temperature for 10 min in trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; GIBCO). After trypsinization, the minced tissue was resuspended in 2 mL granule cell medium (Eagle's MEM with Earle's BSS, 2 mM glutamine (0.292 g/L), 10% heat-inactivated bovine serum (Lifetech), 6 mg/mL glucose, 25 mM KCl, 10 U/mL penicillin, 10 µg/mL streptomycin containing 0.05% DNase-I (final concentrations). The suspension was placed on ice and triturated through a fire-polished glass pipette with the minimal number of strokes needed to obtain a suspension of single cells. Three pipettes of decreasing tip diameters were used and the entire suspension was moved through each pipette tip 2-4 times and triturated slowly without appearance of any bubbles. Granule cells were plated in the presence of media that contains 10% fetal bovine serum. Cultures were incubated in a humidified atmosphere of 5% CO_2 95% air at 37°C.

Seeding density

Cells were seeded at 106 cells / 35 mm Poly-L- Lysine (PLL) (Sigma) coated dishes, each dish containing 2 ml of granule media. Cultures were fed every 2-3 days by replacing half the volume of media. 1µM arabinosylcytosine (SIGMA, USA) (mitotic inhibitor) was added to cultures for 2 days.

2.2 Treatment with H_2O_2

In the treatment phase, neurons were incubated in the presence of 1mM hydrogen peroxide for 24 h. After this the cells were washed twice and added with fresh media to incubate the cells for 24 h (R-24), 48 h (R-48) and 72 h (R-72) post treatment. In this recovery phase the cells were allowed to recover from the insult. week after plating, to suppress the proliferation of mitotic non neuronal cells.

2.3 Co-immunoprecipitation

2x 10^6 cells were seeded per well and treated with H2O2 according to the above mentioned protocol. After this, whole cell extract (100µg) was incubated with TopoIIβ monoclonal antibody for 2 h at 4^0C and protein A/G-agarose beads were added to the mixture and incubated for 1 h at 4^0C. The entire reaction mixture was gently washed three times with PBS with 0.1% tween20 to remove the unbound proteins and the beads were boiled with the loading dye. The immunoprecipated proteins resolved on a SDS–PAGE. Western blotting was done and membranes were probed with monoclonal antibodies directed against PARP-1, Ku-70 and developed with chemiluminiscent reagent (Pierce Biotechnologies).

2.4 CGNs as long term culture:

Cultured CGNs were kept over the period of 7 weeks in culture. In order to maintain similar conditions in the culture medium 2/3rd of the media was replaced

every 2 days until the cells were in culture. Cultures were split in to 1- 7 weeks to carry out experiments.

2.5 MTT assay for Cell viability:

The viability of granule neurons under prolonged period in culture and T cells treated with specific topoisomerase II inhibitors was estimated by the redox activity of mitochondria in reducing MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) in viable cells. Cells were cultured in poly-l-lysine coated 24 well plates (1x105 cells per well). After the cultures reached required age, 500 µl of 0.5 mg/ml of MTT in fresh medium was added to each well and the cells were incubated at 37^0C for 4 h. The plates were then centrifuged at 1,500 rpm for 20 min at room temperature and the medium was carefully removed. Dimethyl sulfoxide (DMSO) (500 µl) was then added to each well to dissolve the formazan crystals. The DMSO-dissolved formazan crystals were read immediately at 540 nm with DMSO as blank on a spectrophotometer.

2.6 Knock down studies

In case of gene specific Knockdown studies, at 3 weeks after culturing , the neurons were treated with gene specific SiRNA (0.1µM - 1µM) using Lipofectamine-2000 (Invitrogen, cat. no. 11668) as tranfection agent and, and also with 0.5 µM of non-silencing siRNA (scrambled) as control. The effect of Topo-II β or Senescence associated gene specific si RNA on the viability of granule neurons in culture was determined by colorimetric quantification of MTT based on assay described above and earlier by (Mosmann, 1983). Cultured CGNs in poly-l-lysine coated 24 well plates after treatment were incubated with 500 µl of 0.5 mg/ml of MTT in fresh medium at 37 °C for 4 h. The plates were then centrifuged at 1,500 rpm for 20 min at 37 °C and the medium was carefully

removed. 500 µl Dimethyl sulfoxide (DMSO) was then added to each well to dissolve the formazan crystals. The DMSO dissolved formazan crystals were read immediately at 540 nm with DMSO as blank on a spectrophotometer.

2.7 Western blot analysis

Cells were harvested by scraping in 25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, and centrifuged at 300g for 7 min at 4^0C. The cell pellet was homogenized in 0.2 ml protein lysis buffer (20 mM Tris–HCl pH 7.5, 0.1 mM β-mercaptoethanol, 1 mM MgCl2 , 0.1 mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.5 mM KCl, 0.5 mM PMSF and 1 µg/µl pepstatin and leupeptin) for 10 min on ice followed by sonication for 15–20s. The protein concentrations in cell lysates were measured using the Bradford method (Bradford, 1976). Twenty micrograms of total protein/lane were separated on 10% sodium dodecyl sulfate (SDS) gels and then transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween-20 for 1 h and then incubated overnight at 40^0C with corresponding protein specific antibodies. After washing and incubating for 1 h at 22^0C with a secondary antibody, conjugated with horseradish peroxidase. These membranes were then washed and immunoreactive bands were visualized either by chemiluminescence (Pierce Western Blot Chemiluminescence Reagent, USA). Relative levels of protein in the different lanes were compared by analyzing scanned images using the NIH IMAGE J program. All studies were performed a minimum of three times using independent cultures.

2.8 Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from granule neurons during the period from 1st to 6th week in culture or after treatments with siRNA of selected senescence associated

or HIV-1 Infected SupT1 cells using TRI-REAGENT (SIGMA, USA). Aliquots of 5 µg were then subjected to reverse transcription using oligo (dT) primer and first Strand Synthesis Kit (Superscript III,SIGMA) to synthesize cDNAs . Aliquots containing an equal amout of cDNA were then used for PCR , with primers for Rat or human β-actin, rat specific topo IIβ, PARP-1, XRCC1, XRCC4 and Ku 70 A2M, GNA14 , GRIA1, MASP1, NPY, SLIT2 and GLB1.and HIV-1 specific Vif, Vpu, Gag-Pol, Env.

Based on sequence homology, primer sequences were designed for Wistar Rat (rattus novergicus) strains and HIV-1 that were aligned using Primer 3 software (available Online). The following primers were used:

S. No	Gene	Forward primer(5'→3')	Reverse primer (5'→3')	Amplicon Size
1	**TOP2B**	GAC AGA GGA AGG TAG TAG AGC CTG	CGT TTT CTT CGG TTT CTT GCT GGC	168
2	**A2M**	TCA CTC ATC CTG TTG TCC GCA ATG CCC TCT	ACC AGC AAG GGC AAA TGC ATA GGC CAA CA	128
3	**GNA14**	ACC AAA GCA AGA TGT CAA AGC TGC CAG GGA	TCC TTG ACA GCA GCA AAC ACG AAG CGG AT	147
4	**GRIA1**	ATG CCA ACC AGT TTG AGG GCA ATG ACC GCT	TTC TCC CAC CAT GCC ATT CCA AGC CTT TGT	167
5	**MASP**	TGC CGA GTG GAA TGC AGT GGC AAT CTC TT	AGG GCA CCT CGG GAT GGT CTT CAA TGT CAA	184
6	**NPY**	TGC TCG TGT GTT TGG GCA TTC TGG CTG A	ATC AGT GTC TCA GGG CTG GAT CTC TTG CCA	167
7	**SLIT2**	AGA ACG GCA CCA GCT TCC ATG GCT GTA T	TGG GCA CAC ACT TTC TTG TGG CAT GGT TCA	133
8	**GLB**	TCA AGG ATG GGC AGC CAT TCC GCT ACA T	TGG ATT GCA TCC AGC CCA GCC ATC TTC AT	118
9	**Ku 70**	TGA AGT GCT CTG GGT CG	CAG CGA TGC TGA TGA TGT CT	142
10	**HIV-1 LTR**	TAC AAG GGA CTT TCC GCG	AGC TTT ATT GAG GCT TAA C	180
11	**XRCC1**	TTC CGA AAA GAA TGC AAT TGC TAT CTC TT	GAG GTA AAC GGT TCT ATA ACG CCT CCT GCA	113

12	**XRRC4**	TAG TTA CAC CCT AAC ATG TCG AAT TTC A	AAG GCA GAC ACT TTC TTG TGG GAT GTT ACA	126
13	**PARP1**	TGG GCA CAC ACT TTC TTG TGG CAT GGT CA	AAA GCA CAC ACT TTC TTG ATG GTT CA	167
14	**Gag-pol**	TAA TCG GCC GAA CAG GGA CTT GAA AGC GAA AG	CCA TCG ATT GCG TCC CAG AAG TTC CAC AAT CC3	
15	**Vif**		CCC ATC TCC ACA AGT GCT GAT ACT TC	
16	**Nef**		CTA GGT CAG GGT CTA CTT GTG TGC	
17	**Env**		TCT GAA GGG ATG GTT GTA GCT GTC C	
18	**β- actin**	CTG ACA GGA TGC AGA AGG AG	GAT AGA GCC ACC AAT CCA CA	119

Cycling conditions were optimized for each primer, and PCR products were run on 1.2% agarose gel stained with ethidium bromide. Gels were digitally photographed, and densitometric analysis was performed. Values are expressed as ratios to β-actin.

2.9 Real time PCR

The ABI Prism 7900 HT sequence detection system (Applied Biosystems, USA) was used for real-time PCR. The synthesized c-DNA was used for quantitative RT-PCR (qRT-PCR) analysis. The real-time qRT-PCR was performed in 96- plate wells (MicroAmp TM Fast optical 96-well reaction plate, Applied Biosystems.) in a 20 µl reaction volume using components of the SYBR green qPCR kit (Applied Biosystems USA). Relative changes in gene expression (fold change) were calculated using the 2-[delta] Ct (threshold cycle) method. Relative gene expression was determined based on the threshold cycles (Ct) of the gene of interest and β-actin as an internal reference gene.

2.10 Comet assay

DNA damage was evaluated using comet assay. Alkaline comet assay (ACA) detects single-strand breaks (SSBs), double-strand breaks (DSBs) and alkali labile sites (Singh et al., 1988)at a pH>13. Neutral comet assay (NCA) is carried out at neutral pH and scores only for double strand breaks (Ostling and Johanson, 1984). Approximately 105 cells were washed and suspended in 500 µL of ice-cold 1x PBS and 1.5 mL of 1% agarose was added to each sample. The agarose-cell suspension was gently layered on a frosted-glass microscopic slide that was pre-coated with 0.75% agarose and was allowed to solidify for 5 min on ice and was immediately transferred to ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0) and 1% Triton X-100) and incubated overnight at 4°C. For ACA, after lysis, slides were equilibrated for 1 h in electrophoresis buffer

2.11 Senescence-Associated ß-Galactosidase (SA-β-gal) assay

Cultured neurons grown on glass cover slips and transfection with siRNA of indicated senescence associated genes was done. After down regulation cells were fixed with 4% paraformaldehyde at room temperature for 3-5 min, cells were washed three times with PBS and incubated in the dark in X-gal staining solution pH-6 (100 mM sodium phosphate, 2 mM MgCl2, 150 mM NaCl, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal). Cells were incubated in the dark for 10 hrs at 37°C. The cells were then washed in PBS, mounted and viewed under a bright field microscope. The positive percentages were determined by counting stained and unstained cells under a microscope at 400X magnification in 4 random fields. A minimum of 200 cells were counted and results were presented as the mean (SEM) for a given number of observations (n) along with mean standard errors.

2.12 siRNA synthesis

We have used double strand siRNA oligos for transient down regulation of indicated genes in rat CGNs. Lipofectamine 2000 (Invitrogen) was used for transfecting the double strand siRNA oligos. Cultures, 1 day after plating were used for transfection as standardized in our lab (Mandraju 2008). Double strand siRNA oligos were synthesized as described earlier. For this, desalted DNA oligonucleotides were obtained from Sigma (India). The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase has been implemented as described. For each transcription reaction, 1 nM of each oligonucleotide was annealed in 50

µl of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) by heating at 95 ºC; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain double stranded DNA. Transcription was performed in 50 µl of transcription mix: 1xT7 transcription buffer (40 mM Tris–HCl, pH 7.9, 6 mM MgCl2, 10 mM Dithiothreitol (DTT), 10mM NaCl and 2mM spermidine) 1mM Nucleotide triphosphates (NTPs), 0.1 U yeast pyrophosphatase (Sigma), 40 U Rnase OUT (Life Technologies) and 100 U T7 RNA polymerase (Invitrogen) containing 200 pM of the dsDNA as template. After incubation at 37ºC for 2 h, 1 U RNase-free DNase (Genetix) was added at 37 ºC for 15 min. Sense and antisense 21-nt RNAs generated in separate reactions were annealed by mixing both crude transcription reactions, heating at 95ºC for 5 min followed by 1 h at 37ºC to obtain 'T7 RNA polymerase synthesized small interfering double-stranded RNA' (T7 siRNA). The mixture (100 µl) was then adjusted to 0.2 M sodium acetate, pH 5.2, and precipitated with 2.5 volume of ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried and resuspended in 50 µl of water.

2.12.1 siRNA oligos

The following oligos were used to synthesize siRNA,

Name	siRNA sense template sequence	siRNA antisense template sequence
TopoIIβ siRNA	5'-AAA GCT TAA CAA TCA AGC CCG CTA TAG TGA GTC GTA TTA -3'	5'-AAC GGG CTT GAT TGT TAA GCT CTA TAG TGA GTC GTA TTA -3'
TopoIIβ scrambled siRNA	5'-ACA CTC GAT CAA TCC AAG GAA CTA TAG GGA ATC GAA ATA -3'	5'-CAC TGG ATT GAT CGA GAT GTT CTA TAG TGA GTC GTA TTA -3'
SLIT 2 siRNA	5'-ATT GAT TCC CCA ACA ATT CAG TAG TAG TGA GTT ATA -3'	5'-AAT TGT CTA CAT CCA GAA TAT ATA TGG AGA GGC ATA ATA -3'
SLIT 2 scrambled siRNA	5'-TAT GGT TCA ACA CCA ATT CCG CAG CCG TTA ATT AAA TAA -3'	5'-CAC TGG ATT GAT CGA GAT GTT CTA TAG TGA GTC GTA CTA -3'
MASP siRNA	5'-GAA AAT CTA ATA CCA ATC TTG AAG AAG CAA ACC CGT ATA -3'	5'-AAC TAG CCT GAC AGC GAT ATT CTA AAG CAA GAC ATA GTA -3'
MASP scrambled siRNA	5'-AAT TAT TCC CCA TCA ACT CTG AAG TTG ATA ATT TTA CCT -3'	5'-GAC TAG AAT GAC AGC TAT ACG CTA CCG CAA GAC CTA CCA -3'
GLB siRNA	5'-CAT TTT TCC TTA TCA ACT CGG CAG TAG ACA AAT CTA GCT -3'	5'-TAC GAC TCT GAC TGC GGT CAT CAA AAG CGG AAC GTA GTA -3'
GLB scrambled siRNA	5'-ATC AAG CTT TAC AGC AAT CCT TTA AAG TGA AAC ATA TTA -3'	5'-CTT AGT AGA CCA TCA ATT ACG CAG TTG ATA ATT CCT TAT -3'
GRIA1 siRNA	5'-AAT TAT TCC CCA TCA ACT CTG AAG TTG ATA ATT TTA CCT -3'	5'-CAC TGG ATT GAT CGA GAT GTT CTA TAG TGA GTC GTA TTA -3'
GRIA1 scrambled siRNA	5'-TAC GTG GTT AAT CCT GAT GTT CTA TAG TGA GTC ATA ATG -3'	5'-TTC TAC CAT TTC ACC GGT ATT CTA AAG CAA GAC GTA GTC -3'
A2M siRNA	5'-CTC AAA CAT AAT AGC GAT AAT CCA AGG GAA GAC ATC TTA -3'	5'-AGA GGT TCA ACA GCA AGT CCG CTG CCG ATA ATA ACC TAA -3'
A2M scrambled siRNA	5'-TAT TCT TCC CTA TCA AAT CTG TGG ATG ATA ATT AGA GCT -3'	5'-GAA AAT CTA ATA CCA ATC TTG AAG AAG CAA ACC CGT ATA -3'

GNA14 siRNA	5'-AAC TAG CCT GAC AGC GAT ATT CTA AAG CAA GAC ATA GTA -3'	5'-TAC AAG CCA GTA ATA ATC CAG AAG TAG CAT TCC GGT GTT -3'
GNA14 scrambled siRNA	5'-GAT GGT TCA ACA CCA ATT CCG CAG CCG TTA ATT ATT TAA -3'	5'-TAT GGT TTC GCA TAA ACT GTG TAG TTG CTA ATT TTA CCT -3'
NPY siRNA	5'-CGA CGT TTA AAG CCA ATG ATC AAG TAG GGA ACA TGT CTA -3'	5'-AAC GGG CTT GAT TGT TAA GCT CTA TAG TGA GTC GTA TTA -3'
NPY scrambled siRNA	5'-AAT AAA TCC CCA TCA ACT CTG AAG TTG ATA ATT TTA GCT -3'	5'-AAT GTT TCA ACA CCA ATT CCG CTG CCG TTA ATT CTT TAA -3'
T7 promoter	5'-TAA TAC GAC TCA CTA TAG -3'	

2.12.2 Strategy to generate T7 siRNAs

The sequence of the gene of interest is shown in red (sense) or blue (antisense), while the two unrelated nucleotides are in black. RISC stands for the RNA-induced silencing complex that targets the mRNA for cleavage.

2.12.3 siRNA transfection

Cultured granule neuron Cells (2 $x10^6$) were transfected by using Lipofectamine-2000 (Invitrogen) with 0.5 µM of non-silencing siRNA (scrambled) and silencing siRNA separately

2.13 Immunofluorescence

CGNs were dissociated and cultured, as described above in 6-well plates containing poly-L lysine coated cover slips at a density of $2x10^6$ cells/well. After 24hr in culture. After 16hr treatment, the neurons were fixed with 4% para formaldehyde containing 0.025% triton X-100, for 15 min at room temperature,

blocked with PBS containing 5% bovine serum, and incubated with monoclonal anti-TopoIIβ (1:100 dilution), polyclonal anti-γH2AX, (ABCAM), anti-PAR (Calbiochem) and anti-neurofilament (NF) (1: 100 dilution). Subsequently the cells were washed 3-4 times with PBS and incubated with anti-mouse- 482 (green), anti-rabbit -595(red) (Invitrogen) at 1:250 dilutions. These cultures on cover slips were mounted on to glass slide and viewed under Fluorescence confocal microscope Lieca (Leach Instruments, Heidelberg, Germany). The excitation/emission wavelengths employed were, 548nm/ 562 nm for (blue) and 650/700 for (red).

2.14 Quantification of Virus

P24 levels were estimated for the quantification of virus using ELISA kits from ABI. Samples were diluted in 500µl of media and 100µl sample was used for the assay. Supernatant from uninfected cells was used as a negative control. Viral quantification was done by plotting the mean of three independent absorbance values with standard deviation

2.14.1 Viral infection

pNL4-3 molecular clone was transfected in 5×10^6 293T cells using Lipofectamine 2000 with medium replacement after 16 hours. Supernatants after 24 hours post infection were used for further infections. The viral titre was calculated as TCID 50/ml.

2.15 Luciferase Assay

Transfection in TZM-bl or 293T cells was done with indicated plasmids and incubated for 40 hours. The cells were washed with phosphate buffered saline (PBS) and lysed with 100µl of cell lysis buffer. The cell lysate was centrifuged at

9500 rpm for 15 min and Protein concentration was normalised for the assays. Luciferase activity assays were performed using substrate (Promega, USA).

2.16 Chromatin immunoprecipitaion assay

Chromatin immunoprecipitation (ChIP) analysis was done according to the Vaughn Jackson with slight modifications. $10x10^6$ SupT1 cells were infected with virus and were crosslinked at indicated time points with 1% formaldehyde solution for 10 min at 37°C. Cells were washed with ice-cold PBS and resuspended in lysis buffer followed by an incubation on ice for 20 min. Sonication was done to produce DNA fragments of 500–1000 bp length . Extracts were then diluted in immunoprecipitation (IP) dilution buffer at 1:10 ratio. 2 ml of diluted sample was used for IP and 10% of the total sample was used as input. Pre-clearing was done by agitation with Protein G agarose for 30 minutes at 4°C. Appropriate antibodies TopoIIβ CHIP grade antibody (Abcam), PARP-1, Ku70 monoclonal anti bodies (BD Biosciences) were added and incubated overnight at 4°C with rotation. Immune complexes were precipitated with Protein A/G agarose mixture and incubated for 2 hours at 4°C under rotation. Beads were pelleted and washed for 3 minutes sequentially with low salt, high salt, LiCl, and Tris-EDTA buffers. The elution of immune-complexes was done with elution buffer, and isolated supernatants were reverse cross linked by incubation at 65°C for 4 hours. The same process was repeated for input control. The mixture was incubated for 1 hour at 45°C by adding proteinase K and the deproteinisation of DNA was done by phenol-chloroform extraction and ethanol precipitation in the presence of 20 mg of glycogen. After washing with 70% ethanol the DNA was dried, and resuspended in Tris-EDTA buffer. 2µl of total DNA was amplified for 32 cycles.

2.17 *Invitro* pull down assay

His-tagged recombinant HIV-1 Tat (4 µg) protein was incubated with 100µl of Ni-NTA matrix at 30°C for 30 min and the matrix was washed thrice with IP buffer to remove unbound protein. 100 µg of SupT1 extract with equal volume of IP buffer (25mM Tris-HCl pH 7.4,150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol) was added to the his Tat bound matrix and incubated overnight at 40C. The matrix was washed thrice with IP buffer and bound proteins were eluted with SDS sample buffer and resolved on 8% SDS-PAGE. Immunoblotting with TopoIIβ, Ku70, PARP-1 antibodies and Ni-NTA (without Tat protein) alone was used as control in the experiment**.**

2.18 Immunoprecipitation

HIV-1 infected SupT1 extracts were prepared by initially harvesting Cells in 25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, followed by centrifugation at 1000g for 10 min at 4 °C. The cell pellet was lysed in IP buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol) for 10 min on ice followed by sonication for 15–20 seconds. The protein concentration was normalised using the Bradford method and lysate was incubated with indicated antibodies over- night at 4°C. The above immune complexes were incubated with 8% protein-A-agarose CL-4B beads (Genei) in IP buffer for 2hours at RT and washed with wash buffer (IP buffer with 0.5% triton-X 100). Samples were identified by Western blotting with the enhanced chemiluminescence kit (G biosciences). All antibodies were obtained from BD Biosciences, with the exception of the anti-Tat antibody [National Institutes of Health (NIH) AIDS Research and Reference Reagent Programme].

2.19 Data analysis

All experiments were repeated three times individually unless otherwise specified. Differences between groups were assessed by One Way Anova followed by student-Newman-Keuls test, p- value less than 0.05 ($p < 0.05$) were considered statistically significant. Data are expressed as the mean ± SD.

Chapter 3

Correlation of TopoIIβ and DNA repair genes in neuronal senescence.

Introduction

Aging has been described as a progressive decline in the ability to withstand stress, damage, and disease resulting in degeneration (Bokov et al.,2004). Age is also a major risk factor in the development of many diseases, although the relationship between the aging process and the etiology of age-related diseases is not fully understood. Previous gene expression studies of aging have primarily concentrated on model organisms or have been confined to specific aging-associated disorders such as progeria syndromes. Ageing of the brain leads to impairments in cognitive and motor skills, and is the major risk factor for several common neurological disorders such as Alzheimer disease (AD) and Parkinson disease (PD). Recent studies suggest that normal brain ageing is associated with subtle morphological and functional alterations in specific neuronal circuits, as opposed to large-scale neuronal loss. In fact, ageing of the central nervous system in diverse mammalian species shares many features, such as atrophy of pyramidal neurons, synaptic atrophy, decrease of striatal dopamine receptors, accumulation of fluorescent pigments, cytoskeletal abnormalities, and reactive astrocytes and microglia2. Ageing resulted in a gene-expression profile indicative of an inflammatory response, oxidative stress and reduced neurotrophic support in brain regions. At the transcriptional level, brain ageing in rat displays parallels with human neurodegenerative disorders.

It is well established that gene expression levels in many organisms change during the aging process, and the advent of DNA microarrays has allowed genome-wide patterns of transcriptional changes associated with aging to be studied in both model organisms and various human tissues. Understanding the effects of aging on gene expression in the human brain is of particular interest, because of its relation

to both normal and pathological neurodegeneration. In humans, many more genes undergo consistent expression changes in the cortex than in the cerebellum.

Studying Cellular senescence mechanisms in *invitro* culture system has proven as an excellent model to replicate the mechanisms in Normal ageing process. Studies on gene expression during cellular senescence *invitro* provide major clues in pathways involved in senescence and crucial genes associated with the process. Topoisomerase IIβ has already been established as an ageing marker (Bhanu et al 2010). Significant decline in the levels of the enzyme during *invitro* and *invivo* ageing process clearly points out its role in ageing (kondapi.et.,al 2008 ,Bhanu.et.,al 2010). Resection of gene networks involved in the process of ageing is of prime interest, due to the fact that there is clear overlap between genes involved in the process of ageing and those involved in neurodegeneration.

Transcriptome analysis of Cerebellar granule neurons in culture was done by (Gupta 2012, PhD Thesis) micro array analysis of 1-4 week cultures. The study characterized the expression pattern of genes during ageing *invitro*. The genes which are significantly down regulated belong to DNA repair, oxidative stress response and many genes related to apoptotic or necrotic stimulus. This investigation resulted in identifying genes – A2M, GNA14, GRIA1, MASP1, NPY and SLIT2 which may be involved in neuronal ageing. These six genes showed up regulation in their expression during ageing CGNs. Hence, specific SiRNA were designed and these genes were down regulated in CGNs on 3rd week and neuronal survival was monitored. Such analysis showed synergistic effects when SLIT2 and NPY were knock down.

This chapter focusses on the characterization of functional importance of genes, which known to be upregulated during the process of neuronal senescence. The

downregulation of these genes such as NPY and SLIT2 during process of cellular ageing resulted in gradual increase in cell viability and prolonged days in culture. The downregulated cells also showed variation in beta galactosidase expression along with marked increase in DNA repair capacity.

Results

Fig 3.1 Down regulation of genes involved in neuronal senescence

A heat map of transcript levels was created for the complete rat genome to display those which are statistically significant (P-value < 0.05), led to identification of potential genes involved in ageing. Semi-quantitative gene expression of A2M, GNA14 , GRIA1, MASP1, NPY, SLIT2 and GLB1 showed significant increase of their expression in 2W, 3W and 4W over 1W (Gupta Ph.D thesis 2012). In this study we have studied the effect of down regulation of these genes on survival of cerebellar granule neurons which were cultured invitro for 3 weeks that would bring delay in senescence. Experiments were designed in such a way that neurons were cultured for 3 weeks and siRNA's specific to the indicated genes were transfected with lipofectamine 2000. Cells were transfected each week with gene specific siRNA of targeted senescence associated genes and non–targeted scramble siRNA as a negative control. RNA extraction was done each week after down regulation and semi quantitative RT PCR was used to check the down regulationoftheindicatedgenes.

Figure 3.1

Fig 3.1 semi quantitative PCR analysis of down regulated CGNs with senescence associated gene specific siRNAs. Untransfected cells were used as negative control and Scrambled (Scr) siRNA was used as non-targeting control.

Fig 3.2 cell viability assay of knock down CGN's

As described by Bhanu et al., 2010, CGNs showed viability until the 5^{th} week. Hence in this study we analyzed the viability of CGNs in which specific senescence associated gene knock down was done in 3^{rd} week of culture. Figure 4.2 shows cytotoxicity detected in terms of reduction of MTT in ageing CGNs. Results are expressed as mean ± SD (3 replicates in two independent experiments). Cell viability in first week was considered as 100%. Values are presented as a percentage of activity in control cells. Cell viability markedly decreased by 40% in 2^{nd} week, 20% by 3^{rd} week and was then stable in 4^{th} week by exhibiting 10% viability. In cells down regulated with SLIT2 and NPY marked increase in cell viability was observed after down regulation. Cell viability is also shown to be increased to 6 weeks, which showed an increase in life span by 1 week in SLIT2, NPY down regulated cells when compared to untransfected control.

Figure 3.2

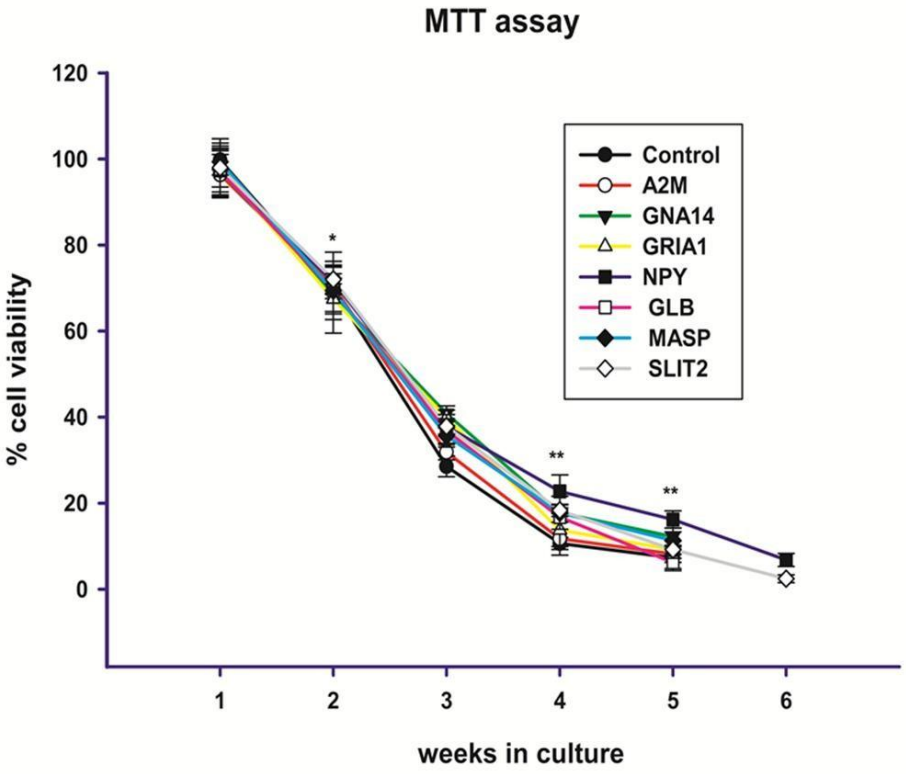


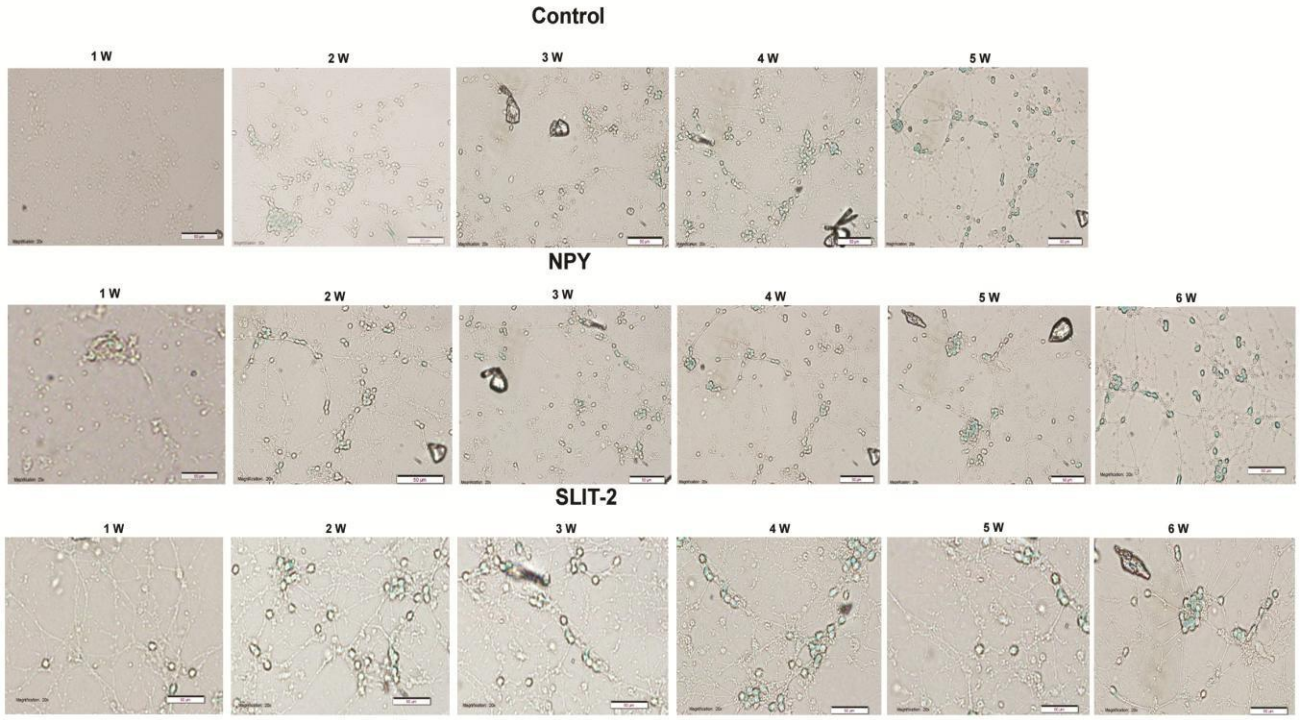
Fig 3.2 Cytotoxicity was detected by reduction of MTT in ageing CGNs. Results are expressed as mean ± SD (3 replicates in two independent experiments). Cell viability in first week was considered as 100%. Values are presented as a percentage of activity in control cells. Cell viability is compared at every week based on MTT values presented. The results showed a marked increase in cell viability in NPY, SLIT2 down regulated cells compared to control, when knock down was done at 3rd week in culture.

Fig 3.3 Senescence associated-β-galactosidase assay

SA-β-Gal is a known marker for senescence (Dimri et al., 1995). Hence, activity and transcript level of this marker was chosen as a standard for this work. The percentage of SA-β-Gal-positive cells increases progressively with age, from 0% in the first week to 99% in 5th week. This increase in SA-β-gal may be due to stress accumulation during ageing in culture. Such significant enhancement in the SA-β gal in cultured CGNs shows in vitro ageing similar to physiological ageing. The delay in senescence, in SLIT2 and NPY knock down CGN's is clearly demonstrated by decrease in progression of SA-β gal activity upon ageing *invitro*. In Figure 4.3, upper panel shows senescence associated β- galactosidase in ageing CGNs in bright field microscopy. The middle and lower panel shows the β-galactosidase activity in SLIT2 and NPY down regulated cells. Figure 3.3 shows that the percentage of SA-β-Gal-positive cells increased progressively with age, from 0% in the first week to 99% in 5th week. The decrease in progression of senescence shows the indirect involvement of SLIT2 and NPY in senescence during ageing *invitro*.

Figure 3.3

A.



B.

Days in culture	% of beta -gal positive cells		
	Control	SLIT2 -	NPY -
7	0	0	0
14	5	4	5
21	64	45	49
28	88	65	59
35	96	86	78
42	---	97	93

Figure 3.3: Senescence associated-β-galactosidase assay

Panel A shows bright field microscopic images of senescence associated β-galactosidase in ageing CGNs. Middle and the lower panels show the delayed senescence in NPY and SLIT2 down regulated cells.

Panel B The percentage of SA-β-Gal-positive cells increased progressively with age, from 0% in the first week to 99% in 5th week. This increase in SA-β-gal may be due to stress accumulation under the prolonged culture period. Such a significant enhancement in the SA-β gal in cultured CGNs shows in vitro ageing

3.4. Assessment of DNA damage with age in in vitro cultured CGNs

DNA repair capacity in SLIT 2 and NPY knock down CGN's was analyzed by Comet assay. The integrity of genomic DNA under the effect of natural ageing in in vitro CGNs was accessed by comet assay in neutral and alkaline conditions (Figure 13).Comets of young CGNs (1W) appeared round and bright suggesting DNA damage to be minimal. However, with progress in age i.e. from 2W to 5W, a significant migration of DNA from nucleus was seen forming a 'comet' tail. Even a naked eye observation of the alkaline comets revealed an increase in tail length which can be directly correlated with an increase in SSBs, DSBs and alkali labile sites. This observation also applied to neutral comets that largely detect DSB. **Figure 3.4 A** shows comets of ageing CGNs (1st to 5th week) in alkaline condition and **Figure 3.4 B** in neutral condition. In Figure 3.4 lower panel shows the comets of ageing CGNs in SLIT2 and NPY dowregulated neurons under alkaline condition which represent both the SSB and DSB. **Figure 3.4.B** depicts the comets under neutral conditions which represent the double strand breaks. Comets corresponding to downregulated CGNs in both conditions showed increase in both the DNA

repair capacity over the period of ageing, which was observed in terms of decrease in tail length when compared to the control untransfected CGNs.

Fig 3.4.A shows corresponding bar charts depicting Mean ± SD. DNA damage was measured in terms of tail moment. There was a concentration dependent production of DNA damage in control batch. DNA damage was quantified using CometScoreTM Freeware v1.5 (TriTek Corporation, Sumerduck, VA, US) in arbitrary units (Scale bar = 100 µm). Tail moment was found to be statistically higher as age progressed in comparison with the control (1W) batch which was done using One Way ANOVA (Tukey post hoc test) (** $P < 0.01$, *** $P < 0.001$).

Figure 3.4.A

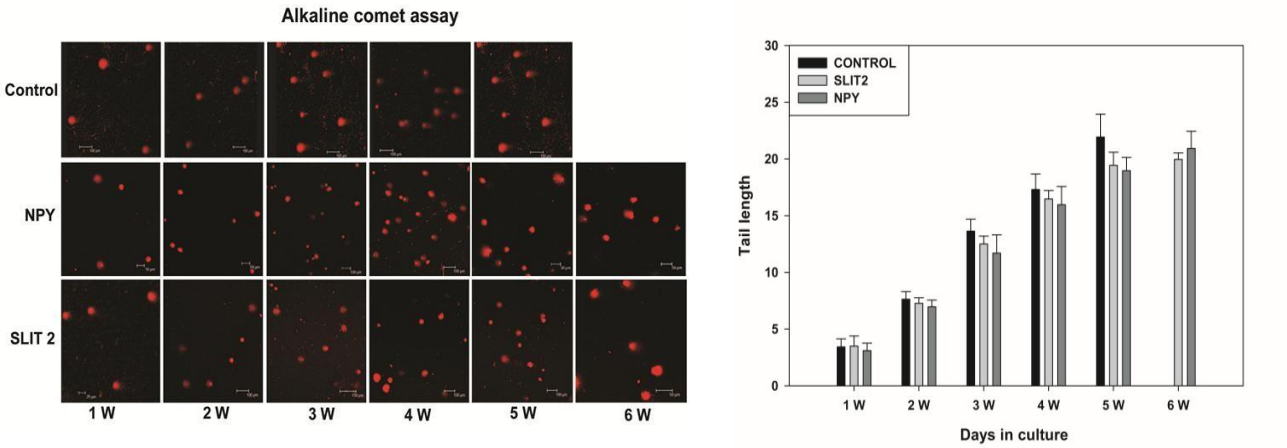


Figure 3.4.A Extent of native DNA damage in control (untransfected) and SLIT2, NPY downregulated in vitro cultured CGNs was evaluated by alkaline comet assay. Upper Panel shows comets of ageing CGNs (1st to 5th week) in untransfected control. Middle and lower panels alkaline comets in NPY and SLIT2 downregulated extracts respectively.

shows corresponding bar charts depicting Mean ± SD. DNA damage was measured in terms of tail moment. There was a concentration dependent production of

DNA damage in control batch. DNA damage was quantified using CometScoreTM Freeware v1.5 (TriTek Corporation, Sumerduck, VA, US) in arbitrary units (Scale bar = 100 µm). Tail moment was found to be statistically higher as age progressed in comparison with the control (1W) batch which was done using One Way ANOVA (Tukey post hoc test) (** $P < 0.01$, *** $P < 0.001$).

Figure 3.4.B Neutral comet assay of knock down CGNs

Figure 3.4.B

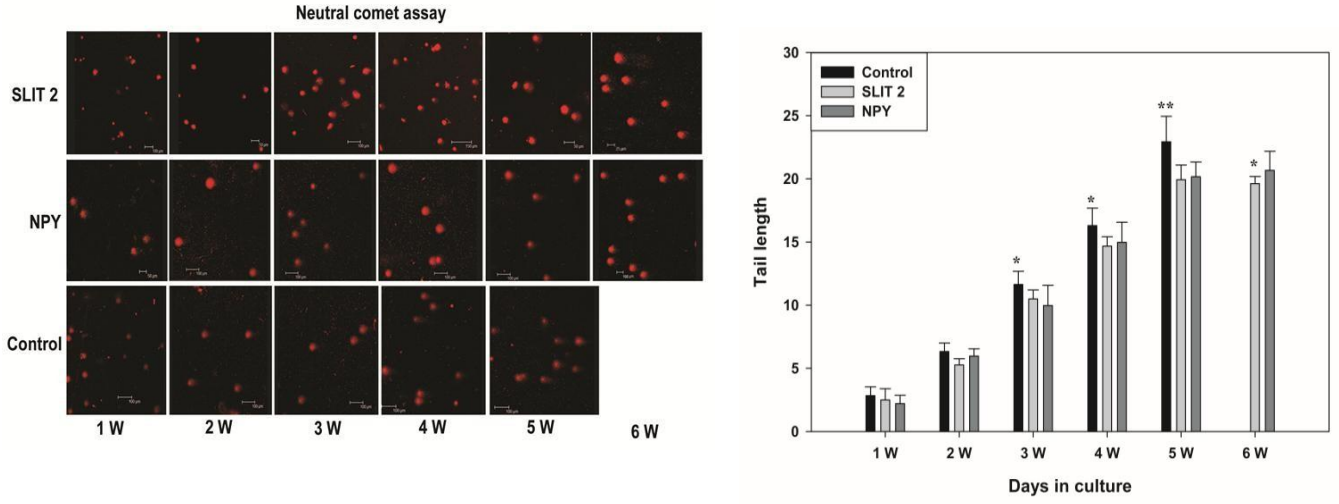


Figure 3.4.B DNA damage in control (untransfected) and SLIT2, NPY downregulated in vitro cultured CGNs was evaluated by neutral comet assay. Upper Panel shows comets of ageing CGNs (1st to 5th week) in (untransfected) control. Middle and lower panels neutral comets in NPY and SLIT2 down regulated extracts respectively. Corresponding bar charts depicting Mean ± SD. Average of 50 cells was chosen for every experiment (n=3). DNA damage was measured in terms of tail moment. There was an age dependent production of DNA damage in control batch. DNA damage was quantified using CometScoreTM Freeware v1.5 (TriTek Corporation, Sumerduck, VA, US) in arbitrary units (Scale bar = 100 µm). Tail moment was found to be statistically higher as age progressed in comparison with the control (1W) batch which was done by using One Way ANOVA (Tukey post hoc test) (** $P < 0.01$, *** $P < 0.001$).

Figure 3.5 Expression analysis of DNA repair genes in SLIT2 and NPY down regulated cells

Senescence-associated changes in the gene expression and repair activities of NHEJ enzymes were analyzed in Control (untransfected) and SLIT2, NPY down regulated invitro CGN cultures. **Figure 3.5 A** shows expression of NHEJ genes – PARP-1, Ku70, XRCC1 and XRCC4 by quantitative Real-Time PCR which reveals significant decrease in relative expression of all genes mentioned in control (untransfected) CGNs on ageing except in SLIT2 and NPY down regulated extracts that showed a slight increase in relative gene expression through the weeks as per statistical analysis, where in, 1W healthy CGNs were taken as control. Slight increase in expression of repair genes correlates which comet assay which showed overall increase in DNA repair capacity of SLIT2 and NPY down regulated extracts upon ageing invitro. Results from gene expression studies of DNA repair genes in down regulated cells demonstrated that SLIT2 and NPY could act indirectly to increase the DNA repair efficiency through slight increase in repair gene expression. The involvement of SLIT2 and NPY in the expression of NHEJ genes during *invitro* ageing needs further study.

Figure 3.5 A

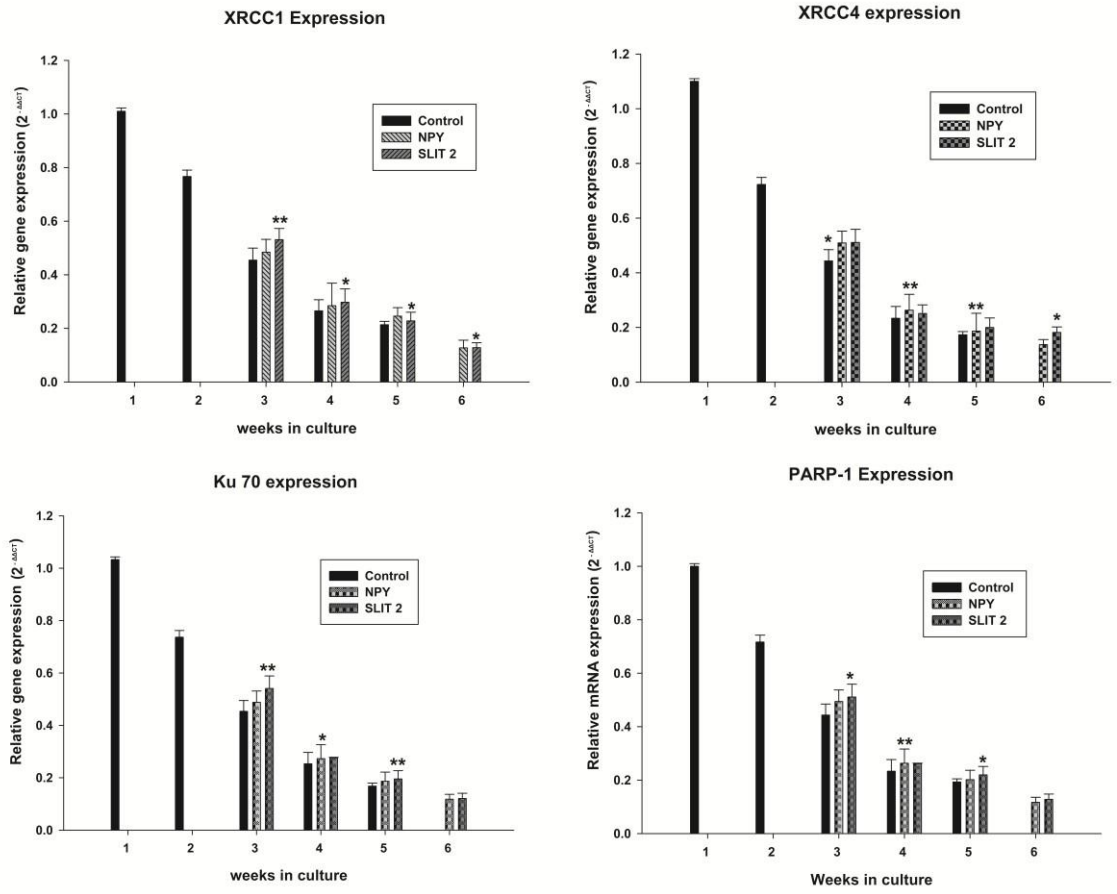


Figure 3.5A: Differential gene expression of DNA repair genes by qPCR

Quantitative Real-Time PCR for NHEJ genes- Ku70, PARP-1, XRCC1 and XRCC4 reveals significant decrease in relative expression of all genes in control (untransfected) cells. SLIT2 and NPY down regulated CGNs shows a slight increase in relative gene expression through the weeks when compared to Control as per statistical analysis, One Way ANOVA (Tukey post hoc test) (*P< 0.05, **P< 0.01, ***P< 0.001). 1W healthy CGNs were taken as control.

Fig 3.5 B Expression of TopoIIβ in SLIT2 and NPY down regulated CGNs

Expression of TopoIIβ has known to be decreased during invitro ageing (Bhanu,et., al 2010). In Control CGNs, fold change expression was done by quantitative Real-Time PCR for TopoIIβ where significant decrease in relative expression of its levels were observed through the weeks as per statistical analysis, One Way ANOVA (Tukey post hoc test) (*P< 0.05, **P< 0.01, ***P< 0.001). 1W healthy CGNs were taken as control. To check whether the expression levels of TopoIIβ changes during downregulation of SLIT2 and NPY, qPCR analysis was done for expression levels of TopoIIβ. There is a slight increase in the level of expression of TopoIIβ in SLIT2 and NPY down regulated cells when compared to control (untransfected).

Figure 3.5B

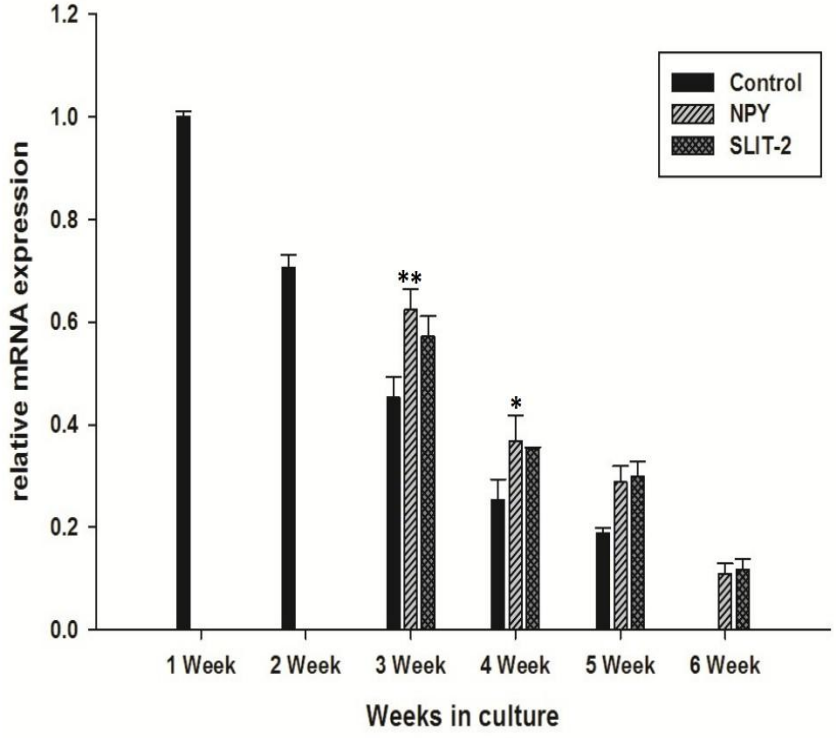


Figure 3.5B Expression of TopoIIβ in SLIT2 and NPY down regulated cells Relative mRNA levels of TopoIIβ in down regulated cells was determined by real time PCR. Genes show a significant increase in relative gene expression through the weeks as per statistical analysis, One Way ANOVA (Tukey post hoc test) (*P< 0.05, **P< 0.01, ***P< 0.001), 1W healthy CGNs were taken as control.

Discussion

Postulated mechanisms of CNS aging include instability of nuclear and mitochondrial genomes, neuroendocrine dysfunction, production of reactive oxygen species, altered calcium metabolism, and inflammation-mediated neuronal damage. Age related changes in gene expression in rat brain have already been reported. Understanding of the molecular basis of aging in mammals has progressed slowly, in part because of lack a large number of biomarkers that can be used to measure the aging process. Gene expression profiling of the aging process and the concurrent generation of transcriptional biomarkers of aging has been pivotal in deduction of biomarkers of ageing and studying intricate biological pathways involved in ageing *invitro*. CGNs are the large homogenous population in rat cerebellum. CGNs were chosen for this study because primary cultures of postnatal rat cerebellum make an excellent model system for molecular and cell biological studies of neuronal development and function. CGNs have been used to study cellular and molecular correlates of mechanisms of survival/ apoptosis and neurodegeneration/ neuroprotection. Identification of potent candidates in progression of neuronal senescence resulted in two genes which were upregulated during invitro ageing namely, NPY and SLIT2. Neuropeptide Y abbreviated as Npy1 encodes a protein which exhibits neuropeptide Y receptor binding with G protein coupled receptor binding (RGD) and is known to be associated with cerebral hemorrhage and may be involved in its pathogenesis. Previous reports have showed the role of NPY in determining predisposition to anxiety disorders. Decreased cerebrospinal fluid in patients with decreased NPY depression implicates that impaired NPY signaling could be involved in the pathophysiology of anxiety and depression. So, it may be explored further as a markerforsenescence.

Since glypican-1 ligands are synthesized by hippocampal-pyramidal cells and CGNs, SLIT 2 family proteins being functional ligands of glypican-1 in nervous tissue,their interaction may be critical for certain stages of CNS histogenesis. Slit 2 homologue showed altered expression in this study.

DNA microarray analysis study from Gupta Ph.D thesis validated few genes which showed increased expression in *invitro* ageing in CGNs. The Study also pointed out few genes by semi-quantitative gene expression of A2M, GNA14, GRIA1, MASP1, NPY, SLIT2 and GLB1. All the genes showed significant increased expression in 2W, 3W and 4W over 1W which clearly showed the significance of these genes in *invitro* ageing. The present study aims at deducing the role of these proteins in ageing. The question is to address in this study that the knock down of these genes at the time of their increase in CGNs would delay senescence. Viability studies showed the importance of SLIT2 and NPY for extended viability in CGNs invitro. Different molecular markers which are established in *invitro* ageing such as β-galactosidase assay and repair efficiency had been used to study the down regulated CGNs. The study showed significant increase in repair gene expression in SLIT2and NPY downregulated cells when compared to Control cells in *invitro* ageing. The expression of TopoIIβ, which is established as a biomarker *invitro* (Bhanu et al 2010), has been checked in SLIT2 and NPY down regulated cells. The expression of TopoIIβ showed a slight increase indicating its importance in regulation of repair gene expression and connection between expression of SLIT2 and NPY in its regulation during ageing *invitro*.

This study aims in evaluation of biological markers in senescence at the molecular level which strengthens the efficacy of interventions designed to retard the aging process. The results from the above study demonstrate the diversity in gene expression of ageing patterns present as well as how rapidly genome-wide patterns

of aging can evolve; they may also have implications for the oxidative free radical theory of ageing, and help to improve our understanding of human neurodegenerative diseases.

Chapter 4

Analysis of association of TopoIIβ in DSB repair in cerebellar granule neurons.

Introduction

Neurons possess a high metabolic activity and are especially vulnerable to damage during continuous exposure of endogenous reactive oxygen species (Naka et.al 2008). Oxidative stress mediated accumulation of DNA damage in CNS has been mechanistically implicated in etiology and pathogenesis of many neurodegenerative diseases (Rolig, etal, 2000) Double strand DNA breaks (DSBs) are one of the most common type of oxidative DNA damages which become deleterious and act as potent inducers of apoptosis as well as chromosomal aberrations when left unrepaired (Jackson,et.al, Norbury,2001). DNA DSBs are known to trigger a cascade of signaling processes that lead to repair and resolution of the DNA intermediates. Basic biochemical pathways for repair of DSBs involve either nonhomologous end joining (NHEJ) or homologous recombination repair HRR. These two major pathways that repair the DNA and maintain its integrity are triggered by endogenous DSBs or those arising from an external agent such as ionizing radiation (IR). NHEJ pathway helps in repairing DNA breaks without the need for homology between the segments to be rejoined. In terminally differentiated cells like neurons, the NHEJ is the sole repair system (Jackson et al.,2001) and this repair process appears to be particularly important in developing nervous system. Studies in vivo show that, lack of Lig4, XRCC4, Ku70 or Ku80, which are components of NHEJ pathway in the knockout mice results in dramatic apoptosis of many types of embryonic post mitotic neurons (Sekiguchi,1999). NHEJ involves Ku heterodimers Ku70 and Ku86 (Doherty et al., 2001), DNA-PKcs (DiBiase, 2000), DNA ligase-IV (LIG4) (Wang 2001), XRCC4 (Koch, 2004) and Artemis (Moshous 2001). One of the proteins that get localized very early to DNA ends at a DSB, is the Ku heterodimer (Yoo.1998) The Ku-DNA complex recruits DNA-PKs to DNA ends and the interaction betweenthetwoleads to formation of a complex that probably serves to hold the

DNA ends in close proximity to one another. The final step in NHEJ process is ligation of the two DNA ends, which is carried out by the LIG4-XRCC4 complex. Additional, but yet unidentified proteins may be required for NHEJ activity, since purified NHEJ proteins are known to fail to recapitulate DNA end joining (Weller et al.,2002).

TopoIIβ deficient neuroblastoma cells are sensitive against hydrogen peroxide mediated insult suggesting the possible role of TopoIIβ in DNA repair. TopoIIβ also has been shown to be involved in dsDNA break mediated activation of the gene transcription in cells by formation of multimeric complex containing TopoIIβ, PARP-1, DNA-PKcs, and also through recruitment of another complex comprising Ku86 and Ku70 during transcriptional activation. Enzymatic activity of TopoIIβ that alters the topology of the DNA (Wang 2002) might be a critical component required for the double strand break repair mechanisms in mammalian cells. The TopoIIβ containing repair protein complex may function as a component of the genome-wide DNA-damage surveillance machinery and also plays a significant role in facilitating dynamic localized changes in chromatin organization during DSB repair in neuronal cells.

As topological changes mediated by TopoIIβ are critical for vital processes in DNA metabolism like transcription and replication. In this chapter we have investigated the role of TopoIIβ in DSB repair process, which is one of the important repair mechanisms in cells. The increased activity and protein levels of TopoIIβ during the senescence and oxidative stress in a neuroblastoma cell line suggest its importance during oxidative damage.The present investigation analyses the repair activity of TopoIIβ in terminally differentiated primary granule neurons in peroxide induced DSBs. NHEJ pathway is mediated by two parallel pathways, one involving Ku70 and the other involving PARP-1. Although the levels of the

proteins may per se indicate the presence of an NHEJ pathway they may not directly establish their possible association with TopoIIβ. The results of co-immunoprecipitation experiments confirmed the direct association of TopoIIβ with the repair proteins. The results of these experiments showed a strong association of TopoIIβ with PARP-1 as well as Ku70. The results show a strong association of TopoIIβ with peroxide induced DSBs repair in primary neurons in both Ku70 and PARP-1 dependent pathways. Recruitment of TopoIIβ to the site of DNA damage has been addressed by co-localization studies with granule neurons under peroxide mediated damage and repair conditions

.Characteristic formation of repair foci along with double strand break marker γ-H2AX was observed. As it was already reported that post translational modifications of TopoIIβ are required for its activity, poly ADP ribosylation and phosphorylation of the enzyme were detected during DNA repair. Furthermore, to address whether the catalytic activity of the enzyme is required for its recruitment to the damaged sites, ICRF-193 was used in the study. The results clearly showed the inhibition of catalytic activity of the enzyme showed loss of recruitment of TopoIIβtothedamagedsites.

Results

4.1 Expression of TopoIIβ along with other repair proteins during peroxide mediated DNA damage

Free radicals produced during cellular metabolism are the major source of DNA damage and such conditions are induced by using hydrogen peroxide (H_2O_2), a potent free-radical generator. Viability of granule neurons has been monitored at 1.0 mM hydrogen peroxide treatment, the results in Fig. 4.1 show the up regulation of TopoIIβ along with NHEJ proteins Ku70 and PARP-1 after peroxide treatment.

Figure 4.1

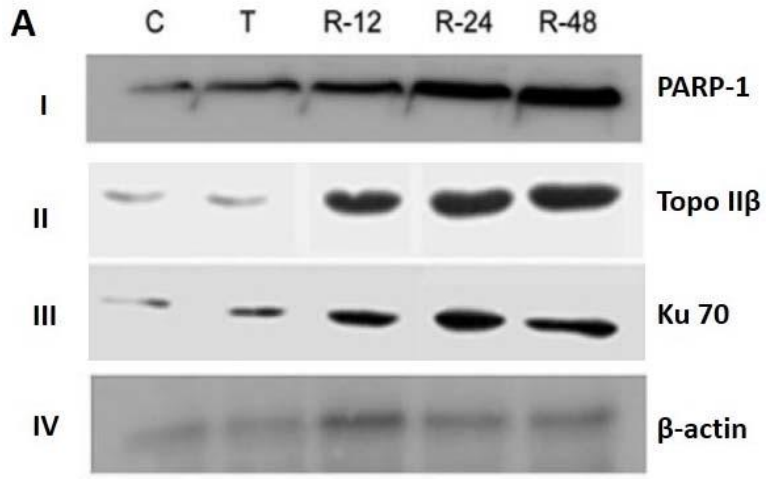
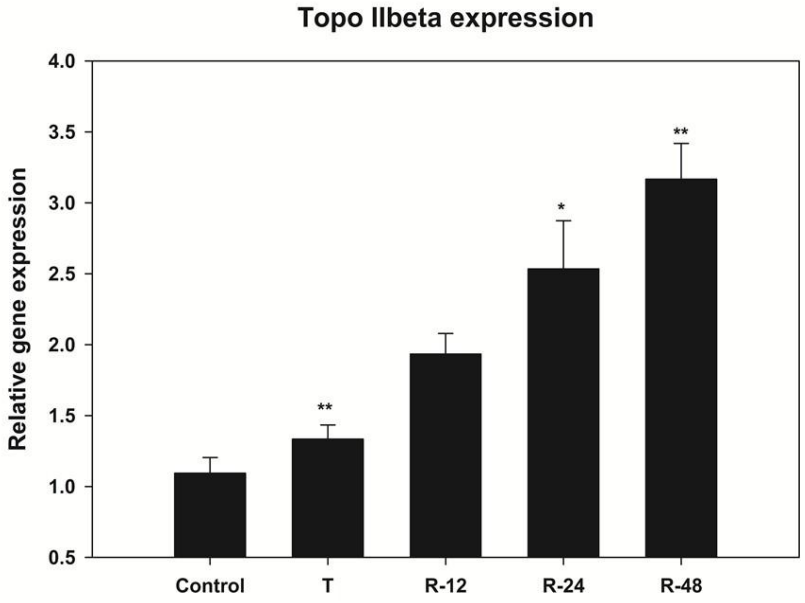


Fig 4.1 PanelA Expression profile of TopoIIβ, PARP-1, Ku70 during peroxide mediated DNA damage and repair

Granule neurons were cultured for 4 days and incubated in the presence of 1 mM hydrogen peroxide for 12 h (Treatment: T). After 12 h the cells were washed with fresh medium and grown in a complete medium for 24 h (R-24) 48h (R-48) and 72h (R-72) respectively. 3A (I,II,III) Levels of PARP-1,TopoIIβ,Ku70 during the treatment and recovery phases as compared with β-actin (internal control) 3A (IV)

B



Panel B Real time PCR to detect mRNA expression levels of TopoIIβ during peroxide mediated DNA damage and repair

mRNA was isolated from neurons treated with hydrogen peroxide as indicated above .the levels of RNA expression indicated as relative florescence units. β-actin was used as internal control in the experiment

Fig 4.2 Recruitment of TopoIIβ to sites of double strand break after peroxide mediated damage

Recruitment of repair proteins to the sites of DNA damage is known to be initial step in the repair of damaged DNA. Recruitment to damaged sites is assisted by many signaling proteins. Co-localization experiments with marker proteins had be proven to initial screening procedure in detection of the sequential recruitment of proteins to the damaged sites Recruitment of TopoIIβ soon after DNA damage is

detected by co-localization studies with a double strand marker protein γ-H2AX. The recruitment was observed within 20, 40, and 80 min time points. TopoIIβ was shown to be recruited along with DSB marker protein γ-H2AX upon peroxide mediated DNA damage.

Figure 4.2

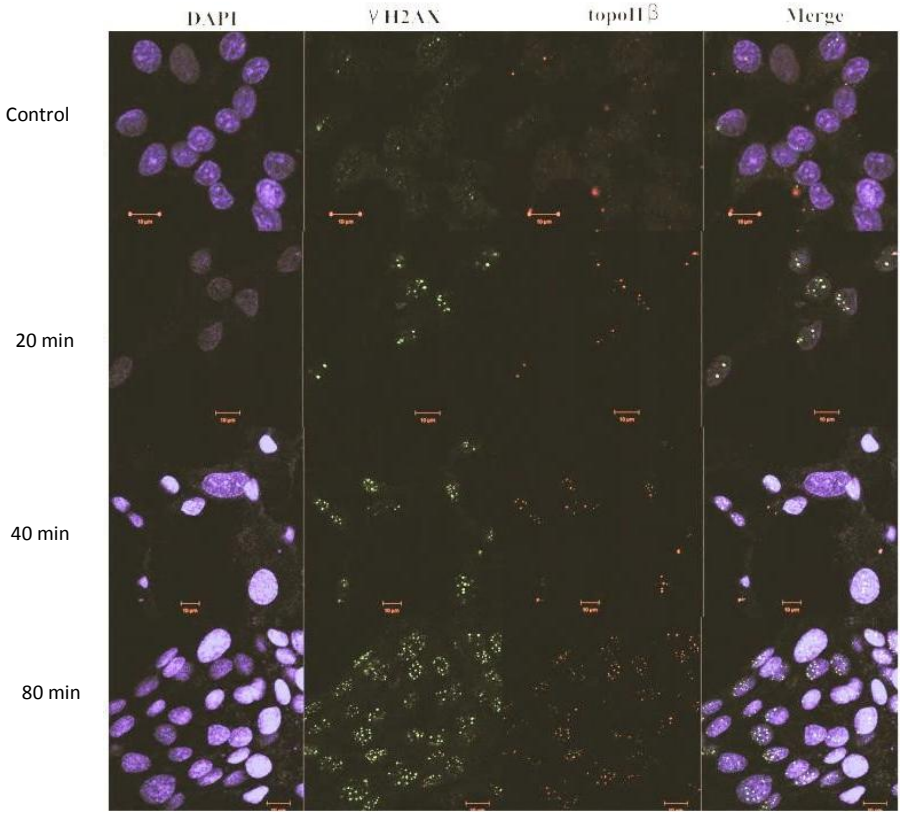


Fig 4.2 Co-localization of TopoIIβ and γ-H2AX soon after peroxide mediated DNA damage Neurons treated with 1mM hydrogen peroxide and recovered after indicated time points (20,40 80 min after damage). Immunofluorescence microscopy was done to detect the co-localization of indicated proteins.

4.3 TopoIIβ Associates with PARP-1 and Ku70 during peroxide mediated DNA damage and repair

An analysis of the TopoIIβ-associated nucleoprotein repair complex using co-immunoprecipitation and colocalization experiments showed a strong association of TopoIIβ with PARP-1 and Ku70 (Fig4.3). Immunofluorescence studies displayed that TopoIIβ and PARP were colocalized during treatment and recovery periods in the nucleus (Fig.4.4). These results demonstrated that TopoIIβ associated with PARP and Ku70 and that this complex is involved in DSB repair through NHEJ pathway.

Figure 4.3

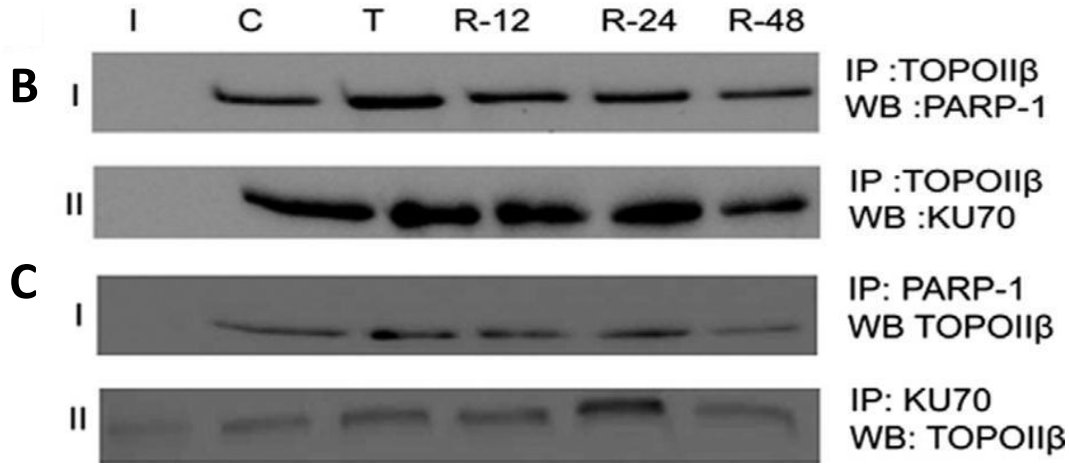


Fig 4.3 Co-immunoprecipitation of TopoIIβ, PARP-1, Ku70 during peroxide mediated DNA damage Granule neurons were treated with hydrogen peroxide and recovered at indicated time points R-12, R-24, R-48. Immunoprecipitations were done with TopoIIβ and western blot was developed with PARP-1,Ku70 antibodies (B-I,B-II). Immunoprecipitations were done vice versa with PARP-1, Ku70 antibodies and western blot was developed with TopoIIβ antibody(C-I,C-II)

Figure 4.4

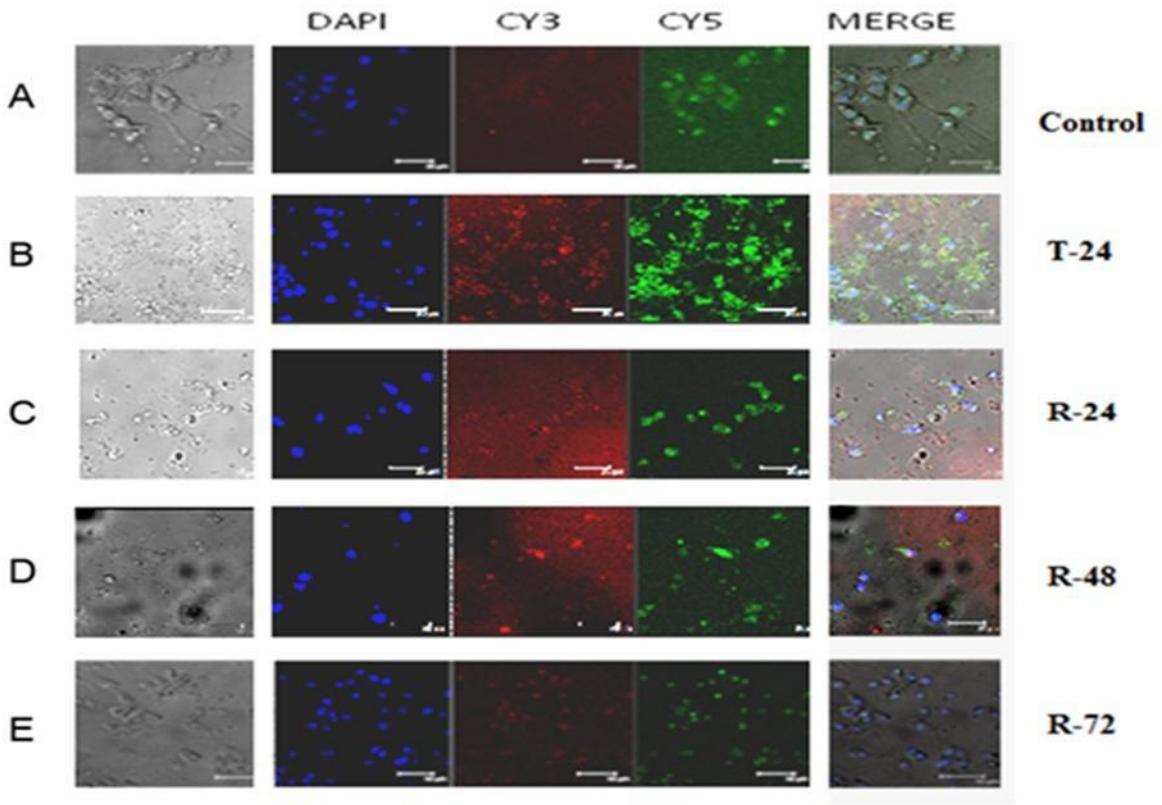


Fig 4.4 Colocalization of TopoIIβ and PARP-1: Granule neurons were cultured and fixed with paraformaldehyde to study the colocalisation of TopoIIβ and PARP-1. A colocalization of TopoIIβ (green-CY5) and PARP-1(red-CY3) was observed in the cell nucleus soon after treatment T-24 (B) and during the recovery periods R-24, R-48 and R-72 (C–E). However, the colocalization of both proteins seems to be decreased after 3 days of post recovery i.e., at R-72. Untreated cells were used as control (A) and DAPI (blue) was used to counter stain the nucleus.

4.5 Post translational modifications of TopoIIβ during NHEJ repair

4.5.1 Poly ADP ribosylation of TopoIIβ

Since TopoIIβ is known to be associated with PARP-1 during peroxide mediated damage it would be interesting to study its post translational modification during repair. Indeed polyADP ribosylation is one of the most common modification of many nuclear proteins especially during DNA damage

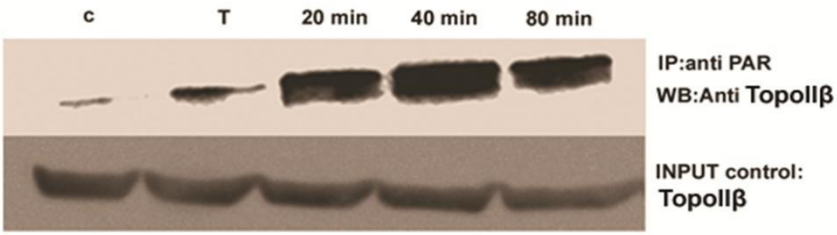
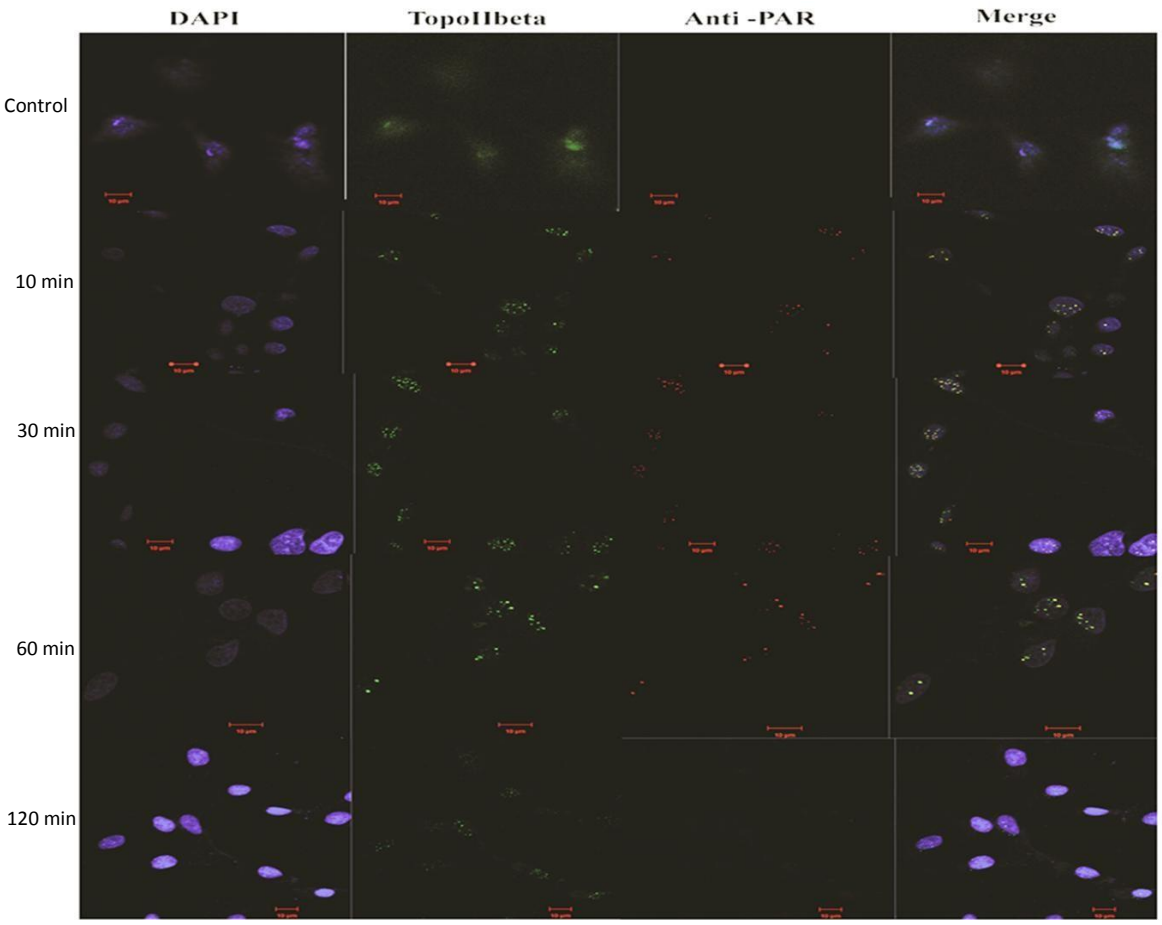


Fig 4.5.1 Immunoprecipitation was done with anti-PAR antibody with control and peroxide damaged extracts at indicated time points and western blot was developed with TopoIIβ antibody. TopoIIβ was used as input control.

The results of **Fig 4.5.1** showed that Poly ADP ribosylated TopoIIβ protein level increased from 20 min, by the level shown at 40 min during DNA damage. PARlyation of TopoIIβ was confirmed by co-immunofluorescence with Anti PAR antibody (**Fig 4.5.2**).

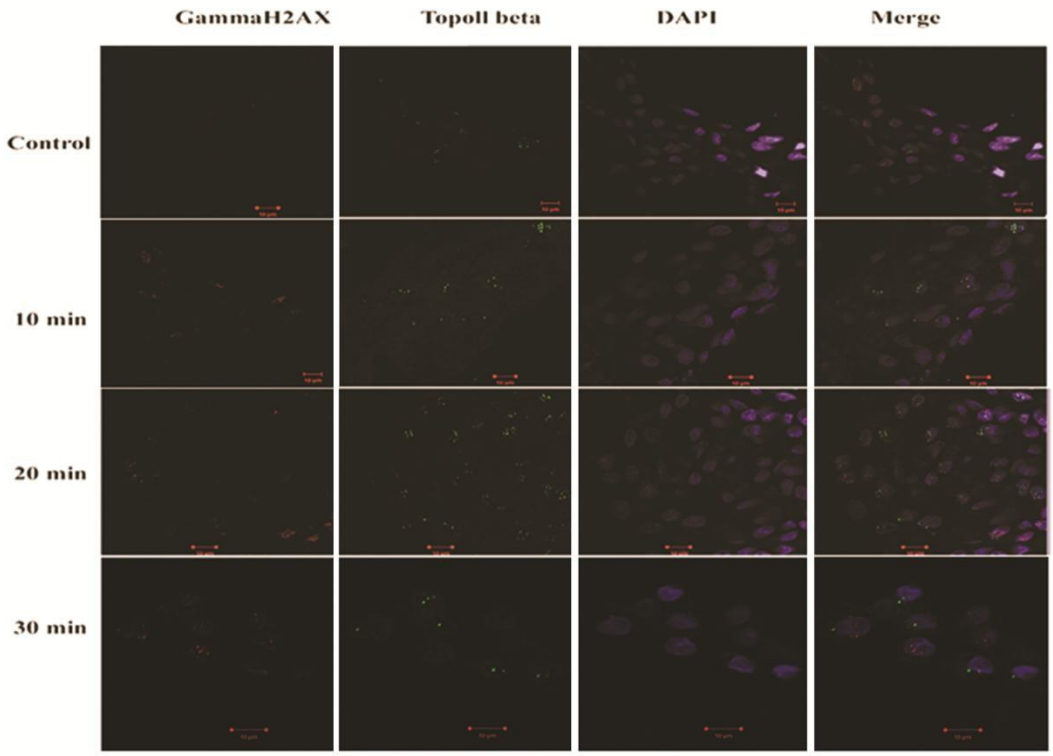
4.5.2 Co-localization of TopoIIβ with poly ADP ribose during peroxide mediated DNA damage



4.5.2 Poly ADP ribosylation of TopoIIβ is confirmed by co-immunolocalization of TopoIIβ with Poly ADP ribose polymer. TopoIIβ (green-alexa flour 458) shown to be colocalized with anti-PAR (Red-alexa flour 695) at 10 30 60 and 120 min after peroxide mediated DNA damage in cerebellar neurons.

4.6 Action of ICRF-193 on recruitment of TopoIIβ to the site of DNA damage

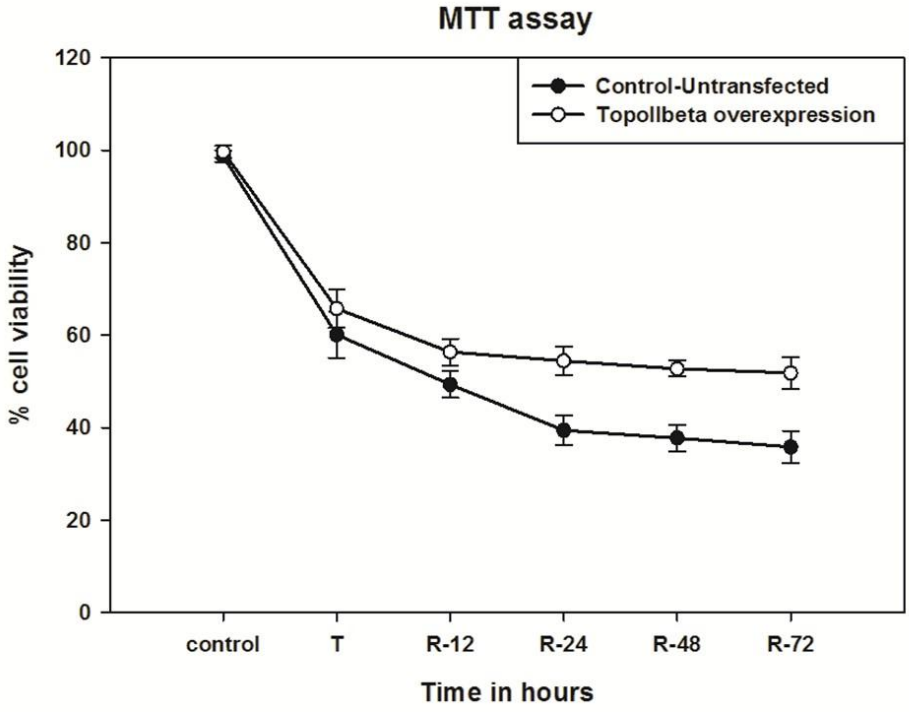
The effect of TopoIIβ catalytic inhibitor on recruitment of TopoIIβ to the damaged sites is analyzed by examining the co-localization of TopoIIβ with γ H2AX in addition of ICRF-193.Results show that catalytic inhibition of TopoIIβ abolished its ability in recruitment at the sites of double strand break.



4.6 Cerebellar granule neurons were treated with 10μM ICRF-193 for 12 hours and damaged with 1mM hydrogen peroxide and colocalization with γ H2AX was examined at different time points**.** TopoIIβ was counter stained alexa flour 498 green and γ H2AX was counterstained with alexaflour 594 (red).

4.7 Reduction in Damage sensitivity of TopoIIβ overexpressing cells

As down regulation of TopoIIβ drastically inhibited DNA repair in cerebellar granule neurons(Mandraju et al 2012), the effect of overexpression of TopoIIβ in damage sensitivity was examined in TopoIIβ overexpressing cerebellar granule neurons .In this gain of function experiment, TopoIIβ overexpression showed significant reduction in damaged sensitivity in cerebellar granule neurons.



4.7 TopoIIβ full length construct was transfected in cerebellar granule neurons and damaged with 1mM hydrogen peroxide .cell viability was examined in transfected and control cells by MTT assay at 12,24,48 and 72 hours after damage induction**.**

Discussion

The two isomers of TopoII, TopoIIα and TopoIIβ exhibit quite contrasting characteristics in that the α isoform is ubiquitous in the nucleus of proliferative cells (Turley 1997) and unlike the TopoIIβ isoform exhibits a developmental role (Tsutsui 2001 , Kondapi 2004). Our earlier result showed the possible roles of the TopoII isoforms in DNA damage and repair in dividing cells.The results in the present study showed that the TopoIIα is present in the neuroblastoma cell line SK-N-SH and in dividing astrocytes but is totally absent in terminally differentiated granule neurons. Further, the results presented here established and defined the distinct role of TopoIIβ in DNA repair of primary neurons through NHEJ pathway. The presence of free radicals is the primary source of damage to DNA and constitutes a major cause for neuronal apoptosis and aging. The damage inflicted by free radicals in vivo is replicated in our experiments *invitro* using H_2O_2. The damage was quantified and the extent of damage, as expected, showed an increase with concentration. The cells could recover themselves completely to normal condition in the case of lower peroxide concentration while the recovery was only partial in the case of higher concentrations. This interaction of TopoIIβ with two critical proteins involved in DSB repair, Ku70 and PARP-1 were analyzed. The recovery of the DNA damage was not evident in TopoIIβ down regulated cells compared to the control (untransfected and scrambled siRNA-transfected) neurons. This confirms the requirement of TopoIIβ in the repair of DNA damage that occurred due to oxidative stress. This is also consistent with earlier studies which showed endogenous oxidative stress leads to increased apoptosis in mice deficient in Ku70 and Polβ. The results of co-immunoprecipitation experiments confirmed the direct repairproteins.The results of these experiments showed a strong association of TopoIIβ with PARP-1 as well as Ku70 (Fig.4.3). This clearly points to the

involvement of TopoIIβ both in Ku70 and PARP-1-dependent pathways and suggests furthermore that both the PARP-1 and Ku70-dependent NHEJ pathways are operational for recovery from H_2O_2-mediated-oxidative damage of primary neurons. In addition, PARP-1 is known to be involved in modification of several proteins like DNA polymerases, DNA ligase, TopoII and PARP-1 itself is a target of post translational modification. TopoII along with Topoisomerase I, DNA polymerase alpha and delta and other proteins undergo poly (ADP) ribosylation modification indeed is shown to decrease the catalytic activity of TopoII. PARP deficient cells have been shown to possess low levels of TopoII). The reported lower activity of poly-(ADP) ribosylated TopoII indicates that TopoIIβ catalytic activity may be by keeping TopoIIβ-PARP association along with repair complex stabilization along with repair protein. The PARylation may inhibitcatalytic activity of TopoIIβ after recruitement of TopoIIβ- PARP may participate in function of these proteins in DSB repair. PARylation itself may not be the only requirement for the repair activity rather the stabilized TopoIIβ in the presence of PARP-1 may play a more significant role in regulation of DNA repair activity or in modulating levels of various repair proteins. This is because both TopoIIβ and PARP-1 have been shown to be critical components of the multi-step serial events required for regulated gene expression in response to many ligand or signal-dependent stimuli. Henceforth, the association of TopoIIβ with PARP-1 and Ku70 as observed in the present study implicates the role of PARP-1-TopoIIβ and Ku70-TopoIIβ complexes in the NHEJ pathway mediated repair process. Such an interaction of PARP-1 and Ku70 in parallel is suggesting the involvement of TopoIIβ in both pathways. Alternatively, PARP-1 mediated poly ADP-(ribosylated) form of Topoisomerase II may be interacting with Ku70 in

promoting NHEJ activity. Further studies are needed to understand the unique mechanism of TopoIIβ involvement in regulation of such repair network.

Chapter 5

Regulatory activities Topoisomerase IIβ in HIV-1 transcription

Introduction

Recently there are increasing evidences about the role of TopoisomeraseIIβ (TopoIIβ) in different cellular processes (Wang, 2002). Adding to this functional diversity of TopoIIβ, we have previously reported its importance in DNA repair, ageing, neuronal development and HIV-1 infection (Bhanu 2012, Mandraju et al 2008, 2009, 2011 Kondapi et al, 2004). The involvement of TopoIIβ in HIV-1 infection was established by a decrease in viral titres upon its downregulation (Bouillé, 2009). Indeed, TopoIIβ was shown to be associated with strand transfer events in HIV-1 reverse transcription (Lokeswara et al, 2013). Earlier report by Ju et al., (2006) have shown the indispensable role of TopoIIβ catalytic activity and its association with the DNA repair proteins in the transcription regulation of few genes (Ju et al, 2006). The double strand break caused by TopoIIβ invokes a plethora of cofactors which includes transcription factors and components of DNA repair machinery leading to a specific gene activation by the modification of chromatin architecture (Ju et al., 2006). Interestingly, a cluster of strong cleavage sites of TopoII were identified approximately 850 bp upstream of the HIV-1 integration site in the host genome and within the HIV-1 5'-LTR, particularly overlying the region that encodes TAR, a key inverted repeat e lement whose transcript is vital for the interaction with Tat (Howard and Griffith, 1993; Pommier et al.,1994). Owing to such diverse functions of TopoIIβ in transcriptional regulation and the presence of topoII cleavage sites at HIV-1 LTR region, we were intrigued at its transcriptional role of integrated HIV-1 proviral DNA.

The transcriptional activation of integrated proviral DNA requires various steps that involve both viral and cellular proteins among which, HIV-1 transactivator, Tat, a 14 KDa viral protein is instrumental in recruiting the entire machinery to bust the nucleosome for the transcription initiation and elongation thereby exerting

its transactivating properties (Berkhout et al.,1989). In this process it engages various host proteins namely P-TEFb, TBP, Sp1, NF-kB, SW1/SNF, p300, PCAF, hGCN5, etc. to HIV-1 LTR (Sheridan et al.,1995, 1997, Herrmann and Rice,1993, Gold et al.,1998, Kashanchi et al.,1994, Veschambre et al.,1995, Chun et al.,1997). Nevertheless, it is evident that some of the host proteins which are involved in this transactivation process are yet to be identified. Further, in the identification of activities exerted by common proteins that are involved in the cellular transcription, DNA repair and transactivation of HIV-1 infection, a more or less identical complexes were found to be operative. Considering TopoIIβ being an integral part in both DNA repair and transcriptional complexes, it may have the role at LTR during HIV-1transcription.

Role of TopoIIβ in transactivation of HIV-1 LTR has been studied in the present study. down regulation as well as catalytic inhibition of TopoIIβ resulted in drastic inhibition. Further, we have identified the direct involvement of TopoIIβ in regulating the HIV-1 transcription by its association with transactivating complex of Tat.

Results

TopoIIβ is essential for HIV-1 replication

Progression of Viral replication was examined in TopoIIβ downregulated SupT1 cells where its levels were confirmed by western blot. The loss of function of TopoIIβ yielded a 50% reduction in the viral replication (Fig 5.1A). To further confirm its role, the level of infection was examined in the SupT1 cells that were treated with increasing concentrations of specific TopoIIβ inhibitors, ICRF-193 and Merbarone. ICRF-193 is a bisdioxopiperazine inhibitor of TopoII, which catalytically inhibit TopoII without causing DNA strand break. For treatment with ICRF-193, we have used 10-50μM of drug where it acts through both TopoIIβ-

specific downregulation and inhibition of TopoIIβ catalytic activity by activating a 26S proteasome pathway (Xiao et al.,2003, Mao et al .,2002, Huang et al., 2001). Merbarone (mer) (5-N-phenylcarboxamido-2-thiobarbituric acid), inhibits the cleavage activity of TopoII without damaging the DNA or the stabilizing DNA-TopoII cleavable Complexes (Fortune et al,1998). The cytotoxicity assays for the drugs were also done to confirm that there is no cytotoxicity at that particular concentration which were used to inhibit TopoIIβ (figure 5.6A and B). Here we have observed a proportionality between the percentage of TopoIIβ inhibition and the viral propagation where there is 33-70% inhibition in viral titers (Fig 3.1B). Similarly, 85% of viral inhibition was recorded when 100 µM of Merbarone was used (Fig 5.1C). Moreover, the specific inhibition of double strand break initiated by TopoIIβ during merbarone treatment implies that the induction of double strand break is essential for the successful replication of virus. These results suggests that TopoIIβ is directly implicated in HIV-1 replication. Since the inactivation and down regulation of TopoIIβ has lead to a decrease in viral load, we tried to address whether gain of function showed the converse effect. Direct role of TopoIIβ in HIV-1 replication is corroborated by the observation of an increased viral load when the cells were transiently transfected with a full length plasmid construct of TopoIIβ. When the full length clone of TopoIIβ was transfected into SupT1 in increasing concentrations ranging between 0.5-5µg, upto 6 fold increase in the viral titer was observed (Fig 5.1D). This confirms the plausible role of TopoIIβ in aidingviralreplication.

Figure 5.1

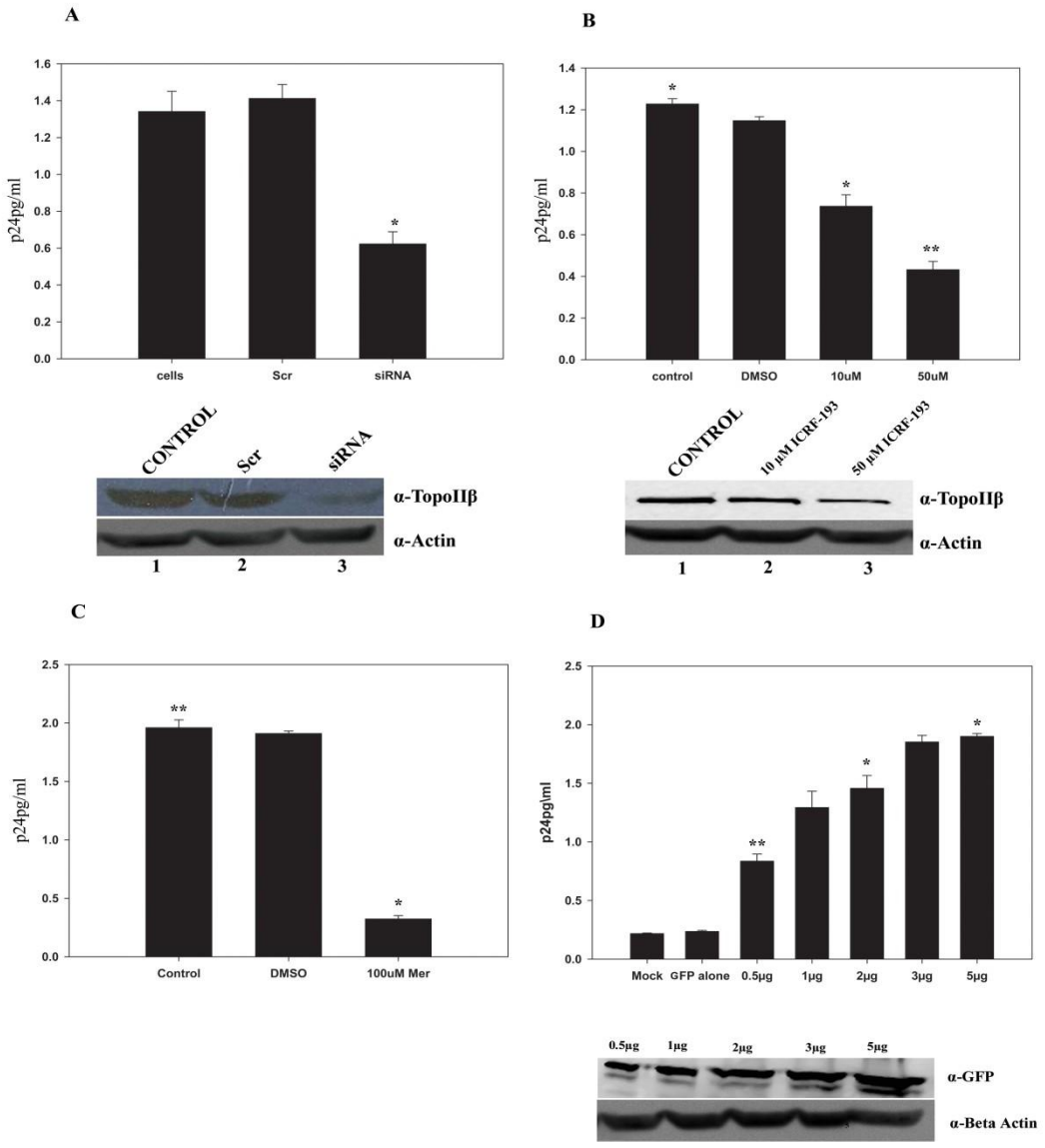


Fig 5.1 TopoIIβ modulates HIV-1 Infection

A.TopoIIβ specific siRNA and Scrambled siRNA were transfected in SupT1 cells 24hours prior to transduction. A decrease in the viral titer was observed that corresponded with the level of TopoIIβ was shown in the immunoblot analysis in the lower panel. B. Transfection with pNL4.3 in SupT1 cells after treatment with

different concentrations of ICRF-193 for 4 hours also resulted in decrease in viral titer with the simultaneous decrease in TopoIIβ levels shown in the lower panel. C. Similarly, when SupT1 cells were treated with 100μM merbarone and transiently transfected with pNL4.3 using Xtreme gene HP (roche), 85% decrease in viral load was observed. D. When SupT1 cells were transiently transfected with increasing amounts of full length TopoIIβ a 6-fold increase was observed in the viral titer after transduction with pNL4.3. Lower panel shows the Immunoblot of TopoIIβ levels in the transfected cell lysates. β actin was used as loading control.

Fig 5.2 Recruitment of TopoIIβ, Ku70 and PARP-1 to HIV-1 LTR during viral replication

As the basic transcriptional machinery during HIV-1 transactivation is recruited at the 5'-LTR, which is the promoter with cis-acting elements contained in it. The recruitment of the TopoIIβ along with the other repair proteins Ku70 and PARP-1 was investigated in this region. This is due to the fact that the same complex is involved in the transcriptional regulation in response to the estrogen stimulation which was reported earlier. The confirmation of this hypothesis was done by Chromatin Immunoprecipitation (ChIP) assay with the antibodies corresponding to these proteins. After immunoprecipitation with the three antibodies, the bound DNA was amplified with the primers that are specific for the 5'-LTR region, during 12-72 hrs post infection in SupT1 cells (Fig5.2). The PCR amplification of the LTR region following immunoprecipitation of genomic DNA with above three proteins along with Tat at 12, 24, 48 and 72hrs implies that the above complex is associated with the viral LTR for subsequent transactivation

Figure 5.2

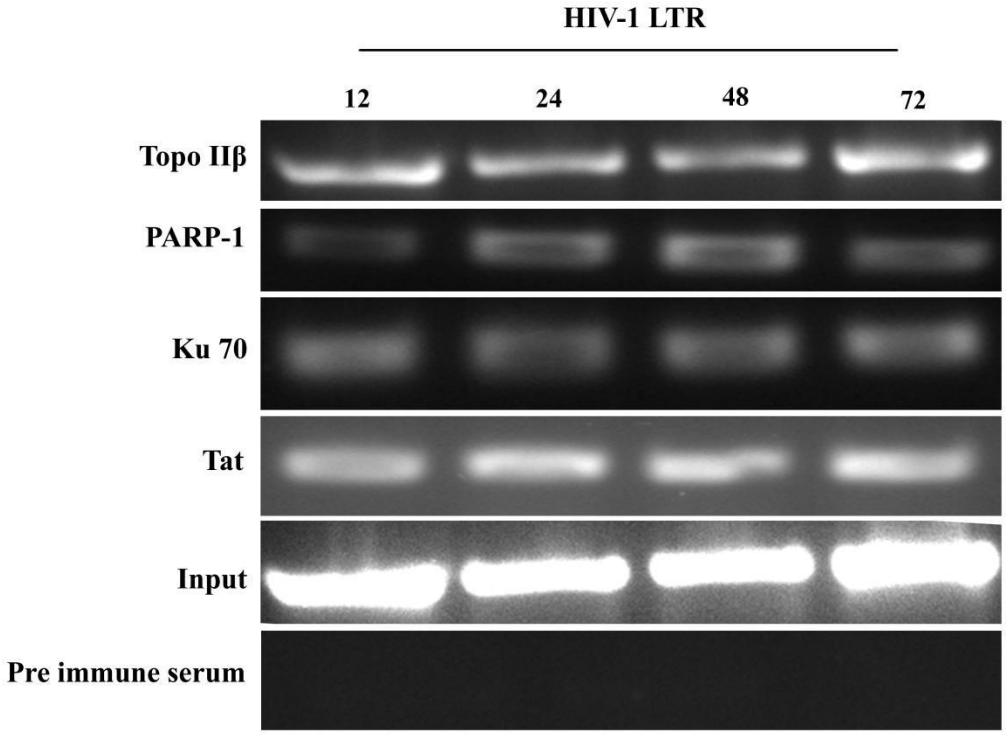


Fig5.2 TopoIIβ/Ku70/PARP-1 binds to HIV-1 LTR during viral replication

3 x 10^6 SupT1 cells were transfected with an episomal pNL4.3 plasmid and ChIPs were performed using antibodies against, TopoIIβ, PARP-1, Ku70 and Tat. A nonspecific mouse antisera (IgG) control was also included. Immunoprecipitated DNA fragments spanning nucleotides -109 to +82 in the vicinity of TAR were amplified by PCR using oligonucleotide DNA primers as described in Hao Ying et al 2012. Amplification of LTR fragment at all the time periods from 12-72hrs in the samples immunoprecipitated with all the antibodies along with Tat shows that the entire complex gets recruited at the LTR

Fig 5.3 TopoIIβ, Ku70 and PARP-1 associates with HIV-1 Tat

Tat is a major viral protein that plays an active role in engaging the mRNA synthesizing machinery after succesful integration of proviral genome and the entire process involves a complex interplay between host and viral factors hence getting its name, the Transactivator protein. Since this entire eventful mechanism needs a complete chromatin reorganisation by Tat, we investigated whether the present complex involving TopoIIβ, Ku70 and PARP-1, which had a role in remodeling of host chromatin for transcription of certain genes, gets recruited by Tat. In this experiment, the His tagged Tat was bound to Nickel-NTA matrix and incubated with100µg of SupT1 lysate where the above three proteins were found to interact with His-Tat which was shown by western blotting (Fig.5.3B). This result was corroborated with co- immunoprecipitation of the whole complex containing TopoIIβ, PARP-1 and Ku70 with Tat during viral infection in SupT1 cells (Fig.5.3A). Hence we propose that this TopoIIβ and its interacting partners are associated invivo with Tat during transcription of HIV-1.

Figure 5.3

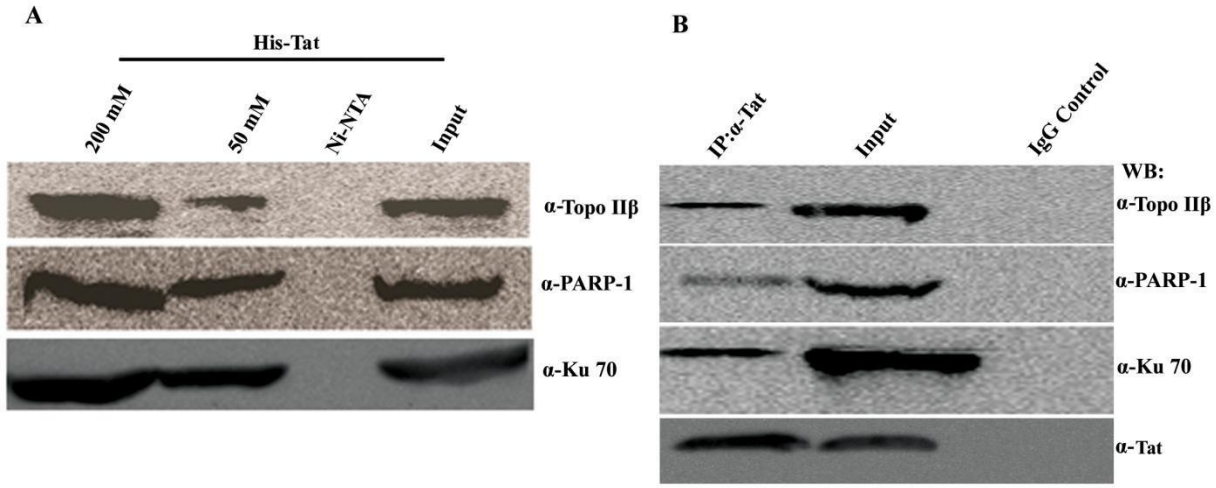


Fig 5.3 TopoIIβ, Ku70, PARP-1 were associated with HIV-1 Tat during viral transcription

A.GST pull-down experiments were performed at 4 °C using purified recombinant His-HIV-1 Tat bound to Ni-NTA. SupT1 lysate was added to the resin and incubated overnight at 4°C. After centrifugation and washing, the bound endogenous TopoIIβ, Ku70, PARP-1 were separated and detected by immunoblotting. All the three proteins were bound to His-tat when eluted with different concentrations of Nacl (lane 1 and 2).Ni-NTA alone was included as control in the experiment.

B. Co-immunoprecipitation was done from HIV-1 infected SupT1 cell extract after 24 hrs of infection by incubating separately with anti-Tat monoclonal antibody for overnight at 4°C. Protein A/G agarose beads were added to the mixture and incubated for 2 h at 4 °C and resolved for blotting with monoclonal antibodies of PARP-1 and Ku-70, TopoIIβ and HIV-1 Tat. 10% of the total sample was loaded as input and mouse IgG was used as isotype control. The binding of all the above proteins to Tat in the infected sample confirms their association invivo.

5.4 TopoIIβ facilitates HIV-1 transactivation

The role of TopoIIβ in LTR-mediated transcription was assessed in TZM-bl cells by overexpression of TopoIIβ. The luciferase activity was taken as the direct measure of HIV-1 transactivation in this reporter cell line. There was upto 3 fold increase in basal transcription of HIV-1 LTR upon TopoIIβ overexpression (Fig.5.4A). Moreover, overexpression of TopoIIβ resulted in enhanced Tat mediated transactivation of LTR, as shown by a 3 fold increase in luciferase activity. (Figs.5.4B). Knock down of TopoIIβ using siRNA or catalytic inhibition using ICRF-193 resulted in a significant reduction in the basal as well as Tat-mediated transcription (Fig. 5.4C,5.4D). There was a 2-fold decrease in basal promoter activity and 1.5-fold decrease in Tat-mediated luciferase activity upon knockdown of TopoIIβ. In addition, we evaluated the effect of TopoIIβ on LTR mediated transcription when the reporter was transfected exogenously. In transient transfection assays, LTR-Luc construct was transfected in 293T cells to quantitate the LTR driven reporter activity upon TopoIIβ expression in the presence and absence of Tat. Our results showed that TopoIIβ overexpression lead to 3-fold increase in LTR-mediated transcription (Fig.5.4E).We further observed that overexpression of TopoIIβ had increased 3-4 fold in Tat mediated transactivation (Fig.5.4F).Treatment with ICRF-193 showed decrease in luciferase activity (Fig.5.4G) and the knockdown of TopoIIβ also lead to a 2-fold decrease in both basal and Tat-mediated LTR-Luciferase activity (Fig.5.4H). Altogether, our results suggest that TopoIIβ triggers viral transctivation of both integrated and unintegrated LTRs.

Figure 5.4

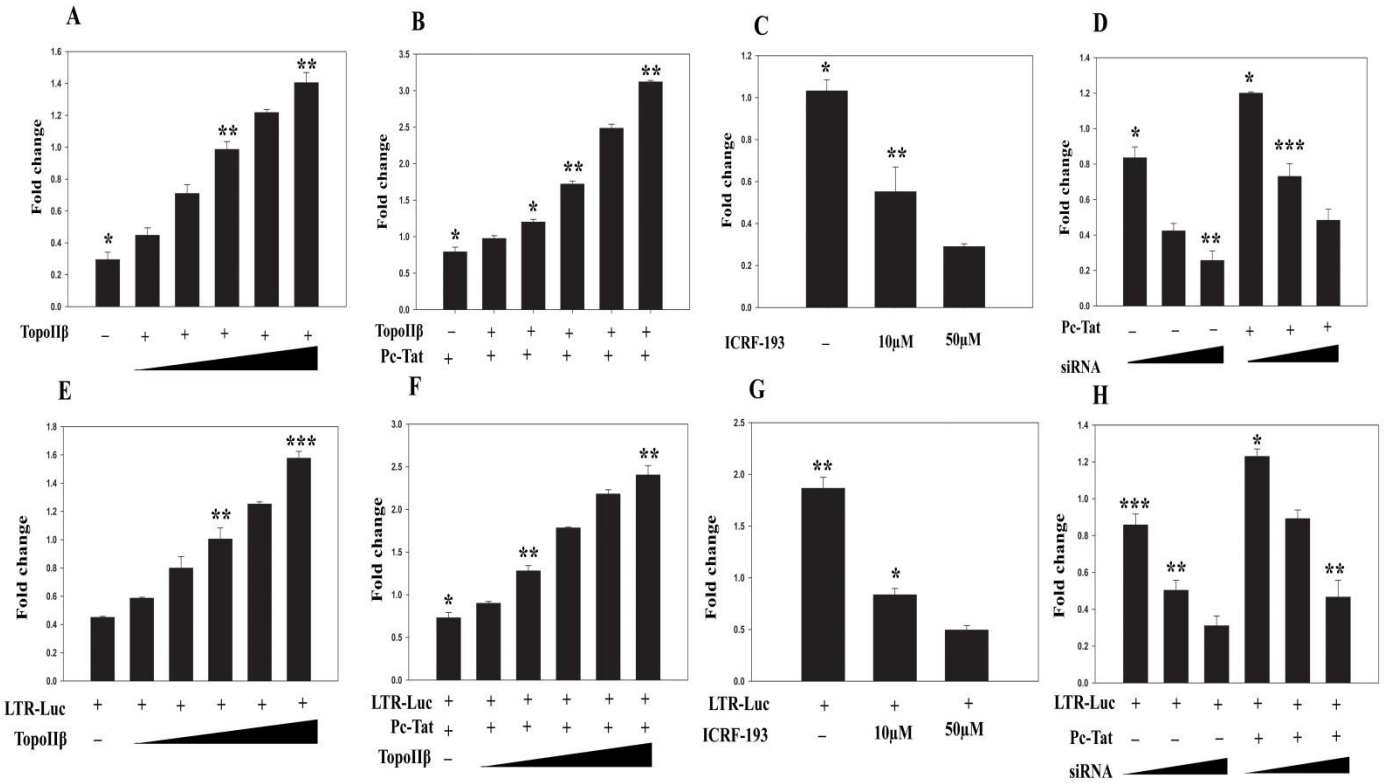


Fig 5.4 TopoIIβ transactivates HIV-1 LTR

Luciferase assays in TopoIIβ overexpression showing elevation of (A) basal,(B) Tat mediated and(C) ICRF-193 down regulated (D) Luciferase assays upon TopoIIβ knockdown in TZM-bl cells. TopoIIβ upregulates basal (E), Tat-mediated (F) and ICRF-193 down regulated (G) Transactivation in 293T cells transiently transfected with LTR-Luc reporter. (H) Luciferase assays upon TopoIIβ knockdown. Experiments were repeated three times and means ± SE was plotted ($*p < 0.05$, $**p < 0.001$, n = 3).

Fig 5.5 Double strand break by TopoIIβ is required for HIV-1 transcription

As the importance of TopoIIβ mediated double strand break for the signal dependent host gene transcription was reported, we investigated whether the induction of this double strand break by TopoIIβ is required for transcription of HIV-1 genes. In this experiment we have used 100μM merbarone to specifically inactivate the double strand break creation by TopoIIβ. Merbarone drastically decreased the Tat dependent transactivation which was confirmed by significant decrease in luciferase activity in HIV- infected TZM-bl cells, where LTR transactivation is a direct measure of luciferase activity. Effect on viral transcription during merbarone treatment was further confirmed by semi quantitative RT-PCR of different viral gene transcripts at 12 hours post infection by the method described previously (Wu and Marsh, 2001)(Fig 5.5A). Merbarone treated cells showed a drastic decrease in viral gene expression when compared to untreated control. The control viral infected cells predominately transcribed nef, env, gag-pol, vif transcripts and there is drastic reduction in the cDNA levels of all the above four viral transcripts upon merbarone treatment, which unequivocally demonstrates that the double strand break induction by TopoIIβ is crucial for the progression of viral gene expression. The catalytic inhibition of TopoIIβ in TZM bl reporter cell line also resulted in decreased luciferase production upon HIV-1 infection when compared to control untreated cells (Fig5.5B)

Figure 5.5

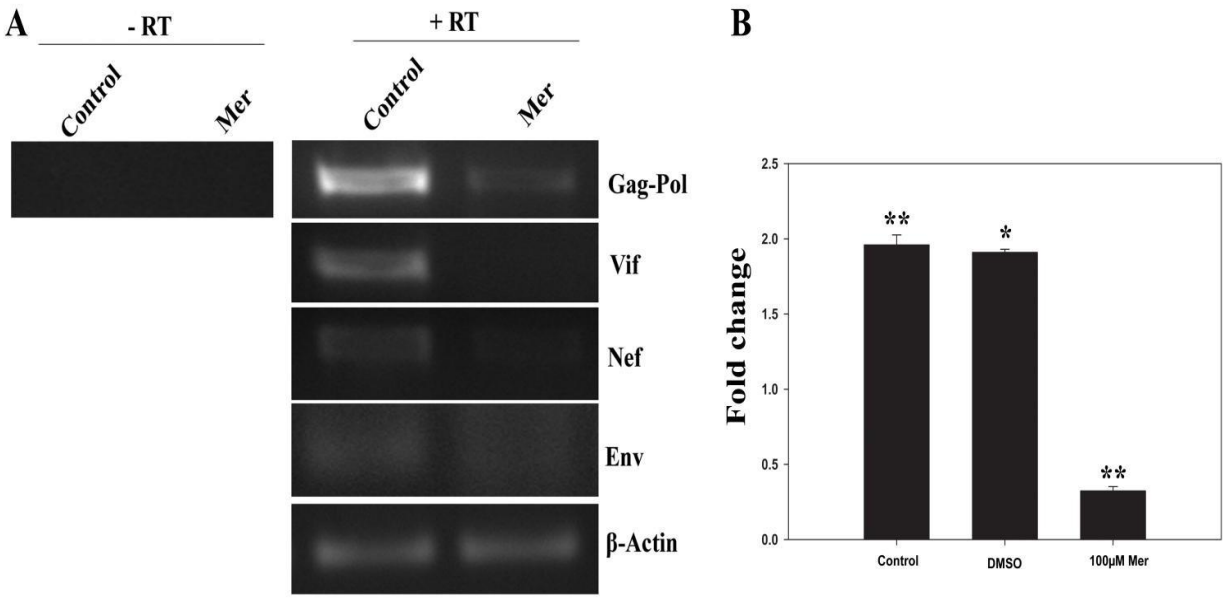


Fig 5.5 TopoIIβ mediated double strand break is required for HIV-1 early gene transcription

A.Detection of early gene products by RT PCR with viral cDNA after merbarone treatment during HIV-1 infection. Total cellular RNA from infected cells (24 h post-infection) was amplified with RT-PCR as described in Methods. Shown are products from RT-PCR using primers specific for gag-pol, vif, env and nef. To confirm that the amplification of the transcripts did not result from DNA contamination, amplification in the absence of reverse transcription (−RT) was included as control in the experiment.

B. Luciferase assay was performed in infected TZM bl cells after treatment with

100µM Merbarone and luciferase quantification was done by luminometry, untreatedcellswereusedascontrol.

5.6 Cytotoxicity of Merbarone and ICRF-193

Cytotoxicity of the drugs on T cells was analysed with addition of different concentrations of drugs, which was analysed by MTT assay.

Figure 5.6

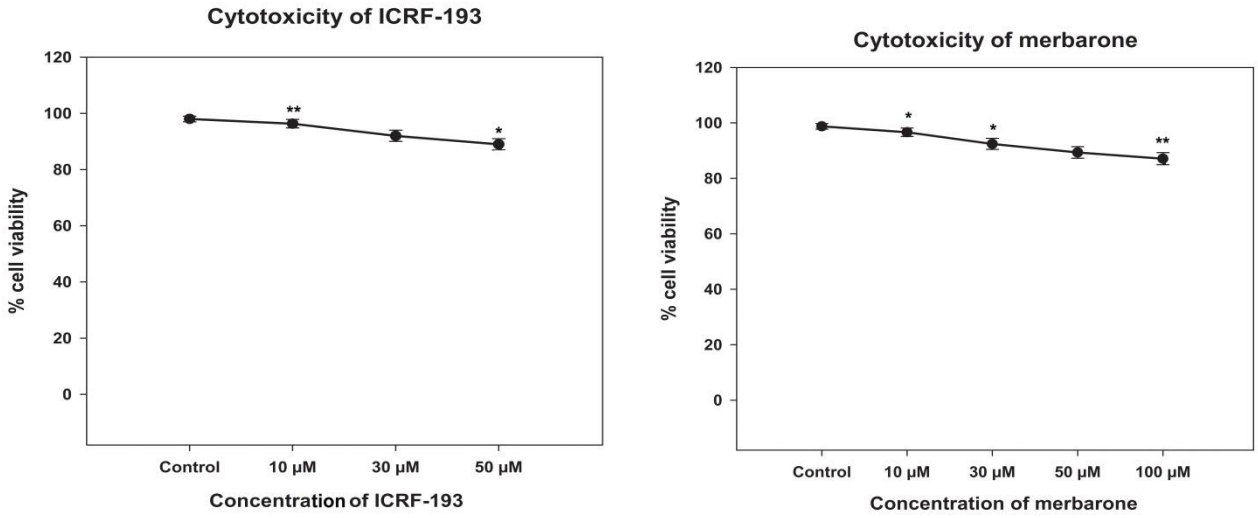


Fig 5.6 MTT assay of ICRF-193 and Merbarone on SupT1ells

Different concentrations of drugs were added to the cells preplated before the addition and incubated for 12 hours. The cytotoxicity was analysed by reduction of dye MTT. Control cells, without addition of the drug were used to calculate percentageviability.

Discussion

HIV-1 transcription is one of the crucial steps in viral life cycle. Host polymerase II complex initiates transcription successfully, but fails to travel far on viral template. This unsuccessful elongation results in short viral transcripts which cannot support viral replication. To overcome this unique problem in viral transcription, HIV-1 encodes a transactivating accessory protein Tat, which activates viral transcription by stimulating elongation from the viral long terminal repeat (LTR) (Eberharter et al. ,2005; Pumferyet al., 2003). Tat orchestrates this viral gene expression through its interactions with various host cellular proteins (Pereira et al.,2003, Benkirane et al.,2008.). Many of these Tat interacting partners are yet to be identified in order to get deeper insights in regulation of viral transcription. Tat recruits many host transcription factors to the LTR region for the complete viral gene expression. Even though many of its interacting partners were identified, the complete mechanism by which it executes transactivation is not completely understood.

It was also found that a TopoIIβ beta mediated double strand break is required for some signal dependent transcriptional regulation. More importantly, treatments with inhibitors and knock out studies established the requirement of TopoII for the replication of various viruses including Human Cytomegalovirus, Adenovirus, Kaposi'sarcoma-associated herpesvirus (KSHV) and Herpes simplex virus type-1(HSV-1) (James Lin 2008, Benson 1998, Kawanishi et al.,1993, Hammersten et al.,1996, Purushothaman et al., 2012.). Adding to this, an antisense mediated repression of TopoII expression leads to the impairment in HIV-1 replicationThe role of TopoIIβ in the formation of PICS and proviral DNA synthesis was also reported from our group (Lokeshwara et al, 2013). These results suggest the involvement of TopoIIβ in various levels of retroviral lifecycle.

In the present study, we have focused on the probable role of TopoIIβ during the post integration events of HIV-1 replication. The results from siRNA down regulation and inactivation by ICRF-193 showed its indispensable role in HIV-1 replication. This was further supported by the overexpression of TopoIIβ which has lead to an enhanced viral titer. TopoIIβ being an integral part of transcriptional regulatory complex and HIV-1 infection, we investigated its direct role in the transcription of HIV-1. In this process we identified the association of Tat with TopoIIβ, PARP-1 and Ku70, which were the same interacting partners observed by Ju et al (2006) during transcriptional regulation of few genes. This was proven by pull down assay with His tagged Tat where all the three protein were found to interact with Tat. Further confirmation was done by the co-immunoprecipitation of the above proteins with Tat during viral infection. The results of ChIP assay have shown that all the three proteins TopoIIβ, PARP-1 and Ku70 were recruited to LTR. Since TopoIIβ has cleavage sites in 5'-LTR and the double strand break created by it in this complex is necessary for the transcription of certain genes, we expected a similar kind of mechanism in this process. For this we have treated cells with merbarone which only inhibits the double strand breaking ability of TopoIIβ but not its protein levels. This showed a decrease in viral titer establishing the role of TopoIIβ cleaved sites in HIV life cycle. Since this might have an impact on transcription we checked for viral mRNA production after siRNA and merbarone treatments which showed a drastic decrease in viral mRNA levels. These results were also confirmed by luciferase reporter assay using TZM-bl cell line. We now propose that this double strand break created by TopoIIβ at LTR might be necessary for the release of torsional stress to facilitate the elongation process executedbypolymeraseIIβ.

In the eukaryotic chromosomes, nucleosomes represent the first level of packing in their higher order structure (Eberharter et al., 2005), the unfolding of which, is a prerequisite to any transcription initiation and this mechanism is executed by chromatin-remodeling complexes that modify histone–DNA interactions (Van Lint. Et al.,1994,1996). Similarly in the case of HIV-1, the provirus gets organized in three specific nucleosomes: nuc-0, -1 and -2 of host genome, independent of the site of integration (Pumfery et al., 2003). Nuc-1 is positioned immediately downstream of the transcription start site and it is in particular that prevents the assembly of transcription complex and gets rapidly disrupted upon activation of the HIV-1 promoter (Van Lint et al., 1996; Verdin et al.,1993; Schröder et al.,,2002 Lewinski et al.,,2005; Jordan et al., 2001) The earlier reported nucleosome specific remodeling of TopoIIβ in which it acts as functional component is due to the cleavage activity it exerts by inducing a double strand break that will resolve the topological barrier finally leading to a structural change in the promoter. By the analysis of earlier reports and our own observations we hypothesise that this topological stress is relieved by Tat mediated recruitment of TopoIIβ, PARP-1, Ku70 to the 5'LTR region facilitating the polymerase II elongation thereby transactivating HIV-1 transcription. Further clarification about the role of TopoIIβ in these chromatin modifying complexes will come by identifying the interaction of TopoIIβ with all probable players in the both remodeling complexes during HIV-1 transactivation which is under study. This in future will cast a light in elucidating the mechanism of the Tat dependent transactivation that probably gives an idea about the role of TopoIIβ in the establishment of viral latency.

Most of the anti HIV compounds currently in use are aimed at viral encoded proteins which have their own limitations (Arora, 2009). The emergence of drug resistant viral strains pose a challenge to the drugs targeting viral encoded proteins, as they mutate to gain resistance to such therapies. The other way to combat HIV is to target host cellular factors involved in regulation of HIV replication. In this case, it will be beneficial to target host cellular factors that are usually expendable for normal cellular functions. Since TopoIIβ is one such protein where its down regulation does not result in cell death, its inhibition will pose minimal off-target effects. More importantly, combinatorial therapy like HAART, which is used to target at various stages of HIV life cycle adds burden to cells leading to toxic side effects. In addition the multiple roles exerted by TopoIIβ in various processes in HIV-1 lifecycle including proviral DNA synthesis, transcription and a suspected role in integration, will place TopoIIβ as an ideal anti-HIV target.

Conclusions

Topoisomerase IIβ, an isoform of type II topoisomerases, involved in various tissue specific cellular processes was found to be functional in various viral infections. Its plausible role in HIV life cycle was also suggested earlier, but not clearly established. In the present study, we have investigated the role of TopoIIβ in HIV-1 infection by its gain and loss of function. Overexpression of TopoIIβ lead to an increase in viral replication, resulting in enhanced virion production.HIV-1 replication was impaired when TopoIIβ was down regulated by siRNA and inhibited by ICRF-193 and merbarone. In recent times, there is an increase in the repertoire of host proteins that are recruited to the HIV-1 LTR, leading to an efficient transcription of viral genome. The role of topoisomerase IIβ in HIV-1 transcription was shown through its interaction with Tat and recruitment to LTR region through co-immunoprecipitation and ChIP assay respectively. Involvement of TopoIIβ in transactivation of HIV-1 LTR was shown through luciferase assay in reporter cell line, TZM-bl. It was also observed that LTR transactivation commensurated with the expression of topoisomerase IIβ in both Tat dependent and independent manner. Additionally, the significance of TopoIIβ induced double strand break was shown through a decrease in viral gene expression when its catalytic activity was inhibited by merbarone. These observations suggest that topoisomerase IIβ is involved in the cascade of coactivator complexes that are recruited at the LTR for regulation of HIV-1 transcription.

Conclusions

The work incorporated in the thesis is derived from the observations of unique biochemical functions of TopoIIβ during different kinds of stress reponses in various cellular processes. The original impetus for studying TopoIIβ came in part from the mysterious and complicated reactions the enzyme carries out. Despite of its isoform IIα, IIβ isoform is expressed in all cell types, with elevated expression found in terminally differentiated cells such as neurons (Capranico et al.,1992). Genetic studies employing mouse models have revealed that TopoIIβ null mutants exhibit a perinatal death phenotype showing multiple defects during neuronal development. Association of TopoIIβ in neuronal DNA repair has been established by the decrease in repair function in TopoIIβ downregulated CGNs upon peroxide mediated DNA damage (Mandraju et al., 2011). Decrease in expression of TopoIIβ in ageing invitro and invivo. (Kondapi.et.al, 2006;Bhanu.,2011). Moreover the invovlement of TopoII isoforms in HIV-1 strand transfer events is also observed (Lokeswara, 2013). These observations led to a conclusion that TopoIIβ might be playing crucial and diverse biological functions during cellular stress response such as oxidative DNA damage, HIV infection and Ageing.

The overall conclusions from the study focusses on biological functions of TopoIIβ during stress response. The expression of TopoIIβ is induced during peroxide mediated DNA damage along with other NHEJ proteins Ku70 and PARP-1 and its recruitement to the sites of DSB suggests its role in NHEJ path way in CGNs. TopoIIβ is shown to be associated with repair proteins such as Ku70 and PARP-1 and further post translational modifications such as phosphorylation and poly ADP ribosylation are important for its function during oxidative DNA damage in neurons.

Involvement of TopoIIβ in HIV infection is studied by its down regulation and inhibition of catalytic activity which inhibited HIV-1 infection. Further studies on transcriptional activity of TopoIIβ at sites of HIV-1 LTR was done by proving its recruitement to LTR regions and interacting with HIV-1 transcriptional activator Tat.HIV-1 transcription was inhibited upon down regulation or inhibition of TopoIIβ which was studied using LTR reporter constructs in different cell lines.

Senescence associated functions of TopoIIβ had been studied by understanding the importance of senecsnece associated genes in invitro ageing of CGNs and the role of TopoIIβ in regulation of their expression.

The overall work incorporated in the thesis strongly points out the important non-replicative functions of TopoIIβ in diverse cellular process.

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Topoisomerase IIb associates with Ku70 and PARP-1 during double strand break repair of DNA in neurons

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abstract

In the present study, the activity of Topoisomerase IIb (TopoIIb) is evaluated during peroxide induced double stranded DNA breaks (DSBs) repair in primary neurons. The results showed that the TopoIIb levels were enhanced during recovery from peroxide mediated damage (PED) along with Ku70, PARP-1, pol beta, and WRN helicase. Furthermore, siRNA mediated knock-down of TopoIIb in primary neurons conferred enhanced susceptibility to PED in neurons. DSBs in neurons are repaired through two pathways, one promoted by Ku70, while the other is by PARP-1 dependent manner. Participation of TopoIIb in both pathways was assessed by analysis of the interaction of TopoIIb with Ku70 and PARP-1 using co-immunoprecipitation experiments in extracts of neurons under peroxide treatment and recovery. The results of these studies showed a strong interaction of TopoIIb with Ku70 as well as PARP-1 suggesting that TopoIIb is associated both in Ku70 and PARP-dependent pathways in DSBs repair in primary neurons. The study has thus established that TopoIIb is an essential component in DSBs repair in primary neurons in both Ku70 and PARP-1 dependent pathways. We suppose that the interaction of TopoIIb may provide stabilization of the repair complex, which may assist in maintenance of tensional integrity in genomic DNA.

Introduction

Neurons posses a high metabolic activity and are especially vulnerable to damage when allowed to continuous exposure of endogenous reactive oxygen species [1]. Oxidative stress mediated accumulation of DNA damage in CNS has been mechanistically implicated in etiology and pathogenesis of many neurodegenerative diseases [2]. Double strand DNA breaks (DSBs)[2] are one of the most common type of oxidative DNA damages which become deleterious and act as potent inducers of apoptosis as well as chromosomal aberrations when left unrepaired [3,4]. DNA DSBs are known to trigger a cascade of signaling processes that lead to repair and resolution of the DNA intermediates.

Basic biochemical pathways for repair of DSBs involve either nonhomologous end joining (NHEJ) or homologous recombination repair HRR [5]. These two major pathways that repair the DNA and maintain its integrity are triggered by endogenous DSBs or those arising from an external agent such as ionizing radiation (IR). NHEJ pathway helps in repairing DNA breaks without the need for homology between the segments to be rejoined. In terminally differentiated cells like neurons, the NHEJ is the sole repair system [6], and this repair process appears to be particularly important in developing nervous system. Studies in vivo show that, lack of Lig4, XRCC4, Ku70 or Ku80, which are components of NHEJ pathway in the knockout mice results in dramatic apoptosis of many types of embryonic post mitotic neurons [7].

NHEJ involves Ku heterodimers Ku70 and Ku86 [8], DNA-PKcs [9], DNA ligase-IV (LIG4) [10], XRCC4 [11] and Artemis [12]. One of the proteins that get localized very early to DNA ends at a DSB, is the Ku heterodimer [13]. The Ku-DNA complex recruits DNA-PKs to DNA ends and the interaction between the two leads to formation of a complex that probably serves to hold the DNA ends in close proximity to one another [14]. The final step in NHEJ process is ligation of the two DNA ends, which is carried out by the LIG4-XRCC4 complex. Additional, but yet unidentified proteins may be required for NHEJ activity, since purified NHEJ proteins are known to fail to recapitulate DNA end joining [15].

Eukaryotic Topoisomerase II (Topo II) is present in two isoforms 170 KDa a and 180 KDa b. TopoIIa is present is proliferating tissues, while TopoIIb is present in all tissues, but predominantly

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[2] Abbreviations used: DSBs, Double strand DNA breaks; NHEJ, Nonhomologous end joining; HRR, Homologous recombination repair; IR, Ionizing radiation; LIG4, DNA ligase-IV; Topo II, Eukaryotic Topoisomerase II; DMEM, Delbucco's modified eagle medium; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; H_2O_2, Hydrogen peroxide; PED, peroxide mediated damage.

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present in terminally differentiated neurons implicating TopoIIb in non-replicating functions of neurons [16,17]. It is indeed TopoIIb deficient neuroblastoma cells are sensitive against hydrogen peroxide mediated insult suggesting the possible role of TopoIIb in DNA repair [21]. TopoIIb also has been shown to be involved in dsDNA break mediated activation of the gene transcription in cells by formation of multimeric complex containing TopoIIb, PARP-1, DNA-PKcs, and also through recruitment of another complex comprising Ku86 and Ku70 during transcriptional activation [18,19]. Enzymatic activity of TopoIIb that alters the topology of the DNA [16] might be a critical component required for the double strand break repair mechanisms in mammalian cells. The TopoIIb containing repair protein complex may function as a component of the genome-wide DNA-damage surveillance machinery and also plays a significant role in facilitating dynamic localized changes in chromatin organization during DSB repair in neuronal cells [19].

As topological changes mediated by TopoIIb are critical for vital processes in DNA metabolism like transcription [19] and replication [20], in the present study we have investigated its role in DSB repair process, which is one of the important repair mechanisms in cells. The increased activity and protein levels of TopoIIb during the senescence and oxidative stress in a neuroblastoma cell line suggest its importance during oxidative damage [21]. The present investigation analyses the repair activity of TopoIIb in terminally differentiated primary granule neurons in peroxide induced DSBs. The results show a strong association of TopoIIb with peroxide induced DSBs repair in primary neurons in both Ku70 and PARP-1dependent pathways.

Materials and methods

Mouse anti-human monoclonal antibodies of TopoIIa, TopoIIb, PARP-1, Ku70, Werner helicase and b-actin antibodies used were from M/s BD Biosciences, Singapore. The rabbit anti-DNA polymerase beta polyclonal antibody was from M/s Santa Cruz, USA while the Goat anti-mouse-HRP conjugated and goat anti-rabbit-HRP conjugated secondary antibodies were from UPSTATE. All the other chemicals and reagents were of molecular biology grade.

Cell lines: SK-N-SH neuroblastoma were obtained from NCCS, Pune India. Cell lines were maintained in DMEM-F12 with 10% FBS.

Primary culture

Cerebellar granule neurons

Cerebellar granule neurons were prepared from the cerebellum of P8 Wistar rats and processed for culture as described in [22,23]. The tissue was treated briefly with trypsin and DNase I and the dispersed cells were cultured at 37 C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 25 mM KCl, penicillin and streptomycin. The cell suspension from one cerebellum was seeded into 6 plastic culture dishes (60 mm in diameter) precoated with poly-L-lysine. After 24 h in culture, proliferation of non-neuronal cells was prevented by replacing the medium by another containing 10 mM cytosine arabinoside. The culture was replenished with fresh media at the end of day 3.

Isolation and culture of astrocytes from young rat cerebellum

The rat pups (6 days) were killed by cervical dislocation and the heads were removed then scrubbed with 70% alcohol and taken cerebellum out carefully. The white tissue and blood vessels were removed. Then tissue was placed in a 100 mm petri dish containing 5 ml of trypsin solution (EBSS/DNase/Trypsin). Chopped the tissue with scalpel blade and added 5 ml of trypsin solution. The suspension was transferred into 15 ml conical tube and incubated at 37 C with gentle agitation/15 min. An equal volume of D/L-valine DMEM was added to terminate trypsinization and spun at 250 g. The supernatant was discarded and the pellet was triturated about 20 times in 5 ml EBSS/DNase-I solution. Ten millilitre of EBS/DNase-I solution was added and allowed to settle for 10 min. Ten millilitre of supernatant was removed into a 50 ml tube carefully then 2 ml of EBS/DNase-I was added to the tissue and triturated again along with 10 ml of EBS/DNase-I solution. The supernatant was removed after 10 min and added to first and is repeated one more time then spun at 250 g/5 min. The pellet was resuspended in 10 ml of DMEM and cells were counted.

Seeding density

1 10^4 cells/T-75 flask, each flask contained 5 ml medium (DMEM Delbucco's modified eagle medium) with 10% FCS and 50 U/ml penicillin–streptomycin, cultures were incubated at 37 C and 5% CO_2 in humidified incubator.

siRNA synthesis

Double strand siRNA oligos were synthesized as described by Donze and Picard [21,24]. Desalted DNA oligonucleotides were obtained from Sigma (India). The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase has been carried out as described in [25] for each transcription reaction, 1 nM of each oligonucleotide was annealed in 50 l of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) by heating at 95 C; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 l of transcription mix: 1 T7 transcription buffer (40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl and 2 mM spermidine) 1 mM NTPs, 0.1 U yeast pyrophosphatase (Sigma), 40 U RnaseOUT (Life Technologies) and 100 U T7 RNA polymerase (Invitrogen) containing 200 pM of the dsDNA as template. After incubation at 37 C for 2 h, 1 U RNase-free DNase (Genetix) was added at 37 C for 15 min. Sense and antisense 21-nt RNAs generated in separate reactions were annealed by mixing both crude transcription reactions, heating for 5 min at 95 C and then for 1 h at 37 C to obtain 'T7 RNA polymerase synthesized small interfering double-stranded RNA' (T7 siRNA). The mixture (100 l) was then adjusted to 0.2 M sodium acetate, pH 5.2, and precipitated with 2.5 volume of ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried and resuspended in 50 l of water.

siRNA transfection

Granule neurons (1 10^6 cells) were transfected by using Lipofectamine-2000 (Invitrogen) according to the manufacturer with 0.5 M of scrambled and TopoIIb siRNA separately. The analysis was performed at 48 h post transfection.

Western blot analysis

Cells were harvested by scraping in 25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, and centrifuged at 300g for 7 min at 4 C as previously described by Angley et al. [45]. The cell pellet was homogenized in a 0.2 ml homogenization buffer (25 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 g/ml leupeptin, 0.1% aprotinin, 1 mM iodoacetamide, 200 g/ml bacitracin and 20 g/ml soybean trypsin inhibitor) for 10 min on ice followed by sonication for 15–20 s. The protein concentrations in cell lysates were measured using the Bradford method. Twenty micrograms of total protein/lane was separated on 7.5%

sodium dodecyl sulfate (SDS) gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 for 1 h and then incubated overnight at 4 C with corresponding protein specific antibodies. After washing and incubating for 1 h at 22 C with a secondary antibody conjugated with horse radish peroxidase, the membranes were washed and developed by chemiluminescent substrate (Pierce Western Blot Chemiluminescence Reagent). Relative levels of protein in different lanes were compared by analyzing scanned images using the NIH IMAGE program. All studies were performed a minimum of three times using independent cultures.

Neutral comet assay

Cells were transfected with ds-siRNA oligos, incubated for 48 h and treated with increasing concentrations of H_2O_2 for 24 h followed by 48 h recovery. Subsequently, 5000 cells were placed in a micro-centrifuge tube containing 1 ml cold PBS, and the neutral comet assay was done as described in [26]. Briefly, cells were centrifuged and resuspended in 500 ml cold PBS, and 1.5 ml of 1% agarose was added to each sample. The agarose-cell suspension was gently layered on a frosted-glass microscope slide, allowed to solidify for 5 min, and were then placed immediately in ice-cold lysis buffer containing 30 mM disodium ethylenediamine tetraacetic acid (EDTA, pH 8.0), 0.5% sodium dodecyl sulfate (SDS) and 0.25 mg/ml proteinase K (Sigma). The samples were lysed for 1 h at 4 C and then kept at 37 C for 12–16 h. After cell lysis and protein–DNA complexes were digested with proteinase K, the agar slides were re-equilibrated in TBE (90 mM Tris–HCl, 90 mM boric acid and 2 mM EDTA, pH 8.0) for 2 h, with a change of buffer every 15 min. The samples were electrophoresed with TBE buffer for 20 min at 25 V. The DNA was then stained with 20 lg/ml of ethidium bromide (Sigma) for 20 min and slides were washed twice for 5 min in TBE. To ensure random sampling, 50 images/slide were captured and in some experiments, the observer was blinded to the conditions. The images were captured on a Confocal microscope (Lieca) and quantified by using Comet-IV software (Perceptive Instruments, UK). The comet moment was calculated by using the equation described in [27]: comet moment R0 n ((intensity of DNA at distance X) (distance))/intensity of total DNA). The mean comet-moment value obtained from control samples was subtracted from the mean comet-moment value for each H_2O_2 dosage. Data shown are the means and SD values from three independent experiments (50 images for each dose of each independent experiment).

Preparation of nuclear extracts

Extracts from TopoIIb down regulated and non-down regulated cells were prepared as described previously [28,29]. Briefly, cells were homogenized in four volumes of homogenization buffer A (10 mM HEPES, pH 7.9, 0.5 mM dithiothreitol, 10 mM KCl, 1.5 mM $MgCl_2$, and protease inhibitor cocktail) using 10 strokes with a Dounce homogenizer (B-type pestle) on ice. The final concentration of protease inhibitors in the buffers was 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 0.05 mg/ml leupeptin, 0.025 mg/ml pepstatin A, and 0.05 mg/ml aprotinin. Homogenates were centrifuged at 5000g for 15 min at 4 C. The supernatents were considered as the cytosolic fraction. Pellets were resuspended in an equal volume of high salt buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1 M NaCl, 0.2mMEDTA, 0.5 mM dithiothreitol, and protease inhibitor cocktail), extracted for 30 min at 4 C with continuous gentle mixing, and centrifuged at 70,000g for 30 min at 4 C. This supernatant constitutes the nuclear protein extract. The nuclear fractions were dialyzed for 6 h at 4 C against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF, centrifuged at 16,000g for 30 min at 4 C, aliquoted and frozen at 70 C. Protein concentrations were determined by the Bradford method.

DNA end joining assay

Large-scale isolation of pUC19 plasmid DNA was performed using standard protocols. For creating a specific double strand break, plasmid DNA was subjected to restriction digestion, generating cohesive (EcoR1) ends. Linear pUC 19 obtained by EcoR1 digestion was labeled with a-^{32}P dCTP using RediprimeTM II kit (Amersham Pharmacia biotech). The labeled DNA was used as a probe in Southern detection of end joined products.

A standard end joining assay was performed in NHEJ assay buffer (45 mM HEPES–KOH buffer pH 7.9, 50 mM KCl, 7.4 mM $MgCl_2$, 0.4 mM EDTA, 0.9 mM DTT, 2 mM ATP, 20 mM dNTPs, 3.4% glycerol, 18 lg BSA), 400 ng of substrate DNA (pUC 19 plasmid DNA digested with indicated restriction enzyme) and 40 ng of nuclear extract. The reaction mixture was incubated for 2 h at 25 C. DNA products were purified by phenol/chloroform extraction and ethanol precipitation. Products were loaded on 0.8% Agarose gel and electrophoresed in 0.5 TBE buffer at 60 volts for 5 h. The gel was stained with EtBr and documented. For Southern analysis, the gel was depurinated and blotted onto a nylon membrane (Hybond N+, Amersham) in a denaturating buffer (alkaline transfer) and the membrane hybridized with the ^{32}P-labeled plasmid probe prepared as described above. The end joined products were visualized as dimers and multimers on the autoradiogram.

Treatment with H_2O_2

In the treatment phase, neurons were incubated in the presence of 0.25 mM, 0.5 mM and 1 mM hydrogen peroxide for 24 h. After this the cells were washed twice and added with fresh media to incubate the cells for 24 h (R-24), 48 h (R-48) and 72 h (R-72) post treatment. In this recovery phase the cells were allowed to recover from the insult.

Immunofluorescence

Granule neurons were cultured on poly-L-lysine coated cover slips. After treating the cells with H_2O_2 according to the above mentioned protocol, they were fixed with 4% paraformaldehyde and probed with polyclonal anti-TopoIIb and monoclonal anti-PARP-1 antibodies at 4 C overnight and incubated with anti–rabbit Cy5 and anti-mouse Cy3 secondary antibodies. DAPI which stains the nucleus was used as a counter stain. Pictures were captured under confocal microscopy (Leica).

Coimmunoprecipitation

2 10^6 cells were seeded per well and treated with H_2O_2 according to the above mentioned protocol. After this, whole cell extract (100 lg) was incubated with TopoIIb monoclonal antibody for 2 h at 4 C and protein A/G-agarose beads were added to the mixture and incubated for 1 h at 4 C. The entire reaction mixture was gently washed three times with PBS with 0.1% tween20 to remove the unbound proteins and the beads were boiled with the loading dye. The immunoprecipated proteins resolved on a SDS–PAGE. Western blotting was done and membranes were probed with monoclonal antibodies directed against PARP-1, Ku-70 and developed with chemiluminiscent reagent (Pierce Biotechnologies).

Results

Homogeneity of cell cultures

The post-mitotic granule neurons and astrocytes were cultured to homogeneity (Fig. 1A) and were ascertained for purity using cell-specific proteins GFAP (astrocytes) and NSE (granule neurons) (Fig. 1B). The cellular protein levels of TopoII and in granule neurons and astrocytes along with the neuroblastoma cell line (SK-N-SH) were analyzed using Western blotting. While the SK-N-SH showed the presence of both the TopoII isoforms, the granule neurons were found devoid of TopIIa and the astrocytes showed only a minimal level of TopoIIb (Fig. 1C).

Upregulation of TopoIIb after DNA damage

Free radicals produced during cellular metabolism are the major source of DNA damage and such conditions are induced by using hydrogen peroxide (H_2O_2), a potent free-radical generator. Viability of granule neurons has been monitored at 0.25, 0.5 and 1.0 mM hydrogen peroxide treatment, the results in Fig. 2A show that about 15% toxicity is observed in control cells at 0.25 and 0.5 mM hydrogen peroxide, which is increased by twice to <30% in presence of 1 mM hydrogen peroxide. Furthermore, evaluation of sensitivity of TopoIIb down regulated cells show that these cells are highly sensitive to hydrogen peroxide, wherein 20% died at 0.25 mM, which increased to 45% at 0.5 mM and 55% at 1 mM hydrogen peroxide suggesting that the presence of TopoIIb is essential for neurons to protect form insults caused due to peroxide.

The intensity of double strand breaks formed was measured in terms of length of tail in comet assay. The treatment concentration showed a proportional effect on the damage induction as revealed from the observed tail length variations. While the cells recovered in the case of lower concentrations, the damage caused in the case of higher concentrations (1 mM) was found to be almost irreversible and irreparable during the period of recovery (Fig. 2B).

The neurons were treated with different concentrations (0.25 mM, 0.5 mM and 1.0 mM) of H_2O_2 for the induction of different degrees of damage. The levels of various DNA repair proteins along with TopoIIb are shown in Fig. 2A. The TopoIIb levels displayed a distinct increase along with Ku70, Polb and WRN helicase during recovery period (Fig. 2C and D). The presence of these proteins was found to be reduced to negligible levels during treatment. The levels of TopoIIa however remained negligible and hence may not possess a role in DNA repair processes. Higher damage and repair caused at 1 mM of hydrogen peroxide could also be due to gross decrease in overall cellular gene transcription as consequences of enhanced oxidative stress, as we see in the case beta actin expression in Fig. 2C.

Decreased repair efficacy of TopoIIb downregulated cells

The above results showed higher level of TopoIIb protein level in neurons during their recovery after the peroxide treatment. To gain more insights if such an elevation is implicated in DNA repair activity, the possible role of TopoIIb in DNA repair was assessed. This was carried out by analyzing the DNA repair activity in TopoIIb downregulated neurons using siRNA oligos, where we

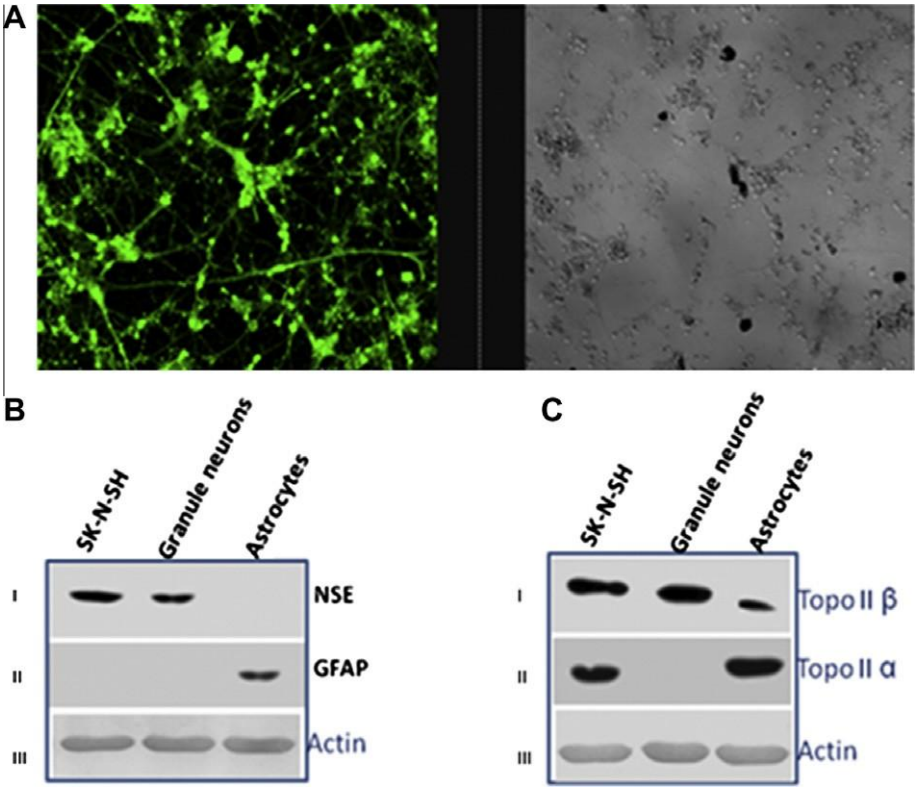

Fig. 1. Homogeneity of granule neurons and expression of TopoIIa and TopoIIb. 1A: Granule neurons were cultured on poly-L-lysine coated cover slips. After 4 days, cells were fixed with 4% paraformaldehyde and stained for NSE (neuronal specific enolase). Pictures were captured with a confocal microscope. 1B: Lysates from granule neurons were resolved by SDS–PAGE and proteins were transferred onto nitrocellulose membrane. Western Blotting was used to detect endogenous NSE 1B (I) and GFAP (marker for astrocytes) 1B (II) protein levels. 1C: TopoIIa (I) and TopoIIb (II) expressions in granule neurons were analyzed in lysates from 4 days post culturing using Western blot analysis ($n = 3$, $p < 0.05$). Data revealed that TopoIIa is absent in granule neurons whereas TopoIIb showed decreased expression in astrocytes compared to granule neurons. Lysates from SK-N-SH cells and astocytes were taken as controls to assess the purity of granule neurons. b-actin was taken as internal control 1B (III) and 1C (III).

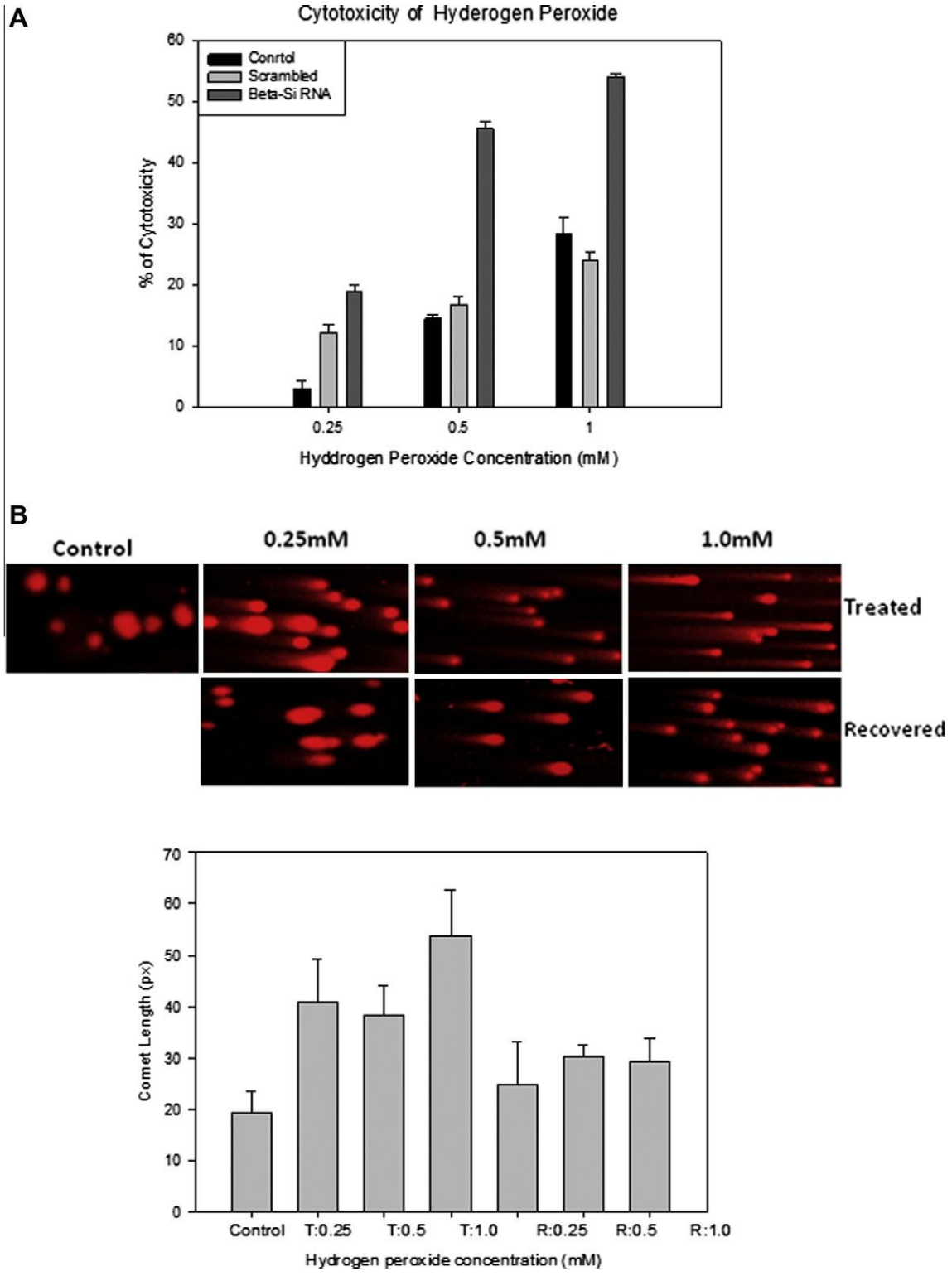

Fig. 2. The expression profile of TopoIIb and repair proteins during peroxide-mediated DNA damage and repair. 2A: Neuronal viability as assessed by MTT assay during treatment of neurons with hydrogen peroxide. 2B: Determination of DNA double strand breaks using a neutral comet assay. Granule neurons were treated with indicated concentrations of H_2O_2 (0.25 mM, 0.5 mM and 1 mM). Double strand breaks were assessed in treated (24 h) and recovered (72 h) cells using neutral comet assay (n = 3). Quantification of DNA strand breaks during peroxide treatment and recovery by neutral comet assay. 2C: Granule neurons cultured for 4 days were incubated in the presence of indicated concentrations of hydrogen peroxide (0.25 mM, 0.5 mM and 1 mM) for 24 h (Treated). After 24 h, the cells were washed with fresh medium and re-cultured in complete medium for 72 h (Recovered). After cell lysis, proteins in whole cell extract were analyzed by Western blot analysis and membranes were probed with antibodies directed against indicated proteins 2A (I–VI). The control lane (C) represents a lysate from cells without H_2O_2 treatment (n = 3). The upregulation of TopoIIb (2A. I) is clearly seen during the period of recovery along with some of the repair proteins which are known to be upregulated during the process of DSB repair (2A. II-VI). 2D: Topo IIb, Polb, Ku70 and WRN proteins on above western blot were quantified shown as bar diagram ($\surd p < 0.05$, $\surd\surd p < 0.001$, n = 3).

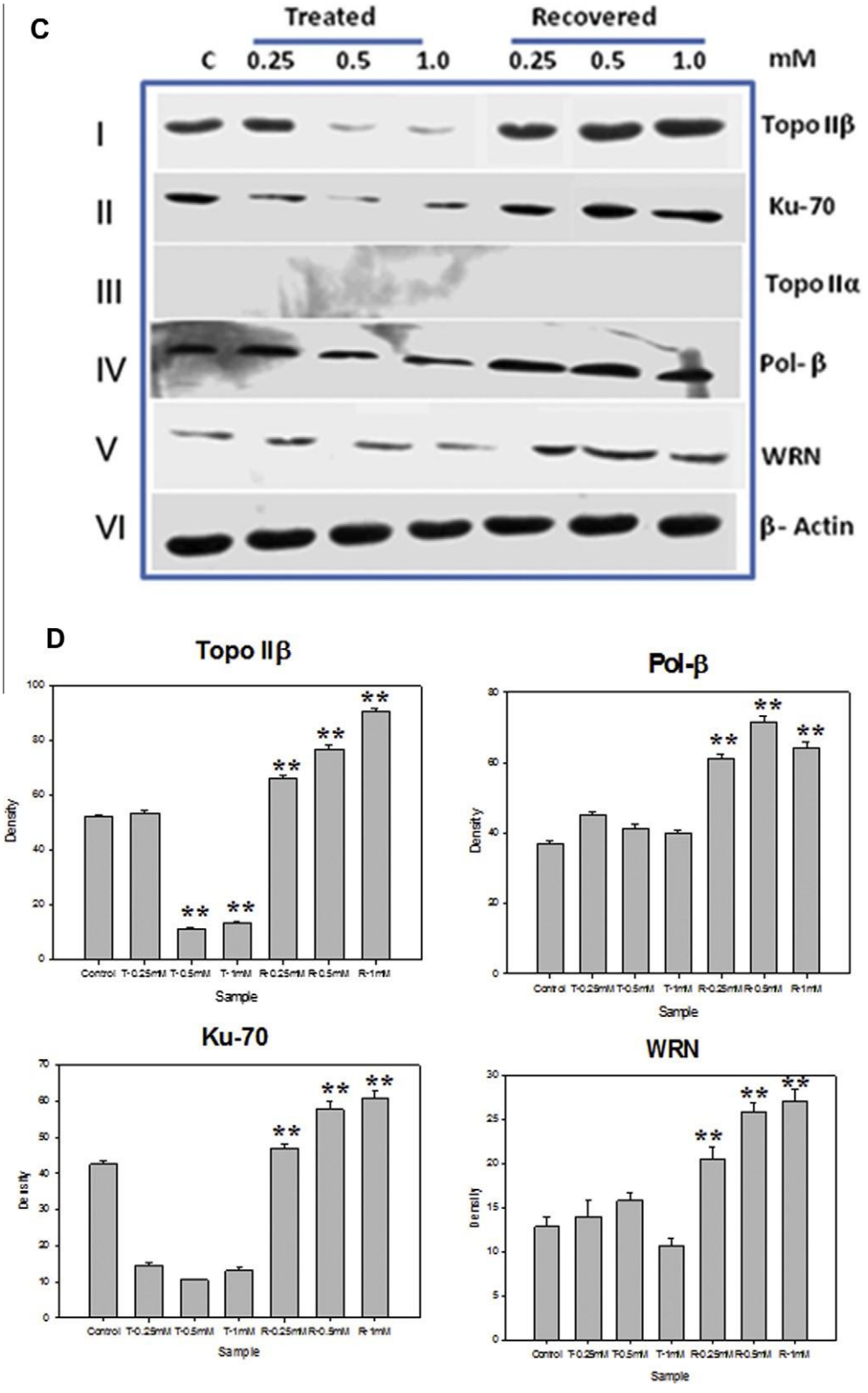

Fig. 2 (continued)

achieved approximately an 80% knockdown of TopoIIb expression (Fig 3).

The neuronal cells transfected with TopoIIb-siRNA scrambled sequence and TopoIIb-siRNA were treated with different concentrations of H_2O_2 as described earlier. The untreated neuronal cells were considered as control samples (Fig. 4A). The tail formation in terms of its length was taken as a direct measure of the DNA damage and was considered to be proportional to the extent of damage. The studies showed that the tail length was significantly longer in TopoIIb knockdown cells compared to scrambled siRNA-transfec-

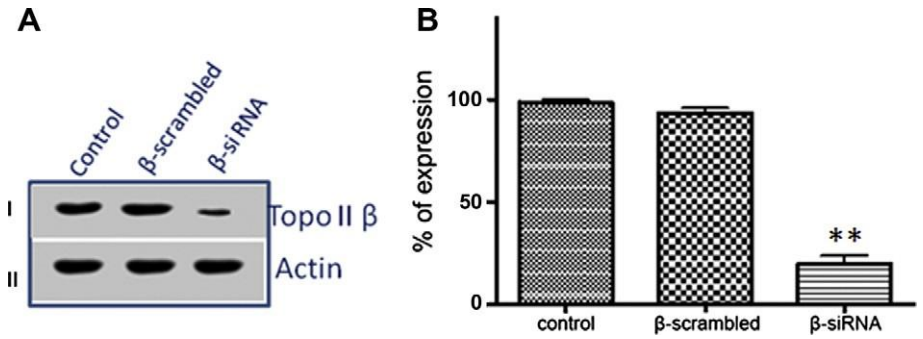

Fig. 3. siRNA-mediated downregulation of TopoIIb in neurons. 3A: TopoIIb-specific siRNA was synthesized by in vitro transcription and was transfected into the granule neurons. 48 h posttransfection the down-regulation of TopoII b was analyzed by Western blotting 3A (I), b-actin was used as internal control 3A (II) and scrambled b-siRNA was used as negative control. 3B: Quantification of the Western blotting experiments (n = 3) ($p < 0.05$, $p < 0.001$, n = 3).

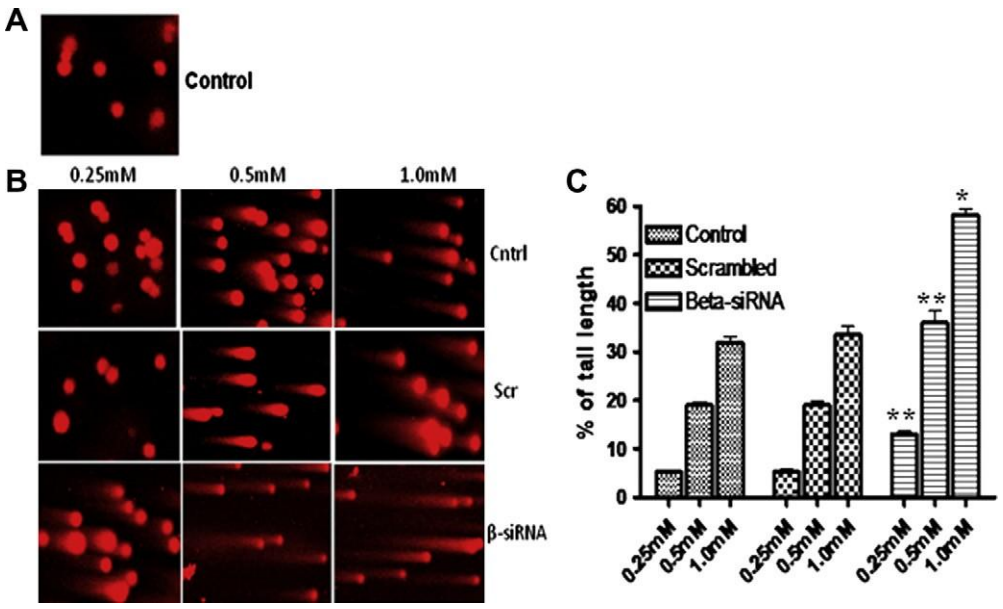

Fig. 4. TopoIIb downregulation drastically affect the double strand break repair. 4A: Untreated cells were used as control. 4B: The TopoIIb down regulated granule neurons were incubated with indicated concentrations of peroxide for 24 h followed by a 72 h recovery. The extent of DNA damage and recovery was analyzed by single cell neutral comet assay. Downregulation of TopoIIb significantly reduced the repair efficacy in neurons which can be seen in TopoIIb knockdown cells in terms of reduction in tail length during recovery compared to control cells. 4C: The percentage of tail length was measured by using Comet-IV software (Perceptive Instruments, UK) and the extent of DNA damage was plotted on Y-axis. Experiments were repeated three times and means ± SE was plotted ($p < 0.05$, $p < 0.001$, n = 3).

ted and control cells which suggest that the deficiency of TopoIIb has led to enhanced damage (Fig. 4B) indicating its importance in double strand break repair. The correlated levels of Ku70, PARP-1, WRN helicase and DNA polymerase beta provided evidence to further look into the NHEJ pathway of double-strand break repair. This was addressed through an in vitro assay and the results demonstrated that multimerisation of the DNA fragment (monomer) occurs in the presence of NHEJ proteins. Here, the formation of multimers was almost inhibited in the TopoIIb-downregulated cells (Fig. 5). In vitro studies on aging neurons show that the levels of TopoIIb decrease with a subsequent decrease in NHEJ activity suggesting the significance of TopoIIb in NHEJ activity in granule neurons [30].

TopoIIb association with Ku70 and PARP-1

An analysis of the TopoIIb-associated nucleoprotein repair complex using co-immunoprecipitation and colocalization experiments showed a strong association of TopoIIb with PARP-1 and Ku70 (Fig 6A and B). Immunofluorescence studies displayed that TopoIIb and PARP were colocalised during treatment and recovery periods in the nucleus (Fig. 7). These results demonstrated that TopoIIb associated with PARP and Ku70 and that this complex is involved in DSB repair through NHEJ pathway.

Discussion

The two isomers of TopoII, TopoIIa and TopoIIb exhibit quite contrasting characteristics in that the a isoform is ubiquitous in the nucleus of proliferative cells [31] and unlike the TopoIIb isoform exhibits a developmental role [32–34]. Our earlier results [21] showed the possible roles of the TopoII isoforms in DNA damage and repair in dividing cells. The results in the present study showed that the TopoIIa is present in the neuroblastoma cell line SK-N-SH and in dividing astrocytes but is totally absent in terminally differentiated granule neurons. Further, the results presented here established and defined the distinct role of TopoIIb in DNA repair of primary neurons through NHEJ pathway.

The presence of free radicals is the primary source of damage to DNA and constitutes a major cause for neuronal apoptosis and

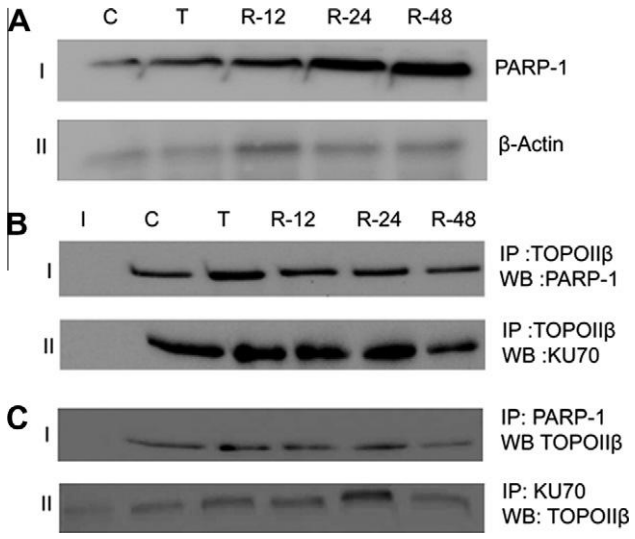

Fig. 6. Association of TopoIIb with Ku70 and PARP-1 during damage and recovery in granule neurons. Granule neurons were cultured for 4 days and incubated in the presence of 1 mM hydrogen peroxide for 12 h (Treatment: T). After 12 h the cells were washed with fresh medium and grown in a complete medium for 24 h (R-24) 48 h (R-48) and 72 h (R-72), respectively. 6A (I) Levels of PARP-1 during the treatment and recovery phases as compared with b-actin (internal control) 6A (II). 6B: whole cell extract from granule neurons was incubated separately with anti-TopoIIb monoclonal antibodies for overnight at 4 C. Protein A/G agarose beads were added to the mixture and incubated for 1 h at 4 C. The beads were boiled with the loading dye and immunoprecipitated proteins are resolved on a SDS–PAGE and Western blotting was done and probed with monoclonal antibodies directed against PARP-1 6B(I) and Ku-70 6B(II). 6C: Extracts were incubated with anti-PARP-1 6C(I) and Ku70 6C(II) monoclonal antibodies and membranes with the immunoprecipitates were probed with antibodies directed against TopoIIb. The control lane (C) represents an extract derived from cells without H_2O_2 treatment, Treatment line (T) is in the presence of H_2O_2 and Recovery lane (R) is cells after washing and re-culturing in fresh medium for recovery. I in panel B and panel C represent IgG control. The data clearly indicates the association of TopoIIb with PARP-1 and Ku70 during DNA damage and recovery.

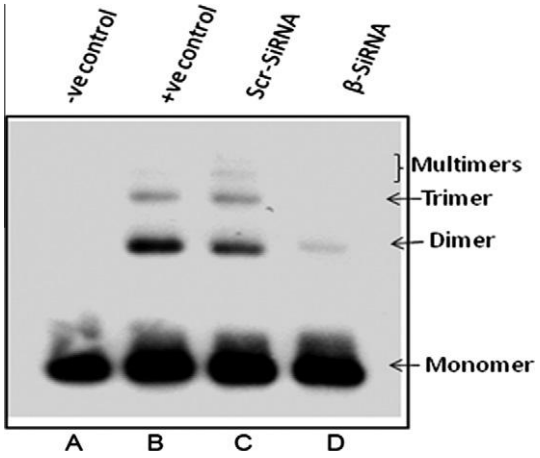

Fig. 5. Confirmation of TopoIIb activity in DNA double strand break repair through in vitro NHEJ assay. Nuclear extracts were prepared from TopoIIb siRNA-treated and wildtype granule neurons and were incubated with EcoR1-digested pUC19 plasmid. After the reaction the plasmid DNA was purified and subjected to southern hybridization and probed with ^{32}P-labeled pUC19 plasmid DNA. Denatured nuclear extract was used as negative control (n = 3) (lane A), untransfected nuclear extract was used as positive control (lane B); lane C and D are b-scrambled and TopoIIb siRNA transfected nuclear extracts, respectively, indicating the reduced end joining activity in TopoIIb downregulated nuclear extracts (lane D).

aging. The damage inflicted by free radicals in vivo is replicated in our in vitro experiments using H_2O_2. The damage was quantified and the extent of damage, as expected, showed an increase with concentration. The cells could recover themselves completely to normal condition in the case of lower peroxide concentration while the recovery was only partial in the case of higher concentrations.

There exist two known distinct mechanisms for repairing double strand breaks, HRR and NHEJ. Mammalian cells repair majority of double strand breaks by NHEJ although there is evidence for coupling with HRR [35]. This may be the reason for the mature neurons to undergo double strand break repair by both HR and NHEJ even though they do not divide [36].

The repair of DNA damage seen during recovery could happen only through NHEJ pathway because the neurons used in the present study are terminally differentiated (post-mitotic) in nature and HR occurs only in developing and differentiating neurons. Moreover, NHEJ is very important in promoting the development of nervous system as may be seen from the earlier studies [7] demonstrating that mice deficient in DNA ligase IV, XRCC4, Ku70 and Ku80 developed massive apoptosis in many types of post-mitotic neurons.

Gao et al. [37] reported the critical requirement of XRCC4 (X-ray repair cross-complementing protein 4) and ligase IV for survival of newly generated post mitotic neurons to DSBs as they occur at higher rates in early post mitotic neurons.

We hence considered the NHEJ-associated proteins like Ku70, WRN helicase & Polb along with both the TopoII isoforms in the present study. Results suggest that TopoIIb and all the three proteins show a similar pattern of expression suggesting their possible interaction. This interaction of TopoIIb with two critical proteins involved in DSB repair, Ku70 and PARP-1 were analyzed. The recovery of the DNA damage was not evident in TopoIIb down regulated cells compared to the control (untransfected and scrambled siRNA-transfected) neurons. This confirms the requirement of TopoIIb in the repair of DNA damage that occurred due to oxidative stress. This is also consistent with earlier studies [38] which showed endogenous oxidative stress leads to increased apoptosis in mice deficient in Ku70 [38] and Polb [39].

To further confirm the role of TopoIIb in NHEJ, in vitro NHEJ assay was conducted using digested pUC19 DNA as a substrate. The nuclear extract from control (scrambled siRNA) cells and those transfected with siRNA could facilitate multimerisation of the digested DNA but the extracts from TopoII-downregulated cells could not support NHEJ activity in vitro suggesting that TopoIIb plays a critical role in NHEJ reaction.

NHEJ pathway is mediated by two parallel pathways, one involving Ku70 and the other involving PARP-1. Although the levels of the proteins may per se indicate the presence of an NHEJ pathway they may not directly establish their possible association with TopoIIb. The results of co-immunoprecipitation experiments confirmed the direct association of TopoIIb with the repair proteins. The results of these experiments showed a strong association of TopoIIb with PARP-1 as well as Ku70 (Fig. 6). This clearly points to the involvement of TopoIIb both in Ku70 and PARP-1-dependent pathways and suggests furthermore that both the PARP-1 and Ku70-dependent NHEJ pathways are operational for recovery from H_2O_2-mediated-oxidative damage of primary neurons. In addition, PARP-1 is known to be involved in modification of several proteins like DNA polymerases, DNA ligase, TopoII and PARP-1 itself is a target of post translational modification [40]. TopoII along with Topoisomerase I, DNA polymerase alpha and delta and other proteins undergo poly (ADP) ribosylation modification indeed is shown to decrease the catalytic activity of TopoII [41–43]. PARP deficient cells have been shown to possess low levels of TopoIIb [44]. The reported lower activity of poly-(ADP) ribosylated TopoII [43] indicates that TopoIIb catalytic activity by itself may not be the only requirement for the repair activity; rather the stabilized TopoIIb in the presence of PARP-1 may play a more significant role in regulation of DNA repair activity or in modulating levels of various re-

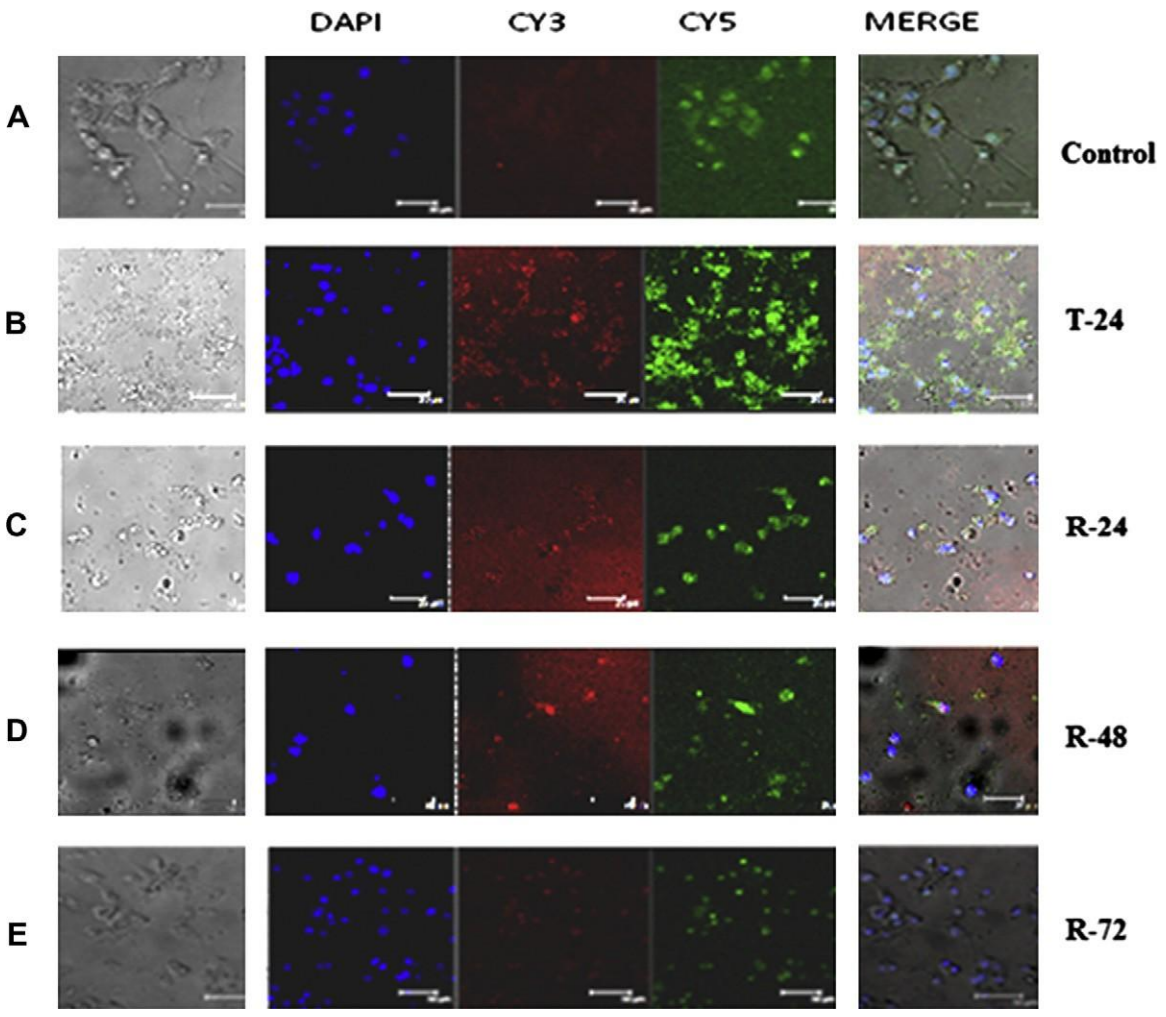

Fig. 7. Colocalization of TopoIIb and PARP-1: Granule neurons were cultured and fixed with paraformaldehyde to study the colocalisation of TopoIIb and PARP-1. A colocalization of TopoIIb (green-CY5) and PARP-1(red-CY3) was observed in the cell nucleus soon after treatment T-24(B) and during the recovery periods R-24, R-48 and R-72 (C–E). However, the colocalization of both proteins seems to be decreased after 3 days of post recovery i.e., at R-72. Untreated cells were used as control (A) and DAPI (blue) was used to counter stain the nucleus.

pair proteins. This is because both TopoIIb and PARP-1 have been shown to be critical components of the multi-step serial events required for regulated gene expression in response to many ligand or signal-dependent stimuli [18,19]. Henceforth, the association of TopoIIb with PARP-1 and Ku70 as observed in the present study implicates the role of PARP-1-TopoIIb and Ku70-TopoIIb complexes in the NHEJ pathway mediated repair process. Such an interaction of PARP-1 and Ku70 in parallel is suggesting the involvement of TopoIIb in both pathways. Alternatively, PARP-1 mediated poly ADP-(ribosylated) form of Topoisomerase II may be interacting with Ku70 in promoting NHEJ activity. Further studies are needed to understand the unique mechanism of Topoisomerase IIb involvement in regulation of such repair network.

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