CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

REGULATION OF TLX EXPRESSION BY p53 FAMILY MEMBERS

DIPLOMA THESIS

in cooperation with

GÖTEBORGS UNIVERISTET

SAHLGRENSKA ACADEMIN

Institutionen för Biomedicin

Supervisors: Prof. Keiko Funa

Erik Johansson, Ph.D.

Doc. PharmDr. Petr Pávek, Ph.D.

Göteborg 2012

Markéta Syslová

KARLOVA UNIVERZITA V PRAZE

Farmaceutická fakulta v Hradci Králové

Katedera farmakologie a toxikologie

REGULACE EXPRESE TLX PROTEINY RODINY p53

DIPLOMOVÁ PRÁCE

Ve spolupráci s

GÖTEBORGS UNIVERSITET

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Vedoucí diplomové práce: Prof. Keiko Funa

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„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu.”

I would like to thank Keiko Funa for accepting me as a foreign student in her group and for her support.

I specially thank Erik Johansson that showed and taught me all techniques essential for my work, for his useful and patient support.

My last thank belongs to all other postgraduate students and postdoctoral staff of the laboratory I worked in for their everyday contribution to my work, their friendly support and the positive atmosphere in the lab.

ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Pharmacology and Toxicology

Candidate:	Markéta Syslová
Supervisors:	Prof. Keiko Funa, Erik Johansson, Ph.D., Doc. PharmDr. Petr Pávek, Ph.D.
Title of diploma thesis:	Regulation of TLX expression by p53 family members

The p53 mutation is associated with poor therapeutic response and prognosis, being observed in almost 60% of human cancers. p53 is kept at low steady-state levels in the absence of cellular stress. In response to various stress, p53 becomes activated. It binds DNA in a sequence specific manner to activate the transcription of a number of genes mostly belonging to cell cycle inhibitors and apoptosis inducers. When p53 is mutated it cannot fulfil its function and regulate target genes. p73, analogue of p53, has two different isoforms with two different functions. In neuroblastoma, TAp73, as well as p53, is infrequently mutated but overexpression of DNp73 is connected with poor prognosis.

TLX (also called NR2E1) is an orphan nuclear receptor, a member of a highly conserved family in both vertebrates and invertebrates. TLX is an essential transcriptional regulator of maintenance and self-renewal of neural stem cells.

In this study I investigated if there is a functional link between p53 family members and TLX. In this thesis is showed that p53 binds the TLX promoter and regulates its activity in both cell lines we tested. These results suggest that TLX interacts with the p53 signalling pathway and is able to regulate the activity of postnatal neural stem cells.

ABSTRAKT

Karlova Universita v Praze
Farmaceutická fakulta v Hradci Králové
Katedra farmakologie a toxikologie

Kandidát:	Markéta Syslová
Vedoucí diplomové práce:	Prof. Keiko Funa, Erik Johasson, Ph.D., Doc. PharmDr. Petr Pávek, Ph.D.
Téma diplomové práce:	Regulace exprese TLX proteiny rodiny p53

Mutace proteinu p53 souvisí se špatnou terapeutickou odpovědí a prognózou. Tato mutace se vyskytuje téměř v 60 % lidských nádorů. V nepřítomnosti buněčného stresu je hladina p53 udržována na ustálené nízké hodnotě. V odpovědi na buněčný stres se p53 stává aktivním a váže se ke specifickým sekvencím DNA. Tím aktivuje transkripci řady genů, často patřích k inhibitorům buněčného cyklu a induktorům apoptózy. Pokud je však p53 mutovaný, nemůže plnit svou fuknci a regulovat tak cílové geny. p73, analog p53, má dvě odlišné izoformy s odlišnými funkcemi. Zatímco TAp73, stejně jako p53, se u pacientů s neuroblastomem nachází v mutované podobě velmi zřídka, mutace DNp73 je spojena se špatnou prognózou.

TLX (take nazývaný NR2E1) je sirotčí jaderný receptor. Je člen vysoce konzervované rodiny protein přítomných u obratlovců a bezobratlých. TLX je nezbytný transkripční regulátor zachování a sebeobnovy nervových kmenových buněk.

Tato studie zkoumala, zda je funkční spojení mezi členy rodiny protein p53 a TLX. Prokázali jsme, že p53 se váže na TLX promotorovou oblast a reguluje jeho aktivitu v obou testovaných buněčných liniích. Naše výsledky naznačují, že TLX ovlivňuje signální dráhy proteinu p53 a je schopen regulovat aktivitu postnatálních nervových kmenových buněk.

CONTENT

1. ABBREVIATIONS

AD1	acidic N-terminus transcription activation domain
AD2	activation domain
BD	binding domain
ChIP	chromatin immunoprecipitation
DBD	central binding core domain
DG	dental gyrus
DMEM	Dulbecco´s modified Eagle´s medium
F	phenylalanine
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H	histidine
HDAC	histone deacetylase
NB	neuroblastoma
NLS	nuclear localization signalling domain
NSCs	neural stem cells
OD	homo-oligomerization domain
PBS	phosphate buffered saline
PRD	proline rich domain
SAM	sterile alpha motif
SDS	sodium dodecyl sulfate
SVZ	subventricular zone
TAD	an acidic N-terminus transcription activation domain
TBS	tris buffered saline
TLX	tailless
wT	wild type

2. INTRODUCTION

2.1. Neuroblastoma

Neuroblastoma (NB) is a type of cancer that most often affects children. Neuroblastoma occurs when immature nerve cells called neuroblasts become abnormal and multiply uncontrollably to form a tumor (Internet 1). NB is a disease of the sympaticoadrenal lineage of the neural crest, and therefore tumors can develop anywhere in the sympathetic nervous system. Most primary tumors (65%) occur within the abdomen, with at least half of these arising in the adrenal medulla. Other common sites of disease include the neck, chest, and pelvis (Brodeur et al. 2006). Neuroblastoma can spread (metastasize) to other parts of the body such as the bones, liver, or skin (Internet 1). Neuroblastoma is the most common and deadly extracranial solid childhood malignancy, accounting for about 15% of all childhood tumor-related deaths (Brodeur et al. 2006). There are about 650 new cases of neuroblastoma every year in the United States. This number has remained about the same for many years. The incidence rate is approximately 7.6 cases per million per year for children aged 0-19 years, 9.5 per million per year for children aged 0-14 (Internet 2).

The average age at the time of diagnosis is about 1 to 2 years. In rare cases, neuroblastoma is detected by ultrasound even before birth. Nearly 90% of cases are diagnosed by age 5. Neuroblastoma is extremely rare in people older than 10 years (Internet 3).

2.2. TLX

TLX is a member of the *tailless* class of orphan nuclear receptors, a highly conserved family in both vertebrates and invertebrates, suggesting this family's importance during evolution. Nuclear receptors form a large family of transcription factors that are evolutionary conserved in species across the metazoans (Mangelsdorf et al. 1995) and have important roles in several biological processes, including cell proliferation, differentiation and cellular homeostasis (Evans 2005). The properties of many nuclear receptors are regulated by small hydrophobic molecules such as steroid hormone and by metabolites like oxysterols, bile and fatty acids. Others are called ‘orphan nuclear receptors’ because their corresponding ligands have not been identified (Gui et al 2011).

The orphan nuclear receptor TLX (also called NR2E1) is an essential transcriptional regulator of neural stem cell maintenance and self-renewal in the adult brain (Shi et al. 2004). Neural stem cells (NSCs) continuously produce new neurons in postnatal brains. Niu et al. have showed that nuclear receptor TLX controls the activation status of postnatal NSCs in mice (Niu et al. 2011). Self-renewal and differentiation are two fundamental properties of stem cells. Neural stem cells are a subset of undifferentiated precursors that retain the ability to proliferate and self-renewal, and have the capacity to give rise to both neuronal and glial lineages (McKay et al 1997, Alvarez-Buylla et al. 1998, Gage et al. 1998, Weiss & Kooy 1998). Stem cell self-renewal is regulated by the dynamic interplay between transcriptional factors, epigenetic control, microRNA (miRNA) regulators, and cell-intrinsic signals from the microenvironment in which stem cells reside (Shi et al. 2008; Qu & Shi 2009). Many transcriptional factors and cell-intrinsic regulators, including TLX, control self-renewal, differentiation and neural stem cell maintenance in both the adult and embryonic nervous system.

TLX is expressed in the periventricular neurogenic zone during mouse embryonic development. TLX expression in the mouse starts at embryonic day 8 (E8), peaks around E12.5, and then declines from E13.5 through neonate. The expression of TLX increases after birth, with high levels detected in the adult brain (Monaghan et al. 1995). TLX knockout mice are viable and appear normal at birth. However, mature TLX knockout mice have significantly reduced cerebral hemispheres and specific anatomical deficits in the cortex and the limbic system (Chiang & Evans 1997, Monaghan et al. 1995, 1997). TLX mutant mice also display severe retinopathies and exhibit increased aggressiveness and reduced learning abilities (reviewed by Gui et al. 2010 and Shi et al. 2008). Late-developing structures such as the upper cortical layers and the hippocampal dentate gyrus (DG) are reduced in size. These phenotypic changes indicate that TLX has an important role for brain development in the young postnatal stage (Liu et al. 2008) and plays a critical role in regulating the development of the visual and nervous system (Gui et al. 2010).

It has recently been shown that TLX is expressed exclusively in astrocyte-like B cells in the adult subventricular zone (SVZ), and recognized to be multipotent neural stem cells. Thus, the TLX promoter is a useful tool to introduce genetic modification specifically

into neural stem cells (NSCs). In fact, inactivation of the TLX gene in the adult SVZ lead to loss of the self-renewal ability of adult NSCs (Liu et al. 2008). The subventricular zone of the lateral ventricle and the subgranular zone (SGZ) of the DG are the largest germinal zones of sustained neurogenesis during adulthood in the mammalian central nervous system (Gage 2000, Alvarez-Buylla and Garcia-Verdugo 2002).

The function of TLX is largely thought to prevent precocious differentiation of NCSs into mature neurons of glial cells during development (reviewed by Niu et al. 2011). TLX is essential for NSC proliferation and neurogenesis in the post natal brain (Shi et al. 2004, Liu et al. 2008, Zhang et al. 2008). The fate of stem cells lacking TLX was not clear, but it was thought that they undergo spontaneous differentiation into mature astrocytes and thus deplete NSCs (Shi et al. 2004). In contrast, Niu et al. showed that deletion of TLX during embryogenesis does not lead to a depletion of cells that have characteristic of NSCs or result in spontaneous differentiation on NSCs into mature astrocytes at the time points examined (Niu et al. 2011). Their data indicate that a loss of TLX function first result in age-dependent decrease of active proliferation, followed by an exit of cell cycle indicated by a non-licensed state.

TLX genetically interacts with p53 signalling pathway in postnatal NSCs. This is supported by the significantly upregulated expression of p21/Cdkn1a, p53 induced cyclin-dependent kinase inhibitor in TLX-null stem cells, indicating a direct link between p21 expression and TLX function (Niu et al. 2011). It has also been shown that TLX directly binds to the promoter region of p21 (Sun et al. 2007). It is well established that the expression of p21 is under the direct control of the p53 signalling pathway in most cellular contexts (Niu et al. 2011). Deletion of p53 alone resulted in a small but significant increase of proliferating cells, which is consistent with a demonstrated role of p53 in adult NSCs (Meletis et al. 2006). Taken together, these data indicate that TLX genetically interacts with the p53 signalling pathway to tightly regulate the activity of postnatal NSCs (Niu et al. 2011).

Niu and collaborators showed for the first time that expressing cells generate both activated and nonproliferative postnatal NSCs and that TLX is required for NSC activation and positioning in the neurogenic niche. TLX genetically interacts with the

p53 pathway to control NSC activation (Fig. 1). It should also be noted in addition to p53 signalling that TLX controls the expression of a plethora of other genes that may play important roles in the regulation of NSC activation (Niu et al. 2011).

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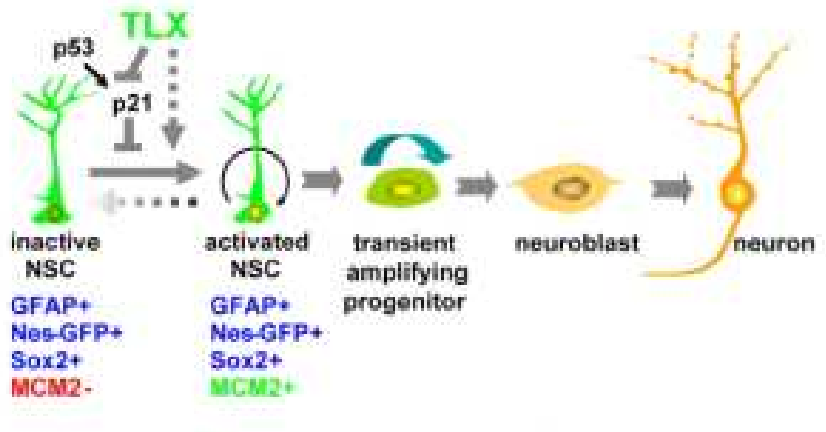


Fig.1 TLX-expressing cells generate both activated and inactivated postnatal NSC, which are identified by marker expression. TLX is required for inactive NSC to proliferate by modulating p21 expression in a p53-dependent manner. Besides p53 signalling, TLX also modulates many other signalling pathways, which may contribute to the regulation of NSC activation (adapted from Niu et al. 2011).

It was recently reported that TLX is overexpressed in various glioma cell lines and glioma stem cells, and that its expression in glioma patients is correlated with poor prognosis (Park et al. 2010). TLX is also expressed in the retina where it is a key factor in retinal development and essential for vision (Yu et al. 2000).

2.3. TLX structure

Being one of nuclear receptor, as pointed out, TLX contains several functional domains, which are characteristic for members of nuclear receptor family such as a DNA-binding domain (DBD) and a conserved ligand-binding domain (LBD). The human and mouse TLX are highly conserved (Fig 2) and are homologous with *Drosophila* tailless. TLX consensus DNA binding sequence is 5´-AAGTCA-3´.

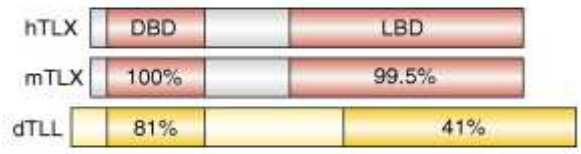


Fig. 2 Structure-function domains and sequence homology of human (h), mouse (m) TLX and (dTLL). (adapted from Shi 2007).

Nuclear receptors are ligand-dependent transcription factors that regulate the expression of genes critical for a variety of biological processes. Nuclear receptors carry out transcriptional functions through the recruitment of positive and negative regulatory proteins, referred to as coactivators and corepressors. One mechanism underlying the repression activity of nuclear receptors is through the recruitment of histone deacetylase (HDAC) complexes (Sun et al. 2007). Sun et al. also showed that TLX interacts with a set of HDACs in neural stem cells. TLX recruits these HDACs to its target genes to repress their expression.

2.4. p53 family members

The p53 tumor-suppressor plays a critical role in the prevention of human cancer. In the absence of cellular stress, the p53 protein is maintained at low steady-state levels and exerts very little, if any, effect on cell fate. However, in response to various types of stress, p53 becomes activated; this is reflected in elevated protein levels, as well as augmented biochemical capabilities. As a consequence of p53 activation, cells can undergo marked phenotypic changes, ranging from increased DNA repair to senescence and apoptosis (Oren 2003). The p53 gene is located on the short arm of chromosome 17 (reviewed Levine et al. 1991).

The p53 protein has a biological function as a G_1 and G_2 checkpoint control for DNA damage (Lane 1992) and also regulates the expression of a large number of target genes (Vogelstain et al 2000).

2. 4. 1 Structure

The p53 protein is composed of 393 residues and contains several structural domains (see Fig. 3).

An acidic N-terminus transcription-activation domain (TAD, AD1) has 42 amino acids and interacts with the basal transcriptional machinery in positively regulating gene

expression. Amino acids 13-23 in the p53 protein are identical in a number of diverse species. The p53 amino acids F19, L22, and W23 have been shown to be required for transcriptional activation by the protein *in vivo* (Lin et al. 1995). It has been shown that p53 uses a hydrophobic interface in its N-terminal domain to interact with the transcriptional machinery of the cell and its negative regulators (Kussie et al. 1996).

Activation domain 2 (AD2) was identified and characterized for p53-dependent apoptosis.

Proline rich domain (PRD) of human p53 is required for induction of apoptosis, transcriptional repression, reactive oxygen species (ROS) production and transactivation. A p53 mutant lacking the proline-rich domain cannot repress a series of promoters efficiently compared with wTp53 (Venot et al. 1998).

Central DNA-binding core domain (DBD) is localized between amino acid residues 102 and 292. It is a protease-resistant and independently folded domain containing Zn^{2+} ion that is required for its sequence specific DNA-binding activity. This domain folds into a four-stranded and five-stranded anti-parallel β sheet that in turn is a scaffold for two α-helical loops that interact directly with the DNA (Cho et al. 1994).

Nuclear localization signalling domain (NLS) within residues 316 to 325**.**

Homo-oligomerisation domain (OD) within residues 334 to 356.

C-terminal basic domain (BD) has 26 amino acids and is suggested to be an important regulatory domain. Residues 353 to 392.

Fig. 3 Schematic structure of p53.

The native p53 is a tetramer in solution, and amino acid residues 324-355 are required for this oligomerization of the protein (Jeffrey et al. 1995). A 3-D model of the tetramer (Fig. 4) is best described as a dimer of dimers held together principally by the β-sheet structure in addition to helix-helix interaction (Clore et al. 1994). In vitro, tetramerization is not essential for DNA binding, and the isolated core domain can bind DNA with approximately one-fifth the affinity of intact p53 (Pavletich et al 1993). In vivo, however, oligomerization-deficient p53 cannot efficiently transactivate from genomic p53 binding sites in transient transfection assays (Pietenpol et al. 1994), and it cannot suppress the growth of carcinoma cell lines. Many mutants of p53 exert their effects through a dominant negative mechanism whereby heteromers of wild-type and

mutant p53 no longer bind DNA sequence specifically or bind with much reduced affinity.

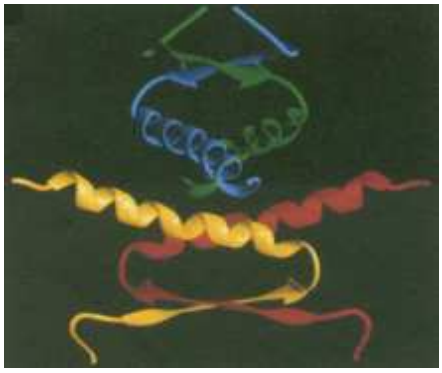


Fig. 4 Schematic structure of the oligomerization domain of p53 (adapted from Clore et al. 1994).

The tumor suppressor p53 is the most commonly mutated gene in human cancers (Olivier et al. 2002). More than 90% of the missense mutations in p53 reside in the central DNA-binding core domain, and these mutations fall into two classes. Mutations in amino acid residues such as R248 and R273, the two most frequently altered residues in the protein, result in defective contacts with the DNA and loss of the ability of p53 to act as a transcriptional factor. A second class of p53 mutations disrupts the structural basis of the β-sheet and the loop-sheet helix motif that acts as a scaffold in this domain. More than 40% of the missense mutations are localized to residues R175, G245, R248, R249, R273, and R282, which play a role in the structural integrity of this domain or the DNA contact sites directly (Cho et al. 1994, Hollstein et al. 1994).

Normal p53 acts as a ‘molecular policeman’ monitoring the integrity of the genome. If DNA is damaged, p53 accumulates and switches off replications to allow extra time for its repair. If the repair fails, p53 may trigger cell suicide by apoptosis (Yonish-Rouach et al. 1991). Tumor cells, in which p53 is inactivated by mutation or by binding to host or viral proteins, cannot carry out this arrest. They are therefore genetically less stable and will accumulate mutations and chromosomal rearrangements at an increased rate, leading to rapid selection of malignant clones (Lane 1992). Wild type (normal) p53 is accumulated in response to cellular stress, such as DNA damage, oncogene activation, hypoxia, and telomere erosion, and triggers several biological responses, including cell cycle arrest, apoptosis, senescence, and differentiation (Vousden et al. 2002) (Fig. 5). In effect, p53 prevents cells from entering or progressing through the cell cycle under

conditions that could generate or perpetuate DNA damage. As the inactivation or activation of p53 sets up life or death decisions, an exquisite control mechanism has evolved to prevent its errant activation at the same time as enabling rapid stress responses (Toledo et al. 2006).

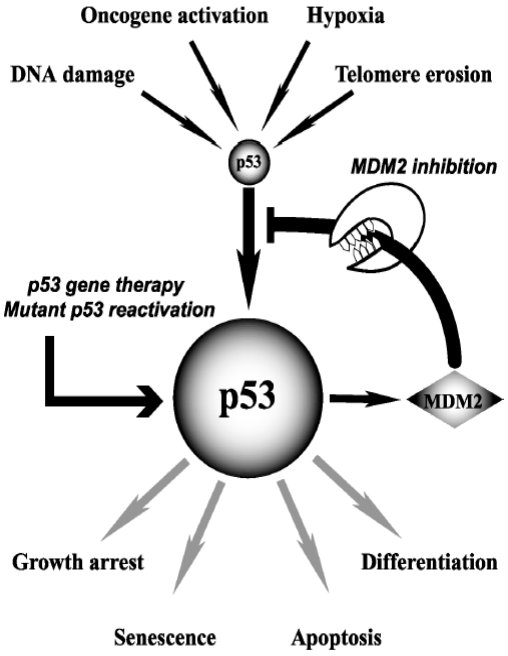


Fig. 5 The p53 signalling pathway and strategies for its restoration in tumors (adapted from Bykov et al. 2003).

The three members of the p53 family share very significant homology both at the genomic and at the protein level (Fig. 6). Each contains a TAD, a DNA-binding domain and an oligomerization domain. p63 and p73, but not p53, contain long C-termini. The determination of the three-dimensional solution structure of the p63 and p73 alpha isoforms C-terminus has shown that this region contains a sterile alpha motif (SAM), which is a protein-protein interaction domain (Chi et al. 1999).

Despite structure homology and substantial sequence among the p53 family, they show significant functional divergence. p53 is a tumor suppressor that is inactivated in a majority of human cancers. In response to cell stress, p53 acts as a sequence specific transcriptional factor, with targets including genes which effect cell cycle arrest, DNA repair, and apoptosis (Stewart et al. 2001). p63 is a putative oncogene, and is required for the development and maintenance of stratified epithelium (Westfall et al. 2004).

p73 has some properties of a tumor suppressor, but unlike p53 or p63, is involved in neurogenesis, neuron survival, and the inflammatory response (Irwin et al. 2001).

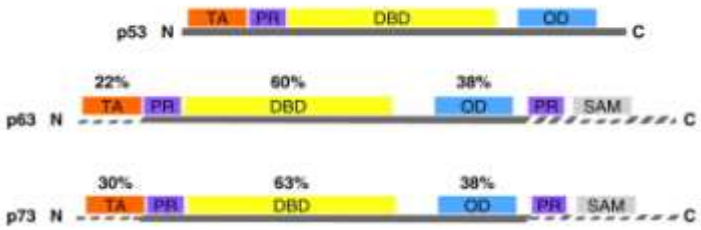


Fig. 6 Schematic representation of the protein modular structure of the p53 family members (adapted from Dötsch et al. 2010).

There are three major domains, which are highly conserved between family members. The TAD is the least conserved with 22% identity between p63 and p53 and 30% between p73 and p53 (Yang et al. 1998). The highest level of homology is reached in the DBD (63% identity between p53 and p73, and 60% identity between p53 and p63), which suggest that the three proteins can bind to the same DNA sequences and transactivate the same promoters (Levrero et al. 2000). The carboxy-terminal oligomerization domain (OD) of p53 is 38% identical with p63 and p73 (Dötsch et al. 2010). The carboxy-terminal isoforms differ in their ability to transactivate gene expression (Zhu et al. 1998, De Laurenzi et al. 1999, Lee & La Thangue 1999, Shimada et al. 1999).

Because of alternative promoter usage and C-terminal alternative splicing, all p53 family members are expressed in a number of isomeric forms. All p53 family genes contain the same modular domain structure, including an amino-terminal transactivation domain (TA), a DNA-binding domain, and a carboxy-terminal oligomerization domain (Dötsch et al. 2010). In addition, p63 and p73 undergo alternative splicing of their C-termini, resulting in three p63 isoforms (α to γ) and seven p73 isoforms (α to η). These isoforms are transcribed from an upstream promoter as well as from a cryptic promoter within intron 3, called the TA and ΔN isoforms (reviewed in Yang & McKeon 2000). Each of these isoforms may (TA) or may not (ΔN) contain the transactivation domain (TAD), depending on whether transcription of the precursor mRNA starts from

exon I (TA forms) or from exon III´ (ΔN forms). The ΔN isoforms of p63 do not activate transcription but instead can act dominant negatively and inhibit transactivation by TA p63 proteins and p53 (Yang et al. 1998). The full-length isoforms (TAp63 and TAp73), containing a transactivation domain (TAD), generally behave similarly to p53 in terms of overlapping target promoters and biological functions. The usage of the alternative promoter produces amino-terminally truncated ΔN isoforms, which are devoid of the TAD and therefore transcriptionally inactivate. As a consequence, these isoforms act as inhibitors of the active family members (Dötsch et al. 2010). TA proteins can directly activate the transcription of ΔN isoforms (Nakagawa et al. 2003, Grob et al. 2001). Full-length and truncated isoforms of the p53 generally exhibit reciprocal biological functions: truncated isoforms support proliferation while TA variants promote cell cycle arrest, cellular senescence and apoptosis. However, it also may depend on intracellular context (reviewed by Dötsch et al. 2010).

2. 4. 2. p73

The p73 gene is expressed as a p53 homologue (TAp73) and also as a negative regulator of p53-dependent apoptosis (ΔNp73) in the control of cell fate. There is evidence that TAp73 proteins can regulate apoptosis and cell cycle arrest, respectively, to induce apoptosis. Both these actions of TAp73 are inhibited by ΔNp73, which is directly transactivated by the TA isoform. Cell fate may therefore be determined both by action of TAp73 and by the relative abundance of TAp73 and ΔNp73 isoforms (Fig. 7). This mechanism is relevant for several cancers, including neuroblastoma (Rossi et al. 2004).
p73 shares the high homology with p53 and p73 maps to chromosome 1p36.1, a region frequently deleted in several tumors, including neuroblastoma, colorectal and breast cancer (reviewed by Ikawa et al. 1999). However, p73 is infrequently mutated in human cancers and has a pro-apoptotic function. The apoptosis-inducing activity of both wild-type p53 and p73 can be inhibited through the induction of ΔNp73, while p53 eliminates the function of both wild-type p53 and p73, through its loss-of-function mutations that frequently occur in many cancers. Thus, death and survival of many cell types in various organs could be regulated by a subtle balance between p53 family members and their isoforms, including the antagonizing variants such as ΔNp73 and ΔNp63, as suggested (Pozniak et al. 2000). Nakagawa et al. has reported that ΔNp73 which was induced by p73, in turn inhibited p73 by a direct interaction. The report has

also shown that in the autoregulatory system of the p53 family members, proapoptotic p73 function is negatively regulated by its own target ΔNp73, whose function is antiapoptotic (Nakagawa et al. 2002).

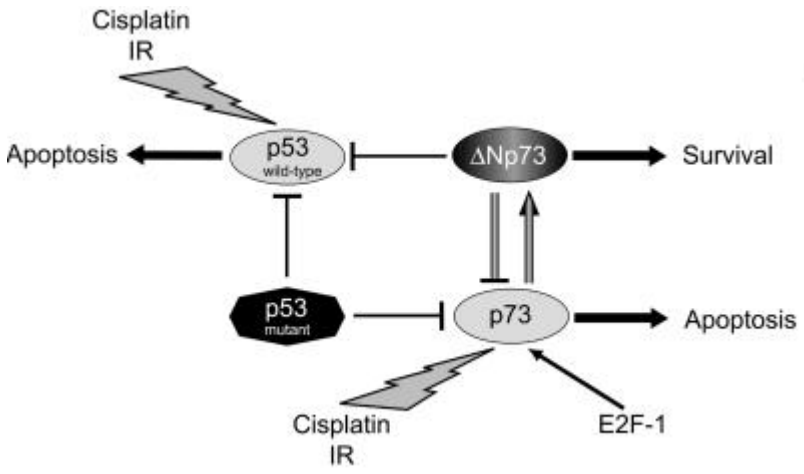


Fig. 7 Schematic representation of interactions between p73, DNp73, wT-p53, or mutant type p53 (adapted from Nakagawa et al. 2002).

2. 4. 3. ΔNp73

ΔNp73 acts as a potent transdominant inhibitor of the wild-type p53 and the transactivation-competent TAp73 and confers drug resistance to the wild-type p53 harboring tumor cells (Zaika et al. 2002). Recently, it has been reported that ΔNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors but not in normal tissues. Thus, it is overexpressed in neuroblastoma (Douc-Rasy et al. 2002), vulval cancer (O'Nions et al 2001), ovarian cancer (Ng et al 2000) and breast cancer cell lines (Fillippovich et al. 2001). Zaika et al. also showed that ΔNp73 can build a complex with wild-type p53 as demonstrated by coimmunoprecipitation from cultured cells and primary tumors (Zaika et al. 2002).

2. 4. 4. p53His175 Mutant

Mutation at amino acid residue 175 is one of the most frequently mutated sites at the DNA binding region of the p53 protein. The p53-R175H is a dominant negative conformational mutant as the mutation may affect the positioning of L2 and L3, 2 amino acid loops that interact with the minor groove of DNA molecule (Tsang et al. 2005). Tsang also showed that p53-R175H mutant is defective for the induction of cell

death by DNA damage. The protective effect of p53-R175H against drug-induced apoptosis was also reported in neuroblastoma cells that express wild-type p53 protein (Cui et al. 2002) and in Hep3B hepatoma cells, a p53-deficient cell line (Stähler & Roemer 1998). The overall structure of p53 relies on arginine 175, which is located in the L2 loop of the DNA binding domain (Cho et al. 1994). Full-length mutant p53 R175H lacks wild-type-like p53 functions and is; therefore, transcriptionally inactive and unable to induce cell cycle or apoptosis (West et al. 2006).

2.5. p53 binding sites on TLX promoter

The DBD of the p53 family proteins carries the greatest homology in between the p53 family members. A fully functional DBD is essential, mainly by the fact that most mutations of p53 reside in this domain. The DBD of p53 family proteins all recognize the p53-responsive element defined by El-Deiry et al. consisting of the decamer RRRCWWGYYY, where R is a purine, Y a pyrimidine and W an adenine or thymine. However, p53 family members can bind to other sequences as well (El-Deiry et al. 1992, Wetterskog et al. 2008). Putative p53 binding sites are shown in Figure 8.

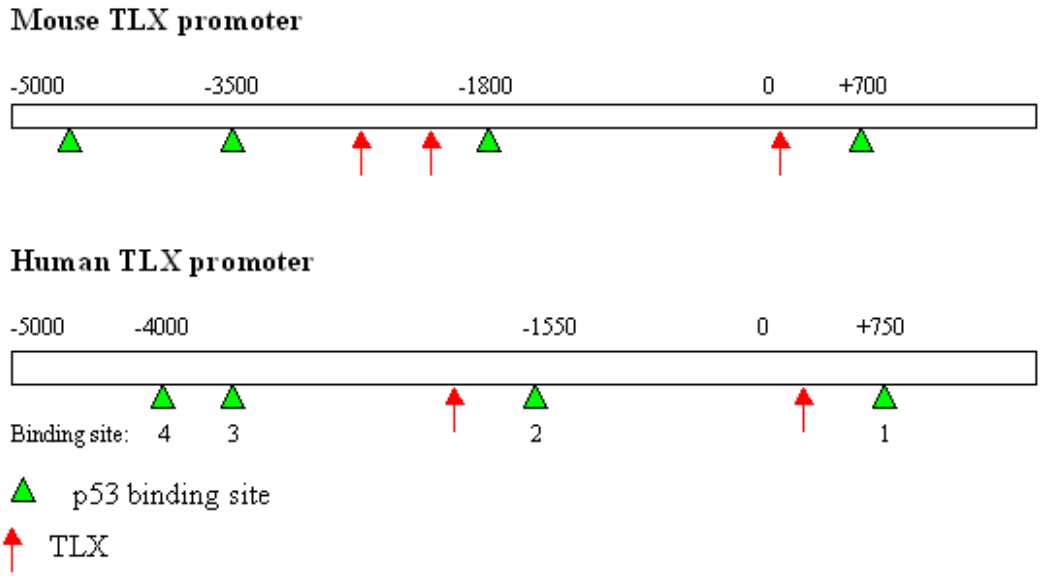


Fig. 8 Mouse and human TLX promoter. Green triangles show possible p53-binding sites and red arrows possible TLX-binding sites.

2.6. p53 in cancer

The p53 transcription factor prevents tumor development through induction of cell cycle arrest and cell death by apoptosis. The nuclear phosphoprotein, p53, is usually present

at low levels in the cell, due to a short half-life (approx. 30 min), but accumulates in response to cellular stress, such as DNA damage from irradiation. It binds DNA in a sequence-specific manner to activate the transcription of a number of genes including p21WAF1, MDM, and BAX. p53 is mutated in up to 60% of human cancers of different types, leading in most cases to accumulation of non-functional p53 (dominant-negative) protein. Mutations occurs most commonly within the DNA binding domain of the protein and the majority of these mutations are missense in nature, leading to an intact, albeit mutant protein (reviewed by Tweddle et al. 2003). This contrasts with other tumor suppressor genes, in which mutations generally lead to a complete loss of function and suggest that mutant p53 is offering a selective advantage to cancer cells. In a subset of non-mutated cancers, p53 is believed to be inactivated by other mechanism including viral inactivation, MDM2 amplification and deletion of the INK4a-ARF gene encoding $p14^{ARF}$ (Vogelstein et al. 2000).

2.7. Neural stem cells

Most adult stem cells are lineage-restricted, also known as multipotent. Thus, neural stem cells (NSCs) generate neural cells: neurons, astrocytes and oligodendrocytes. While progenitor cells already have a degree of differentiation and are committed to differentiating into a specific cell line, for example as neuronal progenitor cells differentiate into neurons (Vieira et al. 2011).

3.THE AIM OF STUDY

The general aim of this thesis was to study the role of p53 family members in the regulation of TLX expression. We wanted to see how overexpression of p53 family members affects TLX-promoter activity and gene expression in different cell lines (normal and neuroblastoma), and to analyse the underlying mechanisms.

4. MATERIALS AND METHODS

4.1. Cell culture

Two different cell lines were used in this thesis. The hTERT RPE-1 cell line is a near-diploid human cell line of female origin. It is established from retinal epithelium. The other cell line used was SH-SY5Y, which is a subclone of SK-N-SH that was established from a bone marrow aspirate of thoracic catecholamine secreting neuroblastoma of a 4-year-old girl (Biedler et at. 1973). Both cell lines were routinely maintained in medium supplemented with 10% fetal bovine serum (FBS), glutamine and 100 units/ml penicillin. The medium used were DMEM/F12 for hTERT-RPE-1 and DMEM for SH-SY5Y.

The cells were kept in 75 cm^2 flask at the temperature of 37°C in a 5% CO_2 humidified under an atmosphere of 5% CO_2. The medium was changed every 3 days and when the cells achieved 80–90% of confluence, the medium was removed, washed with PBS, 3 ml of 0.25% trypsin were added and cells were incubated for 5 minutes under the conditions mentioned above. After this time, cells were washed with medium to stop trypsin action. The detached cells were taken out and centrifuged at 1100 g for 2 minutes at room temperature. The cell pellet was resuspended and seeded into a new flask with medium.

4.2. Promoter Reporter Assay - Luciferase Assay

Promoter reporter assays are used for studying the activity of a specific promoter, enhancer regions and transcription factors. The regulatory sequence of interest is cloned upstream of a reporter gene, in our case the luciferase gene, and the resulting vector is transfected into cells. The cells are treated with drugs or cotransfected with vectors expressing transcription factors or other proteins. If the promoter is active the luciferase enzyme will be expressed. When the luciferase substrate luciferin is added to the cell lysates, the enzymatic reaction produces light that can be quantified and give an indirect measurement of the degree of promoter activity.

The different cell lines were seeded in 24 well plates at a density of 3×10^4 cells/well (RPE-1) and 4×10^4 cells/well (SH-SY5Y) and transfected on the next day using FUGENE®HD (Roche, Basel, Switzerland) transfection reagent following the

manufacturers protocol. Two days after transfection cells were lysed and the luciferase activity was quantified by addition of luciferase substrate solution following the manufactures protocol (Luciferase Assay System, Technical Bulletin, Promega) and the luminescence was recorded by a luminometer (Victor). Transfection of each construct was performed in quadruplicates in each assay.

The luciferase constructs used in this thesis were containing the 4.7, 1.8 and 0.5 kb upstream sequence of the TLX gene. Cells were transfected with 0.3 to 0.4 μg reported plasmid and 0.3 to 0.4 μg expression plasmid.

4.3. Western blot analysis

Western blotting, also known as immunoblotting, is a technique to determine the presence, relative amount and molecular weight of various proteins of interests in cell, by specific antibody detection.

Cultured cells were washed in phosphate buffered saline (PBS) and lysed using lysis buffer containing protease inhibitors. Lysates were sonicated. To remove cell debris, lysates were centrifuged for 20 minutes at 4°C at 14 000 rpm. Equal amounts of proteins were loaded to and separated on 10-12% SDS-PAGE gels and transferred to PVDF membranes that were blocked in 5% BSA and probed by primary antibodies (listed in Table 1) diluted in 5% BSA. The membrane was incubated and shaken overnight at 4°C. The membrane was then incubated in anti-mouse or anti-rabbit IgG secondary antibody diluted in TBS-T for 1 hour. After 3 × 10 min washes in T-BST membranes were developed using the enhanced chemiluminescence (ECL) Advance system (GE Healthcare) and scanned using LAS-1000 Plus (Fujifilm).

Table 1: List of antibodies used in this thesis.

Antibody	Company	Species	Application
p73 (H79)	Santa Cruz	Rabbit	WB (1:1000)
TLX	R&D	Mouse	ChIP
Ac-Histone H3K9/14	Santa Cruz	Mouse	ChIP
p53 (FL-393)	Santa Cruz	Rabbit	ChIP, WB (1:1000)
p53 (Pab-421)	Calbiochem	Mouse	ChIP

4.4. ChIP Assay

Chromatin Immunoprecipitation assays (ChIP assays) is a method used to study *in vivo* the location of DNA binding sites on the genome for a particular protein of interest. It uses cross-linking DNA and proteins by formaldehyde fixation. It is followed by sonication to shear DNA into fragments of 200-1000 base pairs. ChIP validated antibodies specific to proteins are used to immunoprecipitate with protein-DNA complexes, assumed to bind the regulatory region. The DNA fragments bound by proteins are collected, the protein-DNA crosslinking is reversed, proteins and RNA are degraded while the DNA is purified using phenol chloroform extraction. The DNA is amplified with PCR using primers specific against the regulatory region of interest. If the antigen binds the region a band will be detected when run on an agarose gel.

Cells were cultured in 10-cm dishes to almost confluence. Protein and DNA were crosslinked by incubating cells with formaldehyde at a final concentration of 1% for 10 min at 37°C. Cells were lysed in SDS lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS, pH 8.1), the lysate was sonicated and incubated at 4°C with antibodies. The antibody-protein-DNA complexes were precipitated by using ProteinA/G-agarose. Immunoprecipitates were washed once with Low Salt Immune complex buffer (20 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X–100, pH 8.1), High Salt Immune complex wash buffer (20 mM Tris-HCl, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X–100, pH 8.1), LiCl buffer (10 mM Tris-HCl, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, pH 8.1) and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.1). Immunoprecipitates were eluated twice with

elution buffer and incubated with 0.5M NaCl for 3 h at 65°C to reverse the DNA-protein crosslinks. RNA and protein was degraded using Proteinase K for 1 h at 45°C and RnaseA for 30 min at 37°C. DNA was extracted with a PCR Purification Kit (Qiagen) and used for RT-PCR analysis, using promoter-specific primers (Table 2).

Table 2: List of primers used for ChIP in this thesis.

Primer	Forward sequence	Reverse sequence	Species
hTLX p53 1 F1/R1	5´-GGT CGA TCA CAG GGG ATT GG-3´	5´-AGG ACA AGC TTT CCC ATC AGC-3´	H
hTLX p53 1 F2/R2	5´-AAG CTT GTC CTT TCA CCT TCG GT-3´	5´-TCT GAG GTT GAC TGC TAG CCC T-3´	H
hTLX p53 3F2/1R1	5´-CCG GAT CAA CAA GTG GGT ACC TC-3´	5´-AGG ACA AGC TTT CCC ATC AGC-3´	H
hTLX p53 2 F1/R1	5´-GCG TGA ACC AGA ACC TGA GG-3´	5´-CGT AGC GCT TTC TCG AAC TCG-3´	H
hTLX p53 2 F2/R2	5´-CGA GTT CGA GAA AGC GCT ACG-3´	5´-GGG TTT ATT AGG TGA CAG GAC G-3´	H
hTLX p53 3 F1/R1	5´-AGC ATG AGC AAG CCA GCC GGA T-3´	5´-GGC TCA GAT TCG CAG CGC TC-3´	H
HPRT	5´-TGT TTG GGC TAT TTA CTA GTT G-3´	5´-ATA AAA TGA CTT AAG CCC AGA G-3´	H
hGAPDH	5´-GAA GGT GAA GGT CGG AGT C-3´	5´-GAA GAT GGT GAT GGG ATT TC-3´	H
hp53	5´-CCG CAG TCA GAT CCT AGC G-3´´	5´-AAT CAT CCA TTG CTT GGG ACG-3´	H

4.5. RNA isolation, RT-qPCR

Cells were seeded at 1, 5×10^5 (RPE-1) and 2×10^5 (SHSY) cells/well in 6-well plate. On the following day, cells were transfected using FUGENE®HD (Roche, Basel Switzerland) transfection reagent following the manufacturer's protocol. Two days after transfection cells were harvested and RNA isolated using the TRIAzol reagent

(Invitrogen) and subsequent ethanol precipitation. Total RNA was then subjected to quantitative reverse transcription-PCR (qRT-PCR) analysis. Reactions contained 10 pmol forward and reverse primers, 2 × SYBR green super mix (Applied Biosystems) and 2 μL template cDNA. All samples were run in quadruplicate in each experiment. Values were normalized by human HPRT or GAPDH for each sample. The primer sequences used in the qRT-PCR analyses are listed in Table 3.

Table 3: List of primers used for qRT-PCR in this thesis.

Primer	Forward sequence	Reverse sequence	Species
hTLX F1	5´-CAA GAG GTG GTG GCT CGA TTT A-3´	5´-ACC ACT ATG TGT AGG AAC GGC TTT G-3´	H
hTLX-5	5´-GAG GTG GTG GCT CGA TTT AG-3´	5´-GCA TTC CGG AAA CTT CTC AG-3´	H
HPRT	5´-TTT GCT TTC CTT GGT CAG GC-3´	5´-GCT TGC GAC CTT GAC CAT CT-3´	H
p73	5´-GCA CCA CGT TTG AGC ACC TCT-3´	5´-GCA GAT TGA ACT GGG CCA TGA-3´	H
ΔNp73	5´-CAA ACG GCC CGC ATG TTC CC-3´	5´-TTG AAC TGG GCC GTG GCG AG-3´	H

4.6. Statistical analysis

The data presented were stored and analyzed using Excel software (Microsoft).

5. RESULTS

5.1. Luciferase assay

5.1.1. RPE-1 cell line

In order to investigate how the activity of a specific promoter is regulated by transcription factors, the promoter reporter assay (Luciferase assay) was performed. The mouse TLX promoter of three different length, 0.5, 1.8 and 4.7 kb, all inserted in the luciferase-reporter vector (pGL3), were used. Cells were cotransfected with a promoter construct and an empty vector or p53-expression vector. When p53 was transfected into RPE-1 a repression of reporter activity was observed in all TLX promoter constructs. The maximum reduction observed was approximately 35% for the 0.5 kb TLX promoter construct, 40% for the 1.8 kb TLX construct and 60% for the longest 4.7 kb TLX construct (Fig 9). The overexpression of p53 vector was confirmed by qRT-PCR (Fig. 10).

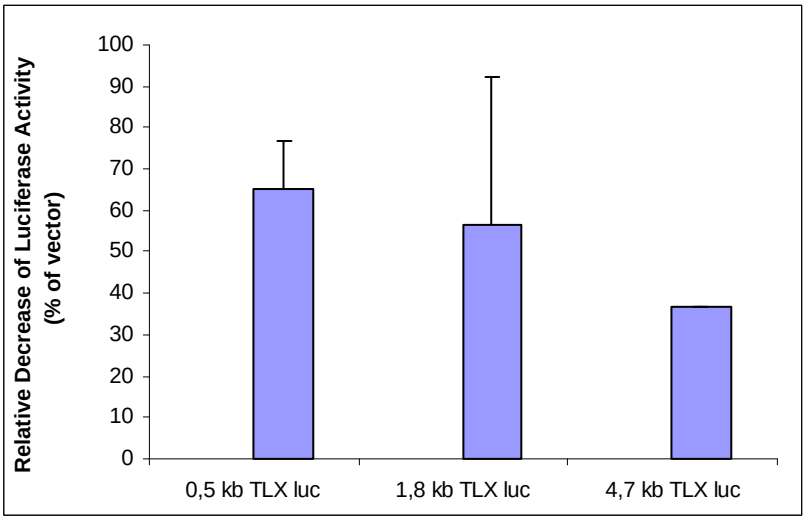


Fig. 9 The TLX promoter constructs were cotransfected to RPE-1 cells with empty vector and p53 overexpressing vector. Data are presented as a percentage of luciferase activity compared to the activity of empty vector control (which was set to 100%). Results are the mean ± SEM of three independent experiments performed in quadruplicate.

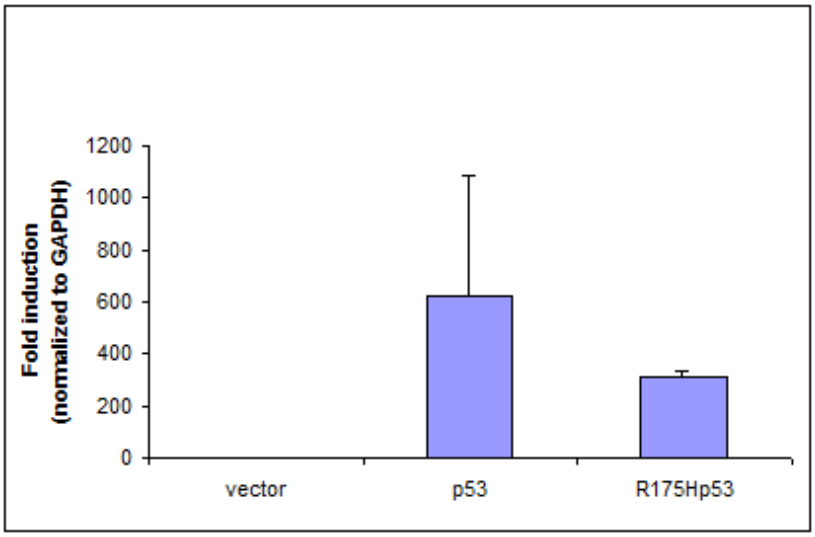


Fig. 10 Overexpression of p53 and R175Hp53 in RPE-1 cells.

5.1.2. RPE-1, 1.8kb TLX luc and p53 family members

Next we examined the 1.8 kb long TLX construct with three different expression vectors: pcDNA3 containing p53, wTp73 or ΔNp73. When we transfected wTp53 and wTp73 into RPE-1 cell line a significant repression of approximately 60% for p53 and 50% for wTp73 was observed (Fig. 11). Interestingly, the transfection with ΔNp73 showed a significant decrease as well. The maximum reduction was approximately 60%. The overexpression of p53 vector (Fig. 10) and wTp73 (Fig. 12A) and ΔNp73 (Fig. 12B) were confirmed by qRT-PCR.

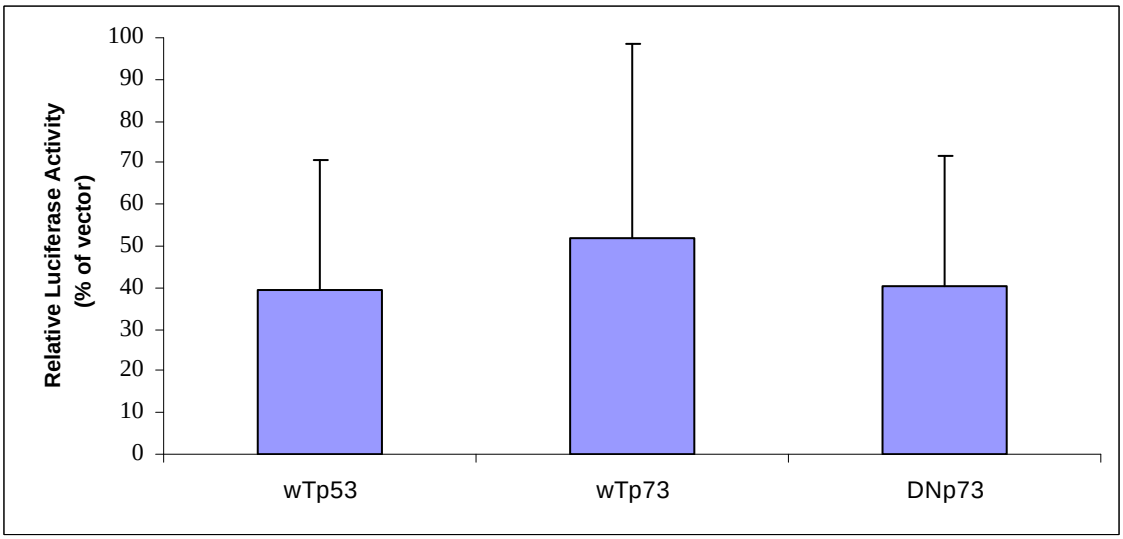


Fig. 11 The TLX promoter constructs were cotransfected to RPE-1 cells with vector alone, vector combined with wTp53, wtTp73 and ΔNp73. Data are presented as a percentage of luciferase activity compared to the activity of empty vector control (which was set to 100%). Results are the mean ± SEM of three independent experiments performed in quadruplicate.

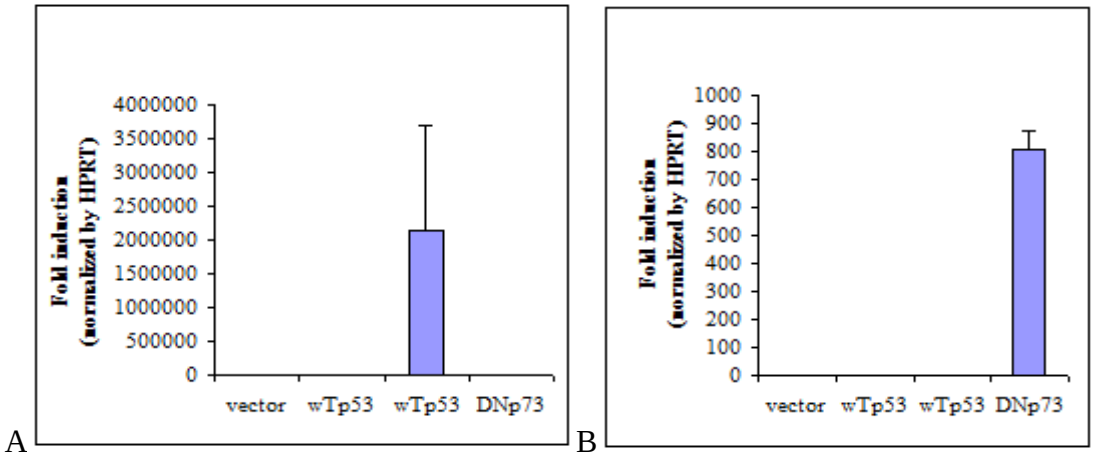


Fig. 12 Overexpression of (A) wTp73 vector and (B) ΔNp73 in RPE-1 cells.

5.1.3. RPE-1, 1.8 kb TLX luc and p53 family members

Another Luciferase assay was performed to examine the 1.8 kb long TLX construct with p53 mutant R175Hp53. Cotransfection of R175H mutant together with wTp53 reduced the inhibitory effect of wTp53 on TLX promoter activity in RPE-1 cells (Fig. 13). The overexpression of p53 vector and mutant R175Hp53 was confirmed by qRT-PCR (viz. Fig. 10).

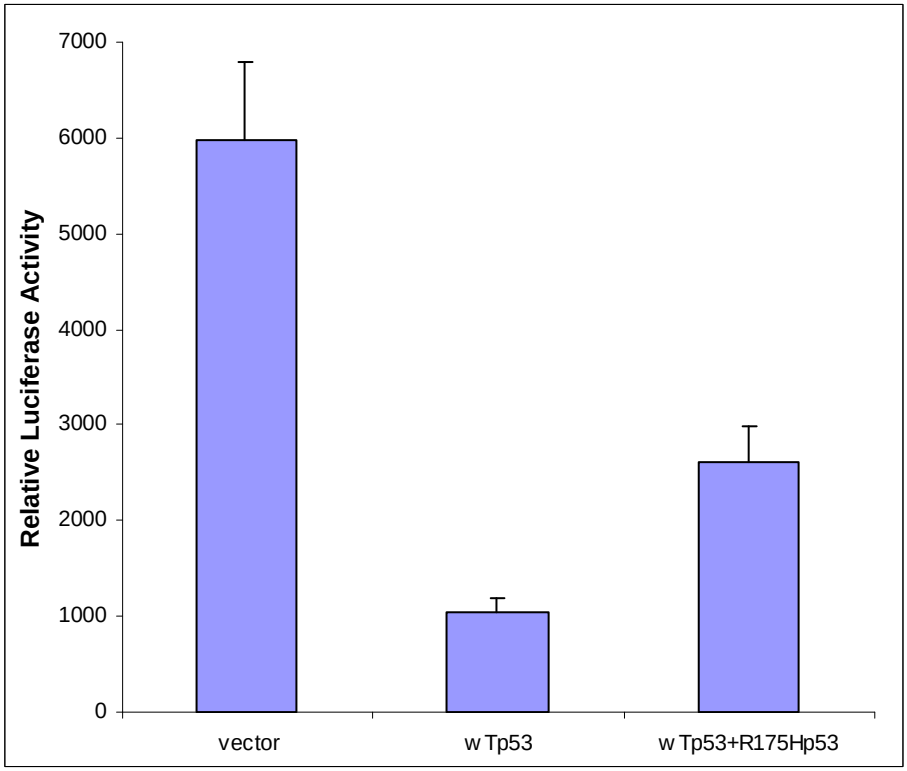


Fig. 13 The 1.8 kb TLX promoter construct was cotransfected to RPE-1 cells with vector alone, wTp53 and H175Rp53. Values represent mean luciferase activity. Error bars indicate standard deviation of quadruplicate samples.

5.1.4. SH-SY5Y cell line

When p53 was transfected into SH-SH5Y a repression was observed in both of the 0.5 kb and 1.8 kb TLX constructs. The maximal reduction is approximately 20% for the 0.5 kb TLX construct, and 70% for the 1.8 kb TLX luc construct (Fig. 14). The overexpression of the p53 vector was confirmed by Western Blot (Fig. 15A).

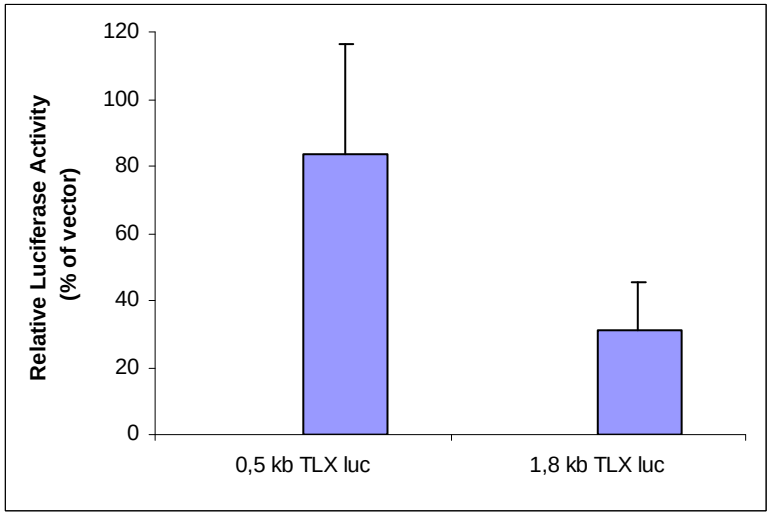


Fig. 14 The TLX promoter construct was cotransfected to SH-SY5Y cells with vector alone and p53. Values represent mean decrease of luciferase activity. Data are presented as a percentage of luciferase activity compared to the activity of empty vector control (which was set to 100%). Results are the mean ± SEM of three independent experiments performed in quadruplicate.

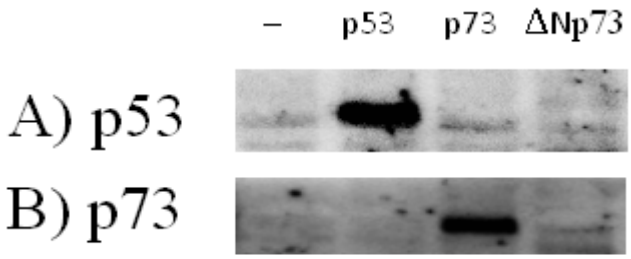


Fig. 15 Overexpression of (A) p53 vector and (B) p73 in SH-SY5Y cells.

5.1.5. SH-SY5Y, 1.8 kb TLX luc and p53 family members

Next we examined the 1.8 kb long TLX construct with three different expression vectors: pcDNA3 containing p53, wTp73, ΔNp73, and p53 mutant R175H. In contrast to the others, co-transfection of wTp53 decreased the activity of approximately 73%. wTp73, ΔNp73 and p53 mutant R175H showed a significant increase when they were transfected. The maximal increases were approximately 60%, 1840% and 275% of the vectors: wTp73, ΔNp73 and p53 mutant R175H (Fig. 16). The overexpression of p53 vector (Fig. 15A) and wTp73 (Fig. 15B) were confirmed by Western Blot. The overexpression of ΔNp73 (Fig. 17B) and also wTp73 (Fig. 17A) was confirmed by qRT-PCR.

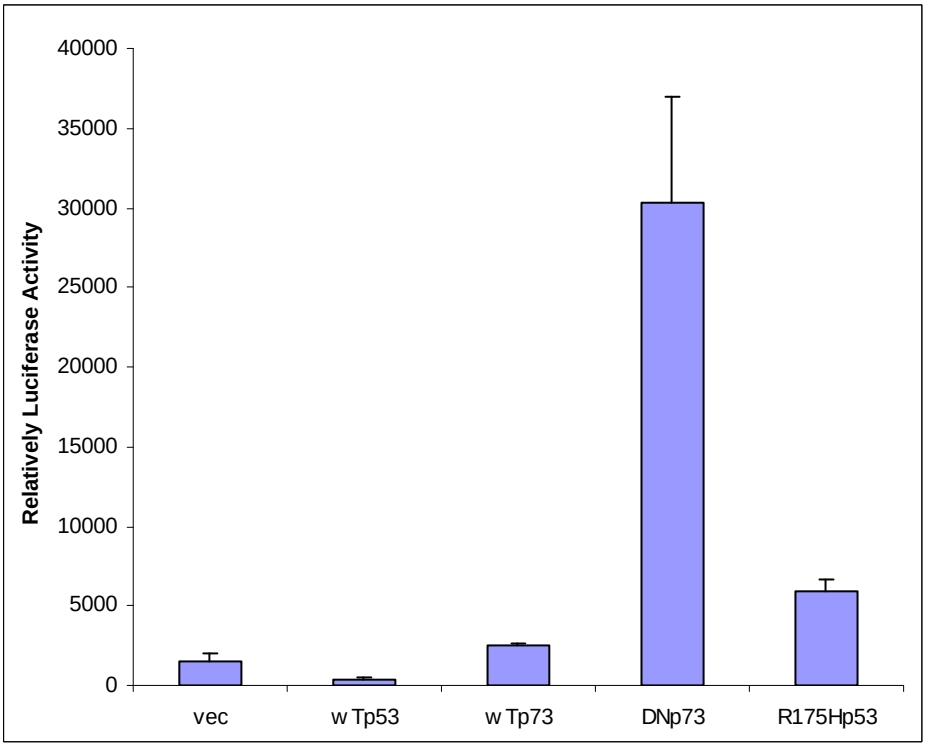
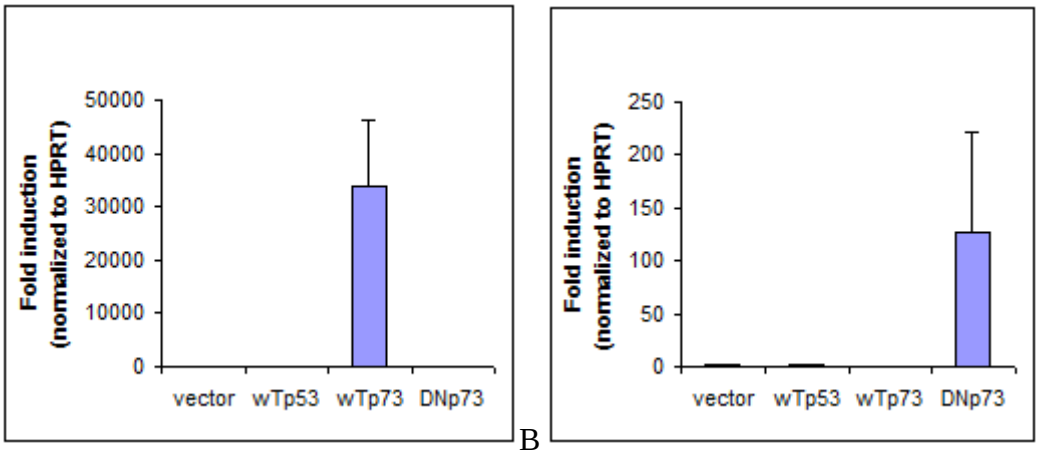


Fig. 16 The TLX promoter construct was cotransfected to SH-SY5Y cells with vector alone wTp53, wTp73, ΔNp73 and mutant H175Rp53. Values represent mean luciferase activity. Error bars indicate standard deviation of quadruplicate samples.



A B

Fig. 17 Overexpression of (A) wTp73 vector and (B) ΔNp73 in SH-SY5Y cells.

5.2. qPCR

Following the repression exhibited by wTp53 on the promoter we confirmed the Luciferase assay results with qPCR. We found that p53 overexpression inhibited TLX mRNA expression in both cell lines RPE-1 (Fig. 18) and SH-SY5Y (Fig. 19).

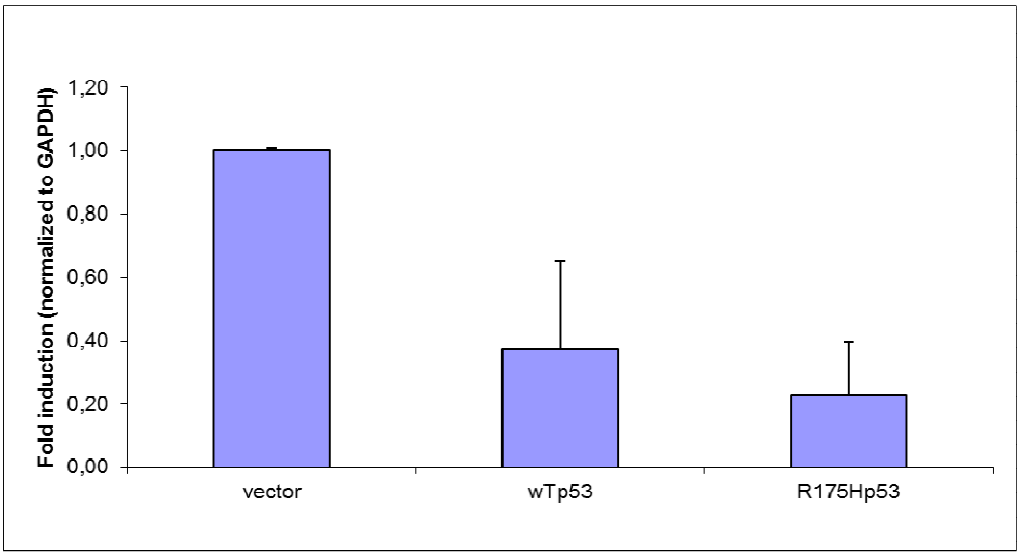


Fig. 18 p53 inhibits mRNA expression of TLX. Error bars indicate standard deviation of two independent experiments performed in quadruplicate.

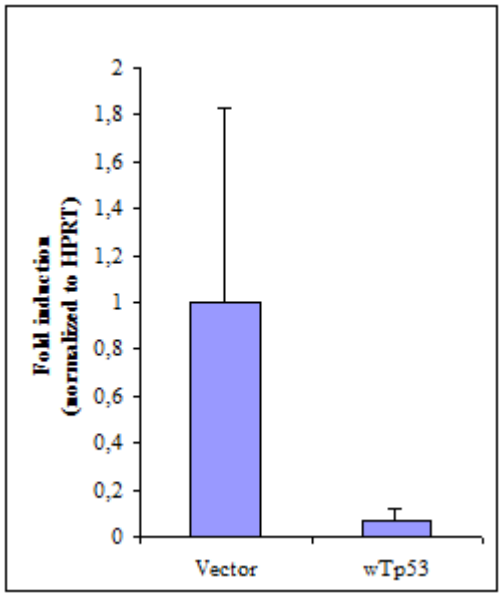


Fig. 19 p53 inhibits mRNA expression of TLX. Error bars indicate standard deviation of two independent experiments performed in quadruplicate.

5.3. ChIP

In order to further analyze the mechanism by which p53 inhibits TLX we wanted to determine if p53 affects TLX expression by direct binding to the promoter region of human TLX *in vivo* using ChIP assay. Since we found several possible binding sites for p53 in the TLX promoter, specific primer pairs were constructed for the different p53

binding sites. The PCR reverse and forward primers flanking the essential regions are shown in Fig. 20.

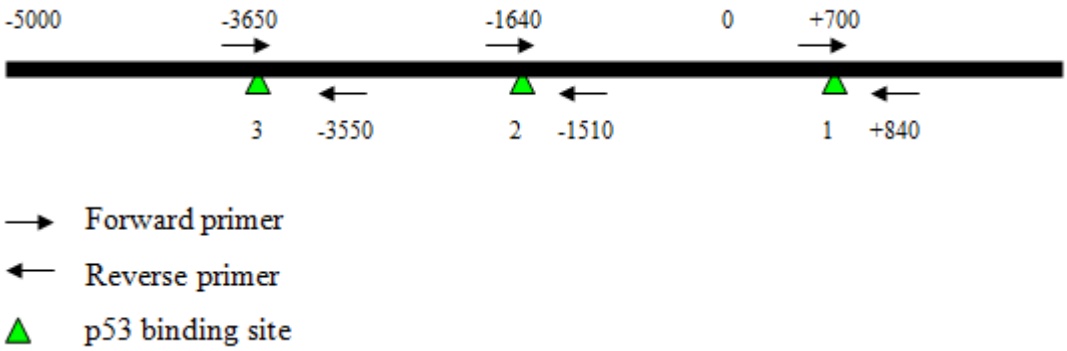


Fig. 20 A diagram illustrating the three pairs of primers used for ChIP analysis and p53 binding sites.

In order to see whether p53 binds the TLX promoter in vivo, ChIP assays were performed for RPE-1 cell line, using 2 different antibodies for p53 (FL393 and AbI), TLX, Acetylated Histone (AcHis), and IgG as non-specific control (Fig. 21). Using chromatin immunoprecipitation and semi-qPCR with primers for the specific promoter regions, we showed that overexpression of p53 in RPE-1 cells led to increased recruitment of p53 to TLX promoter at putative p53 binding site 2 and 3 (but not site 1; data not shown) while histone acetylation (marker for active chromatin) was decreased.

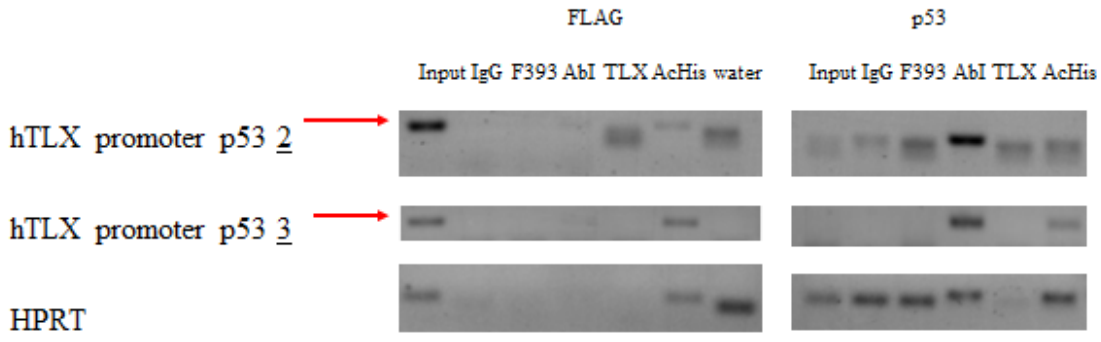


Fig. 21 Overexpression of p53 led to increased recruitment of p53 to TLX promoter at putative p53 binding site 2 and 3, while histone acetylation (marker for active chromatin) was decreased. The arrows show the specific product size.

6. DISCUSSION

Neuroblastoma is the most common extracranial solid tumor in infancy and accounts for approximately 15% of childhood cancer deaths. Disruption of the p53 pathway is a common mechanism leading to defects in apoptosis in cancer cells. Increasing evidence suggest that the p53 pathway may be inactivated in NB. Inactivation of the p53 pathway occurs most commonly at the time of relapse. The p53 family proteins, p73 and p63, can also induce apoptosis, and early studies suggest that p73 may be important in NB pathogenesis and response to treatment (Wolter et al. 2010). The p53 family members and TLX have important roles in neuronal development, but the relationship between them has not been investigated in this context.

The primary purpose of this study was to investigate whether p53 protein can bind to the TLX promoter and if it can affect its activity. We demonstrated herein that p53 binds the TLX promoter and regulates its activity. One putative binding site for p53 is presented on the shortest (0.5 kb) TLX promoter construct, and two and four sites, respectively, for the 1.8 kb and 4.7 kb TLX promoter constructs. Repression of TLX promoter activity by p53 was evident in both cell lines we tested.

Next we examined whether and how other p53 family members and their mutants can affect the promoter activity in RPE-1 and SH-SY5Y. The 1.8 kb TLX construct was used. We expected to see similar expression profiles for the different p53 family members, but we found big differences between the two cell lines. As mentioned above, overexpression of wTp53 was able to downregulate the promoter activity when compared with the vector control in both cell lines.

In NB cell lines, TAp73 is variably expressed in essentially all cells with few exceptions (Kaghad et al. 1997, Kovalev et al. 1998). Conversely, DNp73 was detected only in a subset of cell lines, primarily, but not exclusively, in those with MYCN amplifications (Casciano et al. 2002). The physiological contribution of p73 locus to NB development has been recently shown to be due not to the loss of proapoptotic TAp73, but to the induction of the antiapoptotic form of p73 (Casciano et al. 2002, Douc-Rasy et al. 2002).

DNp73 is an NH_2-terminal truncated isoform of human p73, lacking the transactivation domain, therefore it can bind DNA, but not activate transcription. It is also predicted to be a transdominant inhibitor of wTp53 through direct binding to wT-p53, thus inhibiting its apoptosis-promoting activity (Pozniak et al. 2000). DNp73 was able to activate the TLX promoter in the SH-SY5Y cell line, possibly due to its dominant-negative effect on endogenous p53 repression of TLX promoter activity. Surprisingly, DNp73 repressed the TLX promoter activity in the RPE-1 cell line. This experiment must be repeated to confirm that DNp73 really does not activate the promoter in RPE-1 cells.

Since the p53 family proteins are sequence-specific transcriptional factors where all members can bind to the canonical p53 binding sequence (Wetterskog et al. 2009) and share a high homology with p53, p73 should act similar as p53. However, the opposite effect was seen using p73. Overexpression of wTp73 was able to downregulate TLX promoter in RPE-1 cells, as expected, but slightly upregulated TLX promoter in SHSY5Y cells. This small effect by p73 on TLX may be due to high levels of endogenous p73 in the SH-SY5Y cell line and that overexpression does not give any additional effect. We were unfortunately not able to determine endogenous levels of p73 and DNp73 in any of these cell lines. Since p73 can bind the same consensus sequence as p53 it is possible that DNp73 might compete with p53 and p73 for DNA binding (Wetterskog et al. 2009). Additional mechanism of p53 inhibition might be direct promoter competition, with ΔNp73 displacing p53 from the DNA binding site (Ischimoto et al. 2002). Possible explanation for why regulation of TLX expression shows such differences is that p73 and DNp73 might actually induce p53 nuclear accumulation. DNp73 can possibly induce p53 effects in some case (Goldschneider et al. 2004).

In this study we next examinated a dominant negative 'hot spot' mutant in human cancer (Vousden & Lu, 2002) R175H p53. R175Hp53 is unable to bind to DNA but has the ability to bind to wTp53 and thereby reduce the amount of wTp53 able to bind to DNA by sequestering it. As a result the DNA-binding defective p53 mutant R175Hp53 was not able to repress the promoter activity. Coexpression of R175H mutant p53 with wTp53 inhibited the repressive effect of wTp53 on TLX promoter activity in RPE-1 cells. However, expression of R175H p53 in SH-SY5Y activated the TLX promoter, showing that this DNA-binding deficient mutant has prevented wTp53 from binding to

DNA in both cell lines. The activation occurring in SH-SY5Y might be due to sequestering of TAp73 by the mutant p53, which might have enabled DNp73 to bind DNA. If TAp73 were bound to TLX promoter in SH-SY5Y, release of TAp73 from the promoter by increasing amounts of R175Hp53, which binds and sequesters TAp73, could lead to a relative activation of the promoter.

Having seen the decrease of TLX expression due to p53 overexpression by using Luciferase assay and qPCR analysis, we wanted to verify that the effect of p53 was due to its direct binding to the TLX promoter chromatin in vivo. This was confirmed by using ChIP assay in RPE-1 cells, where we found binding of p53 to the TLX promoter chromatin at two different putative p53 binding sites.

7. CONCLUSION

In summary we showed in this study, that p53 binds the TLX promoter and regulates its activity. Overexpression of p53 was able to downregulate TLX promoter in both tested cell lines. We expected to see similar expression profiles for the different p53 family members. Surprisingly, experiments with other members did not show such uniform results. It is necessary to determine endogenous levels of p73 and DNp73 in these cell lines in order to find the reason behind their different effects in the two cell lines.

We also demonstrated the mechanism by which p53 inhibits TLX. We confirmed that p53 effects TLX expression by direct binding to the promoter region of TLX. From several possible binding sites we proved two of them. Further studies must be performed in order to find out the detailed mechanisms behind the interaction of p53 signalling and TLX in neuroblastoma.

8. REFERENCES

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