CHARACTERIZATION OF THE T122L MUTATION IN *p53* AND ITS PROTEIN PRODUCT IN *Xpc* MUTANT MICE

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"Who is wise? The one who learns from everyone"

"Who is rich? The one who is happy with what he has"

For Tal, Shai, Gil and Yuval,

who make me wiser and richer everyday

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Xeroderma Pigmentosum (XP) is a rare genetic disorder characterized by extreme sensitivity to sunlight, and a profound predisposition to skin cancer due to defects in nucleotide excision repair (NER) of DNA. XP patients can be divided into seven complementation groups (A-G) with corresponding genes *Xpa* thru *Xpg*. I have studied a novel UV-induced hot spot in codon 122 of the p53 gene in mice deficient in XPC protein and heterozygous for the p53 gene. The original Threonine residue is mutated to Leucine as a result of an AC->TT change.

The main goal of this work was to characterize the T122L mutation and its protein product, and to elucidate the mechanism(s) that affect its appearance in XPC deficient skin. I have shown that the T122L mutation is rare in other NER deficient mouse models, suggesting that the XPC protein is required specifically to repair the unidentified damage in this codon. In addition, I have shown that the T122L mutant protein is not a loss of function mutant, but it retains some wild type protein functions including transactivation of p53 regulated genes and promotion of cell cycle arrest in response to UV-induced DNA damage. The altered transactivation properties of the mutant protein might support clonal expansion, giving cells that express it a growth advantage.

In order to determine whether the p53 mutant protein function contributes significantly to the elevated mutation frequency observed in XPC deficient mice I have used a p53 knockout mouse model that retains the genomic region containing codon 122 without expressing p53 protein. I show that the mutation is observed in low frequencies in mice that do not express protein, suggesting that repair deficiency is the key factor for the appearance of the T122L mutation. However, once the mutation is formed its frequency is significantly increased as a result of the mutant protein function. The specific requirement for XPC protein and the location of the damage at a non-dipyrimidine nucleotide site hints at an additional role of XPC in a repair pathway different from NER.

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List of Figures and Tables

Abbreviations

ATP	adenosine triphosphate
BER	base excision repair
BRCA1	breast cancer susceptibility gene 1
CEN2	centrin 2/caltractin 1
CS	Cockayne syndrome
CSA and CSB	Cockayne syndrome A and B proteins
CPD	cyclobutane pyrimidine dimer
DDB	DNA damage binding protein
GGR	global genomic repair
hHR23A and B	human homologue of *S. cerevisiae* repair protein RAD23 A and B
MEF	mouse embryonic fibroblast
MMR	mismatch repair
NER	nucleotide excision repair
PCNA	proliferating cell nuclear antigen
6-4 PP	(6-4) pyrimidine-pyrimidone photoproduct
RE	response element
RPA	replication factor A
TCR	transcription coupled repair
TFIIH	transcription factor IIH
UV	ultraviolet
XP	xeroderma pigmentosum
XPA to XPG	xeroderma pigmentosum groups A to G

Chapter I: Introduction

Nucleotide Excision Repair (NER) in Mammals:

The integrity of genetic material is essential for life, therefore all life forms from bacteria to man have evoloved mechanisms to protect it. Spontaneous DNA damage during normal cellular metabolism, or damage by a wide variety of environmental physical and chemical agents can result in accumulation of mutations with potentially deleterious effects on the cell. One of the main biological responses to DNA damage is DNA repair. There are several repair mechanisms that are able to remove damaged or mispaired bases from the genome (Friedberg 1995): The Base Excision Repair (BER) pathway executes excision of a single damaged base. Mismatch Repair (MMR) removes nucleotides that are incorrectly paired with the nucleotide on the opposite DNA strand (usually during replication). Bulky, helix-distorting lesions are the main targets of a third pathway, Nucleotide Excision Repair (NER). Numerous bulky chemical adducts are eliminated by this versatile process which also serves as the main defense against DNA damage induced by the short-wave ultraviolet (UV) component of sunlight.

Humans deficient in NER suffer from a rare, recessively inherited disorder called xeroderma pigmentosum (XP). XP patients are extremely sensitive to sunlight. Very short periods of sun exposure result in marked skin burning starting as early as a few weeks after birth. The progressive damage to skin and eyes leads eventually to sunlight-induced tumors in sun-exposed areas such as the face, neck and even the tip of the tongue (Friedberg 2001). The frequency of skin cancer in XP patients is more than a 1000-fold higher compared to the general population and the mean age of onset is 8 years, about 50

years earlier. XP has a worldwide distribution. One out of 250,000 individuals has XP in the western world, and one out of 40,000 in Japan.

XP patients can be divided into seven different genetic complementation groups (A-G). The corresponding genes have been cloned and found to have key roles in NER (discussed in detail below). There is another group of patients (called the variant group or XP-V) in which NER is normal. Recently, the XPV gene was cloned and shown to encode a DNA polymerase required for DNA damage bypass during replication (Masutani 1999).

The NER process involves several steps: damage recognition, strand separation at the site of the lesion, dual incision around the lesion to remove the damage-containing oligonucleotide, typically 24-32 nucleotides in length, DNA synthesis to fill the gap and ligation to seal it. The XP proteins participate in damage recognition (XPC, XPA and XPE), strand separation (XPB and XPD) and dual incision (XPA, XPF and XPG).

NER can be divided into two sub-pathways: Global Genomic Repair (GGR), which repairs DNA damage in transcriptionally-silent regions of the genome and the non-transcribed strand of transcriptionally-active genes, and Transcription-Coupled Repair (TCR), which repairs lesions present in the transcribed DNA strands. The damage recognition factor for TCR is believed to be the elongating RNA polymerase II complex that is blocked when it encounters a lesion (Donahue, Yin et al. 1994). The XPC protein, which is required for damage recognition, and the DDB protein, which is encoded by the *XPE* gene, participate in GGR but are dispensable for TCR (Cheo, Ruven et al. 1997; Sugasawa, Ng et al. 1998; Hwang, Ford et al. 1999; Araujo, Tirode et al. 2000; Batty, Rapic'-Otrin et al. 2000; Wakasugi, Kawashima et al. 2002). Proficient TCR in XP

patients that belong to complementation groups C and E can explain why their symptoms are less severe compared to patients from complementation groups A, B, D and G. XP-C and XP-E patients show reduced sensitivity to sunburn and do not suffer from neurological abnormalities. XP-C and XP-E cells are less sensitive to killing by UV irradiation and they can retain up to 50% of the normal repair capacity (Cheo, Ruven et al. 1997; Hwang, Toering et al. 1998; Friedberg, Cheo et al. 1999; Berg, Rebel et al. 2000).

Damage Recognition in NER

Human NER is targeted to sites at which the duplex structure of the DNA is destabilized. It appears that the more a lesion distorts the normal DNA structure, the more efficiently the NER machinery recognizes it and its overall repair rate increases. DNA adducts that distort helical conformation, such as those caused by UV radiation, Acetylaminofluorene (AAF), or benzo [α] pyrene diol-epoxide (BPDE) are repaired by NER (Gunz, Hess et al. 1996). However, conformational change as a result of abnormal base pairing does not appear to be sufficient by itself to promote DNA incision by the NER machinery. A second requirement is altered deoxyribonucleotide chemistry. This bipartite DNA damage recognition mechanism distinguishes NER from the two other DNA repair pathways that have only one requirement for activation: BER requires base damage without duplex destabilization, and MMR acts at sites of mispaired bases without chemical alterations (Hess, Schwitter et al. 1997; Hess, Naegeli et al. 1998).

The proteins involved in damage recognition during NER are the Damaged DNA Binding protein (DDB), which accumulates at DNA damage sites immediately after UV

irradiation (Wakasugi, Kawashima et al. 2002), and the XPC-hHR23B complex that binds DNA independently of DDB and initiates NER (Sugasawa, Ng et al. 1998; Batty, Rapic'-Otrin et al. 2000). Damage recognition by the XPA-RPA complex appears to be important to verify that the lesion is an NER substrate and to orient the rest of the NER machinery around it to achieve incision (Li, Peterson et al. 1995; Nocentini, Coin et al. 1997; Sugasawa, Ng et al. 1998).

The DDB Protein

The Damaged DNA Binding (DDB) protein is composed of two subunits, p127 (DDB1) and p48 (DDB2). The p48 subunit, which encodes a WD40 repeat-containing protein, is mutated in a subset of XP-E patients (Hwang, Toering et al. 1998; Hwang, Ford et al. 1999). DDB absence causes a defect in the removal of cyclobutane pyrimidine dimers (CPDs) from the non-transcribed strand (but not from the transcribed strand) *in vivo* (Hwang, Ford et al. 1999). The p48 subunit of DDB accumulates at locally damaged DNA sites immediately after UV irradiation *in vivo*. The staining pattern merges with the localization of CPD sites, suggesting a specific role for p48 in the removal of CPD sites from the non-transcribed strand (Wakasugi, Kawashima et al. 2002).

DDB is not required for the reconstitution of NER *in vitro* (Araujo, Tirode et al. 2000; Wakasugi, Shimizu et al. 2001). However, it directly stimulates the excision of CPDs, but not (6-4) photoproducts in a cell free system, although its affinity for the (6-4) photoproducts is higher (Batty, Rapic'-Otrin et al. 2000; Wakasugi, Shimizu et al. 2001). DDB forms a complex with XPA and RPA, which results in the CPD excision stimulatory effect (Wakasugi, Shimizu et al. 2001; Wakasugi, Kawashima et al. 2002).

This is in agreement with the observation that removal of (6-4) photoproducts by GGR is normal in cells that lack DDB activity (Hwang, Ford et al. 1999). .

Overall, the data suggest that DDB is an accessory protein that facilitates *in vivo* the identification of lesions that are otherwise poorly recognized by the NER machinery (Batty, Rapic'-Otrin et al. 2000; Batty and Wood 2000). Efficient removal of CPDs *in vivo* requires also the presence of the other two XP factors involved in damage recognition during GGR (Sugasawa, Ng et al. 1998; Emmert, Kobayashi et al. 2000; Kusumoto, Masutani et al. 2001; Sugasawa, Okamoto et al. 2001).

The XPC-hHR23B Protein Complex

XP group C (XP-C) is the most common complementation group in Europe, North Africa and the United States (Friedberg, Cheo et al. 1999). *Legerski et al* first cloned the human *XPC* gene in 1992 (Legerski and Peterson 1992). It contains 823 amino acids and shares limited homology with the product of the yeast DNA repair gene *RAD4.* The mouse cDNA is 75% identical to the human with higher conservation in the C terminus. XPC is constitutively expressed in various tissues, with higher expression levels in liver and kidney (Li, Peterson et al. 1996).

In yeast, the RAD4 protein forms a complex with the *S. cerevisiae* protein RAD23. There are two homologues of RAD23 in humans designated hHR23A and hHR23B, and they both interact with XPC. The XPC-binding domain is well conserved between the homologues, and both proteins have comparable affinity for XPC *in vitro.* Upon complex formation the repair activity of XPC is stimulated (Sugasawa, Ng et al. 1997). XPC mutants that fail to bind the RAD23 homologues also fail to complement *Xpc* mutant cell

lines, showing that the interaction with the RAD23 homologues is important for XPC activity *in vivo* (Li, Lu et al. 1997).

Although *in vitro* there is no significant difference between hHR23A and hHR23B, in living cells most of the XPC molecules are bound to hHR23B (Sugasawa, Ng et al. 1997). The XPC-hHR23B complex has a marked preference for UV-irradiated DNA. It is highly capable to discriminate between damaged and undamaged DNA, based on its ability to recognize regions of distortion in the helix, usually induced by photoproducts, but also ones that are created artificially. For example, the XPC-hHR23B complex binds a bubble structure in DNA, regardless of whether DNA damage is present in that bubble (Sugasawa, Okamoto et al. 2001). Its much higher affinity (~10 fold) for (6-4) photoproducts compared to CPDs is explained by the higher DNA distortion caused by (6-4) photoproducts (Sugasawa, Ng et al. 1998; Batty, Rapic'-Otrin et al. 2000).

Importantly, XPC-hHR23B is necessary for recruitment of TFIIH to damaged DNA both *in vitro* and *in vivo* (Yokoi, Masutani et al. 2000; Volker, Mone et al. 2001). It interacts with the two TFIIH subunits XPB and p62 to initiate the opening of the double stranded DNA around the lesion. Moreover, preincubation of damaged DNA with XPC-hHR23B is sufficient to recruit the entire NER machinery to the lesion, suggesting that the complex is the initiator of the global genomic repair pathway (Sugasawa, Ng et al. 1998; Batty, Rapic'-Otrin et al. 2000). Damaged DNA preincubated with XPC-hHR23B is repaired preferentially compared to DNA preincubated with XPA or XPA and RPA together, supporting the model in which the XPC-hHR23B complex acts before the XPA-RPA complex (Sugasawa, Ng et al. 1998; You, Wang et al. 2002).

The XPA/RPA Protein Complex

XPA protein was the first protein in the XP group to be cloned and demonstrated to have preferential binding to damaged DNA. The *XPA* gene encodes a protein of 273 amino acids with a zinc finger motif, which is required for DNA binding (Asahina, Kuraoka et al. 1994). XPA has a central role in NER. It forms a tight complex with Replication Protein A (RPA), and this interaction is necessary for NER both *in vitro* and *in vivo* (Li, Lu et al. 1995). RPA binds to the zinc binding domain of XPA, leading to increased XPA affinity for double stranded damaged DNA (Li, Lu et al. 1995; Ikegami, Kuraoka et al. 1998). However, compared with the two previous damage recognition proteins DDB and XPC-hHR23B, XPA has a lower affinity for both damaged and un-damaged DNA (Batty, Rapic'-Otrin et al. 2000), and it binds the lesion in a later stage (Evans, Moggs et al. 1997; Sugasawa, Ng et al. 1998; Volker, Mone et al. 2001).

In addition to its ability to recognize various types of helix-distorting DNA lesions (Buschta-Hedayat, Buterin et al. 1999), XPA interacts *in vitro* with many of the proteins that are involved in NER, including DDB (Wakasugi, Shimizu et al. 2001), ERCC1 (Li, Peterson et al. 1995) and TFIIH (Nocentini, Coin et al. 1997). These interactions promote the assembly of the NER complex around the lesion. Moreover, XPA mutant proteins that could bind DNA but had impaired ERCC1 binding failed to complement XP-A cells, indicating that ERCC1 binding is more important than DNA binding for XPA function *in vivo* (Li, Peterson et al. 1995). The available data suggest a dual role for XPA in NER: first, verification that the lesion is an NER substrate and second, organization of the other NER proteins around the lesion, and activation of the two endonucleases to perform dual incision (Sugasawa, Okamoto et al. 2001; Volker, Mone et al. 2001). The requirement for

more than one damage recognition factor during NER contributes to efficient discrimination between lesions that are NER substrates and those that are not.

Assembly of the NER complex

In vitro reconstitution of the NER process requires six core factors: XPC-hHR23B complex, XPA, RPA, TFIIH (core 6 subunits), XPG and ERCC1-XPF (Araujo, Tirode et al. 2000). The order of protein assembly around a lesion is depicted in figure 1. The first complex that binds the damage is XPC-hHR23B, which then recruits TFIIH (Yokoi, Masutani et al. 2000). Mammalian TFIIH includes a 6-subunit core (XPB, XPD, p62, p52, p44 and p34) and three additional components comprising the CDK-activating kinase complex, which is not required for NER *in vitro* (Araujo, Tirode et al. 2000). XPB and XPD are ATP dependent DNA helicases, which are necessary for open complex formation around a promoter during transcription initiation or around a lesion during repair (Evans, Moggs et al. 1997). The first endonuclease that binds the complex is XPG, which is able to bind TFIIH (Iyer, Reagan et al. 1996; Mu, Wakasugi et al. 1997) The XPA-RPA complex binds next with a dual role: guiding the 5' endonuclease ERCC1-XPF to the site of damage via the interaction between XPA and ERCC1 (Li, Elledge et al. 1994; Li, Peterson et al. 1995), and activating XPG to perform 3' cleavage (Volker, Mone et al. 2001). In extracts lacking RPA, XPA or XPG only a limited opening of the double helix can be observed, suggesting that these proteins also take part in stabilizing the NER complex around the lesion (Evans, Moggs et al. 1997; Mu, Wakasugi et al. 1997).

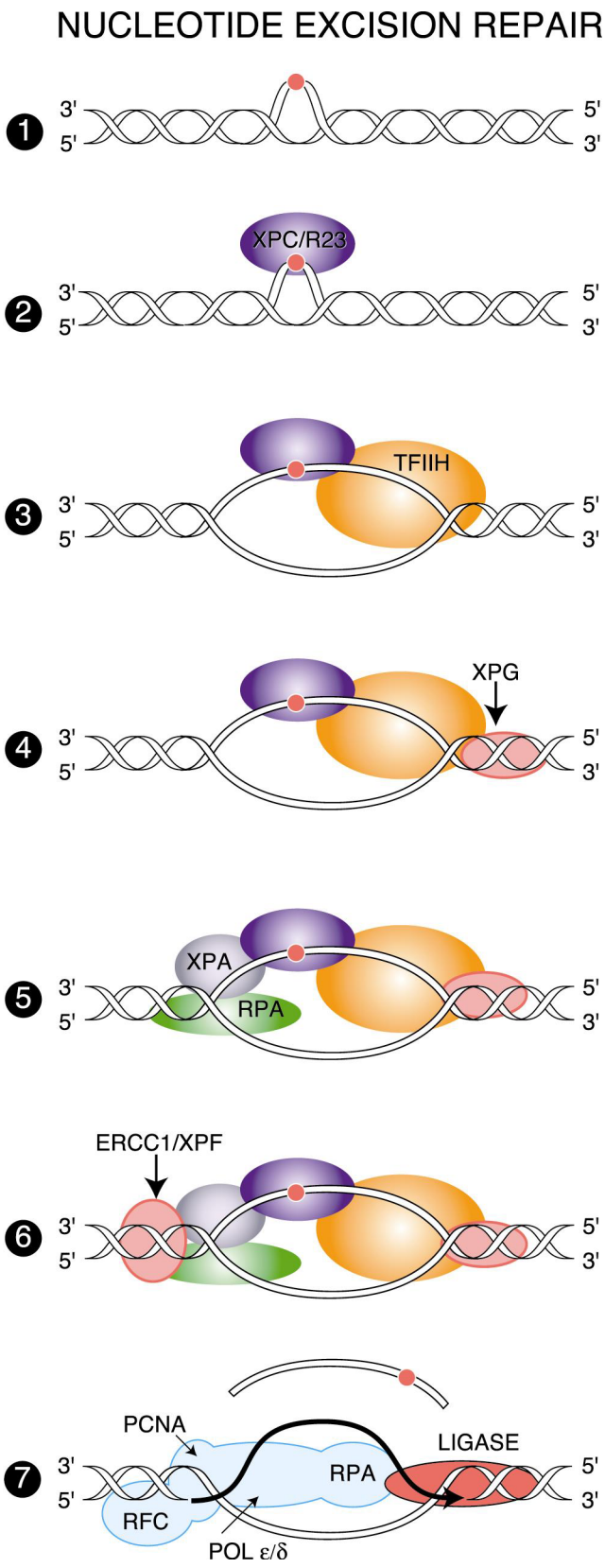
NUCLEOTIDE EXCISION REPAIR
1
3'
5'
5'
3'
2
XPC/R23
3'
5'
5'
3'
3
TFIIH
3'
5'
5'
3'
4
XPG
3'
5'
5'
3'
5
XPA
RPA
3'
5'
5'
3'
6
ERCC1/XPF
3'
5'
5'
3'
7
PCNA
LIGASE
RPA
RFC
POL ε/δ
3'
5'
5'
3'

Figure I-1: Assembly of the NER complex around a DNA lesion During GGR.

The XPC-hHR23B complex recognizes the lesion and binds it. TFIIH is then recruited by XPC followed by the 3' endonuclease XPG, which interacts directly with TFIIH, and the XPA-RPA complex. TFIIH, XPG and XPA-RPA form a stable complex around the lesion. Next the 5' endonuclease ERCC1-XPF complex is recruited to the complex via the interaction between XPA and ERCC1. After the lesion is removed repair synthesis is performed by general replication factors.

The ERCC1-XPF complex, which is the last to be recruited, is not required for opening of the double helix. It can be added to a preformed incision complex, containing all the other factors, to execute incision at the 5' side of the DNA adduct, consistent with its cleavage polarity (Sijbers, de Laat et al. 1996; Evans, Moggs et al. 1997; Mu, Wakasugi et al. 1997; Volker, Mone et al. 2001).

After the removal of the damage containing oligonucleotide by the NER machinery, general replication factors fill the gap and DNA ligase I seals it. The only NER factor that takes part in this step is RPA, which is also involved in replication (Li, Lu et al. 1995; Balajee, May et al. 1998; de Laat, Jaspers et al. 1999).

NER and Cockayne syndrome

Cockayne syndrome (CS) is a rare autosomal-recessive disease that is named after its first describer, E. A. Cockayne (1936) (Friedberg 1996). CS patients suffer from post-natal growth failure and skeletal abnormalities that typically begin within the first year of life. Most of them are also mentally retarded, have impaired sexual development and suffer from a variety of other neurological symptoms and signs. The mean age of death is 12.5 years. Similar to XP patients, CS patients are also hypersensitive to sunlight due to a

defect in NER of transcriptionally-active genes (TCR). However, there is no predisposition to childhood or other cancers apparent in CS patients. They can be divided into two complementation groups, CS-A and CS-B. Both CS-A and CS-B cell lines are proficient in GGR but have no TCR and RNA synthesis is not recovered after UV irradiation, suggesting a role for CS proteins in transcription (Bregman, Halaban et al. 1996; Friedberg 1996; Friedberg 1996; Dianov, Houle et al. 1997; Brosh, Balajee et al. 1999; Citterio, Van Den Boom et al. 2000; Bradsher, Auriol et al. 2002; Kamiuchi, Saijo et al. 2002).

The *Csa* gene encodes a polypeptide of 396 amino acids with a molecular mass of ~44kDa (Henning, Li et al. 1995). The CSA protein belongs to the WD-repeat family of proteins with five consensus WD repeat motifs (Bhatia, Verhage et al. 1996). This family of proteins has a regulatory role in multiple aspects of cellular metabolism including cell cycle, gene regulation and RNA processing. Upon UV irradiation, or hydrogen peroxide treatment, CSA is translocated to the nuclear matrix (Kamiuchi, Saijo et al. 2002), suggesting that it participates in repair of both types of lesions. The translocation is observed in XP-C cells that are TCR proficient and also in XP-A cells, indicating that CSA has a role early in the pathway. It is also colocalizes with RNA polymerase II, which is the damage sensor in TCR. CSA translocation is not observed in CS-B cells.

The *Csb* gene encodes a polypeptide of 1493 amino acids with a molecular mass of ~168kDa. It belongs to the SWI/SNF family of proteins, which is involved in transcription regulation and chromatin remodeling in an ATP-dependent manner (Citterio, Van Den Boom et al. 2000). The protein contains an ATPase domain that is critical for its function. ATPase mutants fail to complement CS-B cell lines for both UV

resistance and recovery of RNA synthesis (Brosh, Balajee et al. 1999; Balajee, Proietti De Santis et al. 2000). The CSB protein interacts with several other repair proteins that are part of the TCR complex: TFIIH (XPB and XPD), XPA and XPG in addition to its interactions with RNA polymerases I and II (Iyer, Reagan et al. 1996; Selby and Sancar 1997; Bradsher, Auriol et al. 2002). The data strongly suggest that CSB has a general role in transcription that is not limited to NER.

A small group of CS patients exhibits also clinical features of XP. These patients have mutations in the NER proteins XPB, XPD and XPG. As mentioned above, all three proteins interact with CSB protein, underlining a connection between them. XPB and XPD are the two DNA helicases that are part of TFIIH. XP-D/CS patients have XPD mutations that appear to be in different locations than those found in patients with only XP group D (Nouspikel, Lalle et al. 1997; Queille, Drougard et al. 2001). XPG is the structure specific endonuclease that nicks damaged DNA 3' to the lesion during NER. XP group G patients produce a full-length protein with no NER activity while XP-G/CS patients produce a severely truncated form of the protein (Nouspikel, Lalle et al. 1997). It appears that some mutations only inactivate the NER activity, resulting in a classical XP phenotype while others generate the more severe XP/CS phenotype. That reflects possible dual roles of the XPD and XPG proteins. In the case of XPB only the severe form is observed.

Cells from all CS patients have defective TC-NER. However, it is difficult to reconcile a defect in a sub-pathway of NER with the distinct clinical features of CS compared to XP. All 5 proteins involved in the disease have been shown to participate in cellular processes other than NER including transcription and chromatin remodeling

(Henning, Li et al. 1995; Bregman, Halaban et al. 1996; Dianov, Houle et al. 1997; Evans, Moggs et al. 1997; Brosh, Balajee et al. 1999; Klungland, Hoss et al. 1999; Balajee, Proietti De Santis et al. 2000; Citterio, Van Den Boom et al. 2000; Le Page, Kwoh et al. 2000; Queille, Drougard et al. 2001; Bradsher, Auriol et al. 2002; Kamiuchi, Saijo et al. 2002). In particular, Le page et al propose a different feature common to CS cells (Le Page, Kwoh et al. 2000). Spontaneous oxidative damage is generated during normal cellular metabolism and especially when cells are proliferating, for example during development. The main pathway involved in repair of oxidative damage is BER. Transcriptionally active cells become a target for oxidative damage when their TCR is defective. Le page et al show that XP-B/CS, XP-D/CS, XP-G/CS and CS-B cells are defective in TCR of oxidative damage, specifically of thymine glycol, a BER substrate, while XP-D and XP-G cells do not have this defect. RNA polymerase II is not only stalled in the non-repaired lesion but its release from the damaged DNA is also much slower. Therefore the actual number of active molecules is reduced, leading to a general transcription inhibition.

Reduced RNA polymerase II activity in extracts of CS cells was previously reported (Dianov, Houle et al. 1997) and all five proteins involved in CS are implicated in transcription (Iyer, Reagan et al. 1996; Dianov, Houle et al. 1997; Evans, Moggs et al. 1997; Bradsher, Auriol et al. 2002; Kamiuchi, Saijo et al. 2002). Taken together, the data is consistent with a model suggesting that defects in transcription, including transcription coupled repair lead to the CS phenotype. The repair pathway primarily involved in oxidative damage is BER. Thus, it is suggested that TC-BER rather than TC-NER is mostly affected, and TC-NER has a secondary affect. Repair deficiency of oxidative

damage in the brain during embryogenesis, leading to decreased neurogenesis and enhanced apoptosis might be the main cause for the many developmental problems observed in CS patients (Murai, Enokido et al. 2001).

NER, apoptosis and predisposition to cancer

DNA repair is an early cellular response to DNA damage. Shortly after UV irradiation the NER recognition factors accumulate at damaged sites to initiate GGR (Sugasawa, Ng et al. 1998; Wakasugi, Kawashima et al. 2002). The TCR pathway is activated by DNA lesions that stall RNA polymerase II (Donahue, Yin et al. 1994). Failure to repair those lesions located in transcriptionally-active regions as a result of too much damage, or due to TCR deficiency leads to transcription inhibition that triggers the apoptotic process, usually in the tumor suppressor gene p53-dependent manner (Balajee, Proietti De Santis et al. 2000; van Oosten, Rebel et al. 2000; Brash, Wikonkal et al. 2001; Lu, Lian et al. 2001; Wijnhoven, Kool et al. 2001).

p53 protein also activates the GGR pathway in response to UV irradiation. It does so by up regulating expression of the NER recognition factors that initiate the process (Hwang, Ford et al. 1999; Adimoolam and Ford 2002). Cells that are p53-deficient have proficient TCR but impaired GGR (Hwang, Ford et al. 1999; Wani, Zhu et al. 1999; El-Mahdy, Hamada et al. 2000; Wani, Zhu et al. 2000). Importantly, GGR deficiency does not lead to enhanced apoptosis, indicating that the signal for the apoptotic process comes solely from lesions in transcriptionally active regions of DNA (Brash, Wikonkal et al. 2001).

TCR deficiency in the skin is associated with higher sensitivity to sunburn, epidermal thickening and development of benign papillomas in hairless mice (Berg, Rebel et al. 2000). However, the increased mutation rate that eventually lead to cancer is balanced by the enhanced apoptotic response that is also associated with the absence of TCR.

On the other hand, increased mutation rate as a result of GGR deficiency is not balanced by an enhanced apoptotic response. Therefore it was suggested that GGR-deficient cells would be more susceptible to transformation compared to TCR-deficient cells. A direct comparison between CSB-deficient mice that have no TCR and XPC-deficient mice that lack only GGR shows that the latter mice are indeed more sensitive to UV-induced skin cancer (Berg, Rebel et al. 2000). TCR deficiency makes CSB-deficient mice and XPA-deficient mice more sensitive to UV and dimethylbenz [α] anthracene (DMBA)-induced apoptosis compared to TCR-proficient XPC mice (van Oosten, Rebel et al. 2000; Wijnhoven, Kool et al. 2001).

This observation has led to a model in which TCR deficiency alone should not result in a particularly high predisposition to cancer. Although DNA damage is not fully repaired, resulting in accumulation of mutations in CS cells, there are less transformed cells due to the increased apoptotic response. This model can explain why CS patients, who suffer exclusively from TCR deficiency, are not predisposed to skin cancer, although they have other more severe clinical features.

In contrast to humans, mice have efficient GGR of 6-4 photoproducts but not of CPDs. Since the GGR pathway appears to be more important to protect against skin cancer than TCR, the differences in the efficiency levels of GGR between mouse and man might explain why CS mouse models suffer from skin cancer while the patients do

not (van der Horst, van Steeg et al. 1997). The balance between enhanced apoptosis and increased mutational rate due to repair deficiency might be altered in mice compared to humans, leading eventually to a higher number of tumors in the mouse. In addition, CS mouse models are chronically exposed to UV irradiation in order to induce skin cancer while the very sick CS individuals are rarely exposed to sunlight. Their average death age is only 4 years higher than the average age for onset on skin cancer in XP patients (Friedberg 1996; Friedberg 2001).

The tumor suppressor gene p53

The tumor suppressor gene *p53* has a pivotal role in protecting cells against carcinogenesis. The gene and its protein product became the focus of a very intensive study when it became clear that *p53* is the most commonly mutated gene in human tumors (Levine 1997). In addition, *p53* knockout mice developed tumors early in life, demonstrating further the importance of loss of wild-type p53 function in tumor formation (Donehower, Harvey et al. 1992; Jacks, Remington et al. 1994). P53 is involved in many different cellular processes, including cell cycle progression, apoptosis and DNA repair. It is activated by different types of cellular stress (DNA damage, hypoxia, introduction of oncogenes, nucleotide deprivation to name a few), and functions as a "guardian" of genomic stability. The outcome of its activation can be either cell cycle arrest or apoptosis. Thus, loss of control by p53 provides a good explanation for tumor development both in humans and in mouse models.

The p53 protein contains 393 amino acids and has been divided structurally and functionally into four domains that are depicted in figure 2 (Ko and Prives 1996; Levine

1997): *(i)* The transcriptional activation domain is located in the N-terminus of the protein. It is 42 amino acids long and is important for p53 interaction with proteins from the basal transcriptional machinery including the TFIIH subunits XPB, XPD and p62 (Murai, Enokido et al. 2001). It contains several phosphorylation sites (serine 15, 20, 33, 37) that appear to be involved in p53 regulation (Steegenga, van der Eb et al. 1996; Shieh, Ikeda et al. 1997; Sakaguchi, Herrera et al. 1998; Shieh, Taya et al. 1999; Unger, Juven-Gershon et al. 1999). This region is highly conserved, and amino acids13-23 are identical in a number of diverse species and in p53 related proteins.

(ii) The sequence-specific DNA-binding domain of p53 is localized between amino acids 102-292. Specific amino acids in this domain either contact the phosphate backbone (K120, S241, R273, A276, and R283) or interact via hydrogen bonds with the DNA bases (K120, C277, R280, and R248). The vast majority of p53 missense mutations in tumors are clustered within this domain, particularly within the four highly conserved regions. Each of the residues that are mutated most frequently in cancer patients makes critical contributions to sequence specific DNA binding. In most cases the mutations result in mutant proteins that can no longer bind DNA and act as transcription factors (Flaman, Robert et al. 1998; Blandino, Levine et al. 1999; Aurelio, Kong et al. 2000)

(iii) The tetramerization domain. Native p53 protein binds DNA as a tetramer, and the region required to form the tetramer is located between amino acids 324-355.

(iv) Non-specific DNA interaction domain. The C-terminal 26 amino acids domain of p53 can function as an autonomous domain capable of binding nonspecifically to different forms of DNA, including damaged DNA and single strands of DNA or RNA. Its

main function is to regulate the ability of p53 to bind specific DNA sequences at its central domain (Avantaggiati, Ogryzko et al. 1997; Zhu, Wani et al. 2001).

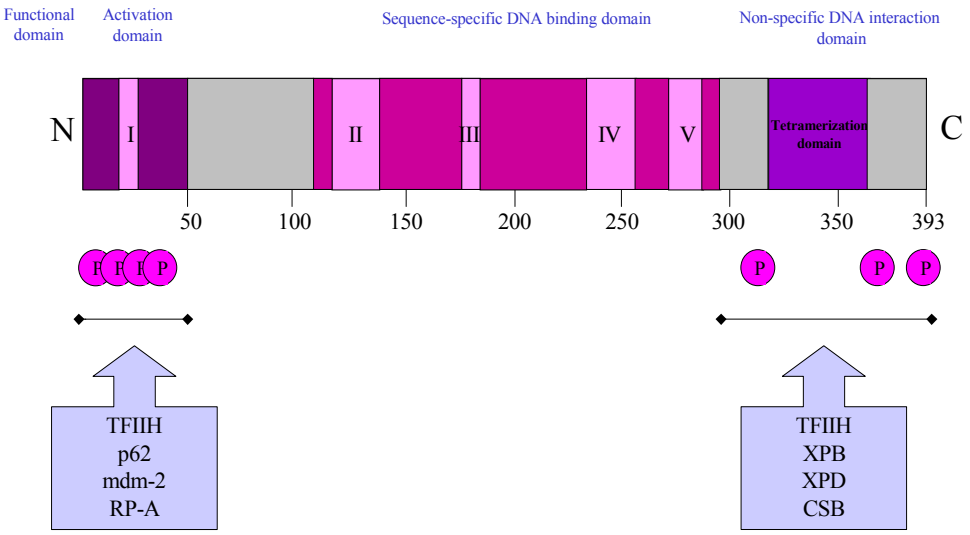

Figure I-2: Structural organization of the p53 protein.

Light purple boxes represent conserved regions. Major phosphorylation sites and relevant protein interactions for each domain are also indicated.

P53 is post-translationally modified by phosphorylation, acetylation and glycosylation (Avantaggiati, Ogryzko et al. 1997; Sakaguchi, Herrera et al. 1998; Zhu, Wani et al. 2001). The possible phosphorylation sites of p53 are located mainly in its N-terminus and C-terminus domains (see above). Importantly, both phosphorylation and acetylation of p53 *in vivo* are regulated by DNA damage (Kobet, Zeng et al. 2000). Both UV and ionizing radiation (IR) induce phosphorylation at Ser15, Ser33 and Ser37.

Phosphorylation on Ser15 and Ser37 stabilizes p53 by disrupting its interaction with the MDM2 protein, an interaction that leads to p53 degradation (Haupt, Maya et al. 1997; Shieh, Ikeda et al. 1997). UV light also induces phosphorylation of Ser392. Acetylation at lysine 382 is induced upon DNA damage and is regulated by N-terminal phosphorylation (Sakaguchi, Herrera et al. 1998).

P53 and Repair pathways

P53 participates in several repair pathways including NER and BER (Bernstein, Bernstein et al. 2002). The role of p53 in NER is separate from its role in cell cycle regulation. p53-dependent cell cycle arrest is mediated mainly by the CDK inhibitor p21 (el-Deiry, Tokino et al. 1993). However p21 deficient cells have normal NER, indicating that NER does not require p21 or other important cell cycle regulators like pRb (Smith, Ford et al. 2000; Zhu, Wani et al. 2000; Wani, El-Mahdy et al. 2002; Wani, Wani et al. 2002). Moreover, the cellular sensitivity to UV irradiation, but not to IR is affected by p53 status in the cell, pointing to an affect on repair of UV induced lesions specifically (Smith, Chen et al. 1995).

In response to DNA damage, p53 interacts directly with three subunits of TFIIH, XPB, XPD and p62, and those interactions are important both for NER and UV induced apoptosis (Wang, Yeh et al. 1995; Zhu, Wani et al. 2000). P53 also interacts with the CSB protein that is required for TCR (Wang, Yeh et al. 1995). P53 deficiency results in significantly reduced GGR (Wani, Zhu et al. 1999; Wani, Zhu et al. 2000; Zhu, Wani et al. 2000).

Three key proteins involved in GGR are up regulated by p53 in response to DNA damage. XPC is induced upon UV irradiation in a p53 dependent manner (Adimoolam and Ford 2002). The promoter region of XPC contains a putative p53 response element that is bound by recombinant p53 *in vitro*. The p48 subunit of the DDB protein that is mutated in a subset of the XP-E patients is activated in response to UV-induced DNA damage and its activation requires p53 (Hwang, Ford et al. 1999). The regulation of two damage recognition factors of GGR by p53 places the protein early in the cellular response to damage. It activates cell cycle arrest allowing time for repair, and independently activates the proteins that are the initiators of the repair process.

A third protein that is activated by p53 and is involved in GGR is GADD45 (Smith, Ford et al. 2000). Cells deficient in GADD45 have a more pronounced defect in GGR of both CPDs and (6-4) photoproducts compared to p53 deficient cells. GADD45 interacts with chromatin and can affect chromatin remodeling of damaged plasmid templates, suggesting a role in coupling NER to chromatin assembly.

On the other hand, p53 expression levels in response to UV irradiation are influenced by the presence or absence of NER proteins. In XPA, XPD or CSB deficient cells where there is no TCR, DNA lesions accumulate on the transcribed strand resulting in blockage of RNA polymerase II and activation of p53 dependent apoptosis. Since TCR deficient cells are highly sensitive to UV, induction of p53 is observed at UV doses that are 10-25 fold lower than the doses required for p53 induction in wild type cells or in TCR proficient XPC cells (Ananthaswamy, Ouhtit et al. 1999; Balajee, Proietti De Santis et al. 2000; Brash, Wikonkal et al. 2001; Queille, Drougard et al. 2001). p53 induction is followed by activation of MDM2, which balances p53 activity by promoting its

degradation (Haupt, Maya et al. 1997). However, treatment with higher doses of UV, which is sufficient to induce p53 and activate MDM2 in wild type and XPC deficient cells, results in suppression of MDM2 and stabilization of p53 in the UV sensitive genotypes (Zhu, Wani et al. 2000; Brash, Wikonkal et al. 2001).

P53 is also involved in damage induced BER: It interacts directly with APE/HAP/Ref-1 which is the major 5' AP endonuclease participating in BER, and with DNA polymerase β to stabilize the interaction between the polymerase and abasic DNA (Gaiddon, Moorthy et al. 1999; Zhou, Ahn et al. 2001).

NER Deficient Mouse Models:

In recent years several mouse models for XP and CS have been developed, in addition to p53 deficient mice (Friedberg and Meira 2000). Targeted gene disruption in mice is a highly useful tool to study the impact of specific gene deficiency, especially in the case of rare genetic diseases such as XP and CS. Although there are some differences in repair capacities between mouse and man, studies with currently available NER deficient mouse models have made major contributions to understanding both fundamental features of NER and the relationship between loss of NER and the predisposition to cancer.

Crossing different mouse models and generating lines deficient in more than one gene allows one to investigate *in vivo* relations between different NER proteins, and between NER proteins and proteins involved in other cellular responses to damage, such as cell cycle arrest and apoptosis. Some knockouts of NER proteins resulted in embryonic lethality, reflecting an additional essential role of these proteins in development. This was the case for the two TFIIH helicases, XPB and XPD. In addition to their role in repair, these proteins are also involved in transcription initiation of genes transcribed by RNA

polymerase II, a fundamental process in the cell. However, more subtle mutations in those genes resulted in viable mice (de Boer, de Wit et al. 1998; de Boer, Donker et al. 1998; Friedberg and Meira 2000).

The NER protein XPG is also implicated in transcription and in BER (Iyer, Reagan et al. 1996; Klungland, Hoss et al. 1999; Le Page, Kwoh et al. 2000). Patients that produce a full-length protein with no NER activity as a result of single amino acid substitution suffer only from XP while patients that produce a severely truncated form of the 3' endonuclease suffer from XP-G/CS (Nouspikel, Lalle et al. 1997). Similarly, an XPG-deficient mouse model producing a truncated form of the protein resulted in retarded mice that die before weaning while a second mouse model producing a longer form of the protein resulted in mice that developed normally. Cells from both XPG mouse models were sensitive to UV and had defective NER (Harada, Shiomi et al. 1999; Friedberg and Meira 2000).

The XPF-ERCC1 complex is the endonuclease that nicks damaged DNA 5' to the lesion during NER (Sijbers, de Laat et al. 1996). Mice lacking the ERCC1 protein are severely runted and die before weaning (Weeda, Donker et al. 1997). There are no human patients with mutations in this gene, suggesting it is essential for life.

The XPA mouse model

Two different groups (de Vries, van Oostrom et al. 1995; Nakane, Takeuchi et al. 1995) have independently generated mice deficient in the XPA protein . XPA-deficient mice develop normally, but are highly susceptible to UVB-induced skin and eye cancer and to DMBA-induced skin tumors. No difference was found between XPA heterozygous

animals and wild type littermates. Cells from homozygous mutant mice are totally defective in NER. The XPA deficient mouse model is therefore a very good tool to study NER deficiency and predisposition to cancer.

Further studies with XPA-deficient mice show that the mice are extremely sensitive to UVB-induced sunburns due to accumulation of DNA photoproducts on the transcribed strand (Brash, Wikonkal et al. 2001). At the same UVB dose they have 2-3 fold more sunburn cells compared to wild type controls. In addition, XPA-deficient mice develop lung tumors in an elevated rate in response to treatment with benzo [α] pyrene, which is known to induce bulky DNA lesions that are substrate for NER (Ide, Iida et al. 2000). The data suggest that NER is biologically significant in the prevention of cancer in internal organs as well.

XPA-deficient mice have higher susceptibility to Aflatoxin B1-induced liver tumors (Takahashi, Nakatsuru et al. 2002). Cell culture studies show that wild type cells, but not XP cells, can rapidly remove DNA adducts caused by Aflatoxin B1, suggesting that NER protects against tumorigenesis in the liver. In addition to DNA damage induced tumors, XPA-deficient mice have spontaneous liver tumors and a low incidence of spontaneous lung tumors (Ide, Iida et al. 2000; Takahashi, Nakatsuru et al. 2002). In each of those cases there was no apparent difference between wild type and heterozygous mice, thus gene dosage effects were not manifested. This is in agreement with cell studies showing that low expression of XPA protein is sufficient for total complementation of UV sensitivity and repair activity. XPA over expression does not increase UV resistance, suggesting all together that XPA concentration is not a limiting factor for repair (Kobayashi, Takeuchi et al. 1998; Muotri, Marchetto et al. 2002).

The CSA and CSB mouse models

Both CSA and CSB deficient mouse models have proficient GGR but no TCR (van der Horst, van Steeg et al. 1997; van der Horst, Meira et al. 2002). CSA and CSB deficient mice develop normally and do not exhibit the severe neurological problems observed in CS patients. In contrast to the patients, both CSA and CSB mouse models are predisposed to UV-induced skin cancer. TCR deficiency is associated with increased mutational rate that can eventually lead to cancer, and in the same time with enhanced apoptotic response, which has a tumor suppression effect. One could speculate that CS mice balance apoptosis and cancer differently than patients. A dominating apoptotic response, leading to decreased neurogenesis in the brain during embryogenesis, might be the main cause for developmental abnormalities observed in CS patients. In the same time it could also explain the abscense of tumors. On the other hand, a stronger survival signal in the mouse might be the reason for both normal development and predisposition to cancer.

Cells from CSA deficient mice mimic the phenotype of human CS-A cells. They are UV sensitive due to their TCR defect. They show normal unscheduled DNA synthesis indicating they have normal GGR, and RNA synthesis is not recovered after UV irradiation (van der Horst, Meira et al. 2002).

CSB-deficient mice are highly sensitive to UV induced apoptosis and to the appearance of UVB-induced sunburn cells. With only TCR deficiency CSB mice induce sunburn cells with a dose response almost identical to the XPA deficient mice that have no NER, indicating that the lesions in the actively transcribed strand initiate the signal for

UVB-induced apoptosis (Brash, Wikonkal et al. 2001). CSB deficient cells are also UV sensitive due to defective TCR, and display elevated expression of p53 resulting in enhanced UV-induced apoptosis (Balajee, Proietti De Santis et al. 2000). The signal is far lower in cells that are both CSB and p53 deficient, confirming that the apoptotic signal is mainly p53 dependent (Lu, Lian et al. 2001). CSB deficient cells are also hypersensitive to ionizing irradiation and to paraquat, indicating that TCR protects the cells also from oxidative damage (de Waard, de Wit et al. 2003).

Mice deficient in both the CSA and CSB proteins have been generated. The double knockout mice are viable and develop normally with no severe neurological problems (Friedberg and Meira 2000). However, the mild neurological phenotype in the CSB deficient mice can be enhanced significantly when they are crossed with XPA deficient mice. Mice deficient in both CSB and XPA proteins display growth retardation and abnormal behavior. A phenotype similar to the one observed in both XP group A and CS patients (Murai, Enokido et al. 2001). Overall, the phenotype for these mice resembles strongly to the phenotype for mice deficient in XPG, a protein that is implicated both in XP and CS (Iyer, Reagan et al. 1996; Nouspikel, Lalle et al. 1997; Harada, Shiomi et al. 1999; Le Page, Kwoh et al. 2000). Similar phenotypes were observed also when crossing CSB or CSA deficient mice with mice deficient in the XPC protein (Friedberg and Meira 2000; van der Horst, Meira et al. 2002).

The XPC mouse model

Mice deficient in the XPC protein were previously generated in our lab using a targeting vector that resulted in a deletion of exon 10 of the gene (Cheo, Ruven et al.

1997). XPC deficient mice develop normally but are predisposed to UVB-induced skin cancer. The mice are also highly predisposed to lung and liver tumors following treatment with AAF, which induces bulky guanine adducts that are substrates for NER (Cheo, Burns et al. 1999). Predisposition to UV-induced skin cancer was observed also in *Xpc* heterozygous mice (Cheo, Meira et al. 2000) After 18 weeks of daily UV irradiation, the latency period for the appearance of skin cancer in 50% of the mice is 50 weeks for heterozygous mice compared to 18 weeks for homozygous mutant mice and 92 weeks for wild type littermate controls.

Similarly to human XP-C cell lines, cells from $Xpc^{-/-}$ mice are sensitive to killing by UV irradiation and exhibit reduced levels of repair synthesis following UV treatment. As expected, the cells are capable of removing (6-4) photoproducts from the transcribed, but not from the nontranscribed strand of a transcriptionally-active gene, indicating that the TCR pathway is intact (Cheo, Ruven et al. 1997). Cells from $Xpc^{-/-}$ mice are also more resistant to p53-dependent UV induced apoptosis compared to TCR-deficient lines such as XPA, XPD and CSB (Ananthaswamy, Ouhtit et al. 1999; Balajee, Proietti De Santis et al. 2000; Queille, Drougard et al. 2001). In fact, $Xpc^{-/-}$ cells show wild type sensitivity to p53-induced apoptosis, since GGR acts downstream to p53 and does not affect p53 induction or p53 induced apoptosis. Reduced apoptotic response can also contribute to the elevated amount of cells with spontaneous DNA mutations in aging XPC deficient mice compared with XPA and CSB deficient mice (Wijnhoven, Kool et al. 2000). Crossing the XPC deficient mice with the XPA deficient line resulted in mice that developed normally and cells showing XPA like UV sensitivity with no NER activity (Friedberg and Meira 2000).

P53 Mutational spectrum in UV-induced skin tumors from XPC deficient mice

Two independent groups (Donehower, Harvey et al. 1992; Jacks, Remington et al. 1994) have generated p53 deficient mouse models. Tyler Jacks (Jacks, Remington et al. 1994) deleted exons 2-6 of the *p53* gene while the Bradley mouse model was created by deleting exon 5 (Donehower, Harvey et al. 1992). In order to test the effect of p53 on tumor formation in XPC deficient ($Xpc^{-/-}$) mice, Jacks mice were crossed with $Xpc^{-/-}$ mice to generate all possible genotypic combinations of *Xpc* and *p53* (Cheo, Meira et al. 1996).

Both $Xpc^{-/-}$ and $Xpc^{+/-}$ mice suffered an enhanced predisposition to UVB-induced skin cancer when the p53 alleles were also defective. Skin tumors in $Xpc^{-/-}p53^{+/-}$ mice appear as early as 9 weeks after the first exposure to UVB light (Cheo, Meira et al. 1996). Thus, it appears that the absence of both XPC and p53 proteins has an additive (or synergistic) effect in the process of tumor formation. The data suggested that the enhanced cancer predisposition in *p53* heterozygous ($p53^{+/-}$) mice is a result of inactivation of the remaining allele following exposure to UV light. Therefore, skin tumors have been collected from the mice and the p53 gene has been sequenced. In agreement with previous studies, sequencing p53 revealed mutations in close to 90% of the tumors. In the $Xpc^{+/+}$ animals the mutational spectrum included mainly classical hot spots located in various dipyrimydine sites in exons 5-8. However, the mutational spectrum of p53 in the $Xpc^{-/-}$ $p53^{+/-}$ animals was significantly different from the one in the $Xpc^{+/+}$ animals. Strikingly, ~ 60% of the mutations in the *p53* allele involved a novel hot spot at codon 122, which is the last codon of exon 4. The original Threonine (T) residue was substituted with either Methionine (M) as a result of a single C→T transition, or Leucine (L) as a result of a double mutation AC→TT or AC→CT (Reis, Cheo et al. 2000).

Significance and Aims of Thesis

Codon 122 is a non-dipyrimidine site located in a highly conserved region of the p53 protein, which belongs to the sequence-specific DNA-binding domain (localized between amino acids 102-292). Mutations in this domain result frequently in p53 proteins that can no longer bind DNA and act as transcription factors (Levine 1997).

Mutations in this codon, or the corresponding human codon 125 have never been reported in either mouse or human cancers of any type. UV-induced mutations appear mainly in dipyrimidine sites resulting in CPDs or (6-4) photoproducts, which are repaired by NER. Codon 122 contains a pyrimidine flanked by two purines, therefore it is unclear why it is mutated in such high frequency or what type of damage leads to mutations in this codon.

Several non-exclusive mechanisms might provide explanations for selection of mutations in codon 122 in skin tumors from $Xpc^{-/-}$ $p53^{+/-}$ mice. First, codon 122 may be a hot spot for DNA damage and/or mutagenesis associated with UVB radiation exposure that requires XPC protein specifically, or NER in general for its repair. Guanine residues located 3′ to cytosine residues such as in codon 122 create CpG sites. The cytosines on those sites in the *p53* gene are methylated and therefore may undergo spontaneous deamination to 5-methyl uracil that generates C→T mutations (Denissenko, Chen et al. 1997; Tu, Dammann et al. 1998). It was recently reported that the XPC protein is involved in the repair of G:T mispairs (which are not NER substrates) via its interaction with thymine-DNA glycosylase (Friedberg 2003). These findings suggest that the absence of XPC protein may enhance the rate of mutations in codons containing CpG

sites including codon 122, and are in agreement with the general notion that XPC protein may participate in DNA repair pathways other than conventional NER.

Selection for mutations in codon 122 may also operate at the level of gene product, i.e., cells expressing the mutant protein ($p53^{T122}$) may have a growth advantage. The mutant is probably recessive since it appears almost exclusively when there is no wild-type p53 protein functioning in the cells. In two cases in which the T122 mutation was found in tumors from *Xpc*$^{-/-}$ *p53*$^{+/+}$ mice, it was demonstrated that the other *p53* allele was also inactivated (Reis, Cheo et al. 2000).

The main goal of this dissertation is to characterize the T122L mutation and its protein product and to elucidate the mechanism(s) that affect its appearance in XPC deficient skin. In chapter II I have characterized the T122L mutation. I have shown that it is a UV specific mutation that appears in both tumor and non-tumor skin cells from XPC-deficient mice but rarely in cells from other NER-deficient mouse models such as XPA and CSA mice. In chapter III I investigated whether cells expressing the mutant protein ($p53^{T122}$) have a growth advantage that selects for cancer. I showed that the mutant protein has altered transactivation functions compared to wild type protein, which might give a growth advantage to cells that express it, especially in the XPC-deficient background. Chapter IV contains studies that compare the mutant protein activity in wild type and *Xpc*$^{-/-}$ cells and preliminary microarray results, indicating that the absence of XPC might have consequences that are unrelated to NER. Chapter V includes summary and concluding remarks.

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Chapter II: T122L is a UV-specific *p53* mutation that appears in XPC deficient skin.

Introduction

Xeroderma Pigmentosum (XP) is a rare human disorder caused by inherited defects in nucleotide excision repair (NER) of DNA. The proteins involved in NER are responsible for recognizing and removing various types of bulky lesions in DNA including those caused by the UV component of sunlight. XP patients have mutations in the genes encoding these proteins and therefore are extremely sensitive to sunlight and have a profound predisposition to various skin cancers. They can be divided into seven complementation groups (A-G) with genes corresponding *Xpa* thru *Xpg* (Friedberg, Cheo et al. 1999).

The NER process includes three major steps: damage recognition, dual incision and repair synthesis (de Laat, Jaspers et al. 1999; Batty and Wood 2000). There are several damage recognition factors available depending on the location of the lesion in the DNA. If the lesion is located in a transcriptionally active region, it can block the transcription machinery and the blockage of RNA polymerase II is believed to serve as the DNA damage sensor (Donahue, Yin et al. 1994). This specific mode of NER is called Transcription Coupled Repair (TCR). If the damage is located in transcriptionally-silent regions of the genome or the non- transcribed strand of transcriptionally-active genes it is recognized by the XPC protein in a mode of NER called Global Genomic Repair (GGR) (Sugasawa, Ng et al. 1998; de Laat, Jaspers et al. 1999). In the cell, most of the XPC molecules are bound to a human homologue of the yeast RAD23 gene, hHR23B

(Sugasawa, Ng et al. 1997). The XPC-hHR23B complex binds damaged DNA in a preferential manner based on its ability to recognize regions of distortion in the helix. Then, it recruits the rest of the NER machinery to the site of damage (Sugasawa, Ng et al. 1998; Batty, Rapic'-Otrin et al. 2000; Yokoi, Masutani et al. 2000; Sugasawa, Okamoto et al. 2001). Five other factors are required to complete the incision and release of a 24-32 nucleotide long damage-containing oligonucleotide: XPA, RPA, TFIIH, XPG and XPF-ERCC1 (Araujo, Tirode et al. 2000). XPA and RPA form a complex that is required to verify the presence of damage and to orient other NER components around the lesion (Li, Lu et al. 1995; Li, Peterson et al. 1995; Nocentini, Coin et al. 1997; Sugasawa, Ng et al. 1998). TFIIH contains two DNA helicases with opposite polarities, XPB and XPD that are required to unwind the double helix (Evans, Moggs et al. 1997). XPG and XPF-ERCC1 are the 3' and 5' endonucleases that cut DNA around the lesion (O'Donovan, Davies et al. 1994; Sijbers, de Laat et al. 1996). The last step of repair synthesis is executed by general replication factors that fill the remaining gap and DNA ligase seals it (de Laat, Jaspers et al. 1999).

Defects in NER are observed also in patients that suffer from another genetically inherited disorder named Cockayne syndrome (CS) after its first describer (Friedberg 1996). CS patients also suffer from photosensitivity but with no predisposition to skin cancer. They have neurological and developmental problems that are less common in XP patients. The patients can be divided into two complementation groups with corresponding genes *Csa* and *Csb*. Cells from CS patients are GGR proficient, suggesting that the CS genes are required specifically for TCR and maybe other transcription related processes.

Several mouse models for both XP and CS, generated by targeted gene disruption, provide informative model systems to study different aspects of NER including cancer predisposition. *Xpc* mutant mice previously generated in our lab are NER deficient and are highly predisposed to UVB-induced skin cancer (Cheo, Meira et al. 1996; Cheo, Ruven et al. 1997; Cheo, Meira et al. 2000).

The tumor suppressor gene *p53* is the most commonly mutated gene in human tumors. The p53 protein is involved in many different cellular processes including cell cycle progression, apoptosis and DNA repair (Levine 1997). In order to assess the effect of p53 on tumor formation in the *Xpc* defective background, *Xpc* mutant mice ($Xpc^{-/-}$) have been crossed with *p53* mutant mice ($p53^{-/-}$).

We previously reported that both $Xpc^{-/-}$ and $Xpc^{+/-}$ mice suffer an enhanced predisposition to UVB-induced skin cancer when one or both *p53* alleles are also defective. Thus, it appears that the absence of the XPC protein has an additive (or synergistic) effect to the absence of the p53 protein in the process of tumor formation (Cheo, Meira et al. 1996). The data suggest that the enhanced cancer predisposition in $p53^{+/-}$ mice is a result of inactivation of the remaining allele following exposure to UV light. Therefore, skin tumors have been collected from UV exposed mice and the *p53* gene has been sequenced. In $Xpc^{-/-}$ $p53^{+/-}$ animals, ~60% of the mutations in the *p53* allele involved a novel, non-dipyrimidine codon, at the end of exon 4. The original Threonine (T) residue at amino acid position 122 was substituted with either Methionine (M) as a result of a single C->T transition, or Leucine (L) as a result of a double mutation AC->TT or AC->CT (Reis, Cheo et al. 2000).

Selection for mutations at codon 122 could conceivably operate through several non-exclusive mechanisms. Codon 122 may be a hot spot for DNA damage and/or mutagenesis associated with UVB radiation exposure that requires XPC protein specifically, or NER in general for repair. Selection may also operate at the level of the gene product, i.e., cells expressing the mutant protein ($p53^{T122}$) may have a growth advantage, leading to $p53^{T122}$-dependent clonal expansion.

In order to determine whether the absence of other NER proteins results in the appearance of the same mutation, we sequenced the *p53* gene in UVB-induced skin tumors in two other NER-deficient mouse models, $Xpa^{-/-}p53^{+/-}$ mice and $Csa^{-/-}p53^{+/-}$ mice. Both of these mouse models are also predisposed to skin cancer (de Vries, van Oostrom et al. 1995; Nakane, Takeuchi et al. 1995; van der Horst, Meira et al. 2002) The results show that mutations in codon 122 occur rarely in tumors from these genotypes. Therefore, either the XPC protein is required specifically for damage repair in this codon, or $Xpc^{-/-}$ cells that express the p53 mutant protein have a growth advantage.

In order to determine whether the p53 mutant protein provides an advantage to cells that express it, we made use of two different models of p53 deficient ($p53^{-/-}$) mice. The experiments described above were performed using the Jacks $p53^{-/-}$ mouse model in which exons 2-6 (which include codon 122 at the end of exon 4) of the *p53* gene were deleted by homologous recombination (Jacks, Remington et al. 1994). On the other hand, in the Bradley $p53^{-/-}$ mouse model only exon 5 of the *p53* gene was deleted and therefore the region containing codon 122 in the genomic DNA is still intact (Donehower, Harvey et al. 1992). This second $p53^{-/-}$ mouse model allowed us to examine the T122L mutation in cells that do not express protein product.

We have developed a Southern-based mutation detection assay that was used to screen non-tumor skin samples, irradiated for short time periods, for the T122L mutation. We have compared the mutation frequency in skin samples from *Xpc*$^{-/-}$*p53*$^{+/-}$ mice, which express p53 protein, and skin samples from *Xpc*$^{-/-}$*p53*$^{-/-}$ Bradley mice that do not produce p53 protein at all. In *Xpc*$^{-/-}$*p53*$^{+/-}$ mice, with one wild type allele that expresses protein, we were able to detect the T122L mutation in skin samples irradiated for 5 weeks in frequencies similar to those observed in skin tumors. Moreover, we detected the mutation in DNA extracted from skin cells as early as 2 weeks after exposure to UV radiation, well before tumors could be detected. These findings are consistent with a direct role of the XPC protein in repairing the unidentified damage at codon 122. The mutation could also be detected in skin cells from *Xpc*$^{-/-}$*p53*$^{-/-}$ Bradley mice that do not express p53 protein, indicating that protein function is not required for the appearance of the mutation. However, the unusually high mutation frequency observed in mice that express p53^{T122} mutant protein was significantly reduced in *Xpc*$^{-/-}$*p53*$^{-/-}$ Bradley mice that do not express p53 protein. We therefore conclude that both the initial repair deficiency and p53^{T122} mutant protein expression are important for the accumulation of the T122L mutation in *Xpc*$^{-/-}$ skin cells, leading to its unusual frequency in skin tumors from *Xpc*$^{-/-}$ mice.

Results

Codon T122 is rarely mutated in NER deficient mice that are not Xpc mutants

In order to determine whether the mutation is specific to *Xpc* mutant mice, *Xpa*$^{-/-}$ or *Csa*$^{-/-}$ mice were crossed with the Jacks *p53*$^{-/-}$ mice (in which exons 2-6 of the *p53* gene were deleted) to generate lines that are either *Xpa*$^{-/-}$ *p53*$^{+/-}$ or *Csa*$^{-/-}$*p53*$^{+/-}$. Mice from those two groups, together with a new group of *Xpc*$^{-/-}$ *p53*$^{+/-}$ mice were UVB-irradiated and UV-induced skin tumors were collected. The *p53* gene was then sequenced in all the tumors. A description of the mutations detected in the *Xpc*$^{-/-}$ *p53*$^{+/-}$ control group is presented in Table 1 and those detected in the *Xpa*$^{-/-}$ *p53*$^{+/-}$ or *Csa*$^{-/-}$*p53*$^{+/-}$ mice are shown in Table 2. With regard to mutations in codon 122, 11 out of 13 tumor samples sequenced in the *Xpc*$^{-/-}$ *p53*$^{+/-}$ control group were mutated in this codon while only 1 out of 13 tumors from the *Xpa*$^{-/-}$ *p53*$^{+/-}$ mice and none of the tumors in the *Csa*$^{-/-}$*p53*$^{+/-}$ mice had mutations in this codon.

We have sequenced the coding region of the *Xpc* gene in the one positive tumor from the *Xpa*$^{-/-}$ *p53*$^{+/-}$ background. We failed to detect mutations in this region; however, we cannot exclude the possibility that the XPC protein was inactivated by some other mechanism(s) during tumor development. The same tumor was also mutated at codon 270, a major hot spot that is very often mutated in skin cancer in wild type mice. Mutations in codon 270 dominated the mutational spectrum observed in skin tumors from *Xpa*$^{-/-}$ *p53*$^{+/-}$ and *Csa*$^{-/-}$ *p53*$^{+/-}$ mice. The sequence at this codon combines neighboring pyrimidines that form CPD upon UV irradiation and a 5' methylcytosine, which enhances the rate of the CPD formation (You, Szabo et al. 2000).

Other mutations in tumors from the *Xpc*$^{-/-}$ *p53*$^{+/-}$ group were mostly located at dipyrimidine sites including a mutation in codon 210 that was previously reported in NER-deficient models (Takeuchi, Nakatsu et al. 1998; Reis, Cheo et al. 2000).

***Trp53* Mutations in mouse skin tumors from *Xpc* mutant mice (UV 7 min)**

Case	Mouse No.	Genotype	Mutations in codon T122	Mutations elsewhere in exons 4-8	DNA sequence
1	8985-T1	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes (~20%)	Arg210→Cys	tttCgc→tttTgc
2	8985-T2	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	Val172→Val	gtCgtg→gtTgtg
3	8985-T3	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	No	tgcACg→tgcTTg
4	8987	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	No	tgcACg→tgcTTg
5	8988	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	No	tgcACg→tgcCTg tgcACg→tgcTTg tgcaCg→tgcaTg
6	8990	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes (~50%)	No*	tgcACg→tgcTTg
7	421-T1	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
	4211-cl		no	Pro92→Ser	gtcCct→gtcTct
8	421-T2	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes (~20%)	No	tgcACg→tgcTTg
9	421-T3	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes (~50%)	Val173→Gly	gtcgTg→gtcgGg
	4213-cl		no	Pro247→Leu	cgacCt→cgacTt
10	9131	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
11	9132	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	No	tgcACg→tgcTTg
12	9134	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	No	tgcACg→tgcTTg
13	9136	*Xpc*$^{-/-}$ *Trp53*$^{+/-}$	Yes	No	tgcACg→tgcTTg

Table II-1: p53 mutational spectrum in skin tumors from *Xpc*$^{-/-}$ *p53*$^{+/-}$ mice

Codon 122 is mutated also in non-tumor skin samples from Xpc$^{-/-}$ *p53*$^{+/-}$ *mice*

In order to assess the contribution of p53^{T122}-dependent clonal expansion to the high frequency of T122L mutations in *Xpc*$^{-/-}$ skin we have developed a southern-based assay that allows us to easily detect mutations that appear in non-tumor skin samples with a sensitivity of 1:10^4. Skin samples are collected at different time points after exposure to UVB radiation. Genomic DNA is extracted and used as a template for PCR to amplify a

~0.5Kb fragment containing a part of exon 4 and intron 4 of the *p53* gene including codon 122 which is the last codon in exon 4.

***Trp53* Mutations in mouse skin tumors from *Xpa* and *Csa* mutant mice**

Case	Mouse No.	Genotype	Mutations in codon T122	Mutations elsewhere in exons 4-8	DNA sequence
1	7164-T1	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
2	7164-T2	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
3	7357-T1	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
4	7357-T2	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
5	7425-T1	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	Gly114→Lys Leu127→Phe	tctGGg→tctAAg cccCtc→cccTct
6	7425-T2	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
7	7270	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$		No	
8	7422	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
9	7425-T3	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
10	7443	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	Yes (~20%)	Arg270→Cys	gttCgt→gttTgt
11	7557-T1	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
12	7557-T2	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
13	8460	*Xpa*$^{-/-}$ *Trp53*$^{+/-}$	No	No*	
14	7194	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
15	7300	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
16	7493	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
17	7544	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	
18	7546	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	Deletion, Gly259-Asp265	tccA(gt)…… …..(a)GCttt
19	7571-T1	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	Arg270→Cys	gttCgt→gttTgt
20	7571-T2	*Csa*$^{-/-}$ *Trp53*$^{+/-}$	No	No	

Table II-2: p53 mutational spectrum in tumors from *Xpa*$^{-/-}$ *p53*$^{+/-}$ and *Csa*$^{-/-}$ *p53*$^{+/-}$ mice

The PCR products are digested with *ApaLI* and then transferred to a nylon membrane and hybridized to a radiolabeled oligonucleotide containing the mutated sequence. The *ApaL1* recognition site (GTGCAC) is lost if the T122L mutation (AC→TT) is present. We have performed experiments of this type using UVB-irradiated skin samples from *Xpc* mutant, *Xpa* mutant, or wild type animals. The results presented in Figure 1 show that the T122L mutation can be detected in non-tumor skin samples from *Xpc*$^{-/-}$*p53*$^{+/-}$ mice after 5 weeks of UVB exposure (a total of 20KJ) but not in the non-irradiated

controls or in samples taken from wild type animals. Quantitative controls include the wild type sequence only, and 1:10^3 T122/ wild type or 1:10^5 T122/wild type mixtures.

We have screened skin samples irradiated for 2, 3 or 5 weeks. A summary of the results is presented in Figure 2. A summary of the results from Table 1 (skin tumors) is also presented for comparison. The T122L mutation appears as early as after 2 weeks of UVB exposure (a total of 8KJ). The mutation frequency is increased as the number of weeks of UVB exposure increases. In samples irradiated for 5 weeks we observed mutation frequencies similar to those observed in skin tumors.

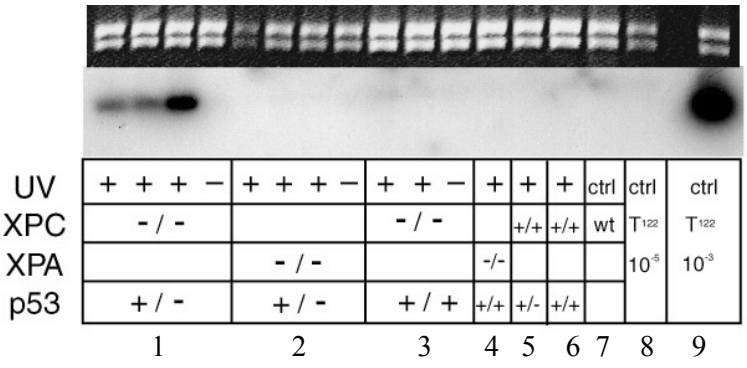

Figure II-1: T122L mutations in non-tumor skin samples

The T122L mutation can be detected in non-tumor skin samples from *Xpc*$^{-/-}$*p53*$^{+/-}$ mice after 5 weeks of UVB exposure (a total of 20KJ) **(group 1)** but not in control samples taken from wild type **(groups 5, 6)** or *Xpa* mutant animals **(groups 2,4)**. Quantitative controls **(groups 7-9)** include the wild type sequence only, 1:10^3 T122/ wild type or 1:10^5 T122/ wild type mixtures.

The early appearance of the mutation and its high frequency in non-tumor samples are consistent with a defect in damage repair at codon 122 in the absence of XPC protein. However, it is still possible that mutations at codon 122 lead to a gain of p53 function that significantly promotes clonal expansion and subsequent tumor development.

We have also irradiated *Xpa*$^{-/-}$ *p53*$^{+/-}$ mice for 2, 3 or 5 weeks and screened skin samples for the T122L mutation. Out of 36 irradiated skin samples we tested none had a signal high enough to have statistically significant difference from the negative wild type control.

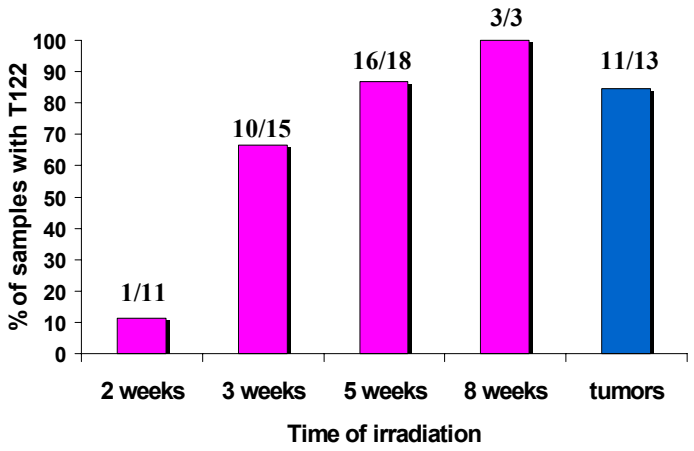

Figure II-2: Accumulation of T122L positive samples in *Xpc*$^{-/-}$*p53*$^{+/-}$ mice as a function of time

The T122L mutation can be detected only in skin samples from *Xpc*$^{-/-}$*p53*$^{+/-}$ mice. The number of positive samples for each time point is shown. The mutation frequency in the *Xpc*$^{-/-}$*p53*$^{+/-}$ skin tumors is shown for comparison.

P53 heterozygous ($p53^{+/-}$) status enhances the frequency of the T122L mutation

We have previously reported that mutations in codon 122 did not appear in UVB-induced skin tumors from *Xpc* mutant mice that were wild type for the *p53* gene ($Xpc^{-/-}p53^{+/+}$), unless the other allele was also inactivated (Reis, Cheo et al. 2000). We have irradiated $Xpc^{-/-}p53^{+/+}$ mice for 5 or 8 weeks. While the frequency of the T122L mutation was ~80% in $Xpc^{-/-}p53^{+/-}$ mice irradiated for 5 weeks, there were no positive samples in the $Xpc^{-/-}p53^{+/+}$ mice irradiated for the same amount of time.

After 8 weeks of irradiation we detected the T122L mutation in $Xpc^{-/-}p53^{+/+}$ mice with reduced frequency compared to $Xpc^{-/-}p53^{+/-}$ mice (data not shown). We cannot exclude the possibility that the other *p53* allele was inactivated in the non-tumor skin cells after 8 weeks of irradiation, leading to *p53* heterozygous status in those samples. Alternatively, the reduced mutation frequency could result from failure of the recessive $p53^{T122}$ mutant protein to promote clonal expansion in the presence of wild type protein. Thus, $p53^{T122}$ protein function contributed to the high mutation frequency in $Xpc^{-/-}p53^{+/-}$ samples. However, the appearance of the mutation in $Xpc^{-/-}p53^{+/+}$ samples suggested that mutant protein function could not be the only mechanism responsible for the unusual frequency of T122L mutations in $Xpc^{-/-}p53^{+/-}$ samples. We have therefore decided to directly test how expression of the protein product affects the original mutation frequency.

The frequency of the T122L mutation is affected by its protein product

The Jacks $p53^{-/-}$ mouse model was generated by deleting exons 2-6 (which include codon 122) of the *p53* gene (Jacks, Remington et al. 1994). Skin cells from $Xpc^{-/-}p53^{+/-}$

mice that were positive for the T122L mutation in our screen also express the mutant protein product, which might contribute to the high mutation frequency we have detected.

In order to investigate the frequency of the T122L mutation in cells that do not express the protein and therefore have no selective growth advantage, we have obtained the Bradley *p53*$^{-/-}$ mouse model in which only exon 5 of the *p53* gene was deleted and therefore the region containing codon 122 in the genomic DNA was still intact (Donehower, Harvey et al. 1992). The homozygous mutant mouse in this model does not express p53 protein as a result of a truncated, unstable mRNA product. These mice were crossed with *Xpc*$^{-/-}$ mice to generate a double knockout line. Non-tumor skin samples from the *Xpc*$^{-/-}$*p53*$^{-/-}$ mice were screened for the T122L mutation using the novel southern-based mutation detection assay described above.

The results presented in Figure 3 show that there is a significant difference between mutation frequencies in the *Xpc*$^{-/-}$*p53*$^{-/-}$ mice, which do not express p53 protein and their *Xpc*$^{-/-}$*p53*$^{+/-}$ littermates controls that have one functional, protein producing allele. In the Bradley *Xpc*$^{-/-}$*p53*$^{+/-}$ control group T122L mutations appear in similar frequencies to those found in the Jacks *Xpc*$^{-/-}$*p53*$^{+/-}$ mice. However, the frequency of the T122L mutation is significantly reduced in the *Xpc*$^{-/-}$*p53*$^{-/-}$ mice, strongly suggesting that the altered p53 protein function has a key role in maintaining this mutation in *Xpc* mutant cells once it has been formed. Importantly, the appearance of the mutation in *Xpc*$^{-/-}$*p53*$^{-/-}$ samples that do not express p53 protein (Figure 3 and data not shown) indicates that the repair deficiency alone is sufficient to generate the mutation in a frequency high enough to be detected by our Southern-based assay.

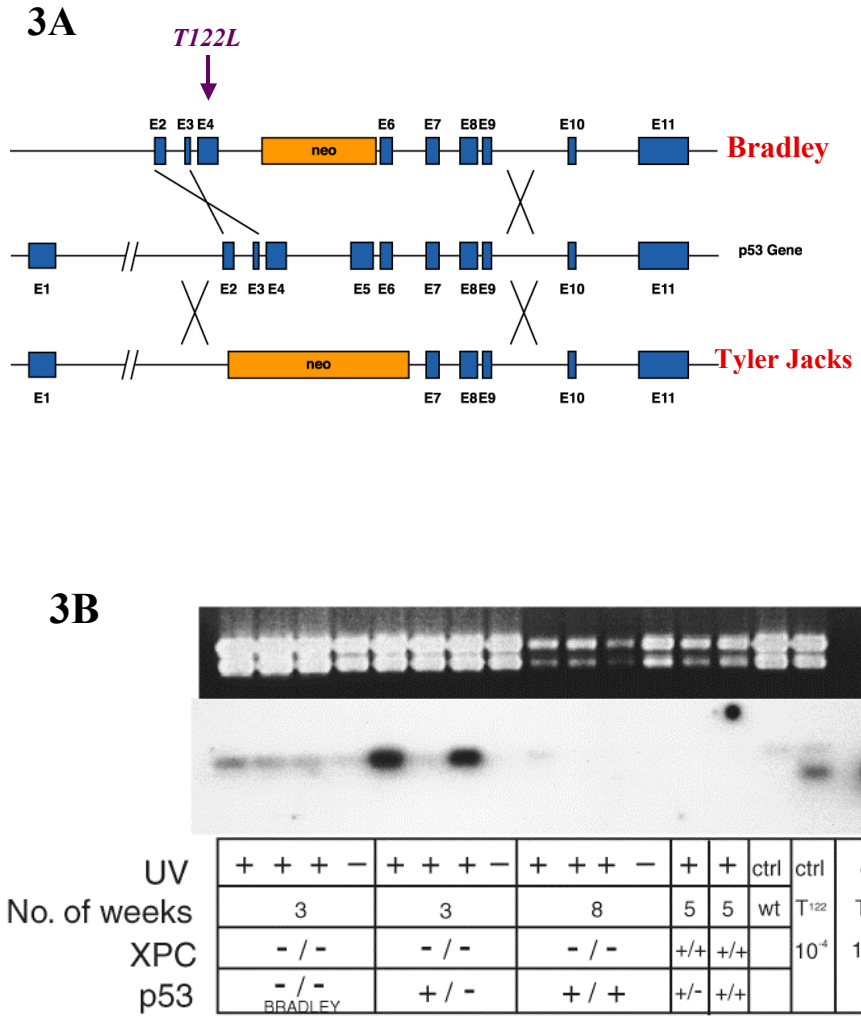

Figure II-3: The effect of the p53 protein function on the frequency of the T122L mutation can be assessed using a second p53 knockout model.

A Depicted are the two target constructs of the two different knockout models, with or without exon 4.

B. Mutation derection assay comparing between samples from an *Xpc*$^{-/-}$*p53*$^{-/-}$ mouse and an *Xpc*$^{-/-}$*p53*$^{+/-}$ one.

Materials and Methods

Mice. *Xpc* mutant mice were generated previously (Cheo, Ruven et al. 1997) and 2 different strains of *p53* mutant mice (Donehower, Harvey et al. 1992; Jacks, Remington et al. 1994) were purchased from The Jackson Induced Mutant Resource (Bar Harbor, ME). *Xpa* mutant mice were kindly obtained from the Tanaka group in Japan and *Csa* mutant mice were generated in our lab in collaboration with Hoeijmakers group in the Netherlands (van der Horst, Meira et al. 2002). Mice heterozygous for *Xpc* and *p53*, *Xpa* and *p53* or *Csa* and *p53* were bred as described (Cheo, Meira et al. 1996). All mice crossed with the Jacks *p53* mutant mice had identical strain background, comprising ~70% 129/Sv and ~30% C57B1/6. The *Xpc* mutant mice crossed with the Bradley *p53* mutant mice have a background comprising ~75% 129/Sv and ~ 25% C57B1/6.

UV-Induced Skin Cancer. The dorsal skin of mice, 8-12 weeks of age, was shaved and irradiated for 5 days/week at a daily dosage of 0.8KJ/m^2 for the $Xpc^{-/-}$ $p53^{+/-}$ and the $Xpa^{-/-}$ $p53^{+/-}$ mice and 1.6KJ/m^2 for the $Csa^{-/-}$ $p53^{+/-}$ mice. UV irradiation was performed using two FS20 erythemal (UVB) lamps (Phillips) filtered by Kodacel sheeting (Kodak, Rochester, NY). Mice were irradiated until either skin tumors were visible to the naked eye or for a maximum of 18 weeks. The mice were monitored for tumors on weekly bases. The tumor samples were frozen in liquid nitrogen after surgical removal and stored at –70°C.

UV-Induced Mutations in Non-Tumor Skin. The dorsal skin of mice, 8-12 weeks of age, was shaved and irradiated for 5 days/week at a daily dosage of 0.8KJ/m^2. After 2, 3 or 5 weeks of irradiation 4 dorsal skin samples and 2 ventral skin samples were collected from each mouse (5 mice were used for each time point), frozen in liquid

nitrogen after surgical removal and stored at –70°C. The ventral skin samples were used as non-irradiated controls.

cDNA Synthesis and Sequencing Analysis. Total RNA was extracted from frozen tumors using Trisol Reagent (Invitrogen). The samples were treated with DNase I (Invitrogen) before subjecting to RT-PCR using the Super Script Preamplification System Kit (Invitrogen). PCR to amplify the p53-coding region was carried out with the following primers: forward, 5'- CATCACCTCACTGCATGGACGATCT-3' (nucleotides 264-288); reverse, 5'- GCAGAGACCTGACAACTATC-3' (nucleotides 1520-1501). PCR reaction were carried out using Platinum Taq (Invitrogen). Sequencing reactions were run on an ABI 310 automated DNA sequencer. Sequencing forward primers were 5'- CCTGTCATCTTTTGTCCCTTC-3' (nucleotides 424-444), 5'-AGCATCTTATCCGGGTGGAAG-3' (nucleotides 723-743) and 5'-AAGTCCTTTGCCCTGAACTGC-3' (nucleotides 1026-1046). Reverse primers that were used to confirm mutations were 5'- GCCTGTCTTCCAGATACTCG-3' (nucleotides 776-757), 5'-AGAGGCGCTTGTGCAGGTG-3' (nucleotides 1093-1075) and 5'-TCTCAGCCCTGAAGTCATAAG-3' (nucleotides 1400-1380).

DNA Extraction and Southern Blot Analysis. Genomic DNA was extracted from frozen samples according to standard protocols. PCR reactions were carried out using a mix of Platinum Taq (Invitrogen) and Pfu Turbo® (STRATAGENE) with the following primers: forward, 5'- CATCACCTCACTGCATGGACGATCT-3' (nucleotides 264-288); reverse, 5'-GGAATGTGAGGGAAGAGAGTTCCAC-3' (nucleotides 172-148). PCR products were digested with *ApaLI* and subjected to electrophoresis in 1.5% agarose gels. DNA was transferred to nylon membranes. A mutated sequence, 5'-

TGTTATGTCTTGGTGAGTG-3' was used as a probe. The probe was labeled with [γ-^{32}P] dATP using a T4 polynucleotide kinase (New England Biolabs). Hybridization was performed over night at 50°C followed by 5 minutes wash at room temperature and 10 minutes wash at 55°C. Hybridization solution contains 5XSSPE, 0.3%SDS, 1% dry milk. Washing solution contains 5XSSPE, 0.1%SDS.

Discussion

We have demonstrated that T122L is a UV specific mutation that appears in a high frequency in XPC deficient mice and rarely in XPA deficient mice. We have also demonstrated that this mutation appears very early after UVB exposure and is gradually accumulated in the skin cells. We have used a p53 knockout model, in which the genomic region that contains codon 122 is still intact but no p53 protein is expressed in order to assess the role of the mutant protein in the appearance of the T122L mutation. We compared the mutation frequency between mice that express the protein and mice that do not. Our results show that the mutation can be detected in mice that do not express the mutant protein, however the frequency of its appearance is significantly lower compared to the frequency in cells with protein expression.

It appears that in XPC deficient mice, expression of the p53 mutant protein product provides the cells with a growth advantage that increases their frequency in the general population. After 5 weeks of ongoing UVB exposure the mutation can be detected in almost every skin sample collected. Skin tumors from these mice, although collected after a larger number of weeks, show the same frequency of T122L positive samples as the samples collected after 5 weeks. We therefore conclude that the presence of the T122L

mutant protein has a major contribution to the high frequency of the original mutation. However, since the mutation can be detected in samples that do not express the mutant protein we suggest that the absence of the XPC protein is another key factor in the appearance of the T122L mutation.

The major UV-induced lesions occur at sites with two adjacent pyrimidines while the sequence coding for threonine 122 is ACG, a pyrimidine flanked by two purines. Guanine residues located 3′ to cytosine residues create CpG sites. The cytosines on those sites in the *p53* gene are methylated and therefore may undergo spontaneous deamination to generate C→T mutations (Denissenko, Chen et al. 1997; Tu, Dammann et al. 1998). Damage at the ACG codon can link the cytosine to its adjacent adenine causing the AC to TT double base pair change observed in the $Xpc^{-/-}$ skin samples.

It was recently reported that the XPC protein interacts with and stimulates the activity of a thymine-DNA glycosylase that is required for the repair of T:G mispairs in DNA (Friedberg 2003), an event initiated by cytosine deamination. Our results do not rule out the possibility that the appearance of the T122L mutation in the XPC deficient background is a result of a rare photoproduct, involving both the adenine and cytosine that requires XPC protein for its repair and is therefore accumulated in DNA extracted from $Xpc^{-/-}$ skin. The mutation appeared only once in a skin tumor from an $Xpa^{-/-}$ $p53^{+/-}$ mouse and was not detected in $Csa^{-/-}$ $p53^{+/-}$ mice or in non-tumor skin samples from $Xpa^{-/-}$ $p53^{+/-}$ mice. The requirment for XPC might be therefore unrelated to its role in NER. Roles in repair pathways other than NER were previously reported also for the XPA and XPG proteins (Klungland, Hoss et al. 1999; Datta, Chan et al. 2001). We will further investigate possible role of XPC in alternative repair pathways.

We have searched the genome for other open reading frames that contain the GTGCACG sequence using an algorithm developed by Dr. Jonathan Wren (personal communication), and found 657 open reading frames containing the 7-nucleotide sequence. We used genomic DNA generated from *Xpc*$^{-/-}$ *p53*$^{+/-}$ skin samples that were T122L positive as templates to analyze two of these genes using gene-specific primers. We found no positive T122L samples in the other open reading frames we screened using our Southern-based assay. The results suggest that the mutation does not appear, or appears in frequencies below our detection level in non-p53 sequences. Alternatively, the DNA sequence context required for the appearance of the AC→TT mutation might be larger than 7 nucleotides. Interestingly, the DNA sequence in human *p53* gene is also slightly different in this region and does not contain the *ApaLI* restriction site.

We have detected several positive samples in *Xpc*$^{-/-}$ *p53*$^{+/+}$ mice that were irradiated for 8 weeks. The significantly reduced mutation frequency in this genotype is in agreement with our previous finding that T122L mutations are rare in skin tumors from *Xpc*$^{-/-}$ *p53*$^{+/+}$ mice (Reis, Cheo et al. 2000), probably because it produces a recessive product that can contribute to the elevated frequency of the mutation only in the absence of wild type protein.

Selection for cells expressing the T122L mutant protein is an ongoing process. The *Xpc*$^{-/-}$ mouse skin must be exposed for a daily dosage of 0.8KJ UVB for at least two weeks before positive samples start to be detected. We have irradiated *Xpc*$^{-/-}$ *p53*$^{+/-}$ mice with a dosage equal to one week of irradiation and then waited for 5 more weeks before skin samples were collected. All 15 skin samples subjected to the southern-based assay were negative (compare to 16 out of 18 positive samples for *Xpc*$^{-/-}$ *p53*$^{+/-}$ mice that had

ongoing radiation for 5 weeks). After 15 days of irradiation the average number of p53 positive foci in the dorsal skin area of UV exposed hairless mice is 9 ± 2 (Rebel, Mosnier et al. 2001). This number is increased to over 100 foci per cm^2 of dorsal skin after 40 days (8 weeks) of UV exposure. P53 positive foci are clusters of epidermal cells that express p53 in mutant conformation and can be detected by immunohistochemical staining long before the appearance of skin carcinomas. All cells in the foci expand clonally from one cell that was originally mutated. The number of p53 positive foci is even higher in $p53^{+/-}$ mice compared to their wild type littermates (Rebel, Mosnier et al. 2001). However, only very few foci progress through the many more steps required to become tumors. The positive T122 signal in the non-tumor skin samples could originate fron such foci, especially in the $p53^{+/-}$ background.

Genotype specific p53 mutants were also reported in patients with mutations in the tumor suppressor genes BRCA1 and BRCA2 that are also involved in DNA repair (Smith, Crossland et al. 1999). Rather than the loss of function mutants commonly observed in sporadic cancers, these mutants retain some of the wild type protein functions. Characterization of the T122L mutant protein also shows that the protein retains wild type properties (see chapters III and IV). The appearance of the T122L mutation is therefore an example of a genotype specific, recessive mutation that was unmasked using the $p53^{+/-}$ background. The investigation of this unique class of p53 mutants is an important tool for studies of p53 dependent events that are critical in the process of tumor formation.

XPC deficient cells have no GGR, which leads to high mutational rate that is not balanced by enhanced apoptotic response (Berg, Rebel et al. 2000; van Oosten, Rebel et

al. 2000). Thus, XPC deficient cells can be highly susceptible to transformation. The absence of the XPA or the CSA proteins results in lost of TCR. The high mutational rate in these cells is balanced by the apoptotic response induced by TCR deficiency. Cells expressing $p53^{T122}$ mutant protein in those genotypes might apoptose before they can clonally expand, which would explain why this mutation cannot be detected in those genotypes in a high frequency. Further investigation of the different responses to UV induced damage in different NER-deficient backgrounds is required.

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Chapter III: A Novel *p53* Mutational Hotspot in Skin Tumors from UV-irradiated *Xpc* Mutant Mice Alters Transactivation Functions

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A mutation in codon 122 of the mouse *p53* gene resulting in a T to L amino acid substitution (T122->L) is frequently associated with skin cancer in UV-irradiated mice that are both homozygous mutant for the nucleotide excision repair (NER) gene *Xpc* (*Xpc* $^{-/-}$) and hemizygous mutant for the *p53* gene. We investigated the functional consequences of the mouse T122->L mutation when expressed either in mammalian cells or in the yeast *Saccharomyces cerevisiae*. Similar to a non-functional allele, high expression of the T122->L allele in *p53*$^{-/-}$ mouse embryo fibroblasts and human Saos-2 cells failed to suppress growth. However, the T122->L mutant *p53* showed wild type transactivation levels with *Bax* and *MDM2* promoters when expressed in either cell type and retained transactivation of the *p21* and the *c-Fos* promoters in one cell line. Using a recently developed rheostatable *p53* induction system in yeast we assessed the T122->L transactivation capacity at low levels of protein expression using 12 different *p53* response elements (REs). Compared to wild-type p53 the T122->L protein manifested an unusual transactivation pattern comprising reduced and enhanced activity with specific REs. The high incidence of the T122->L mutant allele in the *Xpc*$^{-/-}$ background suggests that both genetic and epigenetic conditions may facilitate the emergence of particular functional *p53* mutations. Furthermore, the approach that we have taken also provides for the dissection of functions that may be retained in many *p53* tumor alleles.

Introduction

The tumor suppressor gene *p53* plays an important role in maintaining genome integrity (Prives & Hall, 1999). Upon stabilization and activation, which are achieved mainly by post-translational modifications (Meek, 1999), p53 protein can act in the homotetrameric conformation as a sequence-specific transcription factor (McLure & Lee, 1998). Transactivation is the best characterized and probably most relevant function among the many biochemical activities ascribed to p53 protein {Ko, 1996 #11}. p53-*r*esponse *e*lements (REs) corresponding to a rather loose consensus sequence (two variably spaced palindromic decamers of 5'- RRRCWWGYYY each binding to a p53 dimer) (el-Deiry *et al.*, 1992) have been identified in promoter and intronic regions of over 50 genes. The growing list of p53-regulated genes includes *p21*, *GADD45*, *p53-R2*, *FAS*, *Bax*, *PIG3*, *IGF-BP3*, *Killer/DR5*, *AIP1,* and *MDM2* (Ashcroft & Vousden, 1999; el-Deiry, 1998; Oda *et al.*, 2000; Tanaka *et al.*, 2000). The products of these genes are involved in cell cycle control, induction of apoptosis, modulation of DNA repair, differentiation, senescence, and control of p53 stability/activity.

Recent studies have further validated the view that p53 is a tightly controlled cellular secondary sensor that can integrate many different signals and that regulates the expression of a large number of genes with variable kinetics and intensity (Vogelstein *et al.*, 2000; Yu *et al.*, 1999; Zhao *et al.*, 2000). This key role in signal transduction predicts a strong selection for p53 inactivation in tumors and supports the notion that loss of p53 function disrupts basic cellular functions. An issue that has received relatively little attention is that partial inactivation of p53 activity may be sufficient for, or even favor,

tumor progression, depending on the cell type and physiological state, and the precise function of the mutant *p53* allele in question.

The *p53* gene is frequently (>50%) mutated during tumorigenesis (Hainaut & Hollstein, 2000). About 80% of p53 tumor mutations are missense and result in single amino acid changes that are predicted to interfere with DNA binding and hence impair transactivation (Ko *et al*, 1996). Some mutations can be dominant-negative leading to partially inactive heterotetramers (Aurelio *et al.*, 2000; Brachmann *et al.*, 1996), a feature that may explain the unusual predominance of missense changes in the *p53* tumor mutation spectrum (Hernandez-Boussard *et al.*, 1999).

Several recent reports support the view that certain *p53* mutant alleles can retain partial function. Some mutations are associated with partial transactivation and are capable of inducing G1 arrest, but not apoptosis (Ludwig *et al.*, 1996; Munsch *et al.*, 2000). Furthermore, a recent analysis of 77 different p53 mutant proteins from tumors revealed that >15% could still activate a yeast promoter containing a *p21* RE, but not the *Bax* or *PIG3* RE (Campomenosi *et al.*, 2001), which presumably is important in tumor development. The observation that deletion of the *p21 g*ene does not result in increased spontaneous tumor development and can even decrease tumorigenesis (Pantoja & Serrano, 1999; Wang *et al.*, 1997), suggests that *p53* mutant alleles that retain some transactivation capability might confer a selectable advantage over null alleles under appropriate conditions. For example, apoptosis can be prevented by expression of *p21* (Gorospe *et al.*, 1997; Suzuki *et al.*, 2000).

A few *p53* mutant alleles appear to have acquired functions that may provide a selective advantage to tumor cells, such as the up-regulation of growth promoting genes

(*e.g., Myc, MDR*, *VEGF*) (Frazier *et al.*, 1998; Kieser *et al.*, 1994; Lin *et al.*, 1995). Finally, *p53* mutant alleles that appear normal for transactivation, growth suppression and apoptosis when ectopically expressed at high levels in a tumor cell line, have been detected in breast and ovarian tumors associated with *BRCA1* defects {Smith, 1999 #5}. Hence, detailed functional analysis of multiple tumor *p53* alleles is expected to provide valuable information in predicting tumor state, including aggressiveness and responsiveness to therapy.

We recently reported that codon 122 of the mouse *p53* gene is a novel hotspot for mutation in UV radiation-induced skin cancers in xeroderma pigmentosum (XP) group C mice that are also hemizygous for *p53* ($Xpc^{-/-}$ $p53^{+/-}$) {Reis, 2000 #35}. Surprisingly, the hotspot does not involve adjacent pyrimidines, the major targets for UV damage, but instead occurs at an AC site. The resulting threonine to leucine amino acid substitution (T122->L) accounted for ~40% of all mutations in skin cancers from UV-irradiated $Xpc^{-/-}$ $p53^{+/-}$mice, and was never detected in UV radiation-induced skin cancers in wild type mice. Similarly, the T122->L mutation accounted for only 6% of *p53* mutations in $Xpc^{+/-}$ $p53^{+/-}$ or in $Xpc^{-/-}$ $p53^{+/+}$ mice and was also only rarely observed in $Xpa^{-/-}$mice or mice defective in the *Csa* (Cokayne syndrome group A) gene required for transcription-coupled excision repair (D. Nahari and E.C. Friedberg, unpublished observations). The origin of this mutational hotspot is not known, but is presumed to result from a non-dipyrimidine photoproduct. Its unique prevalence in skin cancers from $Xpc^{-/-}$ $p53^{+/-}$ mice suggests both that the *Xpc* gene product is specifically required for its repair, and that the T122->L mutation is recessive. However, the prevalence of this signature mutation uniquely in the $Xpc^{-/-}$ $p53^{+/-}$ genetic background may additionally or alternatively be

determined by novel specific functional attributes of the mutant p53 protein encoded by the T122->L allele that promote its selection during carcinogenesis in the skin. Furthermore, such selection may be influenced by physiological attributes associated with the *Xpc* homozygous mutant state.

In order to explore the functional consequences of the T122->L mutation we have carried out ectopic expression of this mutant p53 protein in mouse embryo fibroblasts (MEFs) and in human tumor cells that are *p53* null. Additionally, we have exploited a recently developed p53 functional assay in yeast (Inga *et al.*, 2001) that was adapted to precisely assess the transactivation potential of the T122->L allele with various p53 REs under conditions of variable expression. Using this sensitive assay, the T122->L allele exhibits a novel set of functional changes embracing both enhanced and reduced transactivation activity for 12 different p53 REs. We conclude that the precise pattern of T122->L p53-regulated gene expression, rather than a simple loss of p53 function, may be a critical determinant in tumor selection and progression in UV-irradiated mouse skin cells.

Results

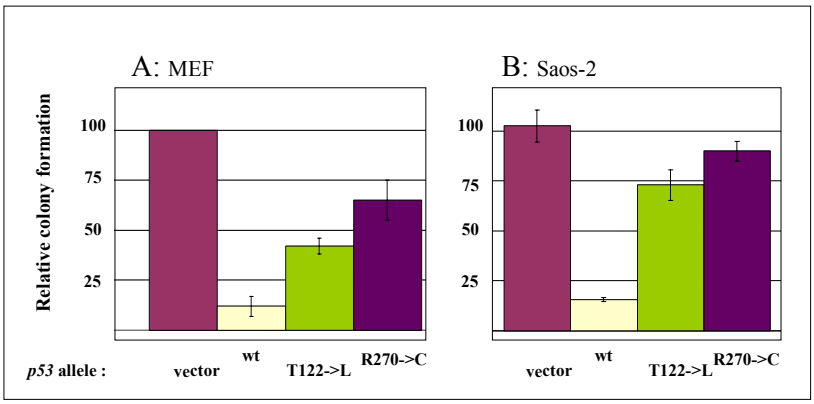
The T122->L allele exhibits limited growth suppression in MEF and Saos-2 cells.

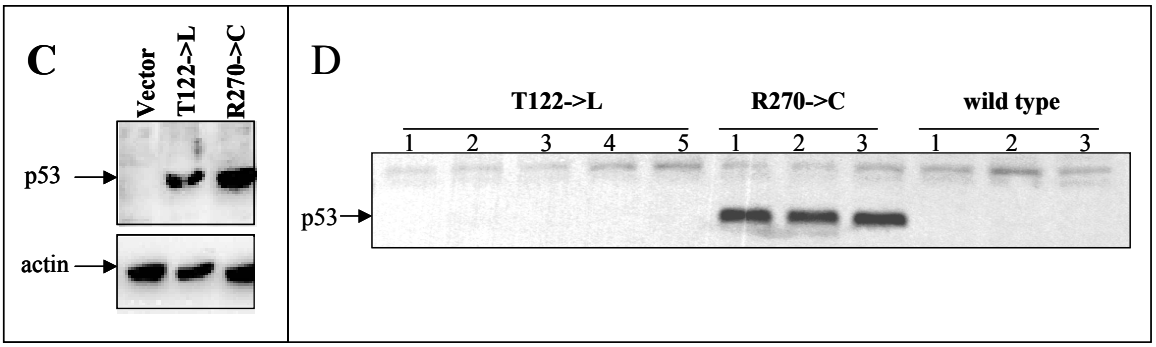
In order to functionally characterize the murine T122->L allele in mammalian cells it was transferred into *p53*$^{-/-}$ MEF cells using a retroviral vector that generates high levels of p53 expression. Responses were compared with expressed wild-type p53 and an R270->C mutant allele. Mouse codon R270 and the equivalent human codon R273 are mutational hotspots in *p53* that are known to inactivate p53 function in many types of tumors.

Expression of wild-type p53 protein reduced the relative colony forming ability to 12% (Figure 1A). As expected, the R270->C allele was much less effective at causing growth suppression. Expression of the T122->L allele had an intermediate effect (65% vs 42% survival, relative to vector control), suggesting some retention of p53 function.

We also examined the effect of high expression of the T122->L allele in the human osteosarcoma-derived Saos-2 cell line which is null for both the *Rb* and the *p53* tumor suppressor genes (Figure 1B). Once again, wild-type p53 reduced relative colony formation while the T122->L and the R270->C alleles had less effect (70% vs 88% survival, respectively, relative to vector). Hence, as relates to growth the T122->L mutant protein has minimal residual function in both cell lines in this assay.

The expression levels of p53 protein were examined in stable MEFs and Saos-2 transformant clones obtained in a limiting dilution experiment. As expected, there was no detectable p53 in the isolates recovered from transfections with wild-type *p53*, while the R270->C transfectants expressed p53 protein at moderate levels (Figure 1C and D, and data not shown). T122->L protein expression was also retained in stable MEF clones (Figure 1C). In contrast, no p53 protein was detected in Saos-2 clones transfected with the T122->L vector (Figure 1D, 5 of the 6 clones examined are presented), suggesting that, similar to wild-type p53, the T122->L protein may not be tolerated in this cell line. Lower levels of expression from the LTR promoter compared to the CMV promoter (data not shown) may explain why stable transfectants expressing T122->L in the MEF cells could be established. However, the result is surprising since the presence of the T122->L vector had little effect on growth suppression in Saos-2 cells (Figure 1B).





Inga, Nahari *et al.,* Fig. 1

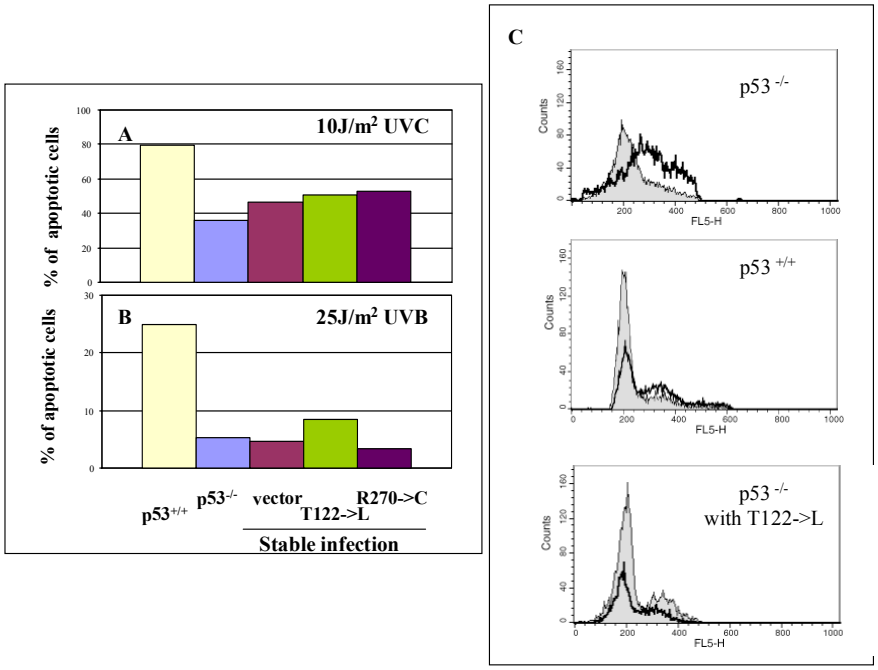
Figure III-1: Growth suppression of *p53*$^{-/-}$ MEF and Saos-2 cells

(A) *p53*$^{-/-}$ MEF cells were infected by the retroviral vector pBabe-PURO expressing the indicated *p53* alleles under the LTR promoter. (B) Saos-2 cells were transfected by lipofectamine with 1μg of pCI-based vectors (*CMV* promoter). Puromycin (A) and G418 (B) resistant colonies were selected and counted after two weeks. The relative mean number and the standard deviations for three independent experiments are presented.

Expression of *p53* alleles in stable transfectants: puromycin resistant MEF clones (C) and G418 resistant Saos-2 clones (D) were isolated and p53 expression was determined by Western Blot analyses (pAb1801 and DO-1). 50 μg of extract were loaded in each lane. Actin was used as control for MEFs extracts while the non-specific band served as control for the Saos-2.

The T122->L protein retains the ability to cause DNA damage-induced G1 arrest but not apoptosis.

p53$^{+/+}$ and *p53*$^{-/-}$ MEF clones expressing either the R270->C or T122->L p53 alleles were irradiated with either 10 J/m^2 UVC or 25 J/m^2 UVB light and the number of apoptotic cells was measured 48 hours later using FACS analysis. These doses resulted in different levels of both p53-dependent and -independent apoptosis (Figure 2A and 2B). The *p53* mutants were defective in apoptosis and exhibited levels of cell death comparable to *p53* null cells (*i.e.*, p53-independent apoptosis).



Inga, Nahari *et al.,* Fig. 2

Figure III-2: Effects of T122->L on UV-induced apoptosis and cell cycle arrest in MEF cells.

p53$^{+/+}$ and *p53*$^{-/-}$ MEF cell lines expressing p53 mutant alleles were mock- or UV-irradiated at the indicated doses. Cells were collected 48 hours after treatment and prepared for FACS analysis by 7-AAD

and Hoechst double staining. (A, B) The histograms show the percentage of apoptotic cells in each cell line after the different UV treatments. (C) Cell cycle profiles of non-apoptotic cells that were mock-treated (solid area) or UVB-irradiated (25 J/m^2) (black line). Presented on the Y-axis are the numbers of cells counted at each point.

Fibroblasts manifest a preference for p53-dependent cell cycle arrest over apoptosis when DNA damage is not extensive (MacCallum *et al.*, 1996). Therefore, UVB radiation-induced cell cycle responses were examined in the non-apoptotic cell population 48 hours after exposure to UV radiation of 25 J/m^2. Many of the MEF cells lacking *p53* were in the S phase (Figure 2C; black line in the top figure), indicating that the G1 arrest was not functional and that irradiation slowed progression through the S phase, leading to an accumulation of cells in this phase of the cell cycle. Mock-irradiated $p53^{-/-}$ cells were largely in G1 at 48 hours, typical for cycling cells of this MEF line (solid peaks). As expected, irradiated normal MEFs ($p53^{+/+}$) that express the wild-type *p53* gene arrested in G1. T122->L-expressing cells similarly retained a large G1 population, consistent with retention of p53 function (Figure 2C). In contrast, cells expressing the R270->C allele exhibited a UV-radiation response similar to $p53^{-/-}$ MEFs (data not shown). Collectively, these results suggest that unlike the R207->C allele, the T122->L allele does not result in a typical loss of function phenotype.

T122->L p53 protein has altered transactivation specificity.

We evaluated transactivation of a luciferase reporter gene under the control of the human *p21*, *MDM2*, *Bax*, or *c-Fos* promoters, or the *RGC* RE. $p53^{-/-}$ MEFs were subjected to transient transfection by either the wild-type, T122->L or the R270->C alleles, along with

a plasmid containing a luciferase reporter downstream from one of the p53-responsive promoters. The luciferase activity was determined in protein extracts 48 hours later. The average activity relative to wild-type p53 and the standard deviation of at least three independent experiments are presented in Figure 3A. Although the large variability in this experiment prevents a reliable assessment of subtle differences, this analysis suggests that the T122->L mutant protein exhibits an altered transactivation pattern. As expected, wild-type p53 protein was able to activate transcription from all the promoters, while R270->C was clearly deficient. The T122->L form failed to activate either the *RGC* cassette or the *p21* promoter. Surprisingly, the protein was able to activate both the *MDM2* and the weaker *Bax* promoter. Over-expression of wild-type p53 represses the *c-Fos* basal promoter [described in (Ginsberg *et al.*, 1991) and Figure 3A]. The T122->L and the R270->C alleles failed to repress the *c-Fos* promoter. The first intron of the *c-Fos* gene also contains a p53 RE and wild-type p53 at near physiological levels of expression can activate a construct that includes the luciferase reporter gene fused to this region (Elkeles *et al.*, 1999). The T122->L allele, but not R270->C, activated this RE, though at reduced levels compared to wild-type p53 protein (Figure 3A). Consistent with the transactivation results both wild-type and mutant T122->L p53 proteins prepared from stably infected $p53^{-/-}$ MEFs bound a p53 consensus RE while R270->C protein did not (data not shown).

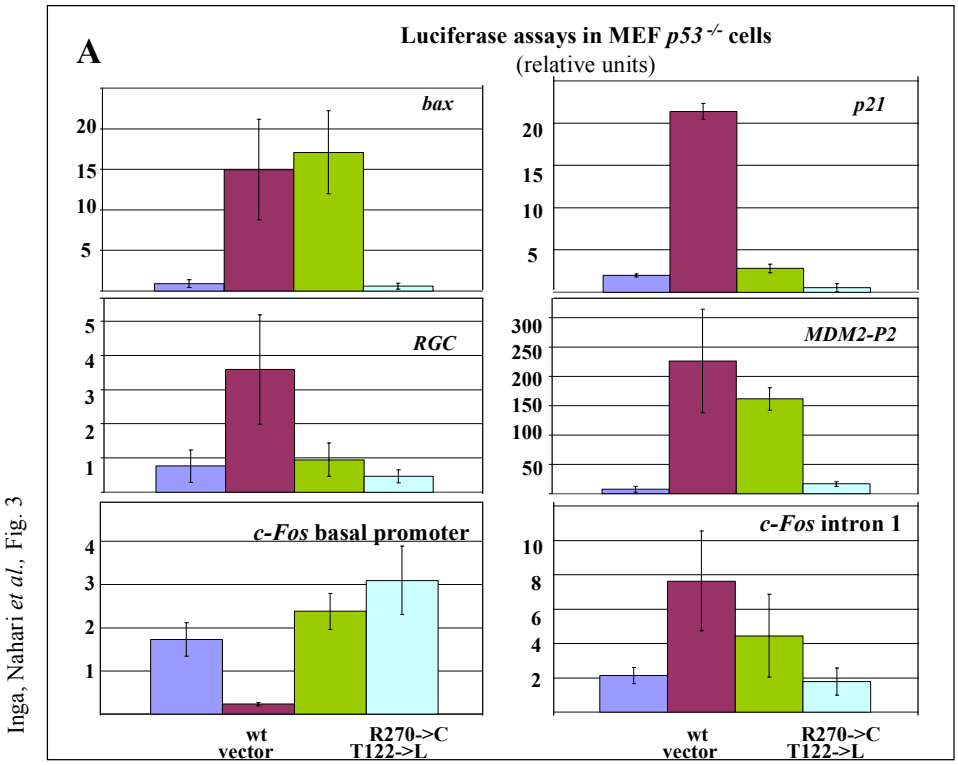
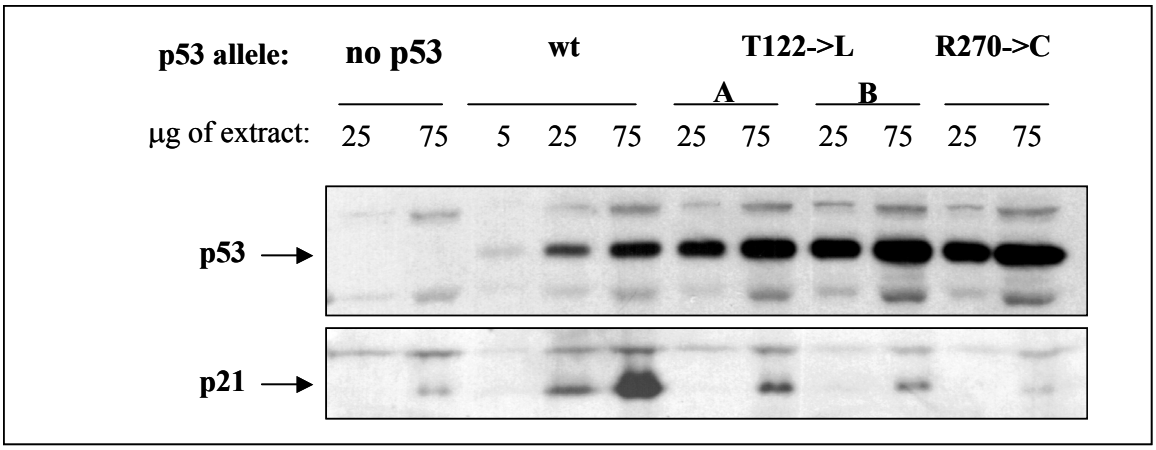


Figure III-3A: T122->L transactivation activity in MEFcells.

MEF cells were transfected using the FuGENE^{TM} 6-transfection reagent, with 2 µg of *p53* expression vector and 4 µg of reporter plasmid and recovered after 48 hours. Relative average luciferase units and standard deviations for at least 3 independent experiments are presented. The six promoters tested are indicated. T122->L p53 transactivation activity is compared to wild type p53 and the R270->C mutant.



Inga, Nahari *et al.,* Fig. 3

Figure III-3B: p53 expression levels and induction of the *p21* gene in Saos-2 transient transfectants. Protein extracts were prepared 24 hours after transfection. p53 and p21 levels (pAb-C19) were determined by Western Blot analysis.

Transactivation of *Bax*, *p21*, *RGC* and *MDM2* was also examined in Saos-2 cells using a similar approach, except that luciferase activity was measured in protein extracts obtained 24 hours after transfection. Levels of p53 protein were high at this time and similar for all three alleles (see Figure 3B). Results were comparable to those observed with MEFs for both the T122->L and the R270->C mutants, except that the T122->L allele also strongly activated *p21* in the Saos-2 cells (data not shown). This difference between MEF and Saos-2 cells for *p21* might reflect cell type-dependent effects. The level of endogenous *p21* induction was measured in Saos-2 cells by Western blot analysis. Wild type *p53* expression led to strong induction of the *p21* gene while the R270->C mutant failed to induce *p21* (Figure 3B). The T122->L allele exhibited a low level of induction compared to wild-type p53. The difference between ectopic and endogenous gene results may be due to the high copy number of the luciferase reporter

and location on a plasmid, which might overestimate the transactivation potential of *p53* alleles that retain partial activity.

The T122->L allele exhibits novel transactivation properties in a yeast-expression assay. As shown above, the T122->L allele falls into the category of p53 mutant proteins that retain partial activity (Ludwig *et al.*, 1996). However, contrary to previously described mutants that showed partial function (Blagosklonny *et al.*, 2001), it appears to retain activity with the weaker *Bax* promoter but not with the stronger *p21*. Furthermore T122->L is the first example of a tumor hotspot mutant that exhibits this type of altered transactivation function as a result of an amino acid change in the L1 loop-S2 strand region, which is part of the highly conserved domain II. Interestingly, similar functional results were reported for the human S121->F p53 mutant (corresponding to S118 in mouse p53), also in the L1 loop (Freeman *et al.*, 1994; Kaeser & Iggo, 2002). However, S121->F has never been found in tumors and its ectopic expression led to enhanced apoptotic response compared to wild-type p53 (Saller *et al.*, 1999).

These results prompted us to characterize extensively the in vivo transactivation capacity of the T122->L protein with a large variety of REs using a yeast-based reporter system. Previously, three yeast strains were developed that contained a copy of the ADE2 color (red/white) reporter gene whose transcription was dependent on p53 protein binding to an upstream RGC, Bax or p21 RE (Flaman et al., 1995; Flaman et al., 1998). More recently we developed a system in yeast that provides opportunities to examine functional features of mutants in the DNA binding domain. p53 expression is controlled by a rheostatable GAL1 promoter (rather than a strongly expressing promoter) so that

relative p53 transactivation ability on various REs can be assessed (Inga et al., 2001). This system was adapted to study the effects of the mouse T122->L allele on transactivation and the number of REs examined was extended to 12 using isogenic strains each containing a different p53 RE (see Table 1).

Table III-1: Transactivation activity of wild type and T122->L p53 with 12 *p53* REs under conditions of variable p53 induction, using the *GAL1* promoter

***p53* RE**	**Minimum galactose level (%) required to detect transactivation with:**		**T122->L activity relative to wild type p53 [b]**
	wild type p53	**T122->L**	
p21-5'	0.004	0.001	↑↑
p21-3'	0.008	(> 0.032)[a]	-
PCNA	0.002	0.004	↓
GADD45	0.001	0.0005	↑
Bax	0.008	0.002	↑↑
PIG3	0.064	0.032	↑ ? [c]
IGF-BP3 box A	0.032	0.002	↑↑↑↑
AIP1	0.001	0.004	↓↓
m-FAS	0[d]	0.001[d]	↓
m-FOS	0.008	0.008	=
MDM2	0.004	(> 0.032)[a]	-
3xRGC	0.004	(> 0.032)[a]	-

[a] The ability to detect transactivation at galactose levels > 0.032% by the T122->L mutant cannot be determined due to general toxicity of the mutant (see Figure 4). There is no transactivation at 0.032% galactose.

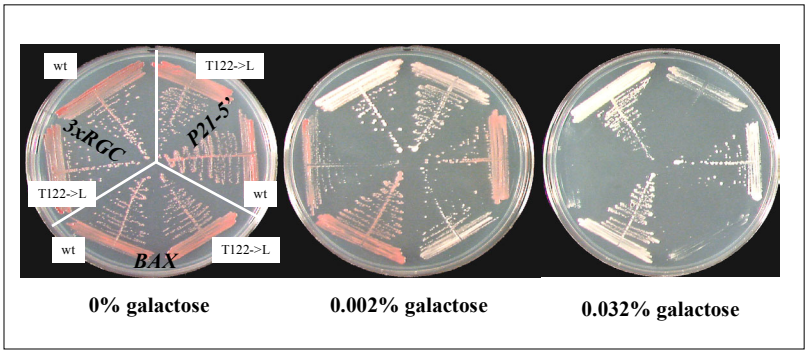
[b] Enhanced, equal, reduced or loss of function is represented by ↑, =, ↓, and -, respectively. Each arrow indicates a two-fold difference in galactose level needed to detect transactivation.

[c] Since wild type p53 shows transactivation with *PIG3* at high expression levels, the poor growth of cells expressing the T122->L allele makes color analysis less reliable.

[d] With expression of wild-type p53, colonies are already white on raffinose plates. With T122->L, colonies become pink at 0.001% galactose, but they remain pink at all galactose concentrations for which there is growth.

While transactivation could be examined at reduced levels of expression, higher expression of the T122->L allele from the *GAL1* promoter completely prevented yeast growth, irrespective of the strain background and the affinity for the p53 RE in the *ADE2* reporter gene (Figure 4, Table 1). Under the same conditions expression of wild-type mouse p53 protein resulted in small colonies (not shown), as did human p53 protein [similar to our previous reports (Inga & Resnick, 2001)]. In contrast, the non-functional mutant allele R270->C had little effect on growth (data not shown). We also constructed yeast expression vectors with *p53* under the control of the moderately expressed, constitutive *ADH1* promoter. As expected from other reports (Scharer & Iggo, 1992), wild-type p53 protein did not affect yeast growth at this level of expression while the T122->L allele prevented growth (data not shown). This strong growth-inhibiting phenotype is similar to that found for *supertrans*/toxic p53 alleles which we previously described (Inga & Resnick, 2001).

We also evaluated the transactivation potential of the T122->L allele with respect to 9 human and 3 murine p53 REs at low levels of induction from the *GAL1* promoter. Each of these response elements, which belong to the p53 consensus but differ in nucleotide sequence (el-Deiry *et al.*, 1992), were derived from the regulatory regions of p53-regulated genes involved in cell cycle arrest, DNA repair and apoptosis induction. While the human REs may not be identical to those in the homologous mouse genes, they provide the opportunity to investigate differences in binding affinity/transactivation capacity between wild-type and mutant *p53* alleles.



Inga, Nahari *et al.,* Fig. 4

Figure III-4: Transactivation potential of wild type and T122->L mutant p53 proteins under condition of variable expression in the *yIG397*, *yPHp21*, and *yPHbax* strains

GAL1::*p53* expression vectors were transformed into strains containing *3xRGC*, *BAX* and *p21::ADE2* reporters. Transformants were streaked out for single colonies on plates containing 2% raffinose as the carbon source plus the indicated amounts of galactose inducer. A low amount of adenine (5 mg/L) in the plates allows the assessment of p53 dependent transactivation (white/pink *vs* red colonies). When grown on medium lacking galactose, all colonies are red. On 0.002% galactose T122->L is defective for transactivation with *3xRGC* (red colonies compared to white colonies obtained with wild type p53 transformants), but it exhibits enhanced activity with both *p21* and *BAX* on the 0.002% galactose plate (pink colonies *versus* the red colonies with wt *p53*). On 0.032% wt p53 efficiently induces all three promoters, while this level of expression prevents growth of T122->L transformants.

Independent yeast transformants containing the *p53* expression vector were streaked for single colonies on plates containing raffinose (which leads to a higher basal level of *GAL1* expression compared to glucose) plus different amounts of the galactose

inducer (*e.g.,* see Figure 4). Growth and levels of transactivation, indicated by red, pink or white colonies, were determined and compared to wild-type *p53* expression (Table 1). Transactivation analysis at low expression levels revealed differences in activity of wild-type p53 with the various REs. For example, transactivation with the *GADD45*, *AIP1*, *p21-5′* REs was observed at lower levels of p53 expression than with the *p21-3′*, *BAX*, and *MDM2* REs. Under conditions of high expression (0.032% galactose) where cells containing T122->L do not grow, wild-type p53 showed detectable transactivation with all REs. However, transactivation of the *PIG3* and *IGF-BP3* REs by wild-type p53 was reduced even at 0.3% galactose (*i.e.,* pink colonies) (data not shown). It is worth noting that human and murine wild-type p53 (that share an overall 89% identity in the DNA binding domain) showed the same relative differences in transactivation capacity with these REs (data not shown).

Multiple differences were noted between the T122->L mutant and wild-type alleles that were revealed only at various low levels of expression. Surprisingly, transactivation was enhanced for the REs *p21-5′*, *GADD45, BAX*, *IGF-BP3-boxA* and possibly *PIG3*, while it was reduced but clearly detectable with the *PCNA, AIP1* and *m-FAS* REs. Wild-type levels of transactivation were observed with *cFOS*. Finally, there was no transactivation (colonies remained red) with the *p21-3′*, *3xRGC*, and *MDM2* REs, indicating a loss of binding to these elements. The increased transactivation with some p53 REs did not correlate with the relative transactivation capacity of wild-type p53 protein towards the various elements. In fact, transactivation was enhanced with both strong (*e.g., GADD45*, *p21-5′*) and weak (*e.g., BAX*, *IGF-BP3*) p53REs.

Since T122->L showed both reduced and enhanced functional activity it is unlikely that differences in protein stability explain the transactivation results. In a separate study with wild-type p53, we established that the amount of p53 is directly proportional to the amount of inducer, so that differences in the levels of galactose required to detect transactivation can be used to estimate relative transactivation capacity towards different REs (Inga *et al.*, in preparation). Based on the amount of p53 required for transactivation, T122->L is ~3 times less active than wild-type p53 for transactivation at the *AIP1* response element and 2 times less active with *PCNA* and *mFAS*. Interestingly, T122->L showed partial function (pink colonies) with this latter RE at low expression, but transactivation did not improve by increasing the protein amount. The mutant protein was ~5 and 3 times more active with the *Bax* and p21-5' REs, respectively The largest difference, ~10 fold increase, was found with *IGF-BP3*. This is one of the weakest REs with wild-type p53 but among the strongest with T122->L. The sequence of IGF-BP3 RE (5' AAACAAGCCacAACATGCTT-3') has two mismatches from the consensus, both at the first position of the p53-monomer binding sites. It is unclear how the T122->L change can improve p53 binding to *IGF-BP3*. Molecular modeling of the equivalent human T125->L change predicted that the mutant protein would be non-functional (Reis *et al.*, 2000).

Since most of the functional assays have been performed in isogenic strains that differ only by the small RE sequences, we conclude that the T122->L amino acid change leads to altered DNA binding specificity towards many REs.

Discussion

Novel features of the T122>L mutation revealed in mammalian and yeast cells.

To better understand the importance of the unusual hotspot T122->L change in p53, we utilized a variety of approaches to assess the functional consequences of this mutation. These approaches in yeast and mammalian cells can be used to characterize the many functionally altered p53 alleles that are likely to appear in human tumors.

Our results indicate that the hotspot T122->L mutant protein has novel functional features relative to wild-type and other *p53* hotspot mutant proteins. The mutant protein exhibited transactivation in luciferase assays, induced partial cell cycle arrest (but not apoptosis) in response to UV radiation, and was poorly tolerated when ectopically over-expressed in Saos-2 cells. These results in mammalian cells suggest a separation of functions since there was loss of apoptosis induction. This specific change in apoptotic response has also been described for a small number of p53 mutations that when over-expressed in tumor cell lines exhibited normal or partial activity toward the *p21* promoter in reporter assays, but lacked transactivation of the *Bax* promoter (Blagosklonny *et al.*, 2001; Ludwig *et al.*, 1996). While the induction of *p21* can be sufficient for cell cycle arrest, the p53-dependent apoptotic response appears to require the concerted activation of several genes including *Bax* (Munsch *et al.*, 2000). Transactivation by T122->L p53 differs from these rare partial function mutations in that it has no activity towards *p21* in MEFs or has greatly reduced activity in Saos-2 cells towards the *p21*, but shows wild-type activity with the *Bax* promoter in both cell lines. Yet, its ectopic expression only leads to G1 arrest and there is no apoptotic response.

Results obtained with over-expressed *p53* alleles at non-physiological levels of protein must be viewed cautiously, especially in light of the complexity of *p53* responses and their regulation, and the activation hierarchy of the many target genes (Vogelstein *et al.*, 2000; Zhao *et al.*, 2000). For example, p53 REs often deviate from the loose consensus sequence in terms of the actual sequence and the number and the arrangement of elements, resulting in variation in transcriptional activation (Szak *et al.*, 2001; Thornborrow & Manfredi, 1999; Wieczorek *et al.*, 1996). In addition, the level of *p53* expression, the extent of specific post-translational modifications in the pool of nuclear p53 proteins and the availability of cofactors might affect the biological outcome of the *p53* response by establishing further discrimination between target genes (Thornborrow & Manfredi, 2001).

The yeast p53 functional assay with moderate p53 expression under the *ADH1* promoter has also been used to characterize many tumor alleles and identify p53 mutant forms retaining function with specific REs (Campomenosi *et al.*, 2001; Flaman *et al.*, 1998). While several alleles appeared wild-type with *p21* and mutant with *Bax* RE, the opposite phenotype was never observed. In addition, none of these mutants appeared to affect yeast growth.

The novelty of the T122->L mutant phenotype led us to characterize the transactivation potential of this allele using the rheostatable *p53* expression system developed in yeast (Inga *et al.*, 2001). This assay previously revealed several *p53* mutations that are toxic in yeast at the moderate expression levels found with the frequently used constitutive *ADH1* promoter (Inga & Resnick, 2001). At low expression levels, the toxic mutants often exhibited enhanced transactivation and altered promoter

specificity. The T122->L allele is similar to these *supertrans/toxic* mutants. It is located in the conserved domain II and it prevents growth of yeast, even at moderate levels of expression.

The transactivation analysis at various low levels of expression identified numerous changes of the T122->L protein relative to wild-type p53. These included enhanced or *supertrans* activity for several REs, and reduced or no transactivation for others (Table1). For example, the human V122->A mutant allele (corresponding to V119 in mouse p53) showed a transactivation pattern similar to T122->L (unpublished results), although there were increased activities with *hFAS* and *mFAS* REs. V122->A was wild-type for growth suppression in Saos-2 cells, and it has never been found in tumors (Inga & Resnick, 2001).

A comparison of transactivation results showed a good correlation for activation of *BAX*, *c-Fos* and *RGC* p53 REs by T122->L in yeast and mammalian cells (Fig. 3 and Table 1), while there were differences with *p21* and *MDM2*. However, the two *p21* REs from the human *p21* promoter were examined separately in yeast and opposite activities were found with T122->L (*i.e.,* enhanced and defective transactivation). The contribution of both elements to the activity of the complete promoter is uncertain. The yeast results suggest that efficient activation of the *p21* promoter requires the interaction of p53 with both REs. Interestingly, the p21-3'RE is about 1Kb closer to the transcription start site and may have an important role in stimulating chromatin modifications near the TATA box (Espinosa & Emerson, 2001) In the case of *MDM2* only one of two REs found in the p53-responsive intronic promoter (Zauberman *et al.*, 1995) was tested in yeast. It is possible that T122->L retains activity with the other *MDM2* RE and hence manifests

activity with the complete promoter in mammalian cells. Gene expression profiling in mouse skin cells at physiological levels of *p53* expression might be informative in characterizing how the T122->L mutation alters the pattern of expression of *p53*-regulated genes.

Preference for the T122->L p53 mutation in $Xpc^{-/-}p53^{+/-}$ cells

Several factors can influence the appearance of mutation hotspots in genes. These include i) the likelihood that a nucleotide(s) is damaged; ii) the ability of one or more DNA repair systems to detect and process the damage; iii) the miscoding potential of the lesion and iv) the functional consequences of the mutation (Holmquist & Gao, 1997). Given the well-established role of *p53* in tumor suppression, the functional consequences of mutations in this gene are expected to significantly contribute to selection (Rodin *et al.*, 1998). The predominance of *p53* tumor mutations located in the DNA-binding domain and their predicted effect in reducing or preventing DNA binding strongly supports the view that p53 transactivation function is critical to tumor suppression (Ko & Prives, 1996). Several *p53* mutation hotspots have been identified in tumors (Hernandez-Boussard *et al.*, 1999), and some have been associated with specific DNA changes such as DNA damage or methylation of CpG sequences (Hussain & Harris, 1999). Some hotspots that are associated with tumorigenesis result in gain of function (Lee *et al.*, 2000; Murphy *et al.*, 2000).

Mutations in *p53* are frequently observed in non-melanoma skin cancers, particularly in squamous cell carcinomas, in which *p53* mutant clones can be detected in normal and pre-malignant cells (Brash & Ponten, 1998). *p53* mutational hotspots in skin

tumors have revealed a distinctive fingerprint that is a hallmark of UV radiation-induced mutagenesis, namely tandem mutations at dipyrimidine sites (Sage *et al.*, 1996). It has been proposed that mutation of one *p53* allele in cells exposed to chronic UV radiation confers a phenotypic advantage due to a partial inhibition of the wild-type protein leading to less apoptosis of damaged "sunburn" cells (Brash *et al.*, 1996).

The T122->L hotspot in *Xpc*$^{-/-}$*p53*$^{+/-}$ mice is clearly different from other *p53* mutation hotspots and is likely the result of altered DNA repair and/or selection for the unique function(s) of this allele. Defective nucleotide excision repair (NER) increases the risk of UVB-induced squamous cell carcinomas in mice (Cheo *et al.*, 2000). While the T122->L amino acid change is infrequent in *Xpa*$^{-/-}$*p53*$^{+/-}$ mice (D. Nahari and E. C. Friedberg, unpublished observations), it is the predominant *p53* mutation hotspot in skin cancers induced by UVB irradiation in *Xpc*$^{-/-}$*p53*$^{+/-}$mice (Reis *et al.*, 2000). This difference could result from a specific requirement for XPC protein for the repair of a minor type of UV-induced lesion at a non-dipyrimidine site. Alternatively, or additionally, the selective pressures for p53 inactivation during skin carcinogenesis might differ between the *Xpc*- and *Xpa*- defective mice. *Xpc*$^{-/-}$ cells are defective only in global genome repair (GGR), while *Xpa*$^{-/-}$ mutants are also defective in transcription-coupled repair (TCR) (Volker *et al.*, 2001).

Stalled RNA polymerase at the site of a lesion, inhibition of transcription and/or prolonged association of p53 proteins with TFIIH might contribute to p53 activation and induction of apoptosis. Differences have been observed in the cellular responses of *Xpa* and *Xpc* mutants to UV radiation. While large amounts of p53 accumulate in the nucleus and enhanced levels of apoptosis are observed in UV-irradiated *Xpa*$^{-/-}$ keratinocytes, the

apoptotic response of *Xpc*$^{-/-}$ keratinocytes is similar to that observed in wild-type cells (Wijnhoven *et al.*, 2000). Moreover, like wild-type cells, UVB-irradiated *Xpc*$^{-/-}$ but not *Xpa*$^{-/-}$ keratinocytes can progress through S phase, a stage of the cell cycle where p53 function might be reduced (Gottifredi *et al.*, 2001). However, the defect in GGR coincides with subsequent arrest in G_2, likely resulting from persistent DNA damage (van Oosten *et al.*, 2000).

Given the potential role of p53 in affecting DNA repair (Offer *et al.*, 2001; Smith *et al.*, 2000), *p53* mutations that result in less apoptotic activity while retaining DNA repair stimulation and cell cycle control may have a higher likelihood of being oncogenic. Interestingly, the transactivation results with T122->L in yeast (Table 1) showed reduced activity with the RE of both *AIP1* and *mFAS*, two important genes for UV radiation-induced apoptosis in skin cells (Hill *et al.*, 1999; Oda *et al.*, 2000), and slightly enhanced activity with the RE of *Gadd45* gene, that instead contributes to DNA repair (Smith *et al.*, 2000).

The reasons for the genetic background contributing to the appearance of the T122->L hotspot are not obvious. However, it is interesting to speculate on this possible contribution in light of the following observations: 1) the levels of p53 protein produced in wild-type and various DNA repair deficient mouse mutants in response to UV are different; and 2) specific responses such as apoptosis can be enhanced at high levels of p53 (Lane, 2001; Zhao *et al.*, 2000). In order to induce skin cancer, mice were chronically UV-irradiated. This is expected to result in p53 stabilization/activation and subsequent downstream p53-regulated responses. Mice with the following six genotypes were examined: wild-type, *p53*$^{+/-}$, *Xpc*$^{-/-}$ *p53*$^{+/+}$, *Xpc*$^{-/-}$ *p53*$^{+/-}$, *Xpa*$^{-/-}$ *p53*$^{+/+}$ and *Xpa*$^{-/-}$

p53$^{+/-}$ (Reis *et al.*, 2000; Takeuchi *et al.*, 1998) (Nahari and Friedberg, unpublished results). Since the amount of unrepaired damage influences the extent of p53 induction, the *Xpa*$^{-/-}$ cells would have the highest levels of p53 and apoptosis. The p53 levels would be expected to be reduced in *Xpc*$^{-/-}$ cells and somewhat lower in wild-type cells where repair is most extensive. The appearance of the T122->L p53 mutation in the Xpc$^{-/-}$ p53$^{+/-}$ background might result in greater viability compared to Xpa$^{-/-}$ p53$^{+/-}$ cells because of less p53 protein and reduced ability to induce apoptosis by the mutant protein. The partial capacity by T122->L for cell cycle arrest and DNA replication/repair functions might further reduce p53-independent apoptosis. Since the mutation appears to be recessive, there would be no selective advantage of a T122->L mutation in a p53$^{+/+}$ background. There have been only two cases of tumor-associated T122->L mutations in *Xpc*$^{-/-}$ *p53*$^{+/+}$ mice and in both the other allele had an independent inactivating mutation (Reis *et al.*, 2000). In p53$^{+/+}$ cells there would be a strong selective pressure for dominant-negative, non-functional p53 mutations (such as R270->C) that may also affect p53-independent (*e.g., p73*-mediated) apoptosis. Interestingly, although the R270->C mutation is frequent in UVB-induced skin tumors from wild type, and *p53*$^{+/+}$ *Xpc*$^{-/-}$ *or Xpa*$^{-/-}$mice, it was not found in the *Xpc*$^{-/-}$ *p53*$^{+/-}$ background (Reis *et al.*, 2000; Takeuchi *et al.*, 1998).

Implications of the T122->L mutation

We have determined that the T122->L mutant p53 is altered in its ability to recognize and transactivate a variety of test REs and have shown that its activity in the yeast functional assay is different from any of the previously studied hotspot tumor *p53* alleles. Our results suggest that it is unlikely that the high incidence of T122->L mutations in tumors

is solely the result of specific DNA damage, a defect in DNA repair, and/or a reduction in wild-type *p53* gene dosage. The altered DNA binding affinity and specificity of the T122->L mutant protein suggests that it would lead to an altered pattern of expression of downstream *p53*-regulated genes *in vivo* in tumor cells and that this contributes to its selection. These results suggest that other *p53* tumor mutations may also reflect functional changes that are uniquely advantageous in the tissue in which they arise. In this regard, it is interesting that in breast cancers identified in BRCA1 families, novel *p53* mutations have been detected (Crook *et al.*, 1998), some of which have subtle transactivation defects (Inga and Resnick, unpublished).

We propose that the yeast functional assay with inducible *p53* expression is a relevant and sensitive screening tool for evaluating *p53* tumor mutations that retain partial function. Transactivation analysis with variable expression of *p53* using different REs under isogenic conditions provides for broad characterization of the relative transactivation capacity of *p53* alleles. This information may be valuable for structure/function studies, tumor diagnosis, prognosis and clinical intervention. When combined with our recent *delitto perfetto* system for rapid *in vivo*, site-directed mutagenesis (Storici *et al.*, 2001), this assay may prove useful for developing a detailed functional classification of tumor-associated *p53* alleles.

Materials and Methods

Plasmids, MEF and Saos-2 cell transfections, growth suppression assays, luciferase assays, DNA binding assays and Western blots.

The mouse wild type *p53* cDNA and the T122->L and R270->C mutant cDNAs were cloned into plasmid pCI-Neo (Promega, Madison, WI) under control of the strong constitutive *CMV* promoter. These vectors were used for transient transfections in MEFs and for all experiments in Saos-2 cells. $p53^{-/-}$ and $p53^{+/+}$ MEFs were derived from 12 day old embryos by culturing homogenized samples in DMEM with 10% fetal calf serum at 37^0C under 5% CO_2. For growth suppression assays, MEF cells were infected by the retroviral vector pBabe-PURO expressing mutant *p53* alleles under the LTR promoter. Puromycin- resistant colonies were selected. For the luciferase assays, $8x10^5$ cells were plated 24 hours before transfection. The cells were then transfected using the FuGENE^{TM} 6 transfection reagent (Roche, Indianapolis, IN), 2 μg of pCI-Neo *p53* expression vector and 4 μg of reporter plasmid and recovered after 48 hours. Luciferase activity from 10 μl of cell lysates was assayed with the Luciferase assay system (Promega) using a Rosysenthos Lucy 2 type luminometer.

The osteosarcoma derived *p53* null cell line Saos-2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in McCoy's 5A medium with 15% FBS serum (Life Technologies, Grand Island, NY). T25 cm^2 cell cultures flasks were seeded and transfected at 60-80% confluence by the lipofectamine reagent (Life Technologies). For stable transfections, 1 μg of purified plasmid DNA was used. Cells were selected by adding G418 (Life Technologies) at 0.5 mg/ml after one day of recovery in complete medium following removal of lipofectamine. Colonies were

stained with Coomassie Blue after 2-3 weeks. Independent stable transfectant clones were obtained by limiting dilution in 96 well plates. Luciferase assays were performed using 20 ng of *p53* expression plasmid and 500 ng of reporter vector in 12 well-plate clusters. Plasmids pGL1012, pGL1138 and pGL-NA containing a 370 bp fragment of the human *BAX* promoter, a 2.3 kb region from the *p21* promoter, and the *MDM2-P2* promoter regulating the luciferase gene, respectively, were kindly provided by Dr. Moshe Oren. Plasmid PG13 containing the luciferase gene under p53 control through 13 copies of the *RGC* sequence was kindly provided by Dr. Bert Vogelstein.

Cells were recovered after 24 hr, lysed, and protein concentration was measured with the Bradford assay (Biorad, Hercules, CA) according to the standard protocol. Luciferase activity was measured with the Luciferase Assay System (Promega, Madison WI) using the single photon monitor program in a scintillation counter (Beckmann, Irvine, CA). After subtraction of the blank reading, arbitrary light units relative to 1 ng of protein extract were determined. The same or similarly obtained protein extracts were used for Western blot analysis. Precast SDS-PAGE gels (PAGE-ONE, Owl Separation Systems, Portsmouth, NH) were used for electrophoresis. Proteins were transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA) using a semi-dry electroblotter (Owl Separation Systems, Portsmouth, NH) according to instructions. p53 was detected using both pAb1801 and DO-1 monoclonal antibodies (Santa Cruz Biotech, Santa Cruz, CA) at a 1:2000 dilution. p21 was detected using C-19 polyclonal antibody at a 1:1000 dilution (Santa Cruz). Immunodetection was performed using the ECL kit (Amersham, Cleveland, OH) according to protocol.

Apoptosis and cell cycle assays in MEFs.

p53$^{-/-}$ MEF cells were stably infected using the retroviral vector pBabe-PURO that was empty or expressed either the T122->L or the R270->C mutant proteins under the LTR promoter. 1x10^6 cells per 15 cm^2 dish were plated and were irradiated or mock-treated 24 hours later. The cells were harvested 48 hours after treatment. Determination of death and DNA content was performed by double staining the cells with 7-AAD (Molecular Probes) and Hoechst (Molecular Probes). Levels of fluorescence staining were assessed by flow cytometry (FACScan Becton Dickinson Immunocytometry Systems, San Jose, CA).

Yeast strains, plasmids, media and reagents.

The haploid *S. cerevisiae* strain *yIG397* (*MAT**a** ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3 3xRGC::CYC1::ADE2*) (Flaman *et al.*, 1995) contains an integrated copy of *ADE2* under control of a minimal *CYC1* promoter. Three copies of the human p53 response DNA element found at the ribosomal gene cluster are inserted in the upstream region of the promoter. Thus, *ADE2* is under p53 transcriptional control so that transactivation by p53 mutant proteins can be easily scored based on the color of yeast transformants on suitable plates. Colonies expressing wild type p53 grow as adenine prototrophs yielding white colonies on plates containing a limiting amount of adenine while small red colonies appear when a nonfunctional p53 is expressed. The haploid strains, *yPH-p21* (*MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3- Δ200 leu2- Δ1, URA3::p21-RE::pCYC1::ADE2*) and *yPH-bax* (*MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3- Δ200 leu2- Δ1 URA3:: bax-RE::pCYC1::ADE2*) (Flaman *et al.*, 1998) allow the evaluation of p53 transactivation function as in *yIG397*, but with the *p21* and *BAX*

p53 REs controlling *ADE2* transcription, respectively. In order to assess the transcriptional activation of the same *ADE2* reporter gene at a fixed chromosomal locus as a function of specific p53 REs, the haploid strain *yIG397* was modified as follows. A *ura3* mutant that had *popped-out* the *ADE2* reporter gene at the *URA3* locus was obtained. The *ade2-1* gene was deleted by a PCR-based method and a promoterless wild type copy of the *ADE2* open-reading frame was integrated replacing the *ade2-1* locus. Isogenic derivatives of this strain, identified as *yAFM-RE*, each containing a minimal *CYC1* promoter that replaces the promoter of the *ADE2* open-reading frame fused to a different p53 RE, were constructed. Expression of wild type p53 in the *yAFM* strains results in the growth of white colonies on plates containing a limiting amount of adenine, since p53 stimulates *ADE2* transcription, while small red colonies appear when nonfunctional p53 is expressed. All the p53 response elements tested in this study are listed in Table 1. The sequence of each response element and a detailed description of the strain construction will be described elsewhere (Inga *et al.*, in preparation).

Plasmids pTGmp and pTAmp are yeast centromeric expression vectors for the mouse wild type p53 under the control of the *GAL1,10* and *ADH1* promoters, respectively. They were constructed from plasmid pLS89 (Flaman *et al.*, 1995) by replacing the human *p53* cDNA with the mouse homologue to obtain pTGmp and then replacing the *GAL1* promoter with the *ADH1* promoter to obtain pTAmp. The T122->L and R270->C mutations were cloned in pTGmp and pTAmp from the pCI-Neo vectors. Yeast strains were cultured in 1% yeast extract, 2% peptone, 2% dextrose (YPD) or in YPD medium containing 200 mg/L adenine (YPDA) or on selective medium. p53 transactivation was generally determined in synthetic medium containing 5mg/l adenine.

Synthetic medium containing 2% raffinose (Sigma, St. Louis, MO) as carbon source and variable concentrations of galactose was used to test transactivation and growth inhibition by various levels of p53.

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Chapter IV: Studies of the T122L Protein in wild type and *Xpc*$^{-/-}$ cells

Introduction

The UV component of the solar radiation is the major carcinogen in skin cancer. UV-induced DNA damage leads to mutations in key genes, which eventually can result in tumor formation. The tumor suppressor gene *p53*, which plays a key role in cell cycle regulation, is mutated in a wide variety of human tumors. The gene can be mutated at over a 100 different amino acids with all classes of mutations occurring (Daya-Grosjean, Dumaz et al. 1995). Many functional changes in p53 activity are correlated with specific mutations that affect protein conformation and p53 transactivation properties (Flaman, Robert et al. 1998; Blandino, Levine et al. 1999; Aurelio, Kong et al. 2000). Most of the p53 mutations are located in the evolutionary conserved DNA binding domain of the protein. The mutational hot spots found in skin tumors are in general similar to those found in other type of tumors. However, some amino acids that are frequently mutated in skin tumors are rarely found in internal tumors. Those mutations are located in sites with two adjacent pyrimidines and are the consequences of the major UV-induced lesions: the cyclobutane dimers (CPDs) and the (6-4) photoproducts.

The majority of these lesions are removed from the DNA by the nucleotide excision repair (NER) process, which is defective in XP patients. In order to investigate the relations between NER deficiency and predisposition to skin cancer our lab has generated mice that are mutated in the *Xpc* gene, and demonstrated that these mice are highly

predisposed to UVB-induced skin cancer (Cheo, Meira et al. 1996; Cheo, Ruven et al. 1997; Cheo, Meira et al. 2000).

Sequencing the *p53* gene in tumors from these mice revealed a novel hotspot at codon 122 of the mouse *p53* gene in *Xpc*$^{-/-}$ *p53*$^{+/-}$ mice (Reis, Cheo et al. 2000). In order to explore the functional consequences of the T122->L mutation we have carried out ectopic expression of the mutant p53 protein in wild type or *Xpc*$^{-/-}$ mouse embryo fibroblasts (MEF cells). We have performed growth suppression, cell cycle and luciferase reporter assays examining wild type and mutant p53 proteins in *Xpc*$^{-/-}$ cells. In addition, we compared *Xpc*$^{-/-}$ and wild type MEF cells using microarrays. We conclude that the precise pattern of T122->L p53-regulated gene expression, rather than a simple loss of p53 function, may be a critical determinant in tumor selection and progression in UV-irradiated mouse skin cells.

Materials and Methods

Plasmids, transfections, growth suppression and luciferase assays

The mouse wild type *p53* cDNA and the T122->L and R270->C mutant cDNAs were cloned into plasmid pCI-Neo (Promega, Madison, WI) under control of the strong constitutive *CMV* promoter. These vectors were used for transient transfections. Wild type, *p53*$^{-/-}$ and *Xpc*$^{-/-}$ *p53*$^{-/-}$ MEFs were derived from 12 day old embryos by culturing homogenized samples in DMEM with 10% fetal calf serum at 37^0C under 5% CO_2. For growth suppression assays, MEF cells were infected by the retroviral vector pBabe-PURO expressing mutant *p53* alleles under the LTR promoter. Puromycin- resistant colonies were selected. For the luciferase assays, $8x10^5$ cells were plated 24 hours before

transfection. The cells were then transfected using the FuGENE^{TM} 6 transfection reagent (Roche, Indianapolis, IN), 2 μg of pCI-Neo *p53* expression vector and 4 μg of reporter plasmid and recovered after 48 hours. Luciferase activity from 10 μl of cell lysates was assayed with the Luciferase assay system (Promega) using a Rosysenthos Lucy 2 type luminometer.

Apoptosis and cell cycle assays

p53$^{-/-}$ and *Xpc*$^{-/-}$ *p53*$^{-/-}$ MEF cells were stably infected using the retroviral vector pBabe-PURO that was empty or expressed either the T122->L or the R270->C mutant proteins under the LTR promoter. 1x10^6 cells per 15 cm^2 dish were plated and were irradiated or mock-treated 24 hours later. The cells were harvested 48 hours after treatment. Determination of death and DNA content was performed by double staining the cells with 7-AAD (Molecular Probes) and Hoechst (Molecular Probes). Levels of fluorescence staining were assessed by flow cytometry (FACScan Becton Dickinson Immunocytometry Systems, San Jose, CA).

Microarrays

Wild type, *Xpc*$^{-/-}$ or *p53*$^{-/-}$ MEFs were irradiated with 25J UVB. The cells were harvested 24 hours later and total RNA was extracted using Trisol Reagent (Invitrogen). RT-PCR to generate cDNA probes, biotin labeling and hybridization with commercially available microarray membranes from SuperArray Inc. (Q series) were performed as recommended by the manufacture (detailed protocols are available at http://www.superarray.com/).

Results and dicussion

T122L is a recessive mutant

In order to functionally characterize the T122L mutant protein in both wild type and *Xpc*$^{-/-}$cells it was transferred into wild type, *Xpc*$^{-/-}$, *p53*$^{-/-}$ or *Xpc*$^{-/-}$ *p53*$^{-/-}$ MEF cells using a retroviral vector that generates high levels of p53 expression. The wild type p53 protein and a second mutant allele, the R270C, were also transferred into the cells for comparison. The R270C is a mutational hotspot that is known to inactivate p53 function in many types of tumors. It is very frequent in UVB-induced mouse skin tumors since it contains a dipyrimidine sequence (You, Szabo et al. 2000). The R270C is a dominant negative mutant. It associates with the wild type protein, and the heterotetramer that is formed is not able to bind DNA (Chene 1998).

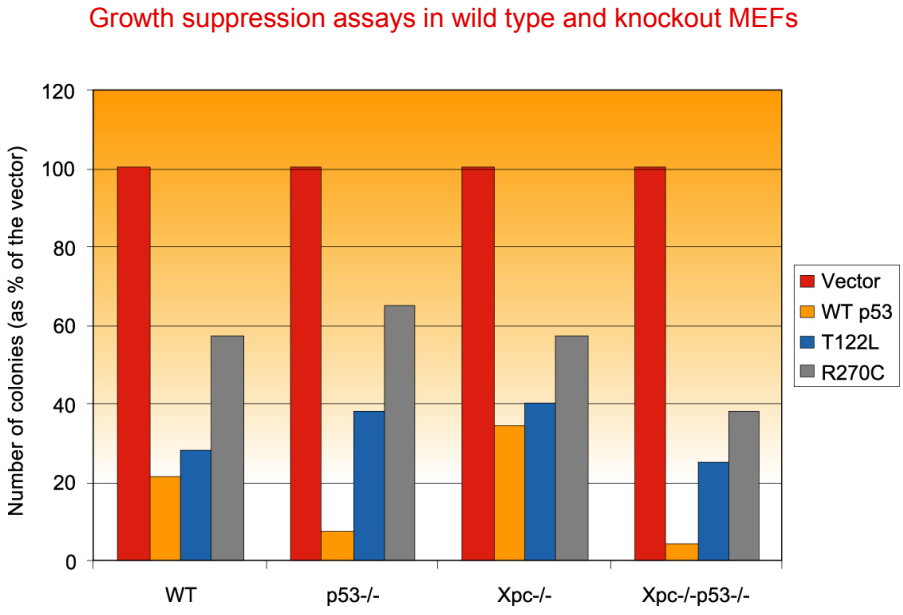


Figure IV-1: Growth suppression of wild type, *Xpc*$^{-/-}$, *p53*$^{-/-}$ and *Xpc*$^{-/-}$*p53*$^{-/-}$ MEF cells

Wild type, *Xpc* $^{-/-}$, *p53* $^{-/-}$ and *Xpc* $^{-/-}$*p53* $^{-/-}$ MEF cells were infected by the retroviral vector pBabe-PURO expressing the indicated *p53* alleles under the LTR promoter. Puromycin resistant colonies were selected and counted after two weeks. The relative mean numbers for three independent experiments are presented.

The results in Figure 1 show that the relative colony forming ability under R270C expression is similar between the wild type and *p53* $^{-/-}$ MEF cells. In XPC deficient cells, expression of R270C resulted in a higher number of colonies when the endogenous p53 protein was present compared to the knockout line. T122L expression in the two lines carrying the endogenous protein resulted in relative colony forming ability similar to the wild type protein expression. Differences between the number of colonies under T122L or wild type protein expression is observed in the lines with no endogenous wild type p53 suggesting that the T122L protein function is recessive. In this assay there were no major differences between wild type and *Xpc* $^{-/-}$cells.

Differences in cell cycle in wild type and Xpc $^{-/-}$ *cells expressing T122L*

We have previously characterized the T122L p53 mutant activity in cell lines that lacked endogenous p53 but were wild type for XP (Inga, Nahari et al. 2002). We have demonstrated that T122L can promote cell cycle arrest but not apoptosis in response to UVB, a feature that might result in the survival of cells that carry mutations, especially in a repair deficient background. Therefore we were interested in investigating the activity of the T122L p53 mutant in cells that are XP deficient (XPC and XPA) in addition to their p53 deficiency. We have generated *Xpc* $^{-/-}$*p53* $^{-/-}$ and *Xpa* $^{-/-}$*p53* $^{-/-}$ cell lines that stably express the T122L mutant and are currently performing cell cycle experiments. While *Xpc* $^{-/-}$ cells are defective in Global Genomic Repair, a mode of NER reparing

lesions in transcriptionally-silent regions of the genome or the non- transcribed strand of transcriptionally-active genes, *Xpa*$^{-/-}$cells are defective in both GGR and Transcription Coupled Repair (TCR), a mode of NER responsible for lesions located in transcriptionally-active regions.

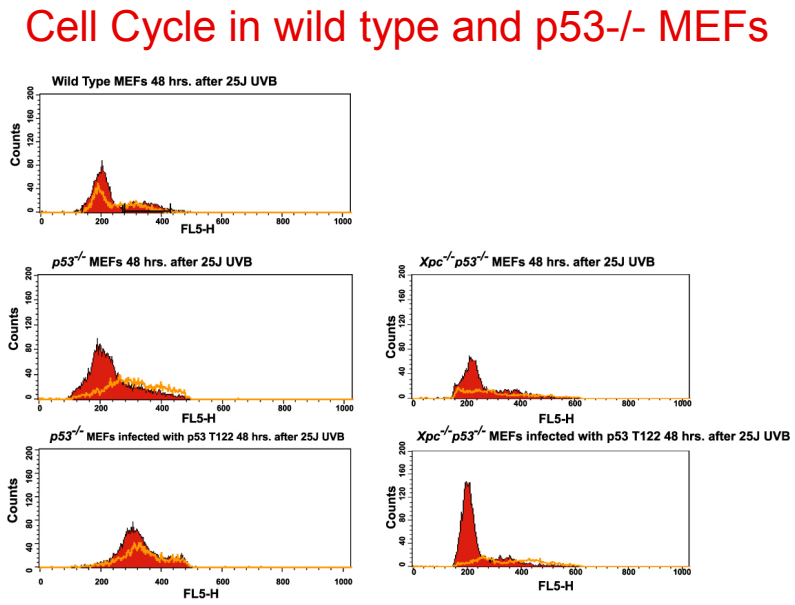


Figure IV-2: XPC can affect the Cell Cycle profile in *p53*$^{-/-}$ MEF cells

p53$^{-/-}$ or *Xpc*$^{-/-}$*p53*$^{-/-}$ MEFs were stably infected with Babe-PURO vector containing the p53^{T122} mutant protein. The cells were then irradiated with 25J of UVB and harvested 48 hours later. Cell cycle analysis of the viable cells before (red area) and after UV (yellow line) is shown.

Preliminary results with the *Xpc*$^{-/-}$*p53*$^{-/-}$ line suggest that p53 does not promote cell cycle arrest in this background. *Xpc*$^{-/-}$ cells have defective cell cycle regulation. The XPC protein interacts with CEN2, which is important for centrosome duplications (Araki, Masutani et al. 2001), an interaction that might couple cell division and NER. UVB irradiated *Xpc*$^{-/-}$ keratinocytes can progress through S phase, but defective GGR leads to

G_2 arrest resulting from persistent DNA damage (van Oosten, Rebel et al. 2000). On the contrary, *Xpa*$^{-/-}$ cells, which are defective also in TCR, accumulate large amounts of p53 in the nucleus and have an enhanced apoptotic response induced by lesions in the transcriptionally-active regions (Brash, Wikonkal et al. 2001). Thus, the very rare appearance of the T122L mutation and its protein product in the *Xpa*$^{-/-}$ background might be related to differences in cell cycle regulation that can affect cells expressing the mutant protein.

T122L has altered transactivation specificity

The threonine of codon 122 is located in a highly conserved region within the p53 protein DNA binding domain, called the L1 loop-S2-strand region. Recent findings suggest that several amino acid changes in this region can alter sequence specificity and affinity of the p53 protein to its response elements (Inga, Monti et al. 2001; Inga and Resnick 2001). Our previous studies show that the T122L mutation alters the transactivation functions of the p53 protein for several key p53 target genes including *p21*, *mdm2* and *Bax* using luciferase reporter assay (Inga, Nahari et al. 2002). We repeated the experiment using *Xpc*$^{-/-}$ MEF cells. The results presented in Figure 3 show that having no XPC protein did not affect T122L activity significantly. Interesingly, in the XPC deficient line, there is no significant different in p21 activation between wild type p53 and the empty vector.

Activation of p53 target genes by wild type and mutant p53 protein.

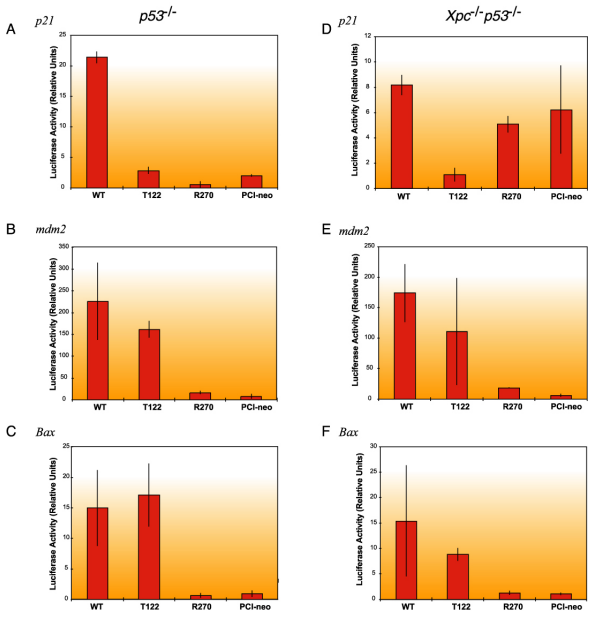


Figure IV-3: Activation of p53 target genes by wild type and mutant p53 protein

Transfection-based reporter assays were carried out using *p53*$^{-/-}$ (A, B, C) or *Xpc*$^{-/-}$*p53*$^{-/-}$ (D, E, F) MEFs. We have used a reporter plasmid with the *p21* (A, D), *mdm2* (B, E), or *Bax* (C, F) promoter region fused to the luciferase reporter gene and expression vectors (PCI-neo) with either the wild-type p53, the T122L mutant or the known dominant-negative mutant, R270C. The values are the average of two or three different experiments and the error bars represent the standard deviation.

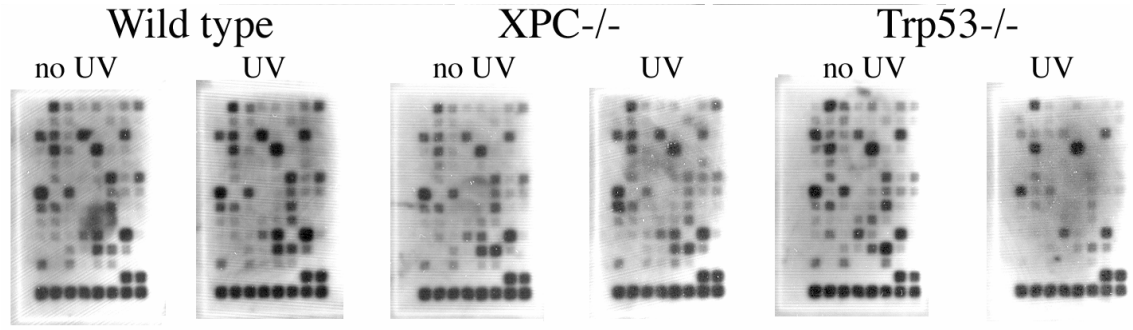
Xpc$^{-/-}$ *cells have altered gene expression profile*

The T122L protein retains the ability to act as a transcription factor and activate p53 target genes. However, the gene expression profiles in cells that express this mutant appear to be different than the one for the wild type protein. DNA microarrays represent a powerful new technology that is being increasingly used in the last several years (Alon, Barkai et al. 1999). The Mouse Cancer PathwayFinder GEArray Q series from

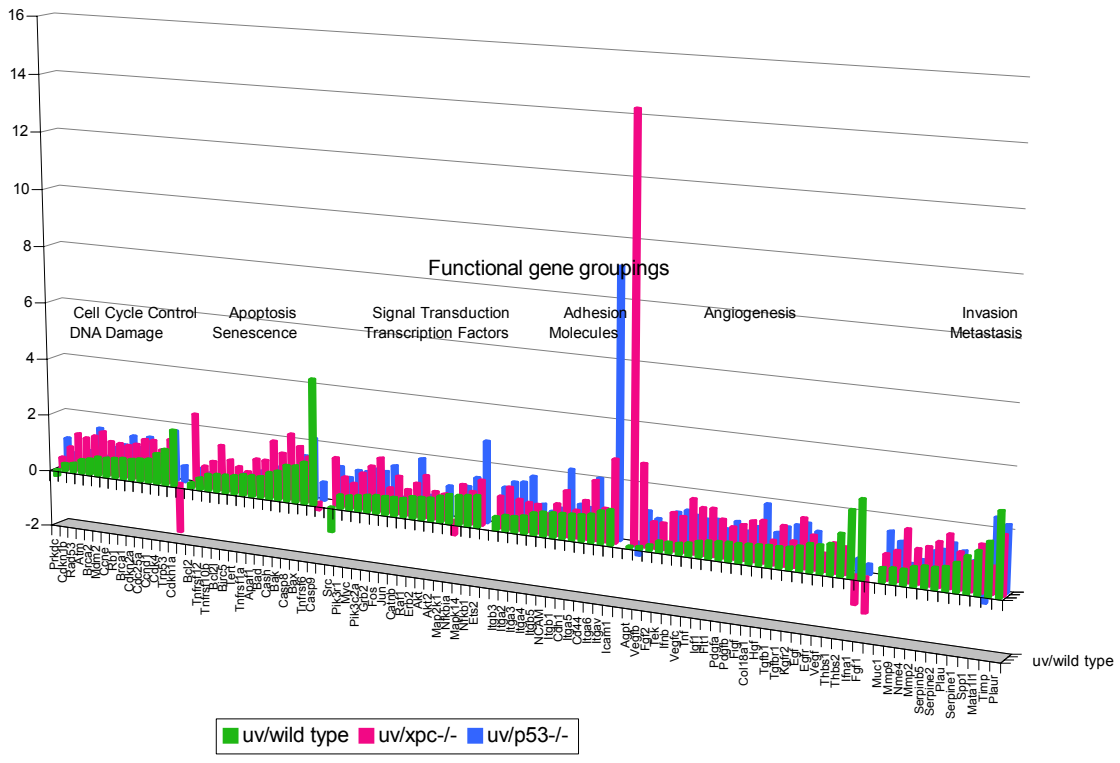
http://www.superarray.com/ focus on specific genes belonging to biological pathways that are frequently altered during cellular transformation and tumorigenesis. This type of experiment allows us to rapidly obtain a gene expression profile of key target genes in cells that express the T122L mutant protein compared to wild type. First, we wanted to determine whether the XPC protein itself influences the gene expression profile. Differences between gene expression profiles in wild type and *p53*$^{-/-}$ cells compared to *Xpc*$^{-/-}$ and *Xpc*$^{-/-}$*p53*$^{-/-}$ cells will suggest that in addition to its role in repair, XPC is important also for cell growth and cell cycle control.

In preliminary experiments we have irradiated wild type, *Xpc*$^{-/-}$ or *p53*$^{-/-}$ cells with 25J of UVB and then collected RNA samples 24 hours after UV treatment. We followed a standard protocol for hybridization of commercially available microarray membranes from SuperArray Inc. (Q series) as recommended by the manufacture. cDNA probes were prepared and labeled with biotin, then hybridized with the microarray membranes. Figure 4A shows the results for the irradiated samples and the non-irradiated controls. Figure 4B presents analysis of the results using the GEArrayAnalyzer software. The fold induction after UV in the three different genotypes is represented. Figure 4C focuses on three genes that had different responses to UV in wild type compared to *Xpc*$^{-/-}$ MEF cells. The first one is the CDK4 inhibitor p21 that is activated by p53 in response to DNA damage to stop DNA replication (el-Deiry, Tokino et al. 1993; Levine 1997). Activation of p21 was reduced in *p53*$^{-/-}$ MEF cells compared to wild type cells as expected. Surprisingly, it was down regulated in *Xpc*$^{-/-}$ MEF cells that are wild type for p53.

4A


Wild type
XPC-/-
Trp53-/-
no UV
UV
no UV
UV
no UV
UV

4B


16
14
12
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Functional gene groupings
Cell Cycle Control
DNA Damage
Apoptosis
Senescence
Signal Transduction
Transcription Factors
Adhesion
Molecules
Angiogenesis
Invasion
Metastasis
uv/wild type
uv/wild type
uv/xpc-/-
uv/p53-/-

4C **4D**

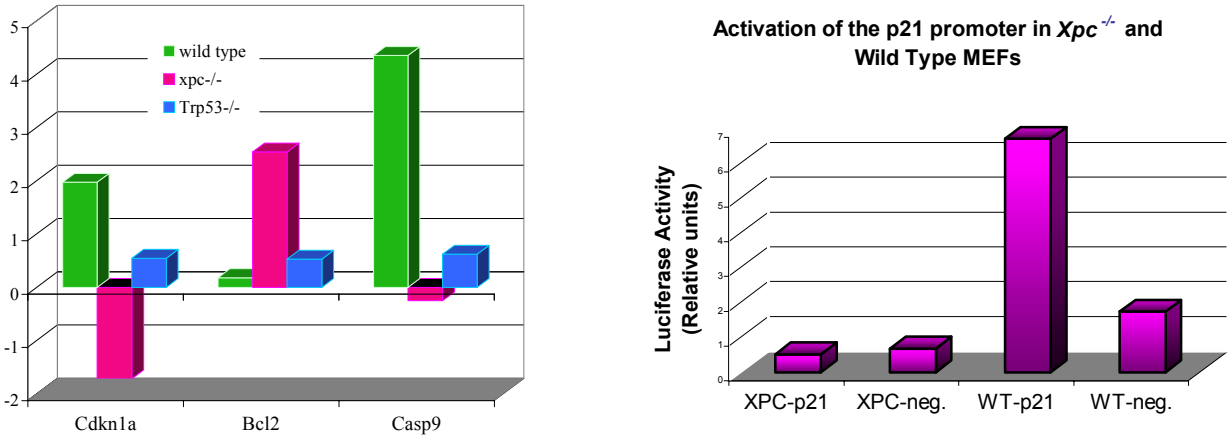

Figure IV-4: Microarray studies in wild type, *Xpc*$^{-/-}$ and *p53*$^{-/-}$ MEF cells.

A. Microarrays probed with cDNAs made from RNA of wild type, *Xpc*$^{-/-}$ and *p53*$^{-/-}$ MEFs exposed to 25J UVB or mock treated and harvested 24 hours later. **B**. Gene profile analysis plotted as fold difference after UV exposure. **C**. Examples of genes with differential expression changes between the 3 genotypes **D.** Activation of the p21 promoter in *Xpc*$^{-/-}$ and wild type cells using luciferase reporter assay.

We followed the microarray experiment with a transient transfection one using luciferase reporter vector containing the p21 promoter (Figure 4D). Wild type or *Xpc*$^{-/-}$ MEF cells were transfected with p21 containing, or an empty vector and recovered after 48 hours to measure luciferase activity. The results show that p21 activation is significantly reduced in *Xpc*$^{-/-}$ background. The data is in agreement with previous reports showing that human XP-A fibroblast do not activate p21 in response to UV irradiation (Conforti, Nardo et al. 2000) and that XP-C human fibroblasts require a higher UV dose compared to wild type fibroblasts in order to activate p21(Zhu, Wani et al. 2000).

The other two genes in Figure 4C, Bcl-2 and caspase-9, have opposite affects on cells. Elevated expression of Bcl-2 suppresses p53 dependent apoptosis, a signal that can be antagonized by Bax, another family member that is p53 induced (Adams and Cory 1998; Evan and Littlewood 1998). On the other hand caspase-9 and its activator Apaf-1 take part in p53 dependent apoptosis (Adams and Cory 1998; Srinivasula, Ahmad et al. 1998; Soengas, Alarcon et al. 1999). Activation of caspase-9 commits cells to apoptosis. Apaf-1 activates Procaspase-9 in the cytochrome c/dATP-dependent pathway via proteolytic cleavage that generates mature caspase molecules. Once activated, caspase-9 can initiate a caspase cascade involving the downstream executioners caspase-3, -6, and -7. Bcl-2 can inhibit the association of Apaf-1 with procaspase-9 and thereby prevent caspase-9 activation.

In response to DNA damage caspase-9 is upregulated in wild type cells. Surprisingly, in *Xpc*$^{-/-}$ cells caspase-9 is down regulated while Bcl-2 is activated. We will first verify the microarray results using conventional methods such as RT-PCR and Northern analysis and continue to investigate the apoptotic response in *Xpc*$^{-/-}$ cells. We plan to utilize the new GEArray Q Series Mouse DNA Damage Signaling Pathway gene array that was designed to profile the expression of key genes involved in DNA damage signaling pathways. The genes featured are transcriptional targets of DNA damage response and can be categorized into three functional groups: cell cycle arrest, apoptosis, and genome stability & repair pathways.

In order to obtain a gene expression profile in cells expressing the T122L mutant protein compared to wild type we will use a well-characterized system for controlled protein expression that utilizes an inducible metallothionein promoter (Ohkubo,

Kawakami et al. 1990; Shaw, Bovey et al. 1992; Burfeind, Chernicky et al. 1996; Lang, Miknyoczki et al. 1998; Alon, Barkai et al. 1999; Zhao, Gish et al. 2000). The system has proven especially informative for the p53 protein (Shaw, Bovey et al. 1992; Lang, Miknyoczki et al. 1998; Alon, Barkai et al. 1999; Zhao, Gish et al. 2000). *p53*$^{-/-}$ cells or *Xpc*$^{-/-}$*p53*$^{-/-}$ cells were stably transfected with constructs that contain a selectable marker and either the wild type protein-coding region or the T122L mutant under the control of the metallothionein promoter. Induced expression is achieved by adding $ZnSO_4$ to the medium. We are currently in the process of selecting Blasticidin resistant clones and analyzing the p53 expression using commercially available p53 antibodies. Once the cell lines are fully characterized we will obtain RNA samples from several time points following UVB induction. Those samples will be hybridized with the microarray membranes described above.

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Chapter V: Summary and concluding remarks

Nucleotide excision repair (NER) is the main cellular defense against the deleterious affects of solar radiation. It removes the major UV induced lesions, CPDs and (6-4) photoproducts, from the DNA. Xeroderma Pigmentosum (XP) is a rare human disorder caused by inherited defects in NER. The hallmark of the disease is hypersensitivity to sunlight and a huge increase in predisposition to skin cancer. The patients can be divided into seven genetic complementation groups (A-G) with genes corresponding to *Xpa* thru *Xpg*.

I have studied a novel UV specific mutation in codon 122 of the *p53* gene that appears in mice deficient in the XPC protein that are also heterozygous for the *p53* gene. Chapter I provided detailed description of the NER process and the clinical consequences of its deficiency. Mouse models for the relevant proteins were also described. In Chapter II I showed that the T122L mutation is rare in other NER-deficient mouse models, suggesting that the XPC protein is required specifically to repair the damage. XPC-deficient ($Xpc^{-/-}$) cells combine a higher mutational rate with reduced apoptosis, which might also explain the frequent occurrence of T122L mutations in this background. I have used a p53 knockout model that retains the genomic region containing codon 122 without expressing the protein in order to show that the appearance of the T122L mutation depends on the p53 mutant protein function. The protein is apparently recessive since the mutation appears mainly in the *p53* heterozygous background.

Detailed characterization of the mutant protein function was presented in Chapter III. The T122L mutant protein retains some wild type protein functions. It transactivates the p53 regulated genes *mdm2* and *Bax* and promotes cell cycle arrest. Genotype specific p53

mutants were also reported in patients with mutations in the tumor suppressor genes BRCA1 and BRCA2 that are also involved in DNA repair. These mutants also retain some wild type protein functions in contrast to the loss of function mutants commonly observed in sporadic cancers. Interestingly, BRCA1 enhances GGR by inducing expression of the GGR damage recognition factors XPC and DDB2 and induction of GADD45 protein, in a p53-independent manner (Hartman and Ford 2002).

Preliminary experiment described in Chapter IV reveals different expression profiles between wild type and *Xpc*$^{-/-}$ MEF cells. It is intriguing that three genes involved directly in activation of cell cycle arrest and apoptosis show different expression levels in UV-treated *Xpc*$^{-/-}$ MEF cells compared to wild type. The XPC protein interacts with CEN2, which is important for centrosome duplications (Araki, Masutani et al. 2001), an interaction that might couple cell division and NER. It was previously reported that UVB irradiated *Xpc*$^{-/-}$ keratinocytes can progress through S phase but the defect in GGR leads to G_2 arrest resulting from persistent DNA damage (van Oosten, Rebel et al. 2000). TCR defective *Xpa*$^{-/-}$ keratinocytes arrest in G_1, and have enhanced apoptosis signal initiated by the stalled RNA polymerase II at lesions in transcriptionally active regions. It will be interesting to compare *Xpc*$^{-/-}$ cells to other GGR-deficient models such as XP-E cells or *gadd45*$^{-/-}$ MEF cells in order to separate GGR-dependent changes from those that are specifically XPC dependent.

In addition to its role in GGR and its possible role in cell cycle regulation, XPC was recently implicated in repair of T:G mispairs, which are not NER substrates. Although we could not identify the type of damage in codon 122, the ACG sequence is unlikely to produce a lesion repaired by NER. Thus, the accumulation of the T122L mutation in the

XPC background might be a result of a different repair pathway deficiency. That will also explain why we could not detect the mutation in the other NER-deficient models we tested.

Two different lines of investigation emerge from studying the mechanisms for the appearance of the T122L mutation in XPC-deficient skin. The first one follows a possible novel repair pathway that involves the XPC protein and is required to repair the unidentified type of damage at codon 122. The second one focuses on possible role of XPC in the interplay between DNA repair, cell cycle regulation and apoptosis. Future studies involving the XPC protein and its different functions in the cell will help us understand why its deficiency results in such an unusual mutational event.

VITAE

Dorit Hanson Nahari was born in Ramat-Gan, Israel on June 16, 1968, the daughter of Dalia and Geoffrey Hanson. After graduating from “Blich” high school, Ramat-Hen, Israel in 1986 she joined the Israeli Defence Forces where she served till 1989 as an educational officer. She entered Israel Institute of Technology at Haifa, Israel in 1990 and received the degree of Bachelor of Science in Food Engineering and Biotechnology, Cum Laude, in June 1994. In September 1994 she entered the graduate school and received the degree of Master of Science in Food Engineering and Biotechnology in October 1996. She joined the graduate school in University of Texas Southwestern Medical Center at Dallas, Texas in August 1997. In 1990, she married Yuval Nahari. Her daughter Tal was born in Haifa, Israel in 1994. Her daughters Shai (1997) and Gil (2001) were born in Dallas, Texas.

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