A MOUSE MODEL FOR TRICHOTHIODYSTROPHY

Een muizenmodel voor trichothiodystrofie

PROEFSCHRIFT

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CONTENTS

voor mijn ouders
voor Madelon

AIM OF THE THESIS

Nucleotide excision repair (NER) is a versatile DNA repair mechanism that safeguards the genome from many types of DNA damages. The importance of NER is highlighted by three inherited human disorders with defective NER: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). XP patients display enhanced susceptibility to sunlight-induced skin cancer, but CS and TTD are not associated with increased cancer susceptibility despite an NER defect. Moreover, CS and TTD patients are characterized by a broad range of neurodevelopmental abnormalities, which are difficult to rationalise as a consequence of a defect in NER. One of the NER genes, *XPD*, is implicated in XP, XP with combined features of CS, and TTD. XPD is a subunit of the dually functional transcription factor IIH (TFIIH) complex, involved in NER and basal transcription initiation. It was hypothesized that mutations in *XPD* may not only affect NER, causing XP and photosensitivity in TTD, but may also impair the transcription function of TFIIH accounting for the neurodevelopmental abnormalities in CS and TTD. The aim of the work outlined in this thesis is to gain insight into the molecular basis of the clinical symptoms associated with defects in the *XPD* gene, and into the enigmatic difference in cancer predisposition between XP and TTD patients. To accomplish this, we aimed at generating mouse models for XP, CS and TTD. Therefore, disease-specific mutations identified in the *XPD* gene of XP, XP/CS and photosensitive TTD patients were mimicked in the mouse *XPD* gene via homologous recombination in embryonic stem cells. **Chapter 1** of this thesis reviews the NER mechanism and the clinical symptoms associated with XP, CS and TTD. In **chapter 2,** literature on NER-deficient mouse models is reviewed and the phenotypical consequence of the role of NER proteins in DNA repair, mitotic recombination and transcription is discussed. **Chapter 3** and **4** describe the generation and characterization of a XPD knockout and TTD mouse model respectively. Further analysis of the TTD mouse model revealed cancer predisposition and signs of premature aging as described in **Chapter 5** and **6**.

CHAPTER 1

NUCLEOTIDE EXCISION REPAIR AND HUMAN SYNDROMES

NUCLEOTIDE EXCISION REPAIR AND HUMAN SYNDROMES

DNA contains the blueprint for the development, proper functioning and reproduction of every organism. Undesired alterations to the chemical structure of DNA molecules (lesions) can arise spontaneously through intrinsic instability of DNA (e.g. deamination, depurination etc.) or they can be induced by chemical compounds and iradiation. DNA lesions are of many different types (see Figure 1) including single strand and double strand breaks, inter- and intrastrand crosslinks and different of base modifications. At the cellular level, DNA lesions hamper processes like transcription and replication resulting in cell cycle arrest, (programmed) cell death and genomic instability (mutagenesis). At the organismal level, DNA lesions have been implicated in genetically inherited disease, carcinogenesis and ageing. A clear example of the deleterious effects of genotoxic agents in man is the strong correlation between sunlight exposure or smoking cigarettes and the development of skin and lung cancer respectively [1]. Both sunlight and cigarette smoke are exogenous sources of DNA damage. DNA base modifications can also arise endogenously through cellular metabolites, for instance oxidative DNA damage can result from free radicals generated as by-product of active oxidative metabolism. Accumulating evidence suggests that modulation of oxidative stress plays a role in ageing [2].

To prevent the harmful consequences of DNA damage, multiple DNA repair mechanisms exist (Figure 1). Briefly, double strand breaks are repaired by homologous recombination dependent repair or in an end-joining reaction, and most small base modifications are removed by base excision repair (BER). Nucleotide excision repair (NER), the repair mechanism investigated in this thesis, removes primarily bulky, helix-distorting adducts. However, considerable overlap exists in substrate specificity of repair pathways, and certain proteins are used in more than one pathway [3].

NUCLEOTIDE EXCISION REPAIR

Elucidation of the core mechanism of NER in the Gramm-negative bacterium *Escherichia coli* served as a paradigm for studies on NER in almost all organisms. The basic principle is removal of a small single-stranded patch of DNA containing the lesion by dual incision of the damaged strand (see Figure 2a). Resynthesis of the gap occurs using the complementary strand as template. The principle is evolutionary conserved from bacteria to man but the proteins involved share little homology. Most mammalian NER genes have been identified now and recently, the NER reaction was reconstituted *in vitro* using purified proteins [4]. This chapter summarizes the substrate specificity and the distinct steps of mammalian NER. For

a more detailed overview on the biochemistry of NER, the reader is referred to refs. 5 and 6.

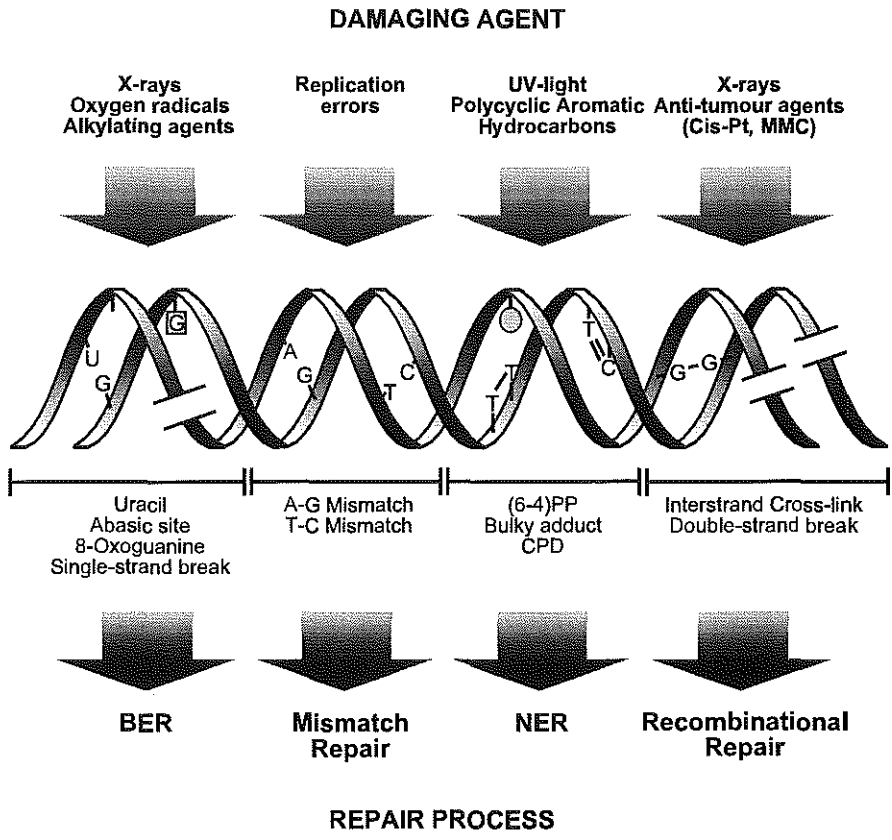

Figure 1 DNA lesions and repair mechanisms. Top: common DNA damaging agents. Middle: examples of lesions that can be introduced by these agents into the DNA double helix. Bottom: the most frequently used repair mechanisms for such lesions. Not depicted but important to realize is that distinct damaging sources can induce similar types of DNA lesions and that also the lesion spectrum of different repair pathways may overlap. Adapted from [6].

Lesions removed by NER

NER is the most versatile of all DNA repair mechanisms because it counteracts the deleterious effects of a plethora of structurally unrelated DNA lesions. The majority of the numerous chemicals to which NER-deficient cells are sensitive share the

capacity to generate bulky base adducts which can cause significant distortion of the DNA helix [7].

The clinically most relevant NER substrates are cis-syn-cyclobutane dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). Both are formed between adjacent pyrimidines, and they constitute the two major classes of lesions induced by solar UV light. The more helix-distorting 6-4PPs are repaired 5-fold faster than CPDs and, although CPDs are more abundant, UV hypersensitivity of rodent and human cell lines correlates better with the capacity to excise 6-4PPs than with CPD removal from the genome [8]. Other NER substrates include bulky chemical adducts such as large polycyclic aromatic hydrocarbons (induced by compounds in cigarette smoke) and the particularly distorting interstrand crosslinks, induced by cisplatin and probably also by certain cellular metabolites [9]. NER has also been implicated in removal of small base damages induced by alkylating and oxidizing agents which are generally not helix distortive [7]. Base excision repair is considered as the main pathway involved in repair of these types of damage, but NER may be considered as a back-up system [10,11]. The relative importance of NER in repair of alkyl and oxidative damage has not been firmly established yet. However, its involvement emphasizes the versatility of the NER mechanism, which also explains how various endogenous and exogenous genotoxic agents may contribute to the large spectrum of clinical symptoms associated with defects in one of the NER components.

The NER mechanism

The NER process involves the action of about 20-30 proteins in successive steps of damage recognition, local opening of the DNA double helix around the injury, and incision of the damaged strand on either side of the lesion. After excision of the damage-containing oligonucleotide the resulting gap is filled by DNA repair synthesis, followed by strand ligation. An in part speculative model for NER is presented below and in Figure 2.

Damage recognition

Binding of the XPC/hHR23B complex is considered as the initial, damage-recognizing step in NER [12], recruiting the repair protein machinery to the damaged site. Since locally premelted DNA is also sufficient the NER machinery to act in the absence of XPC/hHR23B [13], the complex may slightly increase single-strandedness at the site of the lesion. A role for the XPE protein in damage recognition has been proposed, because it has affinity for damaged DNA [14]. Also XPA displays a high affinity for damaged DNA [15], especially in a single stranded context.

Lesion demarcation

The next step is the formation of an open complex, requiring a local unwinding of the DNA helix and demarcation of the lesion. XPA has many interactions with other NER components, for instance with the single strand binding complex RPA [16], the TFIIH complex [17] and the ERCC1/XPF endonuclease [18] hence XPA may orchestrate the repair machinery around the DNA lesion. Full opening of the DNA helix around the lesion is dependent on the presence of ATP [19], strongly arguing that the helicases of the TFIIH complex (discussed below) are actively involved. DNA unwinding by TFIIH may be facilitated by RPA, a heterotrimeric complex involved in NER, replication and recombination [20]. In NER it probably binds to the single stranded region of the undamaged strand with defined polarity [21]. The optimal binding patch of RPA is 30 nucleotides [22], which is approximately the size of the fully opened repair complex and the size of the released damage-containing patch. The orchestration of the different NER proteins in the pre-incision stage of NER is still unclear.

The role of TFIIH in NER and basal transcription

TFIIH is a protein complex of 9 subunits, which was originally identified as an essential factor in basal transcription initiation [23]. Later, the p89 and p80 subunits were rediscovered as the XPB and XPD proteins respectively, involved in NER [24,25]. They contain ATPase-driven 3'→5' and 5'→3' directed DNA helicase activity respectively, required for local unwinding of the DNA helix around the lesion in NER [19] and in the transcription initiation of RNA polymerase II at the promoter [26] (see Figure 2). In accordance with an essential role in basal transcription, mice with inactivating mutations in the TFIIH subunits XPB and XPD are inviable (ref. 27 and G. Weeda, unpublished). Other TFIIH components include Cdk7, cyclin H and MAT1, constituting the cdk-activating kinase (CAK) complex associated with TFIIH. The CAK complex is able to phosphorylate cyclin-dependent kinases (CDKs) involved in cell cycle regulation, and it is required for phosphorylation of the C-terminal domain of RNA polymerase II [28]. The CAK complex is more loosely associated with TFIIH and occurs in free form as well. It is not required for NER *in vitro*, suggesting that TFIIH can be either in a transcription- or in a repair-model [29]. Furthermore, a functional interaction of TFIIH with p53 has been reported [30,31], suggesting that apoptosis is also associated with TFIIH, in addition to NER, transcription, and cell cycle regulation.

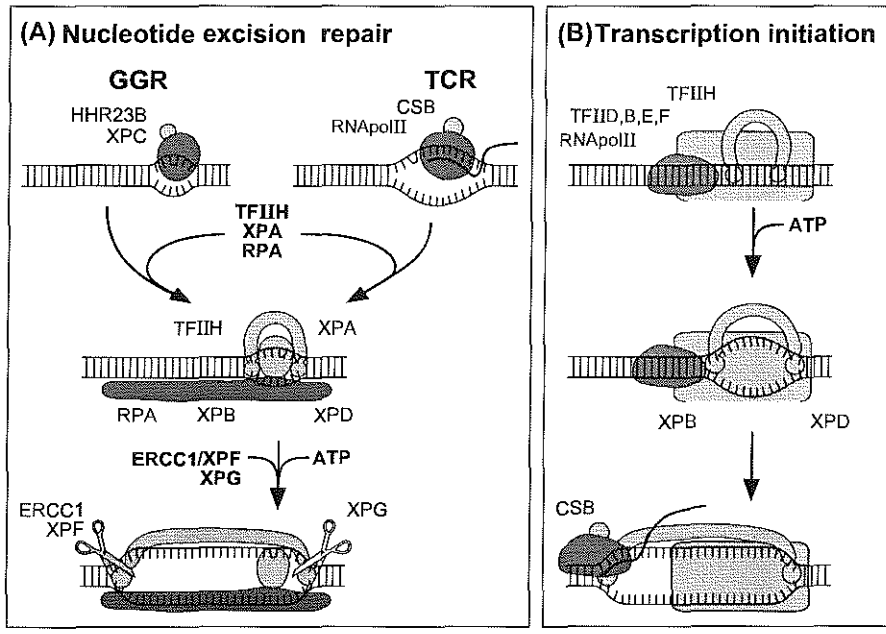

Figure 2. Model for TCR, GGR and the role of TFIIH in transcription and repair.A) Model for transcription-coupled repair (TCR) and general genome repair (GGR). Recognition of DNA damage can occur either by the XPC/HHR23B complex (GGR) or by RNA polymerase and CSB protein in TCR. Subsequently, DNA around the lesion is opened by the concerted action of RPA, XPA and the bi-directional XPB/XPD helicase of TFIIH. This allows incisions of the damaged strand on both sides of the injury by the repair-endonucleases ERCC1/XPF and XPG, excision of the lesion-containing oligonucleotide and gap-filling DNA synthesis. B) TFIIH in transcription initiation of RNA polymerase II. After assembly of the pre-initiation complex-consisting of five basal transcription factors and RNA polymerase II- the promoter region is opened by the XPB and XPD helicases of TFIIH. This allows formation of the first phosphodiester bond, promoter escape of RNA polymerase and transcription elongation. Adapted from [81].

Dual incision

After local unwinding and demarcation of the lesion, an oligonucleotide of 24-32 nucleotides containing the lesion is excised. This requires the structure-specific endonuclease activities of XPG at the 3' side of the open complex [32]. ERCC1/XPF complex cuts at the single strand to double strand transition 5' of the damage [33]. In principle, both XPG and ERCC!/XPF can also incise the undamaged strand, but specificity of ERCC1/XPF seems to be coordinated by RPA, which binds with defined polarity to the undamaged strand. Its 3' oriented side stimulates ERCC1/XPF whereas the 5' oriented side inhibits endonuclease activity of ERCC1/XPF in the undamaged strand [21]. RPA and XPG have a direct interaction, but RPA alone is not sufficient to endow strand specificity to XPG. The

strong interaction between XPG and TFIIH [34] suggests that TFIIH is involved in XPG positioning.

Gap-filling and ligation

The last step in the NER reaction, gap-filling of the excised patch, is used for assaying NER activity *in vitro* and *in vivo* (unscheduled DNA synthesis, UDS). An *in vitro* reconstituted repair reaction showed that efficient repair synthesis occurs after addition of the mammalian DNA replication factors RPA, RF-C, PCNA, and DNA polymerase δ and ε [35]. The NER reaction is completed by ligation of the newly synthesized DNA. DNA ligase I is a likely candidate for this reaction, because mutations in the corresponding gene can give rise to an UV-sensitive phenotype [36].

Two different pathways

The reaction mechanism described above is designated global genome repair (GGR), which removes DNA damage from the whole genome. In contrast, lesions in the transcribed strand of actively transcribed genes are preferentially repaired via the NER-subpathway transcription-coupled repair (TCR) [37]. Both processes are essentially the same except for the initial damage recognition step, which is performed by XPC/hHR23b in GGR. This is the only NER factor dispensable for TCR [38] (see Figure 2a). Instead, the stalled RNA polymerase II complex itself seems to be the damage recognition signal in TCR and attracts the NER machinery [13]. Because a stalled RNA polymerase II sterically hinders accessibility of NER proteins, it has to withdraw or dissociate from the lesion for repair to occur [39]. Cells with a defect in the *CSA* or *CSB* genes are specifically defective in TCR; the CSA protein contains WD-repeats involved in formation of multi protein complexes [40] and CSB is a member of the SWI/SNF family of DNA-dependent ATPases implicated in chromatin remodeling [41]. An interaction between CSB and RNA polymerase II was found [42], suggesting that CSA and CSB are involved in processing of a stalled RNA polymerase complex. However, the precise role of CSA and CSB in the process of TCR remains unclear [43].

NUCLEOTIDE EXCISION REPAIR DEFICIENCY SYNDROMES

The consequences of a defect in one of the NER proteins are apparent from three rare recessive syndromes: xeroderma pigmentosum, Cockayne syndrome, and the photosensitive form of the brittle hair disorder trichothiodystrophy. Seven complementation groups have been identified in XP (XP-A to XP-G), two in CS (CS-A and CS-B) and three in TTD (XP-B, XP-D, and TTD-A). Sun-sensitive skin is associated with skin cancer predisposition in the case of XP, but not in CS and TTD. In addition, the spectrum of clinical symptoms differs considerably between the three syndromes (summarised in Table I) and many of the abnormalities of CS and TTD are difficult to comprehend as a consequence of defective NER.

Xeroderma pigmentosum

Parchment skin (xeroderma) and freckles (pigmentosum) are cutaneous abnormalities of XP patients, which are strikingly limited to sun-exposed areas of the skin, and sun exposure of XP patients generally results in progressive degenerative alterations of the skin and eyes [44]. The mean age of onset of these symptoms is 2 years [45]. Furthermore, XP is associated with a thousand-fold increased risk to develop skin cancers, which are also largely confined to sun-exposed areas like the face, neck, head, and even the tip of the tongue. XP patients mainly develop basal cell carcinomas and squamous cell carcinomas, and less frequently melanomas. The mean age of onset of first skin neoplasm is 8 year, which is nearly 50 years earlier compared to the general population [45]. Many XP patients die of neoplasia, reducing the lifespan by approximately 30 years. Moreover, XP patients have a ten- to twenty-fold increased risk of developing internal cancers under the age of 20 years [46]. Considering the involvement of NER in repair of certain chemically induced DNA lesions, as well as in repair of lesions induced by cellular metabolites, either category of lesions may play a role in these internal neoplasms.

A fraction of XP patients (~18%) displays progressive neurologic abnormalities. The underlying condition seems to be primary neuronal degeneration with loss of neurons. At the severe end of the clinical spectrum are patients with DeSanctis-Cacchione syndrome with microcephaly, progressive mental deterioration, dwarfism and impaired sexual development [47]. It appears that individuals who only have a partial NER defect, like XP-F and XP-C patients, tend to develop neurologic symptoms not at all or later in life compared to patients with more severe NER defects (e.g. XP-A) [44]. A possible explanation for the onset of neurological abnormalities in XP patients is that defective repair in nerve cells of endogenous (oxidative) NER lesions induces neuronal death [48].

XP is characterized by genetic heterogeneity (the seven genes involved are designated *XPA* to *XPG*). Consequently, heterogeneity in severity of the repair defect and of symptoms such as sun sensitivity and neuronal abnormalities is observed. Some XP-A, XP-B, XP-D, and XP-G mutants exhibit a severe NER deficiency [44]. However, a low residual activity is always present in the latter two. In fact, the XP-D complementation group in particular is characterized by heterogeneity of the repair defect, with a UDS over 50% in some cases. Single point mutations are found in the *XPD* gene of XP, XP/CS and TTD patients. An inactivating deletion or truncating mutation in XPD is incompatible with the essential transcription function of the protein. Similarly, the moderate UV sensitivity and intermediate UDS typical of XP-F patients could be due to the anticipated dual function of the XPF/ERCC1 complex in NER and repair of interstrand crosslinks [49]. A null allele for XPF and the consequential defect in crosslink repair may be incompatible with life. Finally, the XPC protein is required only for GGR. XP-C patients display susceptibility to sunburn in the wild-type range because the causative transcription-blocking lesions [50] are removed normally [51]. XP-C cells have a residual UDS of 15-30% due to TCR, and are less sensitive to UV than XP-A or XP-D cells [44].

Cockayne syndrome

Cockayne syndrome (CS) is characterized by cutaneous photosensitivity, and CS cells display increased sensitivity to a number of DNA-damaging agents including UV, due to a defect in TCR. Surprisingly, CS patients are not predisposed to develop skin cancer. Furthermore, CS is a very pleiotropic disorder with physical and mental retardation ([52] for a review). In general, CS patients display skeletal abnormalities such as bird-like face, dental caries and kyphosis of the spinal cord, and osteoporosis in older patients. Furthermore, progressive neurological degeneration is observed with delayed psychomotor development, gait defects and mental retardation. Microcephaly is observed in most CS patients over 2 years old, and nerve biopsies showed myelination abnormalities. Other typical CS symptoms include sensorineural hearing loss, pigmentary retinopathy, wizened facial appearance, thin hairs and cataracts. CS patients display impaired sexual development and postnatal growth failure, and because weight is affected more than length, the condition is termed cachectic dwarfism. The mean age of death is 12½ years and the main causes of death are pneumonia and respiratory infections, which could well be due to the generally poor condition of the patients [52].

Many of the clinical symptoms of CS are difficult to explain via an NER defect, considering the fact that completely NER-deficient XP-A patients do not exhibit them. The transcriptional engagement of CSA and CSB suggests that transcription deficiency, perhaps induced by DNA damage, underlies the clinical symptoms. In addtion to the involvement of transcription, endogenous (oxidative) DNA damage has also been implicated in the onset of developmental defects in CS. CSB knockout

mice display only mild CS symptoms, but completely repair-deficient CSB/XPA double mutant mice suffer from severe growth failure and die before weaning (G.T.J. van der Horst, unpublished data). A defect in transcription-coupled repair of oxidative DNA lesions was detected in CS cells, but not in XP-A cells. Consequently, CS cells are slightly more sensitive to oxidative damage-inducing ionizing radiation than wild-type cells [53]. XP complementation group G contains some patients with the combined features of XP and CS, and defective TCR of oxidative base damage was found to be specifically confined to cells from CS patients within XP group G but not from XP-type XP-G patients [54].

Trichothiodystrophy

Sulfur-deficient brittle hair and ichthyosis (scaling of the skin) in combination with mental and physical retardation, was first described by Pollit in 1968. Later reports use the term trichothiodystrophy (TTD), emphasizing sulfur-deficiency of the hairs as the hallmark of the heterogeneous clinical entity to include TTD patients without ichthyosis and isolated cases of TTD [55]. Owing to its relative rareness and broad clinical heterogeneity, many syndromes have been described that retrospectively belong to the spectrum of TTD. These include Pollit syndrome, Tay's syndrome, Amish brittle hair syndrome, Sabinas syndrome, and Marinesco-Sjögren syndrome. Photosensitivity is reported in most but not all cases of TTD, and Stefanini and co-workers were the first to demonstrate defective NER in cell lines of photosensitive TTD patients [56]. This type of patients can be described by the acronym PIBI(D)S.

Photosensitivity

As mentioned before, photosensitivity in TTD patients is due to a defect in NER, and three genes are involved: *XPD*, *XPB* and the yet uncloned gene *TTDA*. Studies on about 20 UV-sensitive TTD families have shown that the NER defect in all but two families can be assigned to the XP-D complementation group [57, 58, 59]. Although TTDA has not been formally proven to be a subunit of TFIIH, the repair defect in cells of all three complementation groups can be rescued by injection of purified TFIIH complex [60]. Whereas UV-sensitive TTD patients are clearly NER-defective, no cutaneous malignancies have been reported [61]. Moreover, pigmentation abnormalities in sun-exposed areas have been mentioned in only a number of reports and appeared relatively mild compared to XP [61,62]. The repair characteristics of TTD cells will be discussed in more detail below in the paragraph on the XP-D complementation group.

Ichthyosis

Cutaneous symptoms include ichthyosis (scaling of the skin) and histologically, a more prominent cornified layer is observed (hyperkeratosis). Many TTD patients

are diagnosed as a collodion baby (transparent shiny skin, also observed in some keratinization disorders).

Brittle hairs

The molecular biology of the hair abnormalities is the best-understood TTD symptom. TTD hairs are dry and sparse, and the hair shaft breaks easily. Light microscopy reveals clean transverse fractures (trichoschisis) and the distal hair shaft often terminates in "brush breaks". Alternating bands of light and dark ("tiger tail" pattern) is a diagnostic finding seen by polarising light microscopy, and TTD hairs display an incomplete or complete absence of the cuticular layer.
Hairs are composed of proteins of three structural groups: the intermediate keratin filaments (IF), the high glycine/tyrosine proteins (HGTP) and the cystein-rich matrix proteins (CRP) [63]. Within the hair follicle, proliferation takes place in the hair root by the basal layer keratinocytes that are attached to the underlying basal membrane. Once they detach from the basal membrane, keratinocytes undergo a program of terminal differentiation and initiate a cascade of keratin gene expression, in which consecutively the IFs, HGTPs, and finally the CRPs are expressed [63]. IF fibers are crosslinked in a matrix of CRPs, for which the cysteines serve as covalent disulfide crosslinks. At the final stage of terminal differentiation, organelles are discarded; cells enucleate and dehydrate to form the hair fiber. The specific reduction in CRP expression in hair of TTD patients [64] indicates a defect in a late stage of hair keratinocyte differentiation. Reduced CRP contents sever the integrity of the hair shaft, because IFs are not crosslinked properly. A similar defect may also explain hypoplastic and easily breakable nails of TTD patients [61].

Intellectual impairment

Although not as well documented, the TTD syndrome exhibits a pattern of mental retardation that resembles the defect of CS patients. Clinical signs include low IQ, spasticity, hyperreflexia, tremor and ataxia. Microcephaly is often seen and hypomyelination of the cerebral white matter was reported [65]. Myelination in the central nervus system is accomplished by oligodendrocytes, which enwrap nerve fibers to provide electric insulation, and this is accompanied by massive expression of oligodendrocyte specific structural proteins, such as PLP and MBP. Apparently, these proteins are not as abundant in TTD as in normal individuals, bearing some resemblance to the keratinisation defect of TTD hairs.

Decreased fertility

Decreased fertility is not a very prominent TTD feature and mostly encompasses male and female hypogonadism and cryptorchidism (undescended testis) [61].

Short stature

Growth retardation (cachectic dwarfism) in TTD patients is a very heterogeneous clinical symptom, ranging from mild growth retardation [66] to life-threatening cachexia causing death in early childhood [67].
Although not included in the acronym, skeletal abnormalities are observed frequently. Patients have a peculiar bird-like face and receding chin, and most commonly skeletal age is retarded. Radiological analysis revealed axial osteosclerosis (abnormal hardening of the bone) sometimes accompanied by peripheral osteoporosis (demineralization; ref. 66 and references therein). Kyphosis (hunchback) was reported in the Marinesco-Sjögren patients [68]. Although the cutaneous symptoms are unique for TTD, the clinical overlap between CS and TTD is substantial, including growth retardation, decreased fertility, skeletal abnormalities and neurodysmyelination.

XP-D and the repair/transcription-syndrome

The clinical diversity associated with mutations in NER genes culminates within the XP-D complementation group, which is associated with XP, combined XP and CS, and TTD [44]. Generally, patients are compound heterozygotes with one non-functional allele and a disease-specific causative allele, mostly leading to single amino acid substitutions [69] (see Figure 3 for an overview). No clear correlation of disease phenotype and mutations in conserved protein domains is apparent. XP-D patients are classical XP patients with neurologic and pigmentation abnormalities, and are predisposed to develop skin cancer. XP-type XP-D fibroblasts are among the most sensitive of the different XP complementation groups, comparable to XP-A cells. Nevertheless, most XP-type XP-D fibroblasts have residual repair activity of 15-30%. Only two cases of XP-D/CS have been described with severe CS symptoms but also typical XP pigmentation abnormalities [70]. Skin cancer development was observed in one patient, and relatively high UDS (30-40%) in the XP/CS cells is associated with high UV sensitivity [71,72]. The UDS of most photosensitive TTD fibroblast lines is in the same range as XP-type XP-D (15-25%), but in general, TTD fibroblasts are less sensitive to UV-induced cell killing than XP-D cells (see also Chapter 5 for further characterization of the TTD repair defect). A subclass of TTD fibroblasts exists with relatively high UDS (40-55%) and near wild-type UV-survival. So far, no correlation between severeness of repair defect and clinical symptoms has been found. Recently, it was demonstrated by Eveno *et al.* that photosensitive TTD cells have defective CPD repair but (partially) proficient repair of 6-4PPs [73], which was confirmed by Marionet *et al* [74] who showed that CPDs are the predominant mutagenic lesions in TTD cells. Lesion-dependent efficiency of repair may thus underlie the mild sensitivity of NER-deficient TTD cells. Other repair related parameters that differ between XP and TTD are the UV-induced mutation spectrum [75,76], UV-induced reduction of ICAM-1 expression [77], and cellular catalase activity which is reduced in XP but

not TTD cells [78]. Taken together, the TTD repair defect differs from the XP-type XP-D repair defect, which may underlie absence of cancer development in TTD patients. Other factors that may influence cancer predisposition in TTD are the reduced lifespan, frequent hospitalization, and hyperkeratotic skin of TTD patients, which may protect against UV. This topic is discussed in more detail in chapter 5 on cancer-predisposition in TTD mice. To explain the remarkable clinical heterogeneity associated with mutations in the *XPD* gene, the transcription/repair syndrome hypothesis was put forward [79,80]. Mutations in XPD may affect the NER function of TFIIH, resulting in photosensitivity and in cancer development in XP-type patients, but may also affect the basal transcription function of TFIIH, accounting for the typical TTD and CS phenotypes. Non-UV sensitive TTD can be explained by the repair/transcription syndrome as mutations in TFIIH or other transcription-factors, which only affect the transcription function.

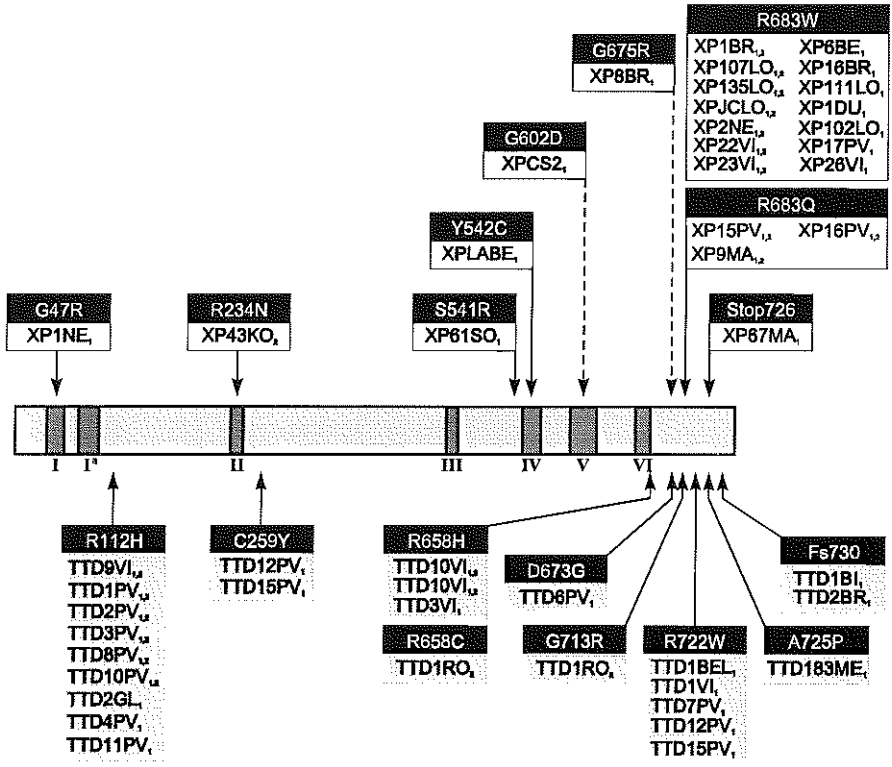

Figure 3. Causative mutations in the XPD protein in TTD, XP and XP/CS patients. The diagram shows the XPD protein with the DNA helicase domains as indicated (I to VI). The amino acid changes resulting from the mutations found in the different syndromes are shown boxed white on black, the cell line designations in black on gray (TTD) and black on white (XP). The dashed lines indicate two cases of XP/CS. Only the changes thought responsible for the pathologic phenotype are shown, deletions likely to affect cellular viability and mutations described as lethal by Taylor *et al.* [69] were omitted in this figure. Adapted from [62].

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CHAPTER 2

MOUSE MODELS TO STUDY THE CONSEQUENCES OF DEFECTIVE NUCLEOTIDE EXCISION REPAIR.

MOUSE MODELS TO STUDY THE CONSEQUENCES OF DEFECTIVE NUCLEOTIDE EXCISION REPAIR.

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Combined biochemical, genetical and cell biological progress in the past decades has culminated in a breakthrough in the insight into the molecular mechanism of nucleotide excision repair (NER). This in turn has provided clues to understanding the molecular basis of the clinical heterogeneity observed in patients with a defect in NER. In recent years, mouse models have been established for the different human NER syndromes. Conventional knockout gene targeting of the mouse *XPA* gene yielded a model for the prototype DNA repair syndrome xeroderma pigmentosum (XP) with a complete NER defect [1, 2]. Similarly, by targeting the *XPC* gene, associated with a specific deficiency in the global genome repair (GGR) pathway [3, 4], a valid model for the group C form of the disease was generated. A mouse model for Cockayne syndrome (CS), with a selective impairment of transcription-coupled repair (TCR) was obtained by mimicking a truncating *CSB* null allele found in a CS group B patient [5]. Recently, a mutant with a partial repair defect and associated remarkable clinical symptoms of trichothiodystrophy (TTD) was established in the mouse by mimicking a point mutation identified in the *XPD* gene of a photosensitive TTD patient [6]. Besides mouse mutants with specific NER defects, knockouts and more subtle mutants have been generated for NER proteins that are involved simultaneously in other cellular processes as basal transcription (XPD and XPB [7 and G. Weeda, manuscript in prep.]), mitotic recombination and cross-link repair (ERCC1 [8, 9]) and ubiquitination (mHR23A and B, J. Ng, K. Sugasawa and B. van der Horst, pers. comm.). The generation of this series of mouse mutants allows *in vivo* investigation of some intriguing questions that have puzzled the field, such as the paradoxical absence of cancer development in TTD and CS despite their NER deficiencies, the pathophysiology of the non-NER related clinical symptoms in TTD and CS patients and the proposed involvement of NER and transcription in the process of aging. This review will focus on data obtained thusfar with established NER mouse models and discuss further utilization of the mouse mutants for unraveling some of the fascinating and medically relevant aspects associated with defects in NER and related processes.

Biochimie, in press

NER DEFICIENCY AND GENOTOXIC SENSITIVITY

A direct clinical consequence of a deficiency in the NER system is the marked UV (sun) sensitivity and, in XP patients, strong predisposition for tumor development on sun-exposed skin [10]. At the cellular level, UV irradiation induces chemical alterations in the DNA, predominantly cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). CPDs are repaired in a fast and complete fashion by the TCR machinery in the transcribed strand of active genes but elsewhere in the genome, repair by GGR is slower and less efficient. The less abundant 6-4PPs are removed very rapidly and genome-wide by GGR and in its absence by TCR in the transcribed sequences. Some important differences exist in the activity of NER between mouse and man. Notably, CPDs (but not 6-4PPs and many chemical adducts) are hardly removed from the non-transcribed sequences in rodents [11]. However, this difference does not appear to have dramatic consequences since repair parameters in mouse embryonic fibroblasts (MEFs) from repair-deficient mice such as unscheduled DNA synthesis (UDS), recovery of RNA synthesis after UV-irradiation and sensitivity to UV-light correlate very well with those of human patient fibroblasts (see Table I).

In vivo, UV-irradiation causes an acute inflammatory reaction in the skin, characterized by cutaneous vasodilatation (erythema), followed by increase in the vascular permeability with exudation of fluid (edema). To man this is best known as sunburn after a sunny day on the beach. Analysis of the minimal UV-dose to induce erythema/edema (MED) in mice demonstrated that persistence of photoproducts, particularly in transcriptionally active DNA, triggers the pathway that leads to erythema and edema in the irradiated skin. Consequently, XPA- and CSB-deficient mice, incapable of repairing photolesions from actively transcribed genes, have a marked reduction in MED [5, 12, de Boer et al., submitted]. For CSB-deficient mice this response may be somewhat more exaggerated when compared with the human disorder because in the mouse model there is no contribution of GGR to the elimination of CPD lesions from transcribed sequences, whereas in the human situation -in the absence of TCR- GGR may still remove a significant fraction of these transcription-blocking lesions albeit more slowly. In contrast, XPC-deficient mice with a GGR defect have a MED in the wild-type range [13, de Boer et al., submitted]. In accordance, nine XP-C patients showed MED within the normal range of healthy humans [14]. When translating the observations from the murine to the human condition one should take into account that the absence of CPD repair by murine GGR makes a wild-type mouse in this regard more like an XPC mutant. Consequently, the difference between the wild-type and XPC states in the mouse is smaller compared to humans.

Table I. Properties of NER mouse models and corresponding human syndromes[a]

Gene	*Mouse Mutation*	*UV-sens.[b] Man*	*Mouse*	*UDS (%) Man*	*Mouse*	*Skin Cancer Man*	*Mouse*	*Relevant clinical symptoms or differences with human syndrome*
XPA	Ko	+++	+++	<5	<5	++	++	Neurodegeneration as in man is not apparent in mouse.
XPB	Ko	n.a.[c]	n.a.	n.a.	n.a.	n.a.	n.a.	Embryonic lethal.
XPC	Ko	+	+	15-30	30	++	++	MED in wild-type range.
XPD	Ko	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Embryonic lethal.
XPD	TTD point mutation	+/-	+/-	25	25	-	+	Like human TTD, except neurodysmyelination.
CSB	Trunc.[d]	++	++	normal	normal	-	+	CS symptoms mildly present, mild growth retardation, retinal degeneration.
CSA	Ko	++	++	normal	normal	-	n.d	As CSB.
ERCC1	ko, trunc.	n.a.	+++	n.a.	<5	n.a.	n.d.	Runted growth, early death through liver/kidney dysfunction. No human syndrome, signs of premature aging.
mHR23a	Ko	n.a.	normal	normal		n.d.		No apparent phenotype.
mHR23b	Ko	n.a.	-	normal		n.a	n.d.	No human syndrome, mice die in utero or premature. Testicular abnormalities. UV-sensitivity and reduced UDS of mHR23a/b double mutant MEFs.

[a] see text for references. mHR23a/b, unpublished data (K. Sugasawa, J. Ng, G.T.J. van der Horst); XPB, unpublished data (G. Weeda); CSA, unpublished data (G.T.J. van der Horst).; [b] UV sensitivity of cultured MEFs and of skin, see text for details.; [c]n.a. not applicable; n.d., not determined.;[d] Trunc, truncating mutation.

Another important short term effect of UV-exposure is a systemic suppression of the immune system, seen for instance as depletion of Langerhans cells in UV-irradiated skin, which is more prominent in XPA mice and XP patients [12, 15]. Probably, immune suppression subsequent to UVB irradiation plays an important role in the development of the highly immunogenic UVB-induced cutaneous malignancies. Evidence for this is the fact that people with kidney transplants who are receiving immunosuppressive medications have a very high frequency of developing squamous cell carcinomas on sun-exposed skin [16]. Studies in NER-deficient mice demonstrate that the GGR status, and consequently the persistence of lesions in the genome as a whole is one of the critical determinants of immune suppression. XPA mice show immune suppression at a much lower UV dose than wild-type mice do [12, J. Garssen, manuscript in prep]. CSB mice display sensitivity to UV-induced immune suppression in the wild-type range, suggesting that persistence of lesions in the global genome is the main determinant triggering the signal transduction cascade to suppress the immune system (J.Garssens, manuscript in prep.). The immune system could therefore contribute to prevention of tumorigenesis in CS, but not in e.g. XP-A or XP-C patients, carrying GGR defects.

Long-term UV exposure induces scaling of the skin, histologically identified as hyperplasia and hyperkeratosis of the epidermis. Compared to wild-type mice, XPA, XPC and CSB mice display enhanced susceptibility to epidermal hyperplasia, indicating that accumulation of photoproducts in both transcriptionally active and inactive DNA can trigger this response. Both epidermal hyperplasia and UV-induced inflammation in TTD mice occur at near wild-type UV levels. Although TTD mice have a residual repair activity of only ~25%, pointing to a substantial repair defect, the low UV-sensitivity of TTD mice and of cultured TTD mouse fibroblasts suggest that considerable repair must still take place [6]. It has been reported that human TTD cells are proficient in repair of the more cytotoxic 6-4PPs but deficient in the repair of CPDs [17]. This may provide an explanation for the UV-resistance of NER-deficient TTD mice, but lesion-specific repair has not been investigated yet. Currently, transgenic mice are developed expressing CPD- and 6-4PP-specific photoreactivating enzymes, allowing assessing the relative contribution of either lesion to UV-induced inflammation, immune suppression, hyperplasia, mutagenesis and carcinogenesis.

UV-INDUCED SKIN CANCER AND MUTAGENESIS IN NER-DEFICIENT MOUSE MODELS: COMPARISON WITH THE HUMAN NER-SYNDROMES

XPA and XPC mice

In XP patients, the age of onset of non-melanoma skin tumors is reduced from 60 to 8 years of age [18]. Similarly, XPA and XPC mice show markedly induced susceptibility to UV-induced skin cancer [1-4]. At a daily exposure of 80 J/m^2 in *hairless* mouse background, the median latency time was reduced by a factor 4.2, from 320 days in wild-type, to 74 days in XPA mice (UV 250-400 nm). A reduction in approximately the same range was seen in XPC mice, suggesting that transcription-coupled repair does not significantly protect against skin cancer, at least in XPC mice (R. Berg, manuscript in prep.). Interestingly, a shift in tumor type and mutational target genes was observed when hairless XPA mice were exposed to a low daily dose of 32 J/m^2 instead of 80 J/m^2 [19]. Whereas mainly squamous cell carcinomas (SCCs) were seen at the high UV-dose, a high frequency of papillomas was found at the low dose. Analysis of genetic alterations in the different tumor types revealed mutations in the *H-ras* gene in papillomas only and a remarkably low number of *p53* alterations in either tumor type, seemingly precluding a significant role of the *p53* tumor suppressor gene in skin tumorigenesis of NER-defective mice. Interestingly, in contrast to the absence of a prominent contribution of p53 mutations in SCC and papilloma's in the XPA mouse, Takeuchi and collaborators [20] found p53 mutations in 48% of UV-induced skin tumors (mainly SCC) in UV-exposed XPA mice and a mutational fingerprint typical for UV and an NER defect. Since the relationship of p53 to genomic (in)stability is very important it is relevant to consider this apparent discrepancy in more detail. Several explanations can be put forward. First, the mice used in both studies were in a different genetic background. Clear differences in the effect of p53 mutations on tumorigenesis have been noted before in different mouse strains. Moreover, other parameters such as the efficacy of immunesuppression could differ between the strains and influence the results. Second, the Takeuchi study involved a dose >2 times that of de Vries *et al.*, explaining also the different spectrum in the types of tumors observed. Moreover, they allowed tumors to develop to a more advanced stage. Finally, Takeuchi cs used microdissection to select specific tumor areas favoring detection of p53 mutations. The above results can be rationalized by assuming that p53 mutations are a relatively late event in UV-induced skin carcinogenesis. [20].

Studies with XPC-/-p53+/- mice show a further reduction in latency of tumorigenesis after UV irradiation compared to XPC-/-p53+/+ mice [4]. Moreover, XPC-/-p53+/- skin showed an aggravated response to UV light; in addition to epidermal hyperplasia and hyperkeratosis the epidermis showed dysplasia, which was even more pronounced in XPC-/-p53-/- animals. In line with this, tumors in

XPC-/-p53+/- mice were more aggressive, including poorly differentiated squamous cell carcinomas. Similarly, benzo[a]pyrene (B[a]P) induced lymphomas appeared faster in XPA-/-p53+/- mice compared to XPA-/-p53+/+ mice but here again a surprisingly low number of LOH of the wild-type p53 locus was observed (H. van Steeg, personal comm.).

These studies show that the ability to manipulate the mouse germline allows genetic dissection of the complex, multistep process of carcinogenesis *in vivo* and the contribution of the NER system in it. Extensive studies of NER-deficient mice with alterations in different tumor suppressor genes, oncogenes and the immune system can be anticipated.

TTD and CSB mouse models

One of the intriguing unsolved issues within the field of NER and associated disorders is the apparent absence of skin cancer predisposition in CS and TTD, despite defective NER. Differences in catalase activity [21], natural killer cell activity [22], UV-induced ICAM-1 expression [23], and lesion-specific repair [17] were reported between XP and TTD patients. Furthermore, the physiology of CS and TTD patients will definitely influence cancer predisposition, e.g. CS and TTD patients have a poor overall condition with reduced life expectancy and frequent hospitalization. In addition, scaling of the skin, characteristic of TTD patients, shields underlying basal keratinocytes from UV. Finally, the partial nature of the NER defect in both CS and TTD may permit the crippled NER system to still deal with low, constitutive levels of DNA damage. However, the relative contribution of each of these parameters on skin carcinogenesis *in vivo* is unclear. The experimental mouse models for the NER syndromes provide a unique tool to investigate the above parameters in a controlled and systematic fashion. Interestingly, both TTD and CSB mice displayed enhanced tumorigenesis in UV- and chemically- induced skin carcinogenesis protocols [5, de Boer *et al.*, submitted]. This finding is in agreement with the dogma that a defect in NER predisposes to cancer but is in seeming discordance with the human clinical data. Very importantly, consistent with expectation, TTD and CSB mice appeared less cancer-prone than the totally NER-deficient XPA mice. Quantification of cancer-predisposition in the *hairless*-mouse model showed that CSB mice were approximately six times less sensitive to UV-induced skin carcinogenesis than XPA and XPC mice in an identical experimental setup (R. Berg, manuscript in prep.). Similarly, preliminary comparison of oncogenesis in TTD versus XPA mouse mutants suggests a lower susceptibility to induced skin cancer in TTD mice when compared with XPA mutants (de Boer *et al.*, submitted). Another indication for an intermediate oncogenic response is the tumor type induced by DMBA. Under the conditions used, skin tumors in XPA mice were exclusively papillomas whereas wild-type mice develop only SCCs. CSB and TTD mice develop a mixture of both tumor types.

Thus, in an experimental setup where animals are exposed to very high genotoxic doses, TTD and CS mice unveiled a clear but intermediate cancer predisposition not noticed with the human syndromes. Several explanations can be put forward to account for this difference. As mentioned, the average life span of CS and TTD patients is reduced compared to XP patients. This combined with frequent confinement to bed may prevent CS and TTD individuals from acquiring the biologically relevant UV dose necessary for completion of the multi-step carcinogenesis process in man. Second, as deduced from the mouse experiments, cancer predisposition caused by the CS and TTD NER defects is not as dramatic as for XPA and XPC mutations and therefore could have gone undetected in the relatively low numbers of human patients. Both above considerations imply that CS and TTD NER *persé* are associated with enhanced cancer susceptibility. Thirdly, the persisting high genotoxic exposure used in the mouse experiments could exceed the repair threshold mounted by the residual NER of CS and TTD. In man, the residual repair may be enough to protect against the much lower levels of UV experienced under natural conditions. Fourth, in the case of CS the TCR defect may mount an efficient apoptotic response after UV exposure [24], which protects against cancer induction. To explain the cancer susceptibility in the CS (and in part TTD) mice one has to assume that this anti-carcinogenic response is less effective in the murine system. Finally, other interspecies physiological differences (such as metabolic rate, immune surveillance etc.) may also influence cancer predisposition. The inability of the mouse GGR pathway to remove CPD lesions is expected to increase the UV-induced cancer predisposition in the wild-type mouse and consequently diminish the relative oncogenic effect of a GGR deficiency in mice. Another consequence of this mouse-human difference is that mice have to rely more on TCR for CPD repair. Thus, a CS defect in the TCR pathway may have more dramatic effects in the mouse when compared with human NER. Hence, this mouse-man variance in repair is anticipated to differentially influence the relative cancer proneness in the two species depending on the nature of the NER defect. Future studies in TTD and CSB mice will undoubtedly focus on quantitation of cancer susceptibility and mutagenesis and on the role of UV-induced immune suppression and apoptosis.

Internal tumors in NER-deficient mice

In addition to UV-induced skin cancer, a defect in NER is anticipated to predispose patients to develop internal tumors because NER lesions are expected to be induced by chemical compounds that enter the body via food, and environmental pollution. Moreover, natural metabolites produced by the cellular metabolism induce NER lesions as well. However, only limited evidence for such a predisposition in XP patients is available [25].

In light of the above, it is of significance to note that 5 out 24 XPA mice, age 1-1.5 years, had developed internal tumors spontaneously (mostly liver adenoma) while

tumor development was absent in a group of 22 control animals [26]. The high frequency of hepatocellular adenomas indicates that the liver accumulates high levels of NER lesions or is particularly sensitive to them, a notion that will be of importance in the discussion of the phenotype of *ERCC1*-deficient mice later. Oral administration of the carcinogen B[a]P to XPA mice induced mainly lymphomas with reduced latency and higher frequency than wild-type mice, establishing XP as a disease with increased risk to internal tumors. To study the correlation between carcinogenesis and mutagenesis, XPA mice were intercrossed with transgenic mice carrying multiple copies of a plasmid containing the *lacZ* marker gene in their genome. After oral administration of B[a]P, plasmids were rescued from the genome and mutation frequencies in the *lacZ* gene were determined, revealing a correlation between lymphoma development and increased mutation frequency in lymphogenic tissue of XPA mice [26]. Similarly, mutagenesis at the endogenous *hprt* locus in B[a]P-treated XPA mice correlated with lymfomagenesis [27].

Because XPA mice are sensitive to a broad range of genotoxic agents (including UV, B[a]P, PhIP, N-OH-AAF, mitomycin C, cis-platin), they are particularly suited for screening genotoxic agents. Therefore, this mouse mutant is now evaluated in a program of the pharmaceutical industry and US government with various carcinogenic and genotoxic compounds, hormones and immuno-suppressing carcinogens. Together with p53+/- mice, transgenic H-ras mice, and several other models, XPA mice are considered as an alternative model for testing carcinogenic and immuno-suppressive compounds. The reader is referred for more information about the test program to web-site Http://ntp-server.niehs.nih.gov/Main_pages/transgen/ILSItbl2.html.

CONSEQUENCES OF MULTI-FUNCTIONALITY OF NER PROTEINS

Besides their role in nucleotide excision repair, many individual NER proteins are simultaneously also involved in other cellular processes. As mentioned before, the TCR subpathway accomplishes preferential repair of transcription-blocking lesions from transcribed DNA sequences, involving the CSA and CSB proteins. Recently, direct interaction between the CSB protein and the (elongating) RNApolII complex has been observed [28]. This implies that a CSB defect may affect the process of transcription elongation as well. The coupling between NER and transcription appeared even more intimate when it was discovered that the *XPB* and *XPD* genes encode the helicase subunits of the dual functional DNA repair/basal transcription factor TFIIH [29, 30] implying that XPB and XPD are also basal transcription initiation proteins. Other NER components are involved in mitotic recombination and replication or have likely connections with base excision repair, ubiquitination and cell cycle regulation (see Table II for an overview). Predictably, the close link of NER with so many different processes will have multiple effects on the physiology of an organism when any of these factors is defective. In the next

section, the phenotype of several of the NER-deficient mice is described, paying special attention to the role of endogenous DNA damage in the onset of some of the pathologic symptoms.

ERCC1, premature aging and the stress response

The *ERCC1* gene was the first human NER gene to be isolated via an NER-deficient Chinese hamster cell mutant as recipient for transfection cloning [31]. In contrast to other human NER genes cloned following the same strategy, subsequent studies excluded that ERCC1 is involved in any known complementation group of the three human NER syndromes. This suggests that mutations in this gene are either very rare, lethal or induce an unexpected phenotype. The ERCC1 protein complexes with the XPF product and the resulting heterodimer has a structure-specific endonuclease activity incising the 3'-extending single strand at a double strand to single strand transition in DNA [32]. In the NER reaction mechanism this corresponds with the 5' incision of the damaged strand. In addition to NER, the ERCC1/XPF complex has a function in a mitotic recombination process that presumably is responsible for repair of intrastrand crosslinks. Chinese hamster ERCC1 and ERCC4 (XPF) mutants are uniquely hypersensitive to DNA crosslinking agents such as mitomycin C (MMC) [33] and ERCC1-deficient mouse embryonic stem (ES) cells carry a defect in gene targeting by homologous recombination when the targeting construct harbors heterologous ends (G. Weeda, unpublished observation). Similarly, MEFs isolated from *ERCC1* knockout mice

Table II. Presumed or established involvement of NER proteins in diverse cellular processes

NER component	*Process*
TFIIH, CSB, CSA	Basal Transcription, Cell Cycle Regulation
CSB, CSA, XPG (TFIIH)	Base Excision Repair
RPA, PCNA, DNA polymerases, ligase I	Replication
RAD23a/b	Ubiquitination
ERCC1/XPF	Cross Link Repair/ Mitotic Recombination

displayed besides a complete NER defect also sensitivity to crosslinking agents and an enhanced spontaneous and induced mutation rate [34]. Weeda et al reported that *ERCC1* MEFs display premature cellular senescence, with large polyploid nuclei in early passages, and many non-cycling cells [8]. Interestingly, neither established ERCC1-deficient hamster cells, nor ERCC1-/- ES cells (Weeda, unpublished data) display this phenotype, suggesting a relation between mortal/immortal status of the cell. Premature cellular senescence is also not observed in any of the MEFs of the other NER mouse models analyzed (J. de Wit, unpublished data). Life span of ERCC1 homozygote mouse mutants is strongly reduced. Two independent studies

[8, 9] reported substantial embryonic lethality, survivors are extremely runted and die before weaning but some live up to 6 months of age (Figure 1). A number of the features point to premature aging. Death resulted from severe progressive liver and kidney dysfunction associated with early onset of polyploidization and aging-related nuclear abnormalities (such as inclusion bodies) in cells of these organs. Mutant mice displayed absence of subcutaneous fat and ferritin deposition in the spleen. Though elevated levels of p53 staining was seen in a fraction of cells in liver, kidney and brain of ERCC1 mice, no indication for increased apoptosis was found. Comparing the ERCC1-/- mice with other NER-deficient mutants suggests that endogenous lesions are generated in the liver which are a substrate for ERCC1, but not for other NER components because none of the other NER mice display this phenotype. The involvement of defective cross-link repair is consistent with the idea that accumulation of interstrand crosslinks contributes to the observed phenotype. A candidate agent could be malondialdehyde, a by-product of fatty-acid metabolism that induces intrastrand crosslinks [35]; other types of DNA lesions may be involved too, such as oxidative lesions. The phenotype of ERCC1 mice may provide clues to a possible human syndrome associated with a severe defect in the ERCC1/XPF functions. Besides ERCC1, several other mouse models exist in which aberrant function of effectors to DNA damage leads to liver dysfunction. First, a mouse model was established with hepatocyte-specific targeted expression of the cyclin-dependent kinase inhibitor p21 [36], involved in DNA damage-induced G_1-arrest.

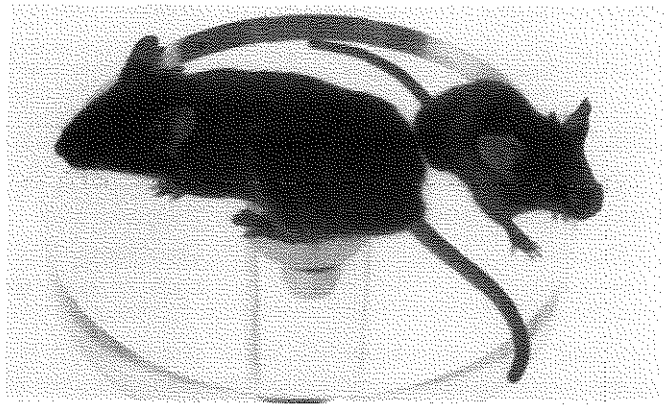

Figure 1. Phenotype of ERCC1 mutant mice. Photograph of a 3-4 week old homozygous wild-type mouse (back) and it's littermate, which is homozy-gous for the truncating *ERCC1*$^{-/\Delta 7}$ allele (front). The *ERCC1* mutation causes runted growth, reduced life span and premature senescence of cultured fibroblasts [8].

This resulted in halting of hepatocyte cell cycle progression, resulting in ERCC1 knockout-like polyploid cells in liver, runted body growth and increased mortality. Secondly, XPA mice develop spontaneous liver tumors, indicative of high genotoxic stress in the liver [26]. Furthermore, embryonic lethality and liver degeneration is observed in mice lacking the transcription factor MTF-1 [37]. MTF-1 is thought to play a general role in cellular stress response. It regulates expression

of the detoxification proteins metallothionein-1 and -2 genes, the expression of which is enhanced by a great number of stimuli like heavy metal ions, reactive oxygen intermediates and X-rays [37 and references therein]. MTF-1 also regulates expression of γ-glutamylcysteine synthetase gene, a key enzyme in glutathion biosynthesis, involved in radical scavenging. The clinical overlap between these mouse mutants is currently only correlative and the role of DNA lesions in the onset of each of the phenotypes has to be analyzed.

TTD, CS and the involvement in transcription

The extreme clinical heterogeneity associated with a NER defect culminates particularly in XP complementation groups -B and -D. Mutations in the *XPB* and *XPD* genes can give rise to XP, XP combined with CS or to TTD features. A clue to the intriguing diversity of symptoms came from the observation that XPB and XPD are subunits of the protein complex TFIIH, which has a dual role in NER and basal transcription initiation [29, 30]. The latter function is essential for all RNA polymerase II mediated transcription. The XPB and XPD proteins are DNA helicases [38] with opposite polarity (3'→5' and 5'→3' respectively) that endow TFIIH with a bi-directional unwinding potential, required for local opening of the promoter region in basal transcription and around the lesion in NER [39]. Because the origin of growth retardation, skeletal abnormalities and neurodysmyelination in CS and the additional brittle hair and ichthyosis symptoms in TTD are difficult to imagine via a NER defect, it was hypothesised that these non-XP features in CS and TTD are due to an impairment of the transcription function of XPD or XPB, whereas the photosensitivity is a consequence of affecting the repair function of XPD or XPB [40, 41]. Non-photosensitive TTD patients can be rationalized by the "repair/transcription-syndrome" model as a mutation that cripples the transcription function of TFIIH but leaves the repair function intact. As expected, targeted disruption of these essential genes in the mouse results in pre-implantation lethality, and development is arrested probably as early as the two-cell stage when zygotic transcription starts [7]. Moreover, mutation analysis of *XPD* in different patients indicated that each causative mutation is syndrome-specific and mostly subtle pointmutations are found [42-45]. Introduction of a TTD-specific point mutation in the mouse *XPD* gene unequivocally demonstrated that the broad spectrum of clinical symptoms in TTD was due to this subtle defect. TTD mice reflect to a remarkable extent the pleiotropic features of the human disorder, including reduction of hair-specific cysteine-rich matrix proteins (CRPs) resulting in brittle hair, growth delay, reduced fertility and life span, and UV sensitivity (Figure 2).

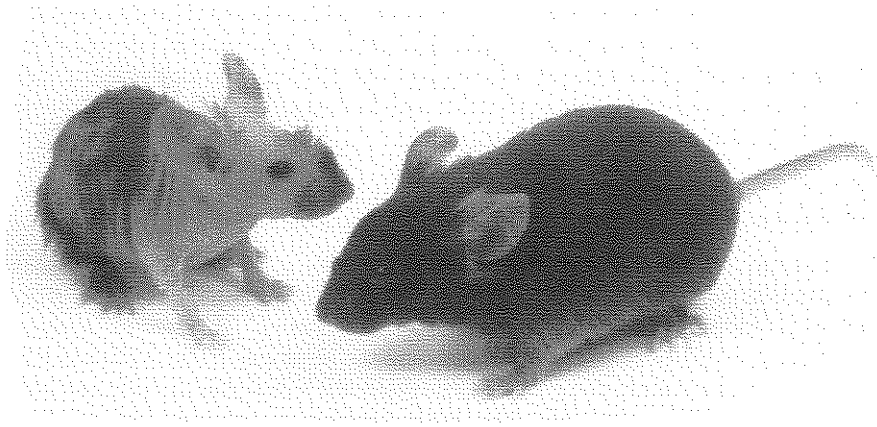

Figure 2. Phenotype of TTD mice. Photograph of a three weeks old wild-type mouse (right) and its littermate which is homozygous for the TTD allele XPD^{R722W} (left). Note the mild growth retardation and sparse, brittle hairs of the TTD mouse. Later in life, the condition of TTD mice declines and TTD mice display features resembling pre-mature ageing.

Analysis of transcription in the TTD mouse model provided evidence that at least the cutaneous symptoms, acanthosis and hyperkeratosis, are associated with reduced transcription of the skin-specific gene *SPRR2* [6]. The *SPRR2* gene encodes a structural component of the cornified envelope and is expressed in the final stage of terminal differentiation (see Figure 3), before keratinocytes dehydrate, enucleate and become solid squames. Reduced SPRR2 expression in TTD skin indicates that transcription is specifically defective in late stages of terminally differentiating cells [46], consistent with the histologically identified defect in late stages of terminal differentiation. Possibly, the TTD mutation in the *XPD* gene interferes with stability of the TFIIH complex, exerting its effect in terminally differentiating cells when cellular metabolism shuts down and TFIIH may become rate-limiting for transcription of abundantly expressed genes such as *SPRR2*. Similarly, dysmyelination in TTD patients may result from a transcriptional defect due to insufficient TFIIH capacity to express the very abundant genes that form structural components of the myelin sheet.

Genetic evidence for the concept of transcription-capacity insufficiency was also obtained from studies in *Drosophila* [47]. The hay^{nc2} allele of the *Drosophila XPB* homologue *haywire* causes recessive male infertility due to a defect in microtubule-based processes in sperm. Similarly, flies heterozygous for a null allele of the β-tubulin gene (Bt2) are fertile at 25°C, but infertile at 18°C, indicating that half the normal level of β2-tubulin is close to the threshold required for normal spermatogenesis. Interestingly, double heterozygosity of hay^{nc2} and *Bt2* also results

in male sterility. As suggested by the authors, β2-tubulin expression may be reduced in *haywire* mutants. Thus in fly, mouse and man, lowered expression of crucial gene products required at high levels in specific tissues or at critical stages of development could explain many of the pleiotropic phenotypes. The TTD mouse may provide an interesting model to study basal transcription in terminally differentiating tissues.

A putative role for endogenous lesions in the onset of TTD and CS symptoms

CS and TTD are segmental progeroid syndromes because they display a number of coherent aging-related symptoms, such as reduced life span, due to early deterioration of the overall condition, early arrest of physical and sexual development, cachexia, skeletal abnormalities, severe progressive neurological dysfunction and an overall 'aged-like' appearance of patients early in life [48, 49]. Similarly, TTD mice have a reduced life span due to starvation and suffer from osteoporosis, early graying, sebaceous gland hyperplasia, and reduced fertility all of which resemble human aging (de Boer, manuscript in prep.). Extensive genetic data from fungi to man indicates that modulation of oxidative stress plays a partial role in relevant aging-phenotypes such as longevity and neuronal death [50]. DNA repair could well be such a modulator and recently, a possible involvement of endogenous DNA lesions in the onset of the progeroid syndromes TTD and CS was found.

TCR-defective CSB mice, that still have normal GGR were crossed into a totally NER-deficient XPA background. Instead of very mild growth retardation observed in male CSB mice, CSB/XPA double mutant mice suffer from severe runting and die before weaning (van der Horst, unpublished data). Similarly, several TTD features were much more dramatic in an XPA-deficient background (de Boer, unpublished data). Two-third of the double mutants die perinatally, and survivors are runted and mostly die at an age of three to four weeks from starvation. The severeness of symptoms in TTD/XPA and CSB/XPA double mutant mice indicates that lesions, which are substrates for NER, cause at least part of the CS and TTD phenotypes. Oxidative lesions are promising candidates because some forms of oxidative damage are substrate for NER; they arise endogenously and have been implicated in the aging process. However, the versatile NER pathway is involved in removal of a plethora of different lesions, some of which may also arise endogenously so other types of lesions cannot be excluded.

The lack of an obvious phenotype of XPA mice [1, 2] shows that NER deficiency *an sich* does not cause TTD or CS-specific progeroid symptoms. The double mutants demonstrate that it is rather a combination of persistence of endogenously generated lesions and a CS- or TTD-specific deficiency. The dual function of TFIIH in repair and basal transcription, and of CSB in TCR in mind, we speculate that endogenously arising DNA lesions may hamper basal transcription in CS and TTD cells, with aging-like clinical symptoms as consequence in affected mice or patients.

Future research should unravel whether a gradual overall decay of transcriptional capacity, whether or not caused by DNA lesions, underlies aspects of human aging.

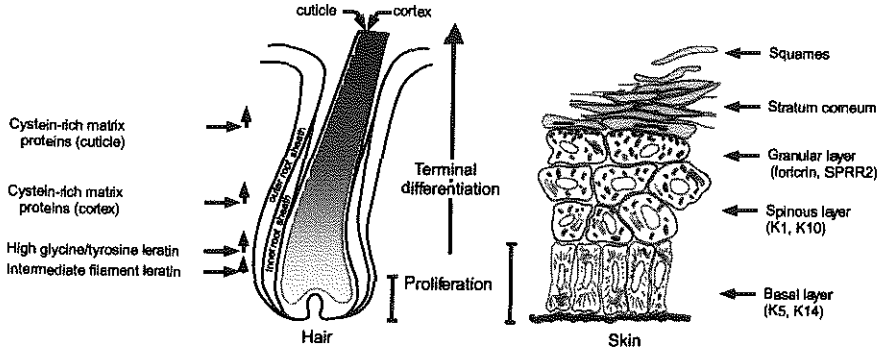

Figure 3. Expression of differentiation specific genes in skin and hairfollicles. Within the epidermis, proliferation takes place in the basal layer of hair and interfollicular keratinocytes that are attached to the underlying basal membrane. Keratinocytes undergo terminal differentiation as they migrate through the suprabasal layers, finally being shed from the tissue surface as dead cornified squames (skin, right) or forming the hair fiber (left). In hair keratinocytes, the process of differentiation initiates a cascade of keratin gene expression, in which consecutively the intermediate filaments, high glycine/tyrosine and finally the cysteine-rich matrix proteins are expressed [51]. The specific reduction in CRP expression in hair of TTD mice indicates a defect in a late stage of keratinocyte differentiation. Similarly, interfollicular keratinocytes follow a process of terminal differentiation in which each distinct layer of skin expresses a unique array of genes that produce characteristic differentiation products. The *SPRR2* gene, encoding a structural component of the cornified envelope, is expressed in the granular layer, which is the last stage before the cells dehydrate and enucleate to become squames. After Powell and Rogers, 1994 [51] and Fuchs, 1995 [52] With permission, from the Annual Review of Cell and Developmental Biology, volume 11 © 1995 by Annual Reviews.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In recent years, an ever-growing series of NER-deficient mouse mutants has been established. Until now these NER-deficient mice appear valid models for the human syndromes with respect to a number of parameters such as genotoxic sensitivity, the nature of the DNA repair defect, and carcinogenesis. Experimental mouse models provide the opportunity to investigate the role of DNA damage and NER in processes like mutagenesis, apoptosis and immune suppression *in vivo*, and thus establish the relative contribution of these phenomena in the multistep process of carcinogenesis. In addition, some mouse mutants may contribute to increase knowledge on the process of aging.

A point of concern in the extrapolation of data obtained from mouse mutants to humans are the significant (interspecies) physiologic differences (metabolic rate, certain DNA repair characteristics, genotoxic pressure, life span), which could exaggerate or underestimate the importance of certain physiologic parameters. A clear example are the manifestations of neurological features in the murine models (both neurodegeneration in XP as well as neurodysmyelination in CS and TTD), which are, if present at all, much milder compared to the human situation. Probably, in the mouse there is simply not enough time for some clinical manifestations to arise, particularly when time is a critical factor in their onset. Alternatively, the time scale between man and mouse is different. Compare for instance the mean age of onset of UV-induced tumors, which is 8 years in XP patients [18] but only 3 to 4 months in XPA mice [19]. The (repair-deficient) mouse latency periods may reach the minimal time intrinsic to the process.

Mice are genetically well defined and allow proper comparison because other genetic differences perturbing any comparison are eliminated. Thus, when the effect of the specific genetic mutation is dominant over the specific genetic constitution the studies with inbred strains are very informative. This certainly applies to the very rare human NER-syndromes for which a relatively small number of clinical reports can establish a dogma for the syndrome. Using NER-deficient mouse models, large-scale experiments and manipulation of experimental conditions may reveal subtle but clinically and scientifically relevant information (e.g. tumor susceptibility of CSB and TTD mice). However, the use of genetically inbred mouse strains needs consideration. Since the genetic make-up and consequently relevant physiologic parameters of different strains of laboratory mice varies this may have significant influence on e.g. tumor incidence and spectrum. Therefore, it is wise to study crucial parameters in more than one genetic background to exclude a possible bias derived from the specific genetic constitution.

Another very promising potential of mice is the ability to search for synergistic effects of genetic loci and pathways by simple genetic means, as it has been successfully applied in yeast and *Drosophila* models. Moreover, manipulation of the mouse genome is becoming more and more sophisticated, offering increased versatility and utility of the mouse as the *in vivo* system amenable to experimental analysis. In conclusion, we are only at the very beginning of the full exploitation of the DNA repair deficient mice for various important areas of science including carcinogenesis, aging, development, genotoxicity testing and mutagenesis.

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CHAPTER 3

DISRUPTION OF THE MOUSE *XPD* DNA REPAIR/BASAL TRANSCRIPTION GENE RESULTS IN PREIMPLANTATION LETHALITY

DISRUPTION OF THE MOUSE *XPD* DNA REPAIR/BASAL TRANSCRIPTION GENE RESULTS IN PREIMPLANTATION LETHALITY

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The xeroderma pigmentosum (XP) group D (XPD) gene encodes a DNA helicase which is a subunit of the TFIIH complex, involved in both nucleotide excision repair of UV-induced DNA damage and in basal transcription initiation. Pointmutations in the XPD gene lead either to the cancer-prone repair syndrome XP, sometimes in combination with a second repair condition, Cockayne syndrome (CS) or the non cancer-prone brittle-hair disorder trichothiodystrophy (TTD). To study the role of XPD in NER and transcription and its implication in human disorders, we isolated the mouse XPD gene and generated a null allele via homologous recombination in embryonic stem cells by deleting XPD helicase domains IV to VI. Heterozygous cells and mice are normal without any obvious defect. However, when intercrossing heterozygotes, homozygous XPD mutant mice were selectively absent from the offspring. Furthermore, we could not detect $XPD^{-/-}$ embryos at day 7.5 of development. In vitro growth experiments with preimplantation stage embryos obtained from heterozygous intercrosses showed a significant higher fraction of embryos that died at the 2-cell stage, compared to wild-type embryos. These results establish the essential function of the XPD protein in mammals and in cellular viability and are consistent with the notion that only subtle XPD mutations are found in XP, XP/CS and TTD patients.

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INTRODUCTION

To counteract the deleterious effects of mutagenic and carcinogenic agents, organisms are equipped with a sophisticated network of DNA repair systems. Nucleotide excision repair (NER), one of the best studied DNA repair pathways, removes a wide diversity of lesions, including cyclobutane pyrimidine dimers and (6-4) photoproducts (induced by UV-light), as well as numerous chemical adducts. The consequences of inborn errors in NER are highlighted by the rare autosomal recessive repair syndromes xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). Complementation tests by cell fusion have demonstrated that the NER syndromes are genetically heterogeneous and comprise at least 10 complementation groups: 7 in XP (XP-A to XP-G), 2 in the classical form of CS (CS-A and -B) and one in TTD (TTD-A), whereas two TTD complementation groups are simultaneously XP groups (XP-B and XP-D) (1, for a recent review).

XP patients display sunsensitivity, pigmentation abnormalities in sun-exposed areas, frequently accelerated neurodegeneration and are predisposed to develop skin cancer. The hallmarks of CS are sunsensitivity, severe mental and physical retardation, skeletal abnormalities and a wizened facial appearance. The mental dysfunction in CS is due to neurodysmyelination (2). In XP complementation groups B, -D and -G, some patients show combined features of XP and CS. TTD is characterized by sulphur-deficient brittle hair and nails, ichthyotic skin, as well as impaired growth and neurodysmyelination like in CS (3). Moreover, approximately 70% of the TTD patients show a NER defect that in most cases is caused by mutations in the *XPD* gene (previously referred to as *ERCC2*) (4, 5). Remarkably, CS patients as well as TTD patients with a defect in NER are not cancerprone.

XPD was found to be identical to the p80 subunit of basal transcription factor TFIIH, involved in transcription initiation of RNA pol II transcribed genes (6). Furthermore, microinjection of the purified TFIIH complex into fibroblasts of other XP, XP/CS and TTD patients appeared to induce selective correction of the NER defect of XP complementation groups B and -D and of TTD complementation group A, but not of the other XP groups (7). Most likely, TFIIH is involved in local unwinding of the DNA duplex at the site of the DNA damage in the NER reaction and of the promoter in transcription initiation, executed by the DNA-dependent ATPase and DNA helicase activities associated with XPB and XPD (7, 8). Although most of the XP features can be explained on the basis of a NER deficiency, a number of symptoms of CS and TTD (such as the neurodysmyelination and the reduced content of cysteine-rich matrix proteins in the brittle TTD hair) are difficult to rationalize in terms of a NER impairment. The association of CS and TTD phenotypes with mutations in the dually functional TFIIH has led to the hypothesis that the unusual symptoms of these diseases are due to subtle impairment of the transcription function of the corresponding proteins, leading to transcription insufficiencies for genes involved in the CS and TTD symptoms. This 'repair/transcription-syndrome' concept also provides a rational for

non-photosensitive TTD patients, in which only the transcription function of TFIIH is crippled, but the repair function is still intact (7, 9). Mutation analysis of the *XPD* and *XPB* gene in patients from different NER syndromes suggests that the causative mutations are syndrome specific and are relatively subtle pointmutations (7, 10, 11, 12, 13, 14).

In order to gain more insight into the dual function of the XPD protein in transcription and NER and into the complex genotype-phenotype relationships for the human syndromes, we decided to inactivate the gene in mouse embryonic stem (ES) cells by gene targeting. We show that loss of XPD function leads to embryonic lethality in the pre-implantation stage, which is consistent with the essential role of the XPD protein in basal transcription.

RESULTS

Isolation of murine *XPD* cDNA and genomic sequences.

To isolate the mouse homolog of the *XPD* gene, a mouse testis cDNA library was screened with a human cDNA probe as described in materials and methods. This yielded a number of overlapping cDNA clones, together covering ~3.5 kb of the mouse *XPD* transcript including a polyadenylation consensus signal AATAAA, 1.2 kb downstream of the stopcodon. Northern blot analysis of RNA from various mouse tissues using a murine *XPD* (*mXPD*) cDNA probe revealed the presence of a messenger RNA of ~4 kb (data not shown, see also (22). In human cells the *XPD* mRNA is ~2.6 kb (15, our unpublished data). Sequencing and restriction mapping indicated that the difference in size between the human and the mouse transcript is due to the different size of the 3'UTR (15, our unpublished observations). Interestingly, the *mXPD* polyadenylation signal (but not the human) maps in intron 7 of the neighboring kinesin light chain 2 (*KLC2*) gene. The *KLC2* gene is member of a large and rapidly growing family of proteins involved in microtubule-base motility (23). The close linkage of the *KLC2* and *XPD* genes is evolutionary conserved between mouse, man and chinese hamster (24) but not fish (25). *KLC2* mRNA is transcribed from the opposite DNA strand and represents a partial antisense transcript of *XPD* in the mouse (24).

The *mXPD* cDNA contains an open reading frame (ORF) of 2280 bp (data not shown), encoding a protein of 760 amino acids. The alignment of the XPD protein with its human homolog, and the postulated functional domains are shown in Fig.1. The overall degree of identity and similarity between the two proteins is 97% and 99%, respectively. Amino acid sequence differences are scattered over the entire ORF, but it is notable that all seven helicase motifs are strictly conserved between man and mouse, except for a minor conservative change (V595→I) in DNA helicase domain V. The high degree of sequence conservation from yeast to man, including

mouse, is in agreement with the function of the protein in both NER and basal transcription (24).

Fig. 1. Comparison of the predicted amino acid sequence of mouse and human XPD. (GenBank accession number: U97572) Only differences of the mouse protein with the human XPD protein have been indicated. Amino acids deleted by the gene targeting are indicated in bold. Previously postulated domains are underlined (15). DNA-BD, DNA binding domain (indicated by italics); I to VI, helicase domains. Similar residues in the mouse sequence (A, S, T, P and G; D, E, N and Q; R, K and H; I, L, V and M; F, Y and W) are given in capitals.

Targeting of the *XPD* gene in ES cells.

The targeting construct used for inactivation of the gene and the probe used for screening and verification of homologous recombinants are outlined in Fig. 2*a*. The knockout construct consists of a ~9 kb *Hind*III/*Sfi*I isogenic mouse genomic DNA fragment covering *XPD* exons 13 to 23 and all 12 *KLC2* exons. A ~4 kb *neo/HPRT* cassette (see materials and methods) replaced a 2.1 kb genomic *Bam*HI fragment containing *mXPD* exons 17 to 21 and part of exon 22 (Fig. 2*a*), but leaves the *KLC2* gene intact. A *tk* cassette (see materials and methods) was inserted in the targeting construct as a counter-selectable marker to select against random integration events. The *neo/HPRT* cassette in this position will truncate the XPD protein at amino acid 524, deleting DNA helicase domains IV to VI and the postulated DNA binding

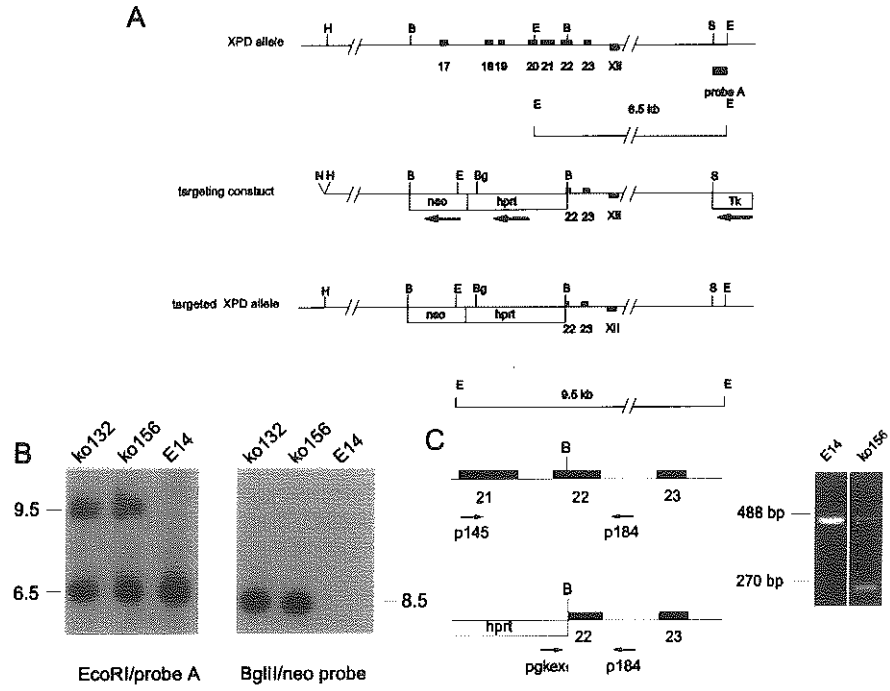

Fig. 2. Disruption of the *XPD* gene by homologous recombination in ES cells. (A) Part of the genomic structure and partial restriction map of the *XPD* gene is depicted. *XPD* exons are indicated by black boxes (exons 17 to 23). The *neo/HPRT* cassette is not drawn to scale. Only the last exon (XII) of the *KLC2* gene is indicated. Arrows mark the transcriptional orientation of the *neo*, *HPRT* and *tk* genes. The 3' external probe A (~0.9 kb *Sfi*I/*Eco*RI fragment) detects a 6.5 kb and 9.5 kb *Eco*RI restriction fragment in the wild-type and targeted allele, respectively. The *neo* probe detects a 8.5 kb *Bgl*II fragment in targeted ES clones. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sal*I. The 5' *Bgl*II restriction site is positioned outside the targeting construct and is not indicated. **(B)** Southern blot analysis of genomic DNA from targeted ES clones. DNA of two targeted ES clones (ko132 and ko156) and untrans-fected ES cells (E14) as control DNA was digested with *Eco*RI and *Bgl*II and probed with probes A and *neo* respectively (see Fig. 2*a*). The length of each hybridizing fragment is indicated. (C) Genotyping by a multiplex PCR assay. Primers p145 and p184 amplify a 488 bp fragment from the wild-type allele (top); primers pgkex$_1$ and p184 amplify a 270 bp fragment from the targeted allele (bottom). The *XPD* exons are indicated by black boxes. Sizes of the obtained DNA fragments are indicated. E14: wild-type ES cells; ko156, targeted ES clone ko156.

domain (15, 26, Fig. 1); potential alternative splicing in the knockout allele from exon 16 to exon 23 causes the ORF to run out of frame. Most likely the deletion of helicase domains will destroy the function of the protein as demonstrated previously for the XPB protein (27), and is therefore considered a functional null allele.

Linearised targeting DNA was introduced by electroporation into *HPRT*-deficient HM-1 ES cells (28). Ten from 150 independent G418/FIAU-resistant clones analyzed had incorporated the targeting vector by homologous recombination based on Southern blot hybridization analysis of genomic DNA (Fig. 2*b*). Two ES clones with a correct karyotype (ko132 and ko156) were checked using a *neo* probe internal to the construct (Fig. 2*b*) and the *tk* cDNA (data not shown) as a probe to verify that a single homologous integration event had occurred.

Generation of mice with a disrupted *XPD* gene.

Chimeric mice were generated by injection of targeted ES clones ko156 and ko132 into C57Bl/6 blastocysts. Germ-line transmission was obtained from chimeras of ES clone ko156, bred to C57Bl/6 females as determined by coat color and Southern blot analysis. To ensure a *HPRT*-proficient background, F1 males were used for further breeding, since they carry the X-linked wild-type *HPRT* gene in contrast to F1 females which are heterozygous for *HPRT*. Mice heterozygous for *XPD* ($XPD^{+/-}$) were healthy without any apparent defect, including the absence of skin tumors until 12 months of age. To determine DNA repair activity, the unscheduled DNA synthesis (UDS) was measured in mouse embryonic fibroblast (MEF) cell lines. $XPD^{+/-}$ MEFs exhibit similar UDS level as wild-type MEFs (data not shown). To obtain mice homozygous for the *XPD* null allele, $XPD^{+/-}$ mice were intercrossed and offspring was genotyped using a multiplex PCR assay of genomic tail DNA (Fig. 2*c*). Among 86 viable offspring, 23 pups analyzed were wild-type, while the remaining 63 where heterozygous for the mutation (Table 1). The close to 2:1 ratio of $XPD^{+/+}$ and $^{+/-}$ mice and the complete absence of homozygous *XPD* mutants among the offspring, indicated that homozygosity of the *XPD* null mutation resulted in embryonic lethality.

Embyonic lethality of homozygous *XPD* mice.

To determine the time of embryonic death, heterozygous mice were intercrossed and sacrificed at embryonic day 7.5 (E7.5). No homozygous tissue was recovered among the 13 embryos analyzed. Importantly, we observed that all decidua of the sacrificed mice contained a normal embryo. Decidua are the placental structures that form at each site in the uterus where implantation occurs. Therefore, absence of empty decidua and 7.5 day old $XPD^{-/-}$ embryos suggests that $XPD^{-/-}$ embryos do not implant, probably because they die at the pre-implantation stage of development. To determine whether XPD is essential for the earliest stages of development, two-cell stage embryos were isolated from the uterus of superovulated $XPD^{+/-}$ females mated with $XPD^{+/-}$ males. Embryos were transferred into M16 medium and cultured *in vitro* for 4 days, when normally most embryos reach the blastocyst stage (21, see Fig. 3). At this time the proportion of embryos in the 2-cell stage compared to embryos in later stages of development was determined. From 202 embryos

Table I. ***Genotype analysis of XPD embryos and progeny***

Cross		Age of progeny	Number of pups per litter	Genotype		
Male	Female			+/+	+/-	-/-
(+/-) x (+/-)		Newborn	6.4	23	63	0
(+/-) x (+/+)		Newborn	6.5	50	54	-
(+/-) x (+/-)		E7.5	-	8	19	0

[a] Both embryos and neonates were genotyped by PCR analysis as described in "Materials and Methods"

analyzed, 63 (31%) where arrested in the two-cell stage. In parallel experiments, control embryos were isolated from matings between heterozygous *XPD* males and wild-type females. From 244 embryos examined, 32 (13%) were arrested in the 2-cell stage (Table 2). Statistical analysis shows a significant difference between the two relative values in a student's T test ($p<0.05$). This indicates that most (if not all) $XPD^{-/-}$ embryos arrest in the 2-cell stage and suggests that XPD is needed during earliest phases of embryonic transcription. However, these data do not exclude that a part of the $XPD^{-/-}$ embryos die at a slightly later stage of development.

DISCUSSION

Mouse *XPD* messenger overlaps the neighboring *KLC2* gene.

Overall comparison of the mouse and the human XPD protein revealed an identity of 97% and 99% similarity allowing for conservative residue differences. This overall homology is consistent with its evolutionary strongly conserved function as a (5'→3') ATP-dependent DNA helicase in NER and basal transcription. The genomic organization of the *XPD* and the *KCL2* gene is conserved: genomic sequence data from this region in mouse and man revealed that the *KLC2* gene is oriented tail to tail with respect to the *XPD* gene (24). In mouse, not more than 195 bp separate the two stop codons of the corresponding genes. In contrast to man, for which the *XPD* polyadenylation signal was previously identified within 70 bp following the stop codon (15), in the mouse a consensus polyadenylation signal (AATAAA) is found ~1.2 kb distal to the stop codon in intron 7 of the *KLC2* gene. (24, our unpublished observations). We demonstrate that this is the functional polyadenylation signal of

the *mXPD* gene. Consequently, the transcription units overlap with each other for about 1 kb. A similar overlap of two genes has been found in the DNA repair gene *ERCC1* as well, which is separated less than 250 kb from *XPD* on human chromosome 19q13.3 (29). However, as is the case with *XPD* and *KLC2*, it is unknown whether this rare phenomenon of gene overlap has any functional relevance (30).

Table 2 ***Growth characteristics of in vitro cultured embryos derived from XPD$^{+/-}$ crossings***[a]

Cross			
Male	Female	Two-cell stage	> Two-cell stage
(+/-)	x (+/-)	63 (31%)[b]	139
(+/-)	x (+/+)	32 (13%)	212

[a] Embryos were grown and analysed as described in "Materials and Methods" [b] Data were pooled from five independent experiments. The relative values of embryos in the two-cell stage between XPD$^{+/-}$ and control crossings are significantly different as determined in a student's T-test (p=0.027)

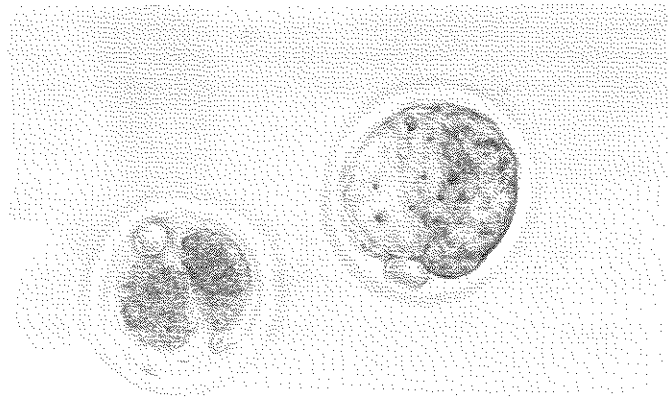

Fig. 3. Preimplantation embryos derived from *XPD*$^{+/-}$ intercrossing. Photograph of a normal blastocyst (right) and a dead 2-cell stage embryo (left) derived from a heterozygous intercross, grown *in vitro*.

Targeted inactivation of the mouse *XPD* gene.

In yeast *Saccharomyces cerevisiae*, the XPD homolog RAD3 is required for NER and has a direct and essential role in RNA polymerase II transcription (31). Both the

RAD3 and RAD25 protein (the latter encodes the yeast counterpart of XPB) are essential components of the yeast basal transcription factor TFIIH and possess DNA-dependent ATPase and DNA helicase activities (32, 33). The presence of the bidirectional helicases suggests that TFIIH as a 'helix opener' is involved in the generation of an open transcription initiation complex, whereas in repair both TFIIH helicases are needed in the pre-incision stage presumably also opening the DNA helix locally to permit dual incision (8, 34, 35). Inactivation of the RAD3 helicase function (by mutating the ATP-binding pocket) does not affect transcription but confers UV sensitivity, suggesting that RAD3 helicase activity is dispensable for transcription and thus for cellular viability. However, deletion mutants of the *RAD3* gene are not viable (36, 37), which is consistent with the idea that the physical interactions of RAD3 is important for TFIIH integrity per sé and thus for cellular viability, but its unwinding capacity is not essential for transcription. In mammals, XPD helicase function is not critical for transcription and is even not totally indispensable for DNA repair synthesis (G.S. Winkler, J.H.J. Hoeijmakers, G. Weeda, unpublished results). We demonstrate here that mice carrying one copy of the mutant *XPD* allele appear healthy and are fertile up to 12 months of age. Apparently, reduction of *XPD* transcription to 50% has no dramatic influence on the TFIIH activity in NER as well as in transcription, which is consistent with the recessive nature of the *XPD* related disorders XP, CS and TTD. Intercrosses of heterozygous *XPD* mice never resulted in liveborn homozygous mutant mice. Thus our findings reveal the essential role of XPD in mammals and rule out functional redundance due to e.g. gene duplication in evolution. Consistent with the essential function of TFIIH, targeted inactivation of the *XPB* gene is also not compatible with life (Weeda *et al.*, submitted). Theoretically, it is possible that the lethality of homozygous mutants is due to a negative effect on the nearby *KLC2* gene. However, three considerations strongly argue against this option. Firstly, the *XPD* knockout targeting construct leaves the *KLC2* gene entirely intact (Fig. 4, middle). Secondly, RNA expression of the *KLC2* gene from the *XPD* targeted allele was unaltered, as determined by semi-quantitative RT-PCR on mRNA of compound heterozygous MEFs with a *XPD* knockout allele and a *KLC2*Δ allele (deletion of a region of the *KLC2* gene containing exon 12 and the 3'UTR, see fig. 4 and data not shown) and MEFs heterozygous for the *KLC2*Δ allele. Thirdly, we have recently found that homozygosity for the *KLC2*Δ allele is compatible with life (de Boer *et al.*, manuscript in prep.). Thus, the remote possibility that lethality of the *XPD* gene is due to distal effects on the neighbouring *KLC2* gene can be ruled out.

Embryonic lethality of *XPD*$^{-/-}$ embryos.

We could neither detect *XPD*$^{-/-}$ embryos, nor empty decidua at E7.5 in the uteri of heterozygous females, mated with heterozygous males. The latter indicates that no embryonic death occurred between E3.5, the time of implantation and E7.5. This

suggests that embryos die at a preimplantation stage of development (E0 to E3.5 days). *In vitro* growth experiments with preimplantation stage embryos suggested that the majority of the *XPD*$^{-/-}$ embryos arrest at the 2-cell stage. This block is consistent with the observation that in the mouse embryo the second, but not the first cell division is dependent on zygotic transcription (38). Mouse embryos grown in the RNA pol II and III specific transcription blocker α-amanitin arrest at the late 2-cell stage (after DNA replication) (39), which is the time when zygotic genome activation occurs. Apparently, there is no pool of maternal XPD protein or mRNA to support transcription sufficiently and drive the embryo through a second cell division. The preimplantation death of *XPD*$^{-/-}$ embryos demonstrates that mutations in the *XPD* gene in XP, XP/CS and TTD patients do not affect the transcriptional role dramatically as this is not compatible with life but rather subtly affect the transcription function of TFIIH, supporting the "transcription syndrome" concept (7, 9).

To get a better insight into how a defect in the NER pathway and a subtle defect in transcription can lead to the clinical manifestations of the different NER syndromes, we are mimicking mutations found in TTD, XP and XP/CS patients in the mouse. Recently, we developed a viable mouse mutant by the introduction of a TTD-specific mutation into the *XPD* gene (manuscript in prep.).

MATERIALS AND METHODS

Isolation of the *mXPD* genomic and cDNA sequences.

A mouse testis cDNA library (λzap) was screened with a human 2.4 kb *Eco*RI *XPD* full length cDNA clone (see also (15). One isolated clone, pME2, contained a 2.4 kb insert corresponding to nucleotide position 1198-2280 of the coding region and the 1.2 kb 3' UTR. Mouse genomic DNA fragments were obtained by screening a mouse 129 genomic cosmid library (kindly provided by Dr. N. Galjart, Rotterdam) with the insert of cDNA clone pME2, yielding clone cos12. The complete cDNA sequence, including the 5' UTR sequences were derived from overlapping mouse genomic sequences and RT-PCR experiments. Sequence analysis was performed by the dideoxy-chain termination method (16) using T7 DNA polymerase (Pharmacia).

Construction of the targeting vector

A knockout targeting construct was prepared by subcloning a ~9 kb genomic *Hind*III/*Sfi*I fragment containing murine *XPD* exons 13 to 23 in pGEM9zf^{+} (Promega). A 2.1 kb *Bam*HI fragment of the *XPD* gene harboring exons 17 to 21 and a part of exon 22 was deleted and replaced by a cassette containing the pMC1-*neo* resistance gene (17) and a *hprt* cDNA driven by the phosphoglycerate kinase promoter (PGK) (kindly provided by Dr. H. te Riele, Amsterdam). A HSV-*tk* gene driven by the *PGK* promoter was inserted into the unique *Sal*I restriction site, obtained by filling the *Sfi*I restriction site by T4 DNA polymerase and ligating *Sal*I linkers. The

architecture of the targeting construct and the probe are illustrated in Fig. 2*a*. Cloning procedures were performed according to Sambrook *et al.* (18).

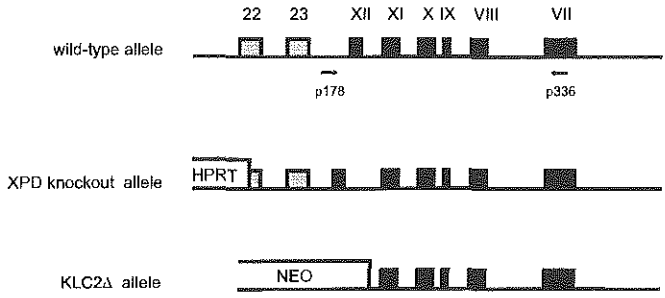

Fig. 4. RT-PCR strategy on the mouse KLC2 gene. Genomic structure (not drawn on scale) of part of the wild-type *KLC2* and *XPD* alleles (top), the *XPD* knockout allele (middle) and the *KLC2Δ* allele (bottom). Primers p178 in the 3'UTR and p336 in exon VII of the *KLC2* gene amplify a 614 bp *KLC2* mRNA fragment transcribed from the wild-type allele and the *XPD* knockout allele, but not the KLC2Δ allele. Coding parts of the *KLC2* exons are indicated with Roman numbers, coding parts of the *XPD* exons are indicated with Arabic numbers. Open boxes indicate the *HPRT* and *neo* cassettes used in the *XPD* and *KLC2* targeting respectively. PCR fragment intensity (data not shown) was quantified using Imagequant after 35 reaction cycles when the amplification of the fragment was in the linear range. Intensities indicated *KLC2* mRNA expression from the wild-type and the *XPD* knockout allele, in *KLC2Δ* heterozygote cells and compound heterozygote KLC2*Δ*/ *XPD* knockout cells respectively. Expression of the mouse *ATR* gene (A. de Klein, unpublished data) was used as a quantitative control.

Gene targeting and ES cell culture.

The targeting construct was linearized with *Not*I and introduced into the *HPRT*-deficient 129/Ola-derived ES cell line HM-1 (kindly provided by Dr. D. Melton, Edinburgh) by electroporation as described (Weeda et al., manuscript submitted). Electroporated cells were seeded onto gelatin coated 10-cm dishes (10^6 cells per plate) and subjected to G418 (200 μg/ml) and FIAU (Bristol Myers, Squibb) (0.2 μM) selection for 7-8 days in DMEM/60% Buffalo rat liver medium (BRL) (19) supplemented with 10% fetal calf serum, 0.1 mM 2-mercaptoethanol and 1000 U/ml leukaemia inhibition factor (LIF, Gibco). Individual clones were picked into 24-well dishes and expanded (no feeder layer). Half of the 24-well dish was expanded and frozen at -80^{0}C in ES medium supplemented with 10% DMSO. The duplicate plate was used to identify targeting events. Chromosomal DNA from expanded cultures was isolated as described earlier (20), and analyzed by Southern blot analysis using a 0.9 kb *Sfi*I/*Eco*RI fragment flanking the targeting construct. Positively targeted clones were reconfirmed by Southern blot analysis using a *neo* probe covering the coding region and a 0.3 kb *Sac*I DNA fragment of the *tk* gene, to verify that a single integration event had occurred by homologous recombination and that no additional random integrated copy of the *tk* gene was present. ES clones used for microinjection into blastocysts were karyotyped.

Generation and screening of *XPD* mutant mice.

Chimeric mice were obtained by injecting 10-15 cells of ES lines ko132 and ko156 into C57Bl/6 blastocysts. An average of 8 injected blastocysts were transferred into pseudo-pregnant female

BCBA recipient mice. Male chimeras, as identified by coat color were crossed with C57Bl/6 females and germline offspring was determined by the transmission of the agouti coat color. Approximately half of the offspring was genotyped as positive for the targeted mutation. To ensure that a *HPRT*-proficient background was maintained, only male F1 offspring were used for further breeding.

For the tail DNA extraction procedure, a piece of tail was lysed overnight at 55^{0}C in buffer containing 50 mM Tris (pH8.0), 100 mM NaCl, 1% SDS and 100 µg/ml proteinase K. A multiplex PCR analysis of tail DNA was used to genotype the offspring using primer p145 (5'cccggctagagtatctgc3') and p184 (5'ttgccggaatacggggcca3') to detect the wild-type allele and p184 and pgkex$_1$ (5'gctgctaaagcgcatgc 3') to detect the targeted allele. Embryos were genotyped via PCR as described above. Embryos were incubated for 5 minutes at 95^{0}C in 25µl of water, and o/n at 55^{0}C after addition of proteinase K (200 µg/ml). After 5 min at 95^{0}C to inactivate the proteinase K, 5 µl was used in a PCR reaction.

In vitro culturing of mouse embryos.

Wild-type and heterozygous *XPD* females (3 weeks old) were superovulated by injecting follignon followed 48 h later by chorilon prior to mating with heterozygous *XPD* males. One-cell stage embryos were isolated from excised oviducts (21). Embryos were placed into 50µl drops of M16 medium in standard microdrop cultures under medium-equilibrated mineral oil in a 5% CO_2 incubator at 37^{0}C for 96 h. At 2.5 days *post coitum* all single-cell embryos were discarded as unfertilised.

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CHAPTER 4

A MOUSE MODEL FOR THE BASAL TRANSCRIPTION/DNA REPAIR SYNDROME TRICHOTHIODYSTROPHY

A MOUSE MODEL FOR THE BASAL TRANSCRIPTION/ DNA REPAIR SYNDROME TRICHOTHIODYSTROPHY

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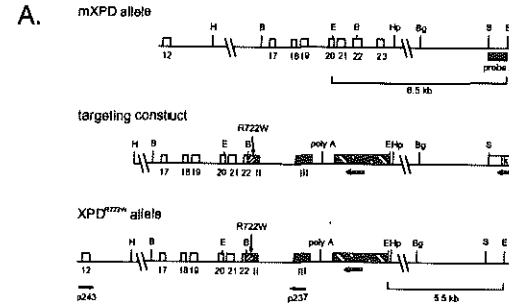
The sun-sensitive form of the severe neurodevelopmental, brittle hair disorder trichothiodystrophy (TTD) is caused by pointmutations in the essential XPB and XPD helicase subunits of the dual functional DNA repair/basal transcription factor TFIIH. The complex disease phenotype is hypothesized to be in part derived from a repair defect causing UV sensitivity and in part from a subtle, viable basal transcription deficiency accounting for the other TTD features. Using a novel gene targeting strategy we have mimicked the causative XPD pointmutation of a TTD patient in the mouse. TTD mice reflect to a remarkable extent the pleiotropic features of the human disorder, including reduction of hair-specific cysteine-rich matrix proteins resulting in brittle hair, growth delay, reduced fertility and life span, UV sensitivity and skin abnormalities. We provide evidence that at least the cutaneous symptoms are associated with reduced transcription of a skin-specific gene. The TTD mouse model strongly supports the concept of TTD as a human disease due to inborn defects in basal transcription and DNA repair.

Molecular Cell 1: 981-990

INTRODUCTION

Ubiquitous genotoxic agents induce a myriad of DNA lesions, that compromise the long-term functioning of the genome. The resulting mutations lead to genetic defects and cancer and probably contribute to aging. To prevent these deleterious consequences, a sophisticated network of genome 'caretaking' mechanisms has evolved of which a system of complementary DNA repair processes constitutes a key component. Nucleotide excision repair (NER) is one of the most versatile DNA repair mechanisms because it eliminates a wide diversity of structurally unrelated DNA lesions, including cyclobutane pyrimidine dimers and (6-4) photoproducts (main DNA damage induced by UV-light), intrastrand crosslinks, bulky chemical adducts and some forms of oxidative damage (reviewed in Friedberg et al., 1995). The NER process involves the action of at least 30 proteins in subsequent steps of damage recognition, local opening of the double helix around the injury and incision of the damaged strand on either side of the lesion. After excision of the damage-containing oligonucleotide, the resulting gap is filled by DNA repair synthesis followed by strand ligation (Friedberg et al., 1995; Wood et al., 1995).

Defects in the NER pathway are associated with at least three rare clinically distinct, autosomal recessive disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (reviewed by Bootsma et al., 1998) . XP patients display extreme photo-sensitivity, pigmentation abnormalities in sunlight-exposed areas, and a > 2000-fold increased frequency of skin cancer. In addition, accelerated neurodegeneration occurs often. CS is a very pleiotropic syndrome characterised by severe developmental and neurological abnormalities. A selective defect in the NER subpathway that very efficiently removes lesions from the coding strand of actively transcribed genes (designated 'transcription-coupled repair' or TCR) underlies the UV sensitivity of CS patients (Venema et al., 1990). Remarkably, CS patients are not cancer-prone. The third NER syndrome is the photosensitive form of TTD, a very pleiotropic neurectodermal dysplasia captured by the acronym PIBIDS: photosensitivity (P), ichthyosis (I), brittle hair (B), impaired intelligence (I), decreased fertility (D) and short stature (S). Interestingly, a subgroup of non-photosensitive, NER-proficient TTD patients (designated IBIDS or BIDS) is also known. Whereas the striking ectodermal symptoms are unique for TTD, the remainder of the clinical picture is highly reminiscent to what is observed in CS, including the characteristic facial appearance and absence of cancer predisposition. The typical brittleness of TTD hair is caused by a reduction in the content of hair-specific cysteine-rich matrix proteins that crosslink the keratin fibers (Gillespie and Marshall, 1983), resulting in a fragile hairfiber. Cell complementation studies have revealed the involvement of a large number of genes in the above NER disorders: 7 in XP (*XPA* to *XPG*), two in CS (*CSA* and *CSB*) and three in TTD (*TTDA*, *XPB* and *XPD*). The extreme clinical heterogeneity associated


A.
mXPD allele
targeting construct
XPD^R722W allele
R722W
probe A
6.5 kb
5.5 kb
B.
E14
1BEL4
1BEL6
6.5 kb
5.5 kb
C.
wildtype XPD
XPD^R722W
G A T C
Amino Acid
L
R→W
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XPD^R722W
wt
+/+ +/− −/−
584 bp
488 bp
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WT XPD
XPD^R722W

Figure 1. Targeting of the mouse XPD gene (A)Targeting strategy. Schematic representation of part of the genomic structure and partial restriction map of the mouse *XPD* gene. The coding parts of the *XPD* exons 12 and 17 to 23 are indicated with open boxes. β-globin exon 2 and 3 are indicated with black boxes and roman numbers. The 194 bp human cDNA fragment is indicated as a shaded box, the site of the TTD1BEL mutation (R722W) is indicated with a vertical arrow. The unique probe A located outside the targeting construct used for Southern blot analysis of ES transformants (Figure 1b) and the primers p243 and p237, used for RT-PCR (Figure 1c) are indicated. The 3' external probe detects a 6.5 kb and 5.5 kb *Eco*RI fragment in the wt and targeted allele respectively. The transcriptional orientation of the *neo* and *tk* selectable marker genes is indicated by horizontal arrows. Restriction sites: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Hp, HpaI; S, SfiI.(B) Southern blot analysis of *Eco*RI-digested DNA from untransfected cells (E14) and two recombinant clones (1BEL4 and 1BEL6) hybridized with probe A (see Figure 1a). The length of each hybridizing fragment is indicated. Blots were hybridized with *neo* and *tk* to verify that no additional integration events had taken place (data not shown). (C) Partial sequence of the wildtype and XPD^{R722W} mRNA amplified from ES clone 1BEL4. The indicated C to T transversion (position 2166) changes amino acid 722 from arginine to tryptophan (R722W). (D) Genomic PCR assay for genotyping progeny. Primers p145 and pβrev amplify a 584 bp fragment from the targeted allele; primers p145 and p184 amplify a 488 bp fragment from the wt allele (top). The sizes of the obtained fragments are indicated. (E) Northern blot analysis of total RNA isolated from testis of wt (+/+), XPD^{R722W} heterozygous mice (+/-) and XPD^{R722W} homozygous mice (-/-). Hybridization with a 2.4 kb human *XPD* cDNA probe detects a ~4 kb wt and 2.7 kb XPD^{R722W} mRNA.

with a NER defect culminates particularly in XP complementation groups B and –D. Mutations in the *XPB* and *XPD* genes can give rise to XP, XP combined with CS, or to TTD features (Broughton et al., 1994; Broughton et al., 1995; Fredrick et al., 1994; Weeda et al., 1997; Weeda et al., 1990). A clue to the intriguing diversity of symptoms came from the observation that XPB and XPD are subunits of the protein complex TFIIH, which has a dual role in NER and basal transcription initiation (Schaeffer et al., 1993). The latter function is essential for all RNA polymerase II mediated transcription. The XPB and XPD proteins are DNA helicases with opposite polarity (3'-5' and 5'-3' respectively) that endow TFIIH with a bidirectional unwinding potential, required for local opening of the promoter region in basal transcription (Holstege et al., 1996) and around the lesion in NER (Evans et al., 1997). Because the origin of growth retardation, brittle hair and neurodysmyelination is difficult to imagine via a NER defect, it was hypothesised that these non-XP features in TTD are due to an impairment of the transcription function of *XPD* or *XPB* , whereas the photosensitivity is a consequence of affecting the repair function of *XPD* or *XPB* (Bootsma and Hoeijmakers, 1993; Vermeulen et al., 1994a). Non-photosensitive TTD patients can be rationalised by the "repair/transcription-syndrome" model as a mutation that cripples the transcription function of TFIIH but leaves the repair function intact. Consistent with this hypothesis, mutation analysis of *XPD* in different patients indicated that each causative mutation is syndrome-specific (Broughton et al., 1994; Takayama et al., 1996; Takayama et al., 1995; Taylor et al., 1997).

In order to gain more insight into the complex clinical symptoms associated with mutations in *XPD* and the observed difference in skin cancer predisposition between XP and TTD patients, we generated mouse models for the various diseases

associated with defects in *XPD*. As reported previously (de Boer et al., 1998), a null allele for *XPD* resulted in a very early embryonic lethality, probably as early as the two-cell stage as anticipated for a gene essential for basal transcription (de Boer et al., 1998). This paper describes the generation and characterization of a mouse model for TTD, by mimicking a point mutation in the mouse germline, as found in the *XPD* gene of patient TTD1BEL.

RESULTS

Gene-cDNA fusion targeting of the TTD1BEL mutation, XPD^{R722W}

To generate a mouse model for trichothiodystrophy, we wished to mimic a causative point mutation, associated with the classical photosensitive form of TTD. The arginine 722 to tryptophan single amino acid substitution (henceforth designated XPD^{R722W}) was selected, which is found in patient TTD1BEL (Broughton et al., 1994) and several other TTD individuals (Takayama et al., 1996, and M. Stefanini personal communication). In view of the absolutely essential basal transcription function of the *XPD* gene (de Boer et al., 1998) introduction of a point mutation is a very delicate operation: the precise protein conformation is likely to be of crucial importance as the protein is only partially inactivated by the mutation and still has to fit into the 9-subunit TFIIH complex. To avoid that mouse/human differences in the region of the mutation could cause complications we decided to "humanize" the part of the *XPD* gene in which the R^{722} amino acid residue resides. Furthermore, the insertion of a dominant selectable marker should not influence the expression of the gene. This reasoning led us to design a novel gene targeting strategy that we term "gene-cDNA fusion targeting". It involves a single step targeting protocol in which part of the coding region of the gene of interest is replaced by the corresponding part of the cDNA, that is cloned in frame and contains the desired point mutation. After gene targeting in embryonic stem (ES) cells the resulting allele will express a mutated, chimeric transcript. The targeting construct used, the probe outside of the targeting construct for screening homologous recombinants and the primers for amplification of XPD^{R722W} mRNA are outlined in Figure 1A. To generate a XPD^{R722W} allele, the most 3' 194 bp of the human *XPD* cDNA including the C2166T mutation and stop codon, was cloned in frame in exon 22 of the mouse *XPD* gene (see Figure 1a). A human β-globin cassette including part of exon 2, intron 2, exon 3, 3'UTR and polyadenylation signal was cloned behind the stop codon to serve as 3' UTR. The neomycin resistance gene as dominant selectable marker was cloned downstream of the polyadenylation signal of the globin cassette so that it would not interfere with XPD^{R722W} transcription. The HSV-*tk* gene was used for selection against random integration. Following electroporation and double-drug selection of ES cell clones,

Southern blot analysis revealed that homologous recombination had occurred in two clones (Figure 1b). Correct expression of the XPD^{R722W} allele was verified by RT-PCR on mRNA of clone 1BEL4, using a primer in exon 12 of the mouse *XPD* gene, which is outside the targeting construct, and a primer overlapping exon 2 and 3 of human β-globin, specific for mRNA of the targeted locus. Sequencing of the obtained 1.1kb fragment (data not shown), showed that no undesired mutations were present in the transcript in addition to the C2166T single nucleotide change (Figure 1c), and that the targeted gene copy is expressed.

ES clone 1BEL4 was injected into blastocysts and transmitted the XPD^{R722W} allele through the germline of chimeric mice. F1 heterozygous offspring were intercrossed and F2 offspring were genotyped by PCR analysis (Figure 1d). All three expected genotypes were detected in F2 litters. The ratio of $XPD^{+/+}$:$XPD^{R722W/+}$:$XPD^{R722W/R722W}$ was 20:56:27, indicating that homozygosity for the XPD^{R722W} allele is not associated with detectable embryonic or neonatal lethality. Expression of the XPD^{R722W} allele was detected via Northern blot analysis performed on testis RNA isolated from mice of all three genotypes (figure 1e). The shorter XPD^{R722W} transcript appeared to be more abundant than the wildtype *XPD* transcript. It was not possible to quantify XPD^{R722W} protein levels as none of the available anti human XPD antibodies recognizes the mouse protein.

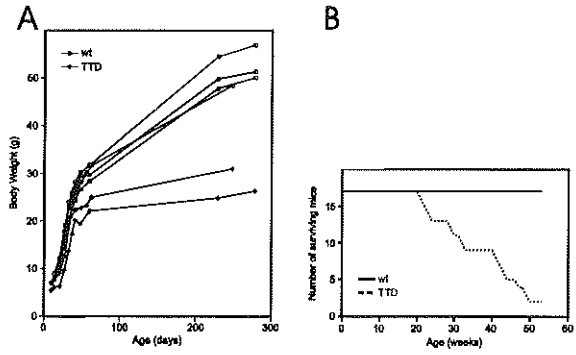

Figure 2. Growth retardation and reduced life span of *TTD* mice. (A) Weight of male wt (4) and TTD (2) mice of two litters was monitored over a period of 250 days. **(B)** Survival of a group of 17 wt and 17 TTD mice plotted against age in weeks.

Because $XPD^{+/+}$ and $XPD^{R722W/+}$ mice were indistinguishable in all aspects investigated, we conclude that the mutant allele has no dominant effect, consistent with the recessive nature of the disease. For reasons of simplicity, $XPD^{+/+}$ and $XPD^{R722W/+}$ mice will further be referred to as 'wt' mice. Next, we crossed $XPD^{R722W/+}$ mice with heterozygous *XPD* knockout ($XPD^{+/0}$) mice (de Boer et al., 1998). $XPD^{R722W/R722W}$ homozygous mice and compound heterozygous $XPD^{R722W/0}$ mice were carefully compared for most parameters that will be described in this paper (see also Experimental Methods). Therefore, in the experiments described below no distinction is made, and mutant mice are henceforth designated 'TTD' mice.

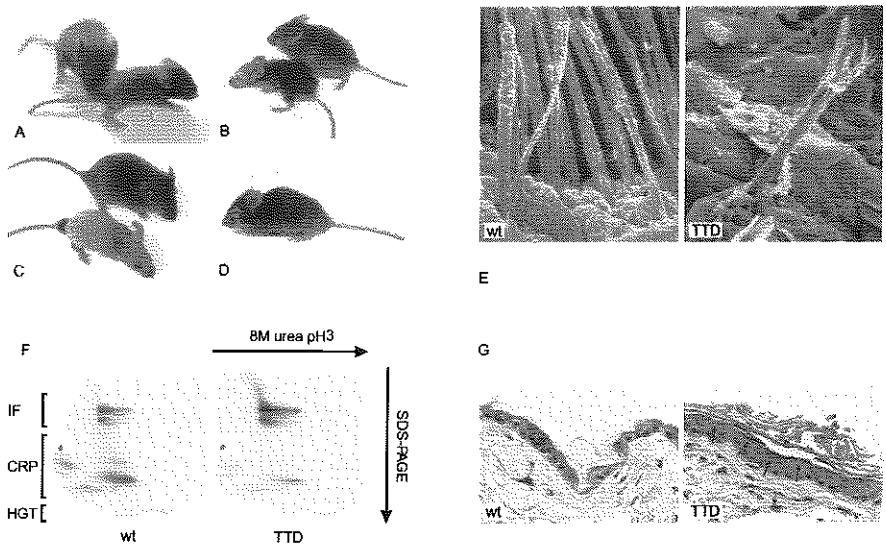

Figure 3. Cutaneous symptoms of TTD mice. (A to D) Consecutive photographs showing the pattern of cyclic hairloss and regrowth on a TTD mouse. Photographs were taken on day 14 (A), day 19 (B), day 28 (C) and day 42 (D). Note also that the TTD mouse is smaller than the wt littermate. **(E)** Scanning electron micrograph of wt and TTD mouse skin and hair. Note the absence of the typical cuticular pattern and the brush-like broken end of the TTD hair and the difference in structure of the skin surface with the normal mouse. Original magnification 700x. **(F)** Two-dimensional gel analysis of wt and TTD mouse hair protein. Equal amounts of ^{14}C-labeled hair from wt and TTD mice were resolved by two-dimensional gelelectrophoresis (pH 3, 8M urea in the first dimension and SDS-PAGE in the second dimension). The gel was fluorographed. The position of the three groups of proteins are indicated. IF, intermediate keratin filaments; CRP, cysteine-rich matrix proteins; HGT, high glycine/tyrosine proteins. A clear example of a protein spot which is strongly reduced in TTD mouse hair is indicated with an asterisk. Relative amounts of the indicated fractions were calculated with ImageQuant (Molecular Dynamics Inc.) and summarized in Table 2. **G)** Histological analysis of the altered skin morphology of TTD versus wt mice. Note that in the TTD mice the epidermis is more than 1-2 cell layers thick (acanthosis) and the cornified layer and granular layer are more prominently visible than in wt control. The granular layer in TTD skin is indicated with arrows. Magnification 40x.

Growth and viability of TTD mice

TTD mice showed a slight but consistent growth retardation from birth, as indicated in Figure 2a. Although subtle at first, weight differences between wt and TTD mice became more pronounced a few months after birth. TTD mice showed adipose tissue hypoplasia, most prominent at older age, when wt littermates became more and more obese (data not shown). These and other signs of starvation and skeletal abnormalities (data not shown) give TTD mice a wizened and prematurely aged appearance. This is strengthened by the clearly reduced lifespan (Figure 2b); most TTD mice die before the age of 1 year, although some get over 1.5 year old.

Death occurs after a short period of physical retardation, characterized by progressive weight loss and cachexia. Pathology of deceased mice failed to reveal gross abnormalities and thus did not provide a clue to a common cause of death. During their life TTD mice behaved quite normally, although repeatedly tremors were noted, that were not observed in wt littermates, hinting at some neurological deficit. We did not detect apparent CNS myelination abnormalities (analysis of the *nervus opticus* and *nervus ischiaticus*).

Table 1. cysteine content and protein composition of wt and TTD hair

	wt	TTD	% of wt
total cysteine content	10[a,b] (14)[c]	7 (8)	70 (57)
IF[d] fraction	27	54	200
CRP fraction	66	39	59
HGT fraction	6	6	100

[a] All values represent percentages of total hair protein. [b] Values for cysteine content represent the mean of three hair samples. [c] Typical values found for control and TTD human hair (Kleijer et al., 1994) are indicated between brackets.[d] IF, inter-mediate filament keratins; CRP, cysteine-rich matrix

Skin and hair abnormalities of TTD mice

The hallmark of TTD is the striking brittleness of hair and nails, due to a reduced content of cysteine-rich matrix proteins, which crosslink the keratin filaments. Newborn TTD mice develop a normal coat (Figure 3a), but spontaneous hair loss was first noted on the head around day 19 (Figure. 3b). Thereafter, a dramatic progressive loss of hair continued untill at the age of four weeks, TTD mice had lost most of their hair except for the whiskers and a small band of fur at the feet and tail (Figure 3c). The naked condition lasted until the normal second cycle of hair growth initiated, starting at the head region until a complete new coat was grown (Figure 3d). A few days later the hair loss began again and this cycle of hairloss and regrowth was maintained throughout their life. However, since hair cycle synchrony deteriorates later in life, hairloss became more patchy and not complete. To further examine the hair defect, we performed scanning electron microscopy on skin of 4 week old TTD mice, when most hairs were shed. Figure 3e shows, that broken TTD hair fibers terminate in brush ends and are (virtually) devoid of the typical cuticular structure of normal hair. Histological examination of the skin revealed follicular plugging and dilation (data not shown). The reduction in cysteine-rich matrix proteins (CRP, made up for up to 40% of cysteines) has a significant impact on the overall cysteine content of TTD hair. As apparent from Table 1, the cysteine content

of hair of TTD mice (of both first and second hair cycle) has dropped to a level found in hair of TTD patients. To determine whether this was due to the reduced synthesis of the class of CRPs, 2D gel electrophoresis of radiolabeled protein extracts of wt and TTD hair was performed. As shown in Figure 3f and the quantitation in Table 1, TTD hair has a substantially lower content of CRPs and a corresponding relative increase of the fraction of intermediate filament (IF) keratins. We conclude that TTD mice exhibit the hallmark of the human disorder: brittle hair due to a selective deficiency in the synthesis of the class of crosslinking CRPs. The scanning micrograph also revealed skin surface abnormalities in TTD mice (Figure 3e). Macroscopic examination of the skin of TTD mice showed ichthyosis (especially in the neck region), and a flexible, oversized and wizened appearance later in life. Histologic analysis (Figure 3g) indicated thickening of the layer of nucleated cells, in particular the granular layer (acanthosis) and a more prominent cornified layer (hyperkeratosis), pointing to an epidermal differentiation defect.

DNA repair characteristics of TTD mice

To examine the effect of the XPD^{R722W} mutation on DNA repair, various NER parameters were analyzed in mouse embryonic fibroblasts (MEFs) derived from TTD and wt embryos. UV survival (Figure 4a) shows that the D37 value of 4 independent TTD MEF lines was approximately 1.5 times lower than wt MEFs (3.6 J/m^2 versus 5.6 J/m^2, respectively), comparable to fibroblasts of patient TTD1BEL (unpublished results). Similarly, TTD MEFs showed a slight but significant hypersensitivity to DMBA, a chemical carcinogen that forms bulky DNA adducts which are substrates for NER (data not shown). Furthermore, the level of UV-induced unscheduled DNA synthesis (UDS) of mouse TTD cells is strongly reduced, reaching only 25 % of wt cells (Figure 4a), comparable to TTD1BEL fibroblasts. To verify whether the partial repair defect exhibited by the cultured TTD mouse cells is also reflected *in vivo,* acute sensitivity of the mice to genotoxic agents was tested. DMBA was applied to the shaven back of wt, TTD and totally NER-deficient *XPA* knockout mice (de Vries et al., 1995). After one DMBA application, *XPA*-deficient mice showed extensive hairloss, crust formation and epidermal hyperplasia on the application site (Figure 4b). Remarkably, these dramatic acute effects were absent in the skin of TTD (as well as wt) mice, even after two applications of 10 μg DMBA. In contrast to DMBA, exposure of TTD mice to increasing UV doses indicated an enhanced erythemic response (unpublished data). In conclusion, the XPD^{R722W} mutation induces a partial NER defect, very similar to that of patient TTD1BEL. Enhanced UV- and DMBA-induced skin cancer susceptibility was noted, as will be reported seperately (de Boer *et al.,* manuscript in prep.)

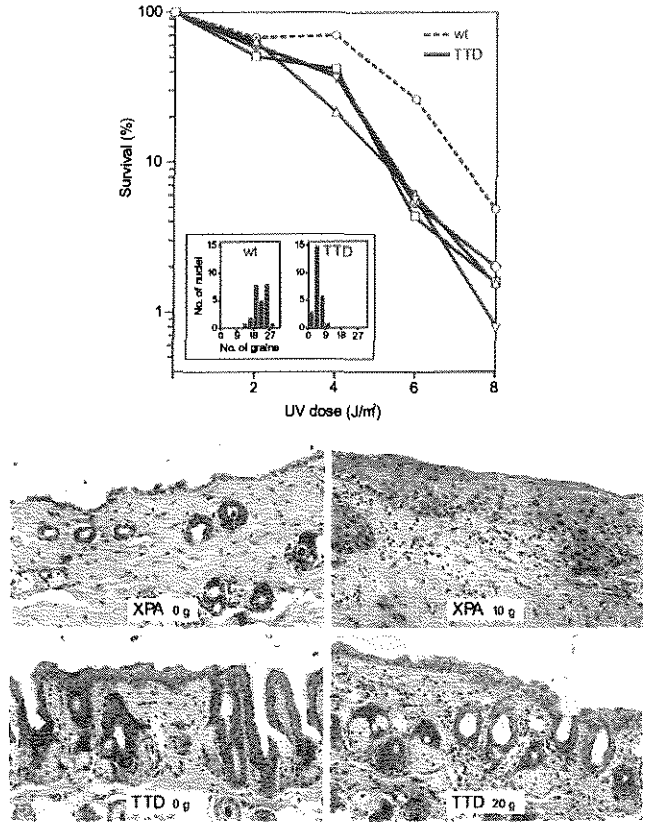

Figure 4. UV and DMBA sensitivity of cultured fibroblasts from TTD mice. (A) UV survival curve of a wt and 4 independent TTD MEF lines. Similar UV sensitivity was observed for cell lines from four human TTD patients with the XPD^{R722W} mutation (TTD1BEL, TTD1VI, TTD7PV and TTD12PV, data not shown). D37 values represent the UV-dose at which 37% survival was observed. The inlay shows the rate of DNA repair synthesis (UDS) of a representative mouse wt and TTD cel line, expressed as the number of autoradiographic grains per fixed square in 25 nuclei. (B) Skin sections of TTD and *XPA*-deficient mice untreated or treated with two and one application of 10 μg of DMBA respectively. Skin samples were taken 24 hours after the final DMBA application. Note the thick epidermis in untreated TTD skin and hyperplasia in DMBA-treated *XPA* skin. Magnification 25x.

Transcription in the skin of TTD mice

The discovery that TFIIH is a dually functional DNA repair/basal transcription factor led us to postulate the "repair/transcription syndrome" model providing a rationale for the pleiotropic clinical symptoms associated with defects in TFIIH (see

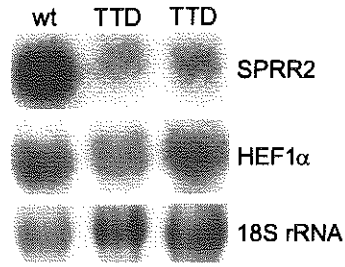

Figure 5. Northern blot analysis of wt and TTD skin. RNA was isolated from dorsal skin of one wt and two TTD mice and hybridized with a *SPRR2* probe. As a loading control the 18S ribosomal RNA band is shown and the blot was hybridized with a human *EF1α* probe. Intensities were quantitated using Imagequant (see Table 2).

introduction). To test whether an overt transcriptional defect is involved in at least part of the disease pathology of TTD mice, we searched for candidate genes the transcription of which might be affected by TFIIH malfunctioning. The expression of *CRP* genes in individual hairfollicles is difficult to quantitatively assess in a reliable fashion. A candidate gene for the skin symptoms is the *SPRR2* gene (Gibbs et al., 1990), expressed late in terminal differentiation of interfollicular keratinocytes (Hohl et al., 1995), similar to the CRPs of the hair (Powell and Rogers, 1994, for a review). The *SPRR2* gene is a member of the family of small proline-rich proteins that constitute structural components of the cornified envelope. Total RNA was isolated from the dorsal skin of one wildtype and two TTD mice, and expression of the *SPRR2* gene was determined via Northern blot hybridization with a human *SPRR2* probe. The intensity of 18S rRNA and the hybridization signal of the constitutively expressed elongation factor *EF1α* mRNA (which has a size similar to *SPRR2* mRNA) served as proper internal controls for RNA loading. As can be seen in Figure 5 and the quantitation in Table 2, transcript levels of the *SPRR2* gene are reduced 2.5 to 3-fold, which is in the same order of magnitude as the CRP reduction in the brittle hair of the TTD mouse (Table 1). Taking into account that the granular layer, in which *SPRR2* is expressed, is increased in skin of TTD mice (figure 3g), one would have expected a proportionally increased *SPRR2* transcript level, strengthening the significance of the decrease found. These results indicate that reduced transcriptional levels of the *SPRR2* gene are associated with the establishment of the TTD skin phenotype.

Table 2. Relative abundance of SPRR2 and EF1α mRNAs in skin of wt and TTD mice.

	wt	TTD	TTD
18S rRNA	1.00[a]	1.29	1.29
EF1α	1.00	0.83	1.06
SPRR2	1.00	0.38	0.41

[a]The intensity of the wt sample is set to one.

DISCUSSION

Gene-cDNA fusion targeting

XPD is essential because of its requirement for basal transcription. This explains why the causative mutations in XP-D patients are predominantly subtle single amino acid substitutions (Taylor et al., 1997). Consistent with this, we found that targeted disruption of *XPD* in the mouse leads to preimplantation lethality, probably as soon as the two-cell stage when embryonic transcription initiates (de Boer et al., 1998). To introduce a single point mutation, without disturbing essential *XPD* functions, we designed the gene-cDNA fusion targeting strategy. This method has several advances above other procedures developed for this purpose, such as the hit-and-run (Hasty et al., 1991) and double replacement protocols (Stacey et al., 1994). First, gene-cDNA fusion targeting requires only a single step to introduce a subtle mutation instead of two, used in the other strategies, thereby reducing the risk of loosing ES cell totipotency upon prolonged culturing. Secondly, the preparation of multiple targeting constructs with different point mutations in the same gene or addition of a convenient tag is more easy with a cDNA than within the context of the complex gene. Thirdly, the gene-cDNA fusion targeting procedure places the dominant marker behind the transcriptional and translational stop of the target gene diminishing the risk that the marker gene influences target gene expression. When this strategy is used as an insertion targeting, the gene itself remains -apart from the insertion- unchanged, thus reducing the chance of inadvertent effects caused by elimination of genomic sequences. Obviously, in contrast to the other strategies it is possible that the insertion of the cDNA and the dominant selectable marker has some unanticipated consequences. The addition of the β-globin intron and 3'UTR to the cDNA fusion apparently did not interfere with proper expression. In fact, the Northern blot shown in Figure 1e suggests that, at least in testis, transcript levels from the targeted allele are more abundant than those of the wt locus. The successful employment of this targeting strategy to introduce a subtle mutation in a ubiquitously expressed essential gene demonstrates the versatility of this method.

TTD mice as a model for the DNA repair defect in TTD patients

Within the population of photosensitive TTD patients, a wide heterogeneity is found with regard to cellular UV survival and residual repair synthesis (Lehmann et al., 1988; Stefanini et al., 1992). However, primary fibroblasts of all 4 TTD cases with the same XPD^{R722W} mutation share a uniform residual UDS of approximately 25% of wt, in striking correspondence to the repair synthesis of MEFs of the TTD mouse. The strong reduction in repair induces nevertheless only a very modest UV sensitivity in MEFs (1.5-fold compared to wt at D37, Figure 4a). Similarly, none of the fibroblasts lines of the four XPD^{R722W} patients is very UV-sensitive (ranging

Table 3. Comparison of the TTD1BEL patient and TTD mice

	TTD1BEL patient	TTD mouse
Cutaneous	Ichthyosis	Acanthosis, hypergranulosis, hyperkeratosis
	Brittle hair, reduced cysteine content	Brittle hair, reduced CRP content
Development	Failure to thrive, developmental delay	Growth retardation, adipose tissue hypoplasia
	Died age 3 from respiratory infection	Most die before age 1 year (mean 35 weeks)
	Hypogonadism, undescended testis	Females reduced fertility, males normal fertility
	Impaired intelligence, cerebellar atrophy	Occasional tremors, normal myelination of *nervus opticus* and *n. ischiaticus*
NER parameters	Very photosensitive skin; UDS 26% of control; fibroblasts 1.5 times more sensitive to UV; no skin cancer	More UV-sensitive skin than wild-type mice; no acute sensitivity to dermally applied DMBA; UDS 25 % of control; MEFs 1.5 times more sensitive to UV; skin cancer prone (UV- and DMBA-induced)

from 1.3-fold to 2.3-fold compared to wt and 10.6-fold for an XP-A fibroblast line, unpublished data). Also after *in vivo* exposure of TTD mice in a UV and a DMBA carcinogenesis experiment, a remarkably mild response was noticed. Acute sensitivity to dermally applied DMBA was strikingly lower in TTD mice compared to the dramatic cutaneous lesions induced in *XPA* and *CSB* deficient mice (de Vries et al., 1995; van der Horst et al., 1997). Similarly, in the UV carcinogenesis experiment, XPA and CSB mice but not TTD mice suffered from severe eye lesions (Bowenoid lesions), pruritis and cutaneous scaling. Despite the low cytotoxicity TTD mice appeared to be prone to UV- and DMBA- induced skin cancer development in both experiments. A detailed analysis of skin carcinogenesis susceptibility in TTD mice will be reported elsewhere (de Boer *et al,* manuscript in prep.). The relative insensitivity of TTD mice may be due to a combination of two factors: the altered skin morphology may provide some protection and the residual repair may be predominantly used for transcription-coupled repair, that has a major effect on UV survival. The latter is confirmed by the reduced residual UDS in MEFs from TTD/CSB double mutant mice, that are totally defective in transcription-coupled repair (unpublished results).

The TTD mouse as a model for non-repair features of the human disorder

The TTD phenotype is extremely pleiotropic (see Table 3) and involves many extra features beyond those of XP-type XP-D patients. In addition to the NER defect and XP characteristics such as photosensitivity and cancer predisposition, the TTD mouse closely resembles the human disorder with regard to these other non-repair

traits (see comparison in Table 3). This includes the typical sulfur-deficient brittle hair, skin abnormalities, retarded physical development, early death, cachexia (and associated metabolic aberrations and adipose tissue hypoplasia), (partially) impaired sexual development and to a lesser extent neurological deficits. The most life-threatening symptom is the progressive cachexia causing a poor overall condition. This makes the patients vulnerable to infections, which is a frequent cause of death. Death in the TTD mouse, grown under laboratory conditions is often preceded by a period of growth stagnation and regression. However, we have not yet elucidated the precise cause of dying. The TTD and CS patients display signs of premature aging. In addition to the strongly reduced life span and cachetic appearance, TTD mice also exhibit other features indicative of premature aging, such as skeletal deformities and early hair graying (unpublished results). The remarkable parallel between the human and mouse disorder demonstrates that the single R722W amino acid substitution in XPD is entirely responsible for the complex TTD phenotype even across the mouse-man species barrier. This rules out significant influence of genetic background on the TTD clinical entity and shows that these features are intrinsic to the TTD-type *XPD* mutation.

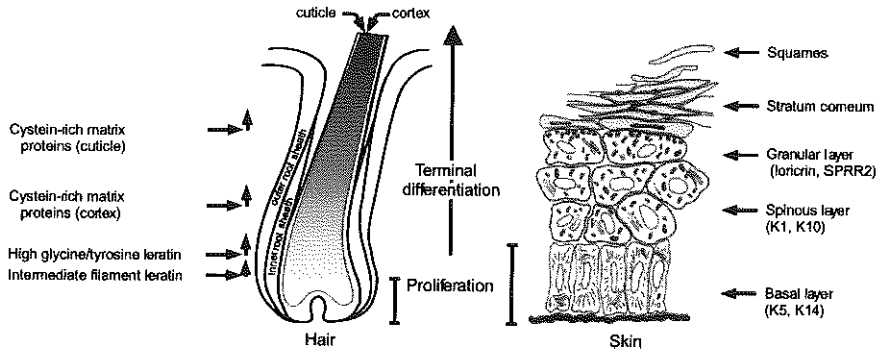

Figure 6. Expression of differentiation specific genes in skin and hairfollicles. Within the epidermis, proliferation takes place in the basal layer of hair and interfollicular keratinocytes that are attached to the underlying basal membrane. Keratinocytes undergo terminal differentiation as they migrate through the suprabasal layers, finally being shed from the tissue surface as dead cornified squames (skin, right) or forming the hair fiber (left). In hair keratinocytes, the process of differentiation initiates a cascade of keratin gene expression, in which consecutively the intermediate filaments, high glycine/tyrosine and finally the cysteine-rich matrix proteins are expressed (Powell and Rogers, 1994). The specific reduction in CRP expression in hair of TTD mice indicates a defect in a late stage of keratinocyte differentiation. Similarly, interfollicular keratinocytes follow a process of terminal differentiation in which each distinct layer of skin expresses a unique array of genes that produce characteristic differentiation products. The *SPRR2* gene, encoding a structural component of the cornified envelope, is expressed in the granular layer, which is the last stage before the cells dehydrate and enucleate to become squames. After Powell and Rogers, 1994 and Fuchs, 1995 (with permission, from the Annual Review of Cell and Developmental Biology, volume 11 © 1995 by Annual Reviews).

The TTD mouse and inborn defects in basal transcription

One of the motivations to generate a TTD mouse was to understand the role of the basal transcription function of TFIIH in the onset of TTD features. We have postulated, that the non-repair traits of TTD (and XP/CS) are due to a subtle impairment of the XPD role in basal transcription (Bootsma and Hoeijmakers, 1993). To explain the characteristic hair and skin abnormalities of TTD we have proposed, that TTD-type *XPD* mutations may alter the XPD conformation and in this way affect the stability of the TFIIH complex. Under normal conditions *de novo* synthesis of TFIIH is thought to compensate for the reduced half-life. However, in terminal differentiating tissues (see Figure 6) where *de novo* synthesis gradually declines, the mutated TFIIH might get exhausted before the transcriptional program has been completed (Hoeijmakers et al., 1996). Indications for reduced levels of TFIIH underlying the CS features associated with defects in TFIIH subunits XPB and XPD have been reported by Satoh and Hanawalt (1997). In the hair this would lead to diminished transcription of genes expressed late in terminal differentiation, such as those for the abundantly expressed CRPs, that perform the final cross-linking between the keratin filaments, before hair keratinocytes dehydrate, enucleate and become solid cuticle or cortex of the hair fiber (see Figure 6, left panel and Powel and Rogers, 1994 for a review). Similarly, in terminal differentiation of the skin, genes such as *SPRR2*, that encode cross linking components of the cornified envelope and are very abundantly expressed late in terminal differentiation (Figure 6, right panel), may suffer specifically from premature lack of TFIIH. Utilizing the mouse model we have investigated *SPRR2* expression in the TTD skin. Northern blot analysis revealed reduced *SPRR2* transcript levels when compared with the constitutively expressed 18S rRNA and EF1α mRNA. Although the 2.5- to 3-fold reduction in *SPRR2* transcript levels may not seem dramatic, it is important to realize that a comparable decrease in CRP levels (Table 1) is associated with the dramatic hair phenotype. These findings link the skin symptoms with a transcription problem and therefor we favour the model of a transcription insufficiency underlying the TTD features. Whether this is entirely due to an intrinsic transcription problem or whether there is an additional contribution from lesions, triggering apoptosis or premature terminal differentiation (Yamaizumi and Sugano, 1994; Ljungman et al., 1996) remains to be investigated. The TTD mouse will be of great value to further explore the surprising concept of human disorders in which the fundamental basal transcription process is affected.

EXPERIMENTAL PROCEDURES

Targeting of the XPD^{R722W} mutation in mouse ES cells

A ~ 9 kb HindIII-SfiI isogenic genomic clone containing exon 13 to 23 of the mouse *XPD* gene in the pGEM9zf(+) vector, was obtained as described previously (de Boer et al., 1998). A human *XPD* cDNA fragment, corresponding to bp 2080 to 2280 of the coding region, was cloned in frame with the mouse gene at the BamHI site in mouse exon 22, deleting a BamHI/HpaI fragment containing part of exon 22 and exon 23 of the *XPD* gene and exon 12 of the nearby *KLC2* gene (Lamerdin et al., 1996). The cDNA fragment contains the C2166T mutation found in one allele of patient TTD1BEL (Broughton et al., 1994), which was introduced via site-directed mutagenesis, resulting in an arginine to tryptophan substitution at amino acid 722. As a 3' UTR and polyadenylation signal, we used a human β-globin cassette (Needham et al., 1992) containing part of exon 2, intron 2 and exon 3 plus the β-globin polyadenylation signal. The pMC-1 *neo* cassette for dominant selection (Thomas and Capecchi, 1987) was cloned 3' of the β-globin polyA signal and a HSV-*tk* gene driven by the *PGK* promoter, was inserted into the unique SfiI site, at the 3' site of the genomic fragment as described previously (de Boer et al., 1998). The architecture of the targeting construct pTC1BEL, and the probe used are illustrated in Figure 1a.

The targeting construct was linearized with HindIII and introduced into 129/Ola derived ES cells. Routinely, 10^7 cells were electroporated with 10 μg pTC1BEL in ES medium as described (de Boer et al., 1998). Cells were seeded onto gelatin-coated 10 cm dishes (10^6 per plate) and after 24 hours subjected to G418 (200 μg/ml) and FIAU (0.2 μM) selection in DMEM medium containing 50% Buffalo rat liver (BRL) conditioned medium (Smith and Hooper, 1996) and supplemented with 10% fetal calf serum, 1% non-essential amino acids (Lifesciences), 0.1 mM 2-mercaptoethanol and LIF (1000 units/ml, Lifesciences). After 6-7 days, individual clones were picked into 24-well dishes and expanded. Chromosomal DNA was extracted from expanded cultures as described previously (Laird et al., 1991), digested with *Eco*RI, and analyzed by Southern blot analysis using a 0.9 kb *Eco*RI/*Sfi*I probe flanking the targeting construct. To verify that the XPD^{R722W} allele was expressed correctly and that no mutations were in the coding region besides the C2166T mutation, total RNA was isolated from targeted ES clone 1BEL4 via the urea/LiCl method (Auffray and Rougeon, 1980) and the XPD^{R722W} messenger was amplified via RT-PCR using primer p243 (5'tcagcacttacgccaaggg3', sense exon 12, outside of the targeting construct) and p237 (5'tgcccaggagcctgaagttc3', antisense exon2/3 β-globin). The resulting 1.1 kb fragment was subcloned into a pBluescriptKS+ (Stratagene) vector and sequenced. ES clone 1BEL4 was examined for correct karyotype prior to blastocyst injection.

Generation of mutant mice and mouse embryonic fibroblast lines

Gene targeted ES cells of clone 1BEL4 were injected into C57BL/6J blastocysts by standard procedures. Chimeric male mice were mated with C57BL/6J females, and transmission of E14-derived germ cells was recognized on the basis of the agouti coat color of the offspring. Heterozygous male and female mice were interbred to generate $XPD^{R722W/R722W}$, $XPD^{R722W/+}$ and $XPD^{+/+}$ mice. $XPD^{R722W/R722W}$ mice showed no phenotypic variance from compound heterozygous $XPD^{R722W/o}$ mice as determined for virtually all aspects of the TTD mouse

model (UDS, UV survival, pathology, gross appearance, life span, brittleness and morphology of hairs, histology of the skin). Furthermore, we showed by semi-quantitative RT-PCR on RNA fom $XPD^{R722/+}$ and $XPD^{R722/0}$ cells that expression of the *KLC2* gene is unaffected in the *XPD* knockout allele (de Boer et al., 1998). The *KLC2* gene resides immediately 3' of *XPD*. Our comparison of $XPD^{R722W/R722W}$ and $XPD^{R722W/o}$ demonstrates that homozygous deletion of exon 12 of the mouse *KLC2* gene in XPD^{R722W} allele does not cause apparent additional symptoms, as discussed previously (de Boer et al., 1998).

Primary mouse embryonic fibroblasts were isolated from E13.5 embryos obtained from matings between $XPD^{R722W/+}$ mice (F1). Cells were grown in DMEM medium as described before (van der Horst et al., 1997). Standard genotyping was performed via PCR on tail DNA with primer p145 and p184 to detect the wildtype allele as described before (de Boer et al., 1998), and p145 and pβrev (5'tctatggttaagttcatgtcataggaaggggagaa3') to detect the targeted allele. For Northern blot analysis, total RNA was isolated from testis of $XPD^{R722W/R722W}$, $XPD^{R722W/+}$ and $XPD^{+/+}$ mice. Equal amounts of RNA (25 μg) were loaded on an agarose gel, blotted to a nitrocellulose filter and hybridized with a 2.4 kb *XPD* fragment. For Northern blot analysis of the skin, total RNA was isolated from dorsal skin of one wt and two TTD littermates and the blot was hybridized with a mouse *SPRR2* probe (Gibbs et al., 1990) and a human EF1α probe (Brands et al., 1986).

Histological studies and electron microscopical analysis.

For histological examination, dissected tissues fixed in 10% formal saline were processed and embedded in paraffin and stained with hematoxylin and eosin using routine procedures.

For scanning electron microscopy, pieces of mouse skin were fixed in a mixture of glutaraldehyde and paraformaldehyde, postfixed in OsO_4, dehydrated in ethanol and critically point dried. After mounting on stubs and coating with gold, the dried skin was examined in a Jeol JSM-25 electron microscope.

Protein analysis of hair samples

Prior to protein analysis, plucked mouse hairs of the second hair cycle were washed successively with ether, ethanol, water, ethanol, air dried and cut into small pieces. Hair protein was extracted essentially as described (Marshall and Gillespie, 1982). Equal amounts of wt and TTD hair protein were S-carboxymethylated with iodo [2-^{14}C] acetic acid (53 mCi/mmol, Amersham) as described (Marshall and Gillespie, 1982) and analyzed by two-dimensional gel electrophoresis. Electrophoresis in the first dimension was at pH3 in 8M urea and in the second dimension by SDS-PAGE (Marshall and Gillespie, 1982). Following electrophoresis, proteins were detected by fluorography (Amplify, Amersham). Identification of protein spots as intermediate keratin filaments, cysteine-rich matrix proteins or high-glycine/tyrosine proteins was based upon their position in the gel by comparison with the well-characterized proteins of man, mouse and sheep (Marshall and Gillespie, 1982; Powell and Rogers, 1990; Powell and Rogers, 1994).

Amino acid analysis was conducted by standard procedures. Prior to analysis hair of the second hair cycle was hydrolyzed in 12 M HCl at 110°C for 24 hours. The separation was performed on a Biotronik 7000 amino acid analyzer by ion exchange chromatography using lithium citrate buffers and ninhydrin detection with a dual wavelength detector at 570 and

440 nm. The amount of each amino acid was expressed as the molar percentage of the total amino acids.

UV-induced UDS and survival assays

For UDS testing, MEFs (passage 4-6) were seeded onto coverslips. The next day, cells were washed with PBS and irradiated at 16 J/m^2 UV-C (Philips, TUV lamp). Subsequently, cells were incubated for 2.5 hours in culture medium containing 10 μCi/ml [^{3}H]-thymidine, fixed and subjected to autoradiography as described before (Vermeulen et al., 1994b).

For survival assays, MEF cultures were exposed to UV and allowed to grow for another 4-5 days, before reaching confluency. Cells were labeled with [^{3}H]-thymidine as described above, rinsed with PBS and lysed. The number of proliferating cells in each dish was estimated by scintillation counting of the radioactivity during a 3 hours pulse-labeling. Cell survival was expressed as the ratio of irradiated over unirradiated cells.

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CHAPTER 5

MOUSE MODEL FOR THE DNA REPAIR/BASAL TRANSCRIPTION DISORDER TRICHOTHIODYSTROPHY REVEALS CANCER PREDISPOSITION.

MOUSE MODEL FOR THE DNA REPAIR/BASAL TRANSCRIPTION DISORDER TRICHOTHIODYSTROPHY REVEALS CANCER PREDISPOSITION.

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Patients with the nucleotide excision repair (NER) disorder xeroderma pigmentosum (XP) are highly predisposed to develop sunlight-induced skin cancer, in remarkable contrast to photosensitive NER-deficient trichothiodystrophy (TTD) patients, carrying mutations in the same *XPD* gene. *XPD* encodes a helicase subunit of the dually functional DNA repair/basal transcription complex TFIIH. The pleiotropic disease phenotype is hypothesized to be in part derived from a repair defect causing UV sensitivity and in part from a subtle, viable basal transcription deficiency accounting for the cutaneous, developmental and the typical brittle hair features of TTD. To understand the relationship between deficient NER and tumor susceptibility we employed a mouse model for TTD that mimics an XPD point mutation of a TTD patient in the mouse germline. Like the fibroblasts from the patient, mouse cells exhibit a partial NER defect, evident from the reduced UV-induced DNA repair synthesis (residual repair capacity ~25%), limited recovery of RNA synthesis after UV exposure and a relatively mild hypersensitivity to cell killing by UV or DMBA. In accordance with the cellular studies, TTD mice exhibit a modestly increased sensitivity to UV-induced inflammation and hyperplasia of the skin. In striking contrast to the human syndrome, TTD mice manifest a clear susceptibility to UV- and DMBA-induced skin carcinogenesis albeit not as pronounced as the totally NER-deficient XPA mice. These findings open up the possibility that TTD is associated with a sofar unnoticed cancer predisposition, and support the notion that a NER deficiency enhances cancer susceptibility. These findings have important implications for the etiology of the human disorder and for the impact of NER on carcinogenesis.

submitted for publication

INTRODUCTION

Genomic instability is an eminent feature in the progression of a normal somatic cell into a transformed cancer cell. To preserve DNA integrity, a network of genome 'caretaking' mechanisms has evolved. An important component of this protection system is a set of complementary DNA repair processes that safeguard the genome from environmentally and endogenously induced mutagenic lesions. The nucleotide excision repair (NER) system eliminates a wide diversity of structurally unrelated DNA lesions, including cyclobutane pyrimidine dimers and (6-4) photoproducts (main DNA damage induced by UV-light), intrastrand crosslinks, bulky chemical adducts and some forms of oxidative damage (13), making NER the most versatile DNA repair mechanism known to date. The NER process involves the concerted action of approximately 30 proteins in subsequent steps of damage recognition, local opening of the double helix around the injury and incision of the damaged strand on either side of the lesion. After excision of the damage-containing oligonucleotide, the resulting gap is filled by DNA repair synthesis followed by strand ligation (13, 43). The importance of NER is illustrated by three rare, autosomal recessive human NER-deficiency syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (3).

XP patients, with a defect in one of the NER components (XPA-XPG), are very sensitive to sunlight and have a ~1000-fold increased risk of developing skin cancer. In XP patients, the age of onset of non-melanoma skin tumors is reduced from 60 to 8 years of age (17). Additionally, pigmentation abnormalities in sunlight-exposed areas are a hallmark feature and frequently accelerated neurodegeneration occurs (reviewed in 3). CS is characterised by photosensitivity and several additional symptoms, which are difficult to rationalise via a defect in NER, such as severe growth retardation (referred to as cachectic dwarfism), neurodysmyelination and skeletal abnormalities. On the basis of the progressive nature and resemblance to aging phenotypes of several of these features, CS is recognized as a segmental progeroid syndrome. A mutation in the *CSA* or *CSB* gene is associated with a selective defect in transcription-coupled repair. This NER subpathway accomplishes very efficient removal of transcription-obstructing lesions from the template strand of active genes, which are less efficiently repaired by the complementary NER process, global genome repair (GGR) (35). Remarkably, CS patients appear not cancer-prone. Moreover, patients with combined features of XP and CS were identified with defects in the *XPB*, *XPD* or *XPG* genes (6, 36, 42). Adding to the clinical complexity, *XPB* and *XPD* are also involved in the photosensitive form of the third NER syndrome: TTD (30, 41).

TTD shares many features with CS, including (neuro)developmental and skeletal abnormalities. In addition, TTD patients display ichthyosis (scaling of the skin) and a specific defect in the expression of a group of cysteine-rich matrix proteins underlying the striking brittle hairs and nails (14), the hallmark of the disease. TTD

patients have a reduced life expectancy, but extensive clinical heterogeneity exists, ranging from mild growth retardation to life-threatening cachexia. Just like CS, TTD appears to be not associated with skin cancer predisposition despite the overt NER defect. Moreover, although considerable heterogeneity in severity of the NER defect is seen (18, 29), no clear correlation exists between severeness of many TTD features and the DNA repair defect. In fact, a subgroup of non-photosensitive, NER-proficient TTD patients is also known, suggesting that the NER impairment and the typical TTD phenotypes are clinically and perhaps molecularly unrelated. In support of this idea, it was discovered that *XPB* and *XPD* are essential DNA helicase subunits of the dually functional DNA repair/basal transcription initiation factor TFIIH (26, 27). Previously, we proposed that mutations in those genes may not only affect NER, causing XP and the photosensitivity in CS and TTD, but depending on the mutation may also subtly impair basal transcription explaining the typical CS and TTD features (15, 38). Consistent with this hypothesis, mutation analysis of *XPD* in different patients indicated that each causative mutation is syndrome-specific (4, 5, 31-33). Mostly subtle point mutations are found, consistent with the essential role of *XPD* in basal transcription initiation. Moreover, by gene targeting we showed that a *XPD* null allele is lethal in mice, in a very early stage of embryogenesis (8).

To study the complex clinical symptoms and the paradoxical absence of skin cancer in NER-deficient TTD patients, we generated a mouse model for TTD by mimicking the XPD^{R722W} allele in the mouse germline as found in five TTD patients (7). TTD mice reflect to a remarkable extent the pleiotropic features of the human disorder, including growth delay, reduced fertility and life span, cutaneous abnormalities and UV sensitivity of cultured fibroblasts. Like in patients, TTD mice displayed the remarkable brittle hair phenotype due to a reduction of hair-specific cysteine-rich matrix proteins. Moreover, we found that the keratinization defect in TTD mice is associated with reduced transcription of the late terminal differentiation marker *SPRR2*, consistent with the idea that reduced transcription potential explains part of the TTD features. Having established a valid mouse model for TTD, this paper presents further characterization of the repair defect of TTD mice, and examines the crucial issue of cancer predisposition.

RESULTS

Repair characteristics and genotoxic sensitivity of NER-deficient cells

To provide a detailed account of the DNA repair defect in the TTD mouse model at the cellular and organismal level a number of DNA repair parameters was systematically examined and compared with DNA repair characteristics of other NER-deficient mouse mutants. We first studied repair parameters in primary mouse

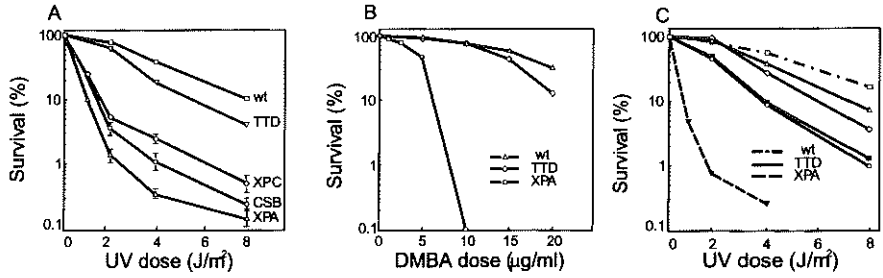

Figure 1 Comparison of genotoxic sensitivity of various NER-deficient cells. A) UV-survival of primary MEFs of NER-deficient mice as indicated. For proper comparison, all survivals were performed in one experiment. For each genotype, identical results were obtained with several other lines (data not shown). UV-sensitivity has been described before for all cells used (7, 10, 25, 34). B) DMBA survival curve of a wild-type, TTD and XPA MEFs. Values represent average of four wild-type, four TTD and two XPA cell lines. Cells were grown for 2 hours in the presence of the indicated doses. Sensitivity of TTD and wild-type was not significantly different. C) UV-survival of proliferating fibroblasts of a normal subject C5RO (open square), four TTD patients with the XPD^{R722W} allele (TTD1BEL, circle; TTD1VI, filled square; TTD7PV, diamond; TTD12PV, triangle) and from a XPA patient XP25RO (closed triangles). See materials and methods for details on experimental protocols.

embryonic fibroblasts (MEFs) established from wild-type mice, XPA mice with a complete NER deficiency (10), XPC mice carrying a selective defect in GGR (25), CSB mice with a specific TCR defect (34) and TTD mice (7). To dissect the TTD repair-defect in either repair sub-pathway, first UV-induced unscheduled DNA synthesis (UDS) was measured, which is considered a parameter mainly for GGR activity. TCR-defective CSB cells have UDS levels in the wild-type range (table 1), but GGR-deficient XPC MEFs display ~30% residual repair synthesis indicating that TCR makes a small but significant contribution to UDS as well. TTD MEFs exhibit only ~20-40% UDS similar to cells of the corresponding patients. A significant proportion of this is derived from TCR, since inactivating this NER subpathway in CSB/TTD double mutant MEFs reduces residual UDS to less than half (data not shown). TCR activity was assessed indirectly by analysis of the cellular capacity to perform RNA synthesis 16 hours after UV irradiation. As expected, persistence of lesions in transcriptionally active DNA reduces RNA synthesis in TCR-deficient XPA and CSB MEFs (Table 1), while XPC MEFs exhibit a response in the wild-type range. In TTD MEFs, RNA synthesis recovery is only ~20 % of wild-type, comparable to what we found in TTD1VI cells (data not shown). This indicates that a small but significant level of residual TCR is present in TTD. Thus, both GGR and TCR are clearly affected in TTD MEFs, but substantial residual repair activity for both subpathways persists.

To study the toxic consequences of defective repair, UV survival of TTD MEFs was compared to that of XPA, XPC, CSB and wild-type MEFs (Figure 1a). The relative sensitivities of the different cell lines (XPA>CSB>XPC>TTD>wt)

correlates very well with literature on the corresponding NER-deficient human cells (3, 13) and our own unpublished data. It is worth noting that the sensitivity of TTD MEFs to UV-induced cell killing is very mild (see Figure 1a and Table 1). The modest hypersensitivity of TTD MEFs was confirmed using DMBA, a compound that induces bulky DNA adducts which are also substrates for NER (13). Whereas XPA MEFs are very sensitive (Figure 1B, and ref. 10), TTD MEFs appeared only slightly more sensitive when exposed to increasing doses of DMBA in several independent experiments (the difference between wild-type and TTD survival was even not statistically significant). To confirm that the XPD^{R722W} allele is associated with mild genotoxic sensitivity, both in mouse and man, we performed UV survival experiments with four human TTD fibroblast lines harboring the XPD^{R722W} allele. Figure 1c shows that under the conditions used, UV-sensitivity was in the same order as for TTD MEFs, again very mild when compared to XPA cells.

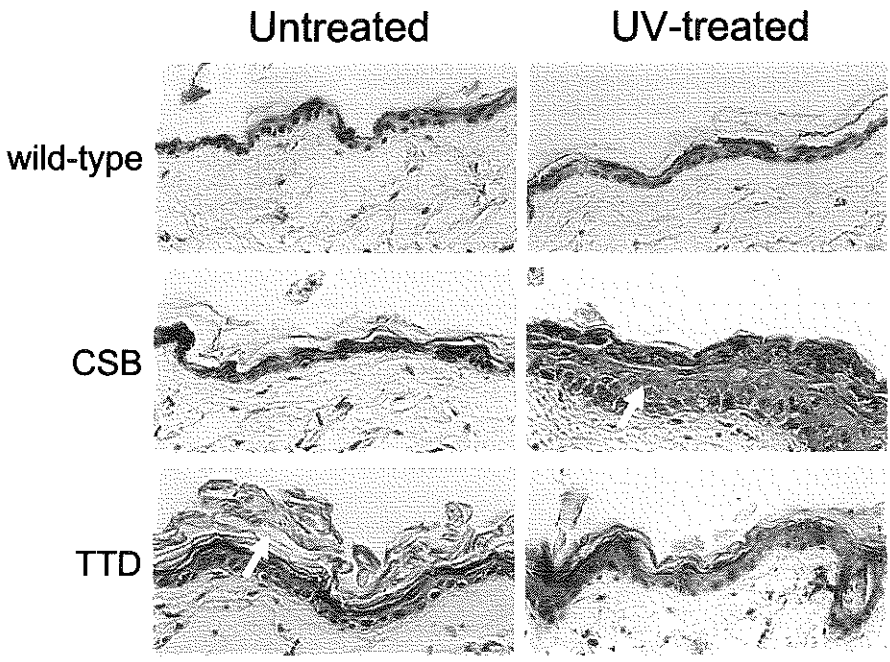

Figure 2. UV-induced hyperplasia in CSB, but not in wild-type and TTD mice. Skin sections of TTD, wild-type and CSB mice as indicated, untreated or treated with UVB (100 J/m^2/day) for 4 consecutive days on the shaven back. Skin samples were taken 24 hours after the final irradiation. Note the thick cornified layer in TTD skin (indicated with an arrow) and hyperplasia in UV-irradiated CSB skin (indicated with an arrow). The difference in thickness between treated and untreated TTD skin lies within the normal range observed.

In vivo sensitivity of NER-deficient mice

UV irradiation has two very distinct effects on the skin. First, acute UV-induced inflammation occurs, macroscopically characterized by erythema (redness) and edema (swelling) of the skin. This effect is predominantly caused by lesions in actively transcribed DNA (2) and thus serves as a parameter for TCR. Long-term exposure causes scaling of the skin, histologically characterized by hyperkeratosis and hyperplasia of the epidermis, which is due to persistence of lesions in transcribed genes as well as the genome overall. Though photosensitivity is reported in TTD patients, the severity of either symptom is not known. TTD mice allowed us to characterize these parameters *in vivo*.

UV-induced inflammation is expressed as the minimal UV dose required to induce edema (MED), determined via application of a series of single UV-doses on the ears of TTD as well as wild-type, XPA, XPC and CSB mice (see Materials and Methods for further information). As shown in Table 1, the TCR defect of XPA and CSB mice is associated with low MED, and TCR proficiency in XPC mice with a response in the wild-type range. TTD mice have a slightly reduced MED compared to wild-type and XPC mice (1200 J/m^2 versus 1500 J/m^2) but not nearly as outspoken as XPA and CSB mice (150 J/m^2), consistent with the idea that TCR is only partially affected in TTD mice.

Table I DNA repair characteristics of NER-deficient mice and MEFs

Strain	UDS (%)[a]	RNA synthesis Recovery (%)	D10 (J/m^2)[b]	MED (J/m^2)	Hyperplasia [c]	Cancer-prone[c]
wt	100	100	8	1500	-	n.a.
XPA	<5	<5	0.9	150	++	++
CSB	>95	<5	1.3	150	++	+
XPC	30	>95	1.4	1500	+	++
TTD	25	~20	4.9	1200	+/-	+

[a] The UDS values represent the mean of several independent experiments. For proper comparison, the data of all cell lines in the text are from one experiment. [b] Deduced from Figure 1a [c] Data are compiled (10 , 25, 34 and this study)

Susceptibility to UV-induced hyperplasia of TTD mouse epidermis was examined by irradiating the shaven backs of wild-type, TTD and CSB mice on 4 consecutive days with 100 J/m^2 UVB. As depicted in Figure 2, epidermis of irradiated CSB mice displayed hyperplasia as reported before (34), while both wild-type and TTD mice were not detectably affected at this low UV-dose. Additionally, no effect of UV irradiation on the skin was observed in chronically exposed TTD mice in the UV-induced carcinogenesis protocol (daily doses of UVB increasing from 80 J/m^2/day to 670 J/m^2/day UVB for a period of 29 weeks, data not shown). In contrast, XPA, CSB as well as XPC mice displayed marked pruritis, scaling of the skin and eye

lesions when chronically exposed to variable doses of UVB (9, 10, 25, 34). Only mild hyperplasia and inflammation was noted on histological sections of TTD skin, but not wild-type skin after 6 treatments with 10 μg DMBA (data not shown).
In conclusion, comparison with NER-deficient mice demonstrates that TTD mice are mildly sensitive to genotoxic agents *in vivo*.

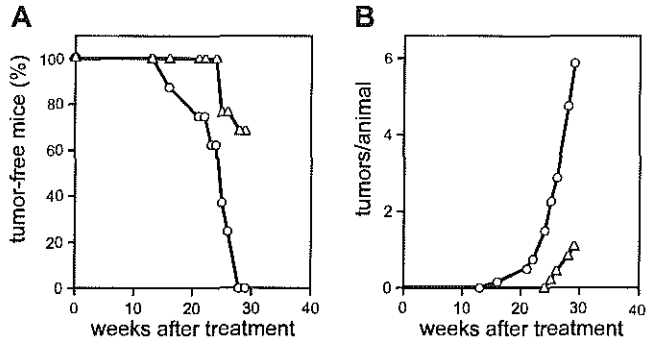

Figure 3. UV- induced skin tumor development in wild-type and TTD mice. Shaven mice were subjected to an UV-B carcinogenesis protocol (see Materials and Methods). Triangles, wild-type mice; open circles, TTD mice. A) Incidence, latency and B) yield of skin tumor formation after UV treatment. The cumulative dose is 103 kJ/m^2 (250-400 nm)

UV-light induced skin cancer susceptibility

It was of special interest to investigate cancer predisposition under experimental conditions in the TTD mouse model in view of the notion, that TTD patients despite their NER deficiency do not appear to be cancer-prone. To this end, 8 TTD mice and 13 wild-type mice were chronically exposed on the shaven back to low daily doses of UV-light. As the experiment continued it became apparent that the TTD mice failed to develop clear cutaneous scaling and eye lesions as registered in XPA and CSB mice, despite the fact that the cumulative UV dose for the TTD mice was much higher than that employed in the XPA and CSB experiments (103 kJ/m^2 versus 25 and 50 kJ/m^2, respectively (10, 34)). After 18 weeks, TTD mice started to develop multiple tumors in UV-exposed areas (Figure 3a and b), which were histopathologically identified as squamous cell carcinomas (SCCs). From week 27 onwards tumors appeared in wild-type mice. The significantly reduced latency time of developing tumors ($p<0.01$) together with the overt increased tumor yield demonstrate that TTD mice are more susceptible to UV carcinogenesis than wild-type mice.

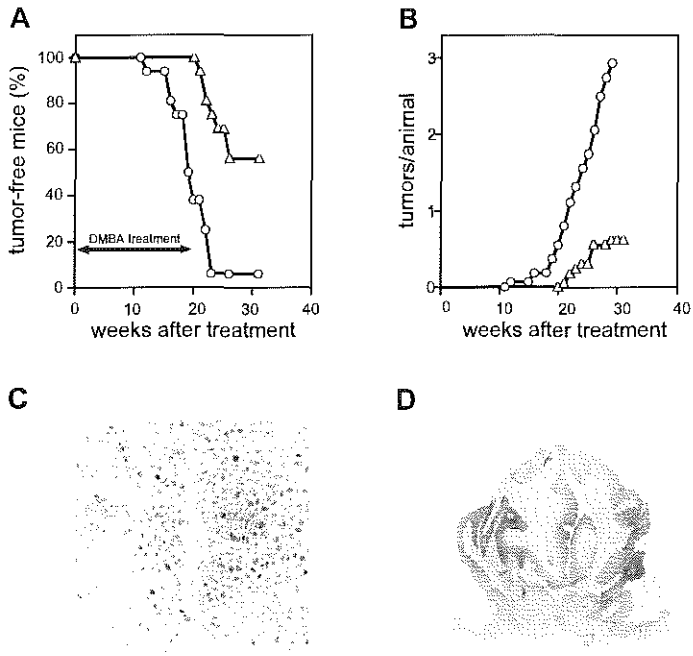

Figure 4. DMBA-induced skin tumor development in wild-type and TTD mice. Shaven mice were subjected to a DMBA carcinogenesis protocol (see Materials and Methods). Triangles, wild-type mice; open circles, TTD mice. A) Incidence, latency and B) yield of skin tumor formation after DMBA treatment. Histopathological examination of DMBA-induced skin tumors in TTD mice shows mixture of squamous cell carcinomas (C) and papillomas (D).

DMBA-induced skin cancer susceptibility

To confirm the cancer proneness of TTD mice, we subjected 15 wild-type and 15 TTD mice to a complete DMBA carcinogenesis protocol, by weekly applying 10 μg of DMBA to the shaven back for a period of 20 weeks. After 12 weeks TTD mice started to develop skin tumors whereas the first tumor in wild-type mice was only observed after 21 weeks (see Figures 4a and b). Despite the very weak cytotoxic effect of DMBA application to the skin, the clear decrease in latency time and the dramatic increase in tumor yield demonstrate that TTD mice are prone to chemical-induced skin cancer.

Tumors on the skin of TTD mice were histopathologically identified as papillomas and SCCs (Figure 4c) at a ratio of 2:3 (Table 2) and one tumor was classified as a fibrosarcoma. In contrast, wild-type mice developed predominantly SCCs whereas

using a similar protocol, XPA-deficient mice only developed papillomas (10, and this study).

DISCUSSION

Since the identification of CS and TTD as DNA repair disorders, the paradoxical absence of skin cancer predisposition in these NER-deficient patients has been puzzling. The set of NER-deficient mice generated in our, and collaborating laboratories allows a quantitative and *in vivo* approach towards elucidating the role of DNA damage and repair in the multi-step process of carcinogenesis. This paper describes cellular repair parameters, quantitation of UV-induced inflammation of the skin and carcinogenic properties of TTD mice compared to other NER-deficient mouse mutants. In contrast to the human syndrome, TTD mice are clearly predisposed to develop skin cancer although not as cancerprone as XPA mice.

Repair defect in TTD mice

The TTD-specific *XPD*^{R722W} allele, which we mimicked in the mouse genome, is associated with a clear but partial DNA repair defect in human fibroblasts. RNA synthesis recovery was severely but not completely abolished (Table 1). Moreover UDS, UV-induced mutagenicity levels in a plasmid-based study (22) and UV-

Table II DMBA-induced tumorigenesis in TTD, wild-type and XPA-deficient mice

	Papilloma[d]	Squamous cell carcinoma
XPA[a]	100 %	n.d.[c]
TTD[b]	40 % (18)	60 % (28)
wild-type	10 % (1)	90 % (9)

[a](10); [b] This study. [c] Not detected. [d] Number of tumors between brackets.

survival employing a protocol with non-cycling cells were comparable between TTD/XPD^{R722W} fibroblasts and fibroblasts from XP patients of complementation group D (4, 29).

Despite the repair defect, TTD mouse skin appears not very sensitive to either UV-induced inflammation (only slightly more sensitive than wild-type mice do) or hyperplasia induced by UV or DMBA. In TTD patients, photosensitivity of the skin has been reported, but ethics constrain experimental quantitation of this symptom. In addition, mild pigmentation abnormalities upon sunlight exposure, one of the hallmark features of XP, have been reported sporadically in TTD patients (4, 16), suggesting that the TTD repair defect resembles XP in this respect, albeit to a mild extent. *In vivo*, photosensitivity in TTD mice and patients may be moderated by UV-shielding by the thick hyperkeratotic epidermis. However, also the eyes of TTD

mice, which are histologically normal (T. Gorgels, unpublished data) fail to display the extreme UV-induced lesions of chronically exposed XPA mice (9). Instead, the mild genotoxic sensitivity in TTD seems to be established at the cellular level for several reasons. First, under the experimental conditions used here, the TTD MEFs and the fibroblasts of four TTD patients carrying the same XPD^{R722W} allele display a comparable mild UV sensitivity (Figure 1). Mild UV sensitivity appears to be a more common feature among TTD fibroblasts although some heterogeneity is apparent (N.G.J. Jaspers and A. Raams, personal communication). Second, the mutational spectrum in TTD cells, considered as a fingerprint of the repair defect, resembles more that of wild-type than of XP-D cells (19, 22) and TTD cells are less sensitive to UV-induced transcription inhibition of the ICAM-1 marker gene than XP-D cells (1). A possible rational for the milder consequences of the TTD repair defect was provided by Eveno and coworkers (11) who showed that photosensitive TTD cells have defective CPD repair but (partially) proficient repair of 6-4PPs. This was confirmed by Marionnet *et al* (21) who demonstrated that CPDs are the predominant mutagenic lesions in TTD cells. Lesion-dependent efficiency of repair may thus underlie the mild sensitivity of NER-deficient TTD cells, but this has not been analyzed in the TTD mouse yet. Mouse models are being generated expressing 6-4PP- and CPD-specific photolyases in the skin, allowing us analysis of the role of either lesion in UV sensitivity and skin carcinogenesis in general.

Trichothiodystrophy syndrome and cancerproneness

An important and intriguing enigma associated with TTD is the observation that repair-deficient TTD patients seem to be free of cancer despite their NER defect, which is in striking contrast to XP and XP/CS cases from the same XP-D complementation group (3). Previously, differences in catalase activity (39), natural killer cell activity (20), and apoptotic response (40) have been reported between TTD en XP-D patients but the relative importance of each of these parameters has not been studied in detail yet. Obviously, they may be addressed *in vivo* in the TTD mouse mutant. The most significant observation reported here is that TTD mice exhibit enhanced UV- and chemical-induced cancer susceptibility. This shows that TTD does not somehow intrinsically protect against skin cancer and is in agreement with the dogma that a defect in NER predisposes to cancer but is in apparent contrast with clinical data. Notably, as much as the experimental setup allowed, TTD mice appeared less cancer-prone than XPA mice. The reduction in latency of UV-induced tumors was less pronounced in TTD compared to XPA mice (10) and the tumor type induced by DMBA resembled the wild-type spectrum: XPA and wild-type mice develop predominantly papillomas and SCCs respectively whereas TTD mice develop a mixture of both tumor types. We propose that the molecular characteristics of the TTD repair defect, reflected by low UV-sensitivity, imposes less severe predisposition to UV-induced skin cancer in TTD mice and patients than in XP. Furthermore, possible and established physiological differences between

mouse and man, e.g. in metabolic rate, immune system, apoptotic response, spontaneous and induced mutation rates and certain repair parameters may influence the difference in cancer proneness differently in the two species. However, additional factors may also explain the paradoxical absence of skin cancer development in TTD patients.

XP and TTD are clinically very different syndromes of which a defect in basal transcription is thought to participate in the clinical outcome of TTD. Discussion of the difference in cancer predisposition between XP and TTD should include possible consequences of repair- and transcription-related phenotypes on tumor development. For instance, the thick cornified layer of TTD patients will shield the underlying proliferating keratinocytes of the basal layer significantly from UV-irradiation and thus reduce the mutagenic dose. Furthermore, TTD keratinocytes, the target cells for skin tumorigenesis, have a defect in late stages of terminal differentiation (7) which might have an impact on transformation to a tumor cell. At least as relevant is the severity of the disease and the young age at which many TTD patients die, which probably does not allow time to accumulate enough damage to develop skin tumors, as suggested previously for Cockayne syndrome (34). This is certainly likely for the five patients with the R722W mutation, because they were all severely affected and died very young (at least four before age 5 (4)). In conclusion, the experimental mouse model reveals that TTD syndrome in man may be associated with hitherto unnoticed cancer proneness although the residual repair activity of TTD cells protects largely against the cytotoxicity and carcinogenicity of UV. This may be particularly relevant under normal conditions when the low damage load does not exceed the limited DNA repair capacity of the TTD cells.

Mouse models for human NER syndromes

A set of mouse mutants is now available for all three NER syndromes and for most of the possible NER deficiencies: a complete NER defect (XPA mice), a selective GGR impairment (XPC mice) or TCR defect (CSB mice) and the subtle XPD/TTD mutation (ref. 12 for an overview of established NER-deficient mouse models). In these mice, we and others systematically compared a number of DNA repair parameters in an identical experimental setup and with mice of defined genetic background to obtain a comprehensive understanding of the role of NER and it's subpathways in preventing cells from cell death, UV-induced inflammation of the skin and carcinogenesis. We conclude that repair characteristics like UDS, RNA synthesis recovery and the relative sensitivity of the different NER-deficient MEFs to UV light are comparable between man and mice. Differences in repair between human and rodent cells are known (notably lack of CPD repair from non-transcribed DNA in rodent cells (24)). Apparently, this does not significantly influence most of the parameters we analyzed, although it may explain the very small difference in MED between wild-type and TTD mice because at least in TTD patient fibroblasts, repair of CPD is affected more than repair of 6-4PPs (11). The degree of cancer

proneness in mice corresponds with the nature of the repair defect, mainly that of the GGR pathway of NER. TTD mice with a partial GGR and TCR defect as well as CSB mice with a TCR deficiency are more mildly cancer-prone than XPA and XPC mice (10, 25, 34, R.B. manuscript in prep.). Thus, the TTD defect does not *per sé* protect against tumorigenesis, which has important implications for the surveillance of TTD patients. Furthermore, quantitation of sensitivity to UV-induced inflammation of the skin confirmed that lesions in actively transcribed DNA cause sunburn as mentioned before (2). In contrast, lesions in both the global genome (XPC mice) as well as in transcribed DNA induce hyperplasia at comparable UV doses. Other processes evoked by induction of lesions in the DNA may also play a role in carcinogenesis. Studies on the relations between persistence of DNA lesions on the one hand and apoptosis and systemic suppression of the immune system are in progress in NER-deficient mice.

MATERIALS AND METHODS

NER-deficient mice and cell lines

For simplicity, the term TTD mouse is used for mice homozygous for the XPD^{R722W} allele (7). Similarly, XPA, CSB and XPC refers to mice homozygous for the targeted allele in the respective genes (10, 25, 34). Since no heterozygous effect was observed in any experiment, both heterozygous mutant and homozygous wild-type mice are referred to as wild-type. Mice used for generation of mouse embryonic fibroblasts (MEFs) and determination of UV-induced hyperplasia were in mixed 129-C57Bl/6 background. Mice in the MED assay were mixed 129-C57Bl/6 (XPC and TTD) and pure C57Bl/6 (XPA and CSB). No effect of genetic background on MED in wild-type mice was noted (J. G., unpublished data)
Human TTD fibroblast cell lines TTD7PV and TTD12PV, TTD1VI and TTD1BEL were kindly provided by drs. M. Stefanini, A. Sarasin, and A.R. Lehmann respectively.

DNA repair characteristics of MEFs

Mouse embryonic fibroblast (MEFs) for all genotypes were obtained in our laboratory as described before (7).
For UDS testing, MEFs were seeded onto coverslips. The next day, cells were washed with PBS and irradiated at 16 J/m^2 UV-C (Philips, TUV lamp). Subsequently, cells were incubated for 2.5 hours in culture medium containing 10 μCi/ml [^{3}H]-thymidine, fixed and subjected to autoradiography as described before (37).
RNA synthesis recovery was measured according to (23). In short, coverslip-grown cells were exposed to 10 J/m^2 of 254 nm UV light, labeled with [^{3}H]uridine, and processed for autoradiography. The relative rate of RNA synthesis was expressed as the ratio of grains over UV-exposed to unexposed nuclei. In general, UDS values are very well comparable within an experiment but show variation between experiments.
DMBA survival was performed by incubating cells in 7,12-dimethylbenz[a]anthracene (DMBA)-containing medium for 2 hours (day 1). To activate DMBA, a microsomal S9-

fraction (TNO-voeding Zeist) was added. Cells were washed twice and grown until day 6. Cell survival was measured by the addition of the tetrazolium salt XTT (final concentration 0.12 mg/ml) to the culture medium. The amount of formazan dye formed after 2 hours incubation was measured with an enzyme-linked immunosorbent assay reader. Cell survival of DMBA-treated cells was calculated as the percentage of absorbance in relation to the absorbance of untreated cells. The procedure followed was as described by the manufacturer (Boehringer, Cell Proliferation Kit II). The experiment was performed with four independent TTD MEF cell lines, two XPA MEF cell lines and four wild-type MEF cell lines.

For UV-survival assays, cell cultures were exposed to UV and allowed to grow for another 4-5 days, before reaching confluency. Cells were labeled with [^{3}H]-thymidine as described above, rinsed with PBS and lysed. The number of proliferating cells in each dish was estimated by scintillation counting of radioactivity during a 3 hours pulse-labeling. Cell survival was expressed as the ratio of irradiated over unirradiated cells.

Quantitation of UV-induced inflammation

To determine the minimal erythema/edema dose (MED), mice were exposed to broadband UVB radiation from a filtered (Schott-WG305 filter) Hanovia Kromayer Lamp Model 10S (Slough, UK). This is a hand-held lamp that allows short exposures to limited skin areas (such as the ears) by placing the circular port (approximately 2 cm^2) in close contact to the skin (28). The dose rate was 150 J/m^2/second; 280-400 nm and each strain of mice was examined at least at 5 different doses in triplo.

Besides macroscopic evaluation of edema and erythema reactions, the increase in skin thickness was determined as a value for acute UVB effects. Ear skin was exposed to the Kromayer lamp because ears do not contain a fur (shaving was not necessary). Ear thickness was measured prior to and 24 hours after UVB exposure using an engineer's micrometer (Mitutoyo model 193-10, Veenendaal, The Netherlands). The lowest dose that was able to induce a significant swelling response (i.e. edema reaction) was denoted to be the MED for that strain of mice.

UV- and DMBA-induced skin effects and carcinogenesis

Acute effects in the skin of shaven wild-type, CSB and TTD mice was assessed by exposure to 100 J/m^2/day UV-B light (250-400 nm, American Philips F40 sun lamps) during 4 consecutive days. Skin-samples were obtained from two mice per genotype, 24 hours after the last exposure and were routinely processed (hematoxylin-eosin staining) for histopathology.

UV-induced carcinogenesis was studied by exposing the shaven back of 8 TTD and 13 wild-type mice (starting age 8 weeks) to UV-B light using an incremental-dose protocol starting at 80 J/m^2/day and gradually increasing to 670 J/m^2/day (250-400nm). Timer-controlled American Philips F40 sunlamps were positioned 33 cm above the cage and yielded a dose rate of 13.3 J/m^2/min (250-400nm). Chemically-induced carcinogenesis in the mouse skin was tested with 7,12-dimethylbenz[a]anthracene (DMBA) in the complete carcinogenesis protocol (10). Shaven TTD mice and wild-type littermates (15 per genotype, age 8-12 weeks) received 20 weekly applications of 10 µg of DMBA dissolved in 100 µl acetone.

Mice were checked for tumor appearance once a week. Skin tumors were routinely processed for histopathologic examination.

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CHAPTER 6

SYMPTOMS OF PREMATURE AGING IN A MOUSE MODEL FOR THE DNA REPAIR/BASAL TRANSCRIPTION SYNDROME TRICHOTHIODYSTROPHY

SYMPTOMS OF PREMATURE AGING IN A MOUSE MODEL FOR THE DNA REPAIR/BASAL TRANSCRIPTION SYNDROME TRICHOTHIODYSTROPHY

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The sun-sensitive form of the severe neurodevelopmental, brittle hair disorder trichothiodystrophy (TTD) is caused by point-mutations in the essential XPB and XPD helicase subunits of the dual functional DNA repair/basal transcription factor TFIIH. The complex disease phenotype is hypothesized to be in part derived from a repair defect causing UV sensitivity and in part from a subtle, viable basal transcription deficiency accounting for the other TTD features. Previously we established a TTD mouse model by mimicking the causative XPD point-mutation of a photosensitive TTD patient. TTD mice reflect to a remarkable extent the pleiotropic features of the human disorder. In this report we describe a comprehensive range of progeroid symptoms observed in aging TTD mice. These include reduced life span and fertility, cachectic dwarfism, early graying, sebaceous gland hyperplasia, spinal kyphosis and osteoporosis, reminiscent of human aging. TTD mice reveal an important link between DNA repair and transcription capacity in the onset of age-related symptoms.

manuscript in preparation

INTRODUCTION

The genome is under continuous genotoxic stress due, to endogenous and environmental DNA damaging agents such as solar, and ionizing irradiation, chemical compounds and oxygen radicals derived from cellular metabolism. DNA lesions affect the functioning of the genome and are implicated in mutagenesis and carcinogenesis. Moreover, they have also a suspected role in aging [1]. To counteract the obviously deleterious effects of DNA injury, a network of DNA repair pathways has evolved. The most versatile mechanism is nucleotide excision repair (NER) which removes a wide diversity of DNA distorting lesions, among which the main UV-induced lesions, bulky chemical adducts and some forms of oxidative damage [2]. The NER process involves the action of at least 30 proteins in subsequent steps of damage recognition, local opening of the double helix around the injury and incision of the damaged strand on either side of the lesion. After excision of the damage-containing oligonucleotide, the resulting gap is filled by DNA repair synthesis followed by strand ligation [3,4]. Two NER subpathways are known. Global genome repair (GGR) removes lesions genome wide, while transcription-coupled repair (TCR) removes transcription-blocking lesions very efficiently but exclusively from the transcribed strand of active genes. The clinical consequence of a complete defect in NER is evident from the rare recessive disorder xeroderma pigmentosum (XP). Seven genes are involved in XP, designated *XPA* to *XPG*. XP patients are very sun-sensitive, display accelerated photoaging of the skin and have a 1000-fold increased risk of developing skin tumors. Accelerated neurodegeneration is noticed in a subset of XP patients [5]. A second human NER disorder, Cockayne syndrome (CS) is characterized by photosensitivity, which is remarkably not associated with cancer predisposition [5,6]. Moreover, CS is considered a segmental progeroid (aging) syndrome because patients have an overall aged appearance, suffer from postnatal growth failure (cachectic dwarfism), skeletal abnormalities (osteoporosis and sclerosis), progressive neurologic dysfunction (due to dysmyelination), and reduced life expectancy. These symptoms are absent in XP patients and it is difficult to envision that these are caused by a defect in NER alone. CS is specifically associated with a defect in the transcription-coupled repair (TCR) sub-pathway [7] due to mutations in the *CSA* or *CSB* gene [8,9]. However, a sole defect in TCR cannot explain the CS features, as some forms of XP carry a total NER defect and yet fail to express the severe CS symptoms. An interaction has been found between CSB and RNA polymerase II, suggesting that impaired transcription in cells of CS patients may be involved in the onset of CS symptoms. Recently, Leadon *et al.* provided evidence for a defect in TCR of oxidative DNA damage in cells of CS patients [10]. This opens up the possibility that oxidative lesions in actively transcribed DNA contribute to the developmental defects associated with CS.

DNA repair and transcription are also intimately linked in a third human NER syndrome, the photosensitive form of the pleiotropic disorder trichothiodystrophy (TTD). The disease is very reminiscent of CS, including neurodevelopmental and skeletal abnormalities and remarkable absence of skin cancer [5,11]. In addition, TTD patients display ichthyosis (scaling of the skin) and the typical brittle hairs and nails, which are due to a specific defect in expression of the group of cysteine-rich matrix proteins. Most patients have point mutations in the *XPD* gene [12], which is also involved in patients with XP [13] and in patients with combined features of XP and CS [14]. A clue to the extreme clinical heterogeneity associated with mutations in *XPD,* was the discovery that the gene encodes one of the helicase subunits of the multi-protein TFIIH complex [15]. TFIIH is involved in local unwinding of the DNA helix around the lesion in NER [16] and of the promoter region in transcription initiation of RNA polymerase II [17]. Thus, *XPD* mutations may not only impair NER, causing photosensitivity, but may also subtly affect the transcription process, providing a rational for the non-repair phenotypes of TTD such as growth retardation and the reduced expression of cysteine-rich matrix proteins in TTD hair
[18,19]. To obtain insight into the pathophysiology of the TTD syndrome and to study the molecular intricacies of repair and transcription *in vivo* we developed a mouse model for TTD by mimicking the causative point mutation of a photosensitive TTD patient in the mouse *XPD* gene (XPD^{R722W}). TTD mice display a remarkable resemblence to the human disease including brittle hair and ichthyosis [20]. A partial NER defect was associated with mild UV sensitivity in cultured fibroblasts but surprisingly also with a hitherto unnoticed predisposition to UV-induced skin cancer (de Boer *et al.*, submitted). Here we report that TTD mice reveal a consistent picture of coherent features of premature aging. In addition to reduced life span and cachectic dwarfism, TTD mice display osteoporosis,

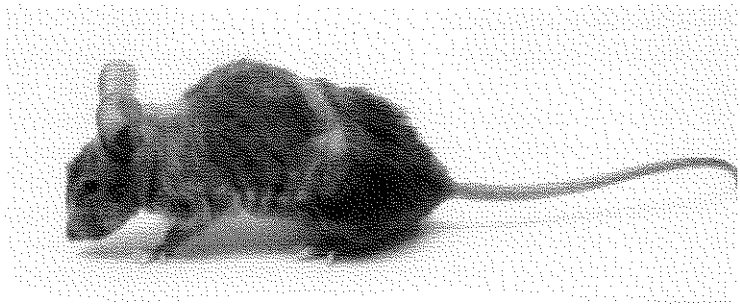

Figure 1. Aged appearance of TTD mice. Progeroid symptoms (cachexia, kyphosis) appear at age 3-4 months but become more pronounced later in life. The picture shows a TTD mouse of 16 months old.

osteosclerosis, early depigmentation of hair, sebaceous gland hyperplasia, reduced female fertility and starvation. TTD mice are a valuable experimental model to study a possible link between compromised genome functioning (transcription) induced by DNA damage and the process of aging.

RESULTS

Through regular observation of a large group of aging TTD mice and wild-type littermates, we noticed that TTD mice develop an overall aged appearance (see Figure 1) at a young age. This, together with the reduced lifespan and characteristic stagnation of development triggered us to conduct a more systematic analysis of specific parameters indicative of premature aging. For proper comparison all mice were in a 50% C57BL/6 and 50% 129 background.

Cutaneous features of premature aging

As reported previously, both skin and hair keratinocytes display abnormalities at late stages of the terminal differentiation process [20]. A more prominent granular and cornified layer in the skin is associated with reduced expression of the *SPRR2* gene which is considered a marker for late stages of terminal differentiation of skin keratinocytes [21]. Similarly, TTD hairs are brittle due to a deficiency in the group of cystein-rich matrix proteins. These proteins crosslink hair keratin filaments and are expressed at the end of the differentiation program [22]. Another hair abnormality we observed frequently in aging TTD mice was depigmentation, for which the onset and manifestation was heterogeneous but which was significantly earlier and more frequent than the sporadic cases of graying we noticed in wild-type littermates. A clear example is shown in Figure 2a with a large patch of gray hairs, which are completely devoid of melanin granules (not shown). Other TTD mice exhibited more subtle depigmentation (e.g. brown instead of black).

Besides brittle and depigmented, TTD hairs also had a greasy appearance. Histologic analysis showed that hyperkeratosis in the outer and inner root sheet resulted in follicular plugging and dilatation. Moreover, hyperplasia of the sebaceous gland was observed frequently (Figure 2b). Sebaceous gland cells are indistinguishable between wild-type and TTD mice indicative of their benign nature. Moreover, no malignant conversions were detected, neither in aging mice nor in skin of mice subjected to an UV- or a chemical-induced skin cancer protocol (de Boer *et al.*, submitted). Both graying and sebaceous gland hyperplasia are features of human aging.

Ovarian dysfunction

Although sexual behaviour of most female TTD mice appeared to be unimpaired, as evidenced by the presence of copulatory plugs, full term pregnancy was observed only sporadically. For instance, a group of six female TTD and six wild-type mice were test bred for a period of 6 months to wild-type stud males (starting age 2 months). Only one litter of three pups was observed in the TTD group while all six wild-type females yielded several litters in the same period. To study ovarian function in TTD females in more detail, eight TTD and eight wild-type control females (both age 16 months) were placed with stud males, checked for copulatory plugs and ovaria were analysed. On histological basis, two groups of TTD animals could be discerned. In the first group (three animals) no signs of an active estrous cycle were found. Ovaries were very small and contained immature preantral and small antral follicles, but no preovulatory follicles, little interstitium and absent corpora lutea, implying complete anovulation (Figure 3A). In these animals a copulatory plug was never found, probably resulting from the absence of an estrous cycle. In the other group (five TTD females) a more heterogeneous picture was found. In two animals antral follicles were present together with new corpora lutea indicating that ovulation had occurred. In addition, ova were present in the oviduct. However, ovulation in these females probably occurred very infrequently, since corpora lutea from previous cycles were absent (Figure 3B). In another female, an ovulatory LH peak probably had taken place, since large preovulatory follicles were present with an

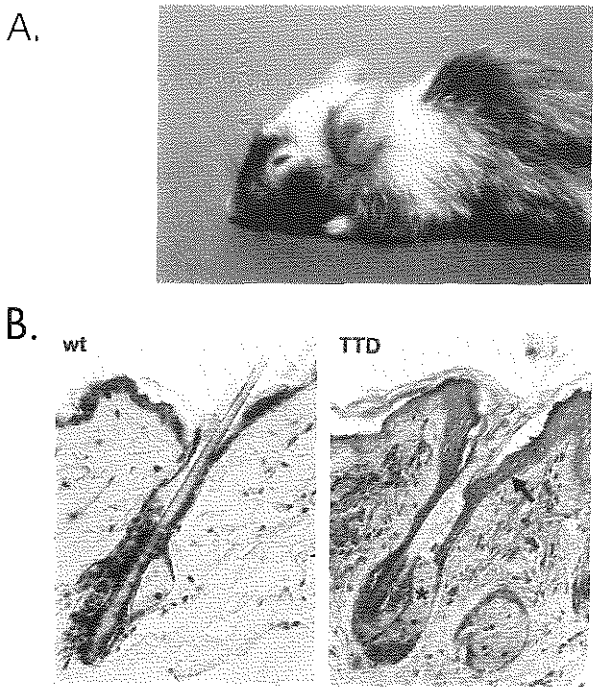

Figure 2. Cutaneous symptoms of aging A) Typical example of early depigmentation in fur of a 13 months old TTD mouse. No depigmentation was found in a group of 10 aged-matched wild-type mice. **B)** Follicular dilation and sebaceous gland hyperplasia (asterix) in TTD compared to wild-type (wt) skin. Mark the hyperkeratotic TTD epidermis (indicated with an arrow).

oocyte that showed germinal vesicle breakdown (*i.e.* resumption of meiosis) and cumulus dispersion. However, ovulation had not completed because no ova were found in the oviducts (Figure 3C). In two other animals, growing preantral and large antral follicles were present, in the absence of indications of previous ovulations (Figure 3D). These ovaries were similar in appearance to the ovaries of the first anovulatory group, but the follicles and interstitium in these animals were developed much further. The ovaries from wild type females showed a normal picture, *i.e.* preantral and large antral follicles with new corpora lutea and ova in the oviduct (Figure 3F).

Overall, these observations indicate a broad range of ovarian dysfunction in TTD mice ranging from complete anovulation, via intact follicular growth to preovulatory stages to sporadic, normal ovulation. Because fertility could very well be a function of physical fitness of the individual, it is important to note that no strict correlation was found between severeness of cachexia (as apparant from bodyweight) and degree of anovulation. The heterogeneity in ovarian dysfunction as well as other TTD features may be due to the mixed 129-C57BL/6 background of the mice. Whether acyclic TTD females resemble reversible infertility as in anorecic females or irreversible infertility as in mammalian menopauze awaits further analysis.

We also compared fertility of a group of six TTD males to six wild-type littermates by mating to wild-type females. At least up to seven months of age, TTD males displayed normal fertility.

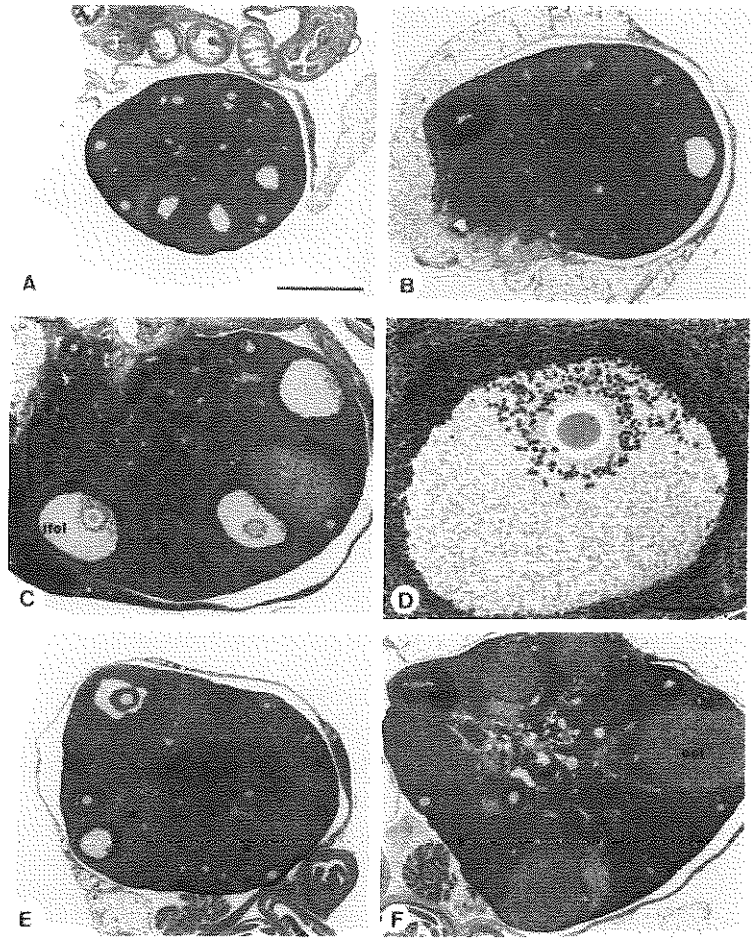

Figure 3. Heterogeneous ovarian dysfunction in TTD females Ovaries from TTD mice (A,B,C,D and E) and from control mice (F) at estrous. **A)** Note the absence of large preovulatory follicles and corpa lutea. **B)** Note the presence of a recently formed corpus luteum. **C)** Note the presence of large preovulatory follicles with cumulus dispersion and an oocyte showing resumption of meiosis. **D)** Larger magnification **E)** growing follicles are seen but corpora lutea of previous ovulations are absent. **F)** Ovary from a wild-type control animal at estrous. Note the presence of old and new corpora lutea. Lfol: large preovulatory follicle; ncl, newly formed corpus luteum; ocl, old corpus luteum. Bar A, B, C, E and F are 500 μm. Bar D is 100 μm.

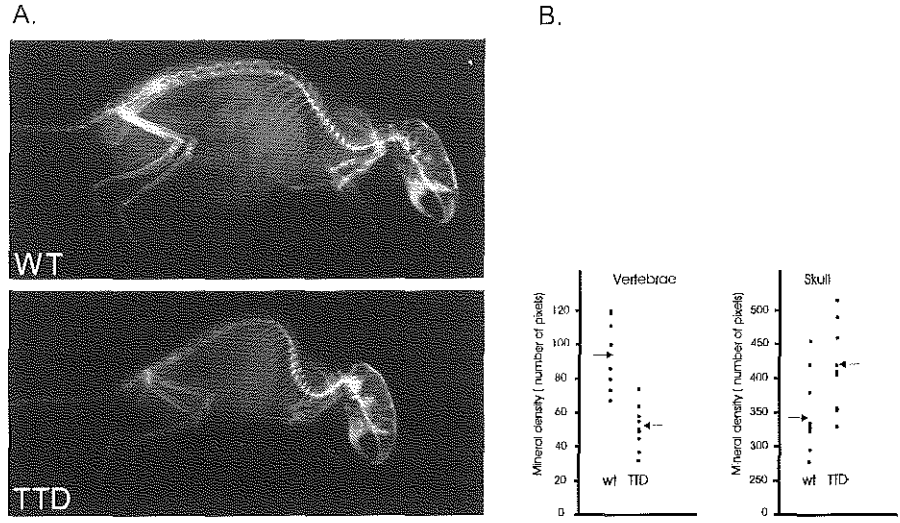

Figure 4. Skeletal abnormalities in TTD mice A) Radiological picture of a wild-type and TTD mouse. Note the kyphosis of the spinal column, decreased mineral density in vertebrae and limbs, and increased mineral density of the skull of TTD mice. **B)** Quantitation (see Methods for details) of mineral density of the vertebrae and skull in wild-type and TTD mice. Both average for skull and vertebral density (indicated by arrows) was significantly different ($p<0.01$).

TTD mice display several features of starvation.

The most life-threatening symptom of TTD patients is failure to thrive, causing a cachectic and poor overall condition. This makes the patients vulnerable to infections, which is a frequent cause of death [11]. Cachexia in TTD mice is heterogeneous in onset and severity but is clearly progressive and associated with premature death [20]. While excessive fat is observed in the abdominal cavity of aging wild-type mice it is only sporadically detectable in a TTD littermates (figure 5), indicative of starvation. Analysis of blood cell parameters of TTD mice (age 6 months) revealed mild normochrome anaemia in TTD mice (Table 1), a feature associated with starvation in man. This was further substantiated by the significantly decreased levels of the branched-chain amino acids (valine, leucine and isoleucine) in blood of TTD mice (Table 2). Prolonged starvation has a profound effect on the concentration of these plasma amino acids, because they are used in the process of gluconeogenesis when energy supplies are sub-optimal. Reduced levels of branched-chain amino acids are therefore a clear indication of starvation.

Table 1. Blood cell values of wild-type and TTD mice.

		wild-type mice	TTD mice	p-value
red blood cell count	(10^{12}/L)	10.3	8.7	< 0.01
hemoglobin	(mmol)	9.4	8.1	< 0.01
hematocrite	(L/L)	0.56	0.47	< 0.01
mean cell volume	(fL)	54.5	54.3	>0.05

A significant difference of plasma concentration between wild-type and TTD mice was also found for 1-methyl-histidine and phenylalanine (all other amino acid concentrations, as well as albumin, glucose, creatinin, LDH and urea levels were normal, see table 2 and data not shown). We determined whether abberant food uptake or malabsorbtion of food in the intestines could account for starvation in TTD mice. First, dental abnormalities were excluded. Faeces of TTD mice appeared normal and even in severely cachectic animals, food was found in stomach and intestines, indicating that TTD mice fed normally. Furthermore, we analysed the intestines histologically but no gross abnormalities were found. It must be noted however that TTD mice suffered more frequently from prolapse of the rectum than wild-type mice so an intestinal abnormality cannot be excluded completely.

To investigate whether malabsorbtion of food occurred in TTD mice we analysed the organic acids of urine because certain changes are typical for intestinal bacterial degradation when malabsorption occurs [24]. However, no aberrant organic acids in urine of TTD compared to wild-type mice were detected (data not shown), arguing against malabsorbtion as the cause of starvation in TTD mice. Further pathology of TTD mice did not specify abnormalities in other vital organs as liver, kidney or spleen. In summary, TTD mice suffer from progressive failure to thrive due to starvation, which is likely to be the cause of premature death, but we failed to identify one major cause of this feature.

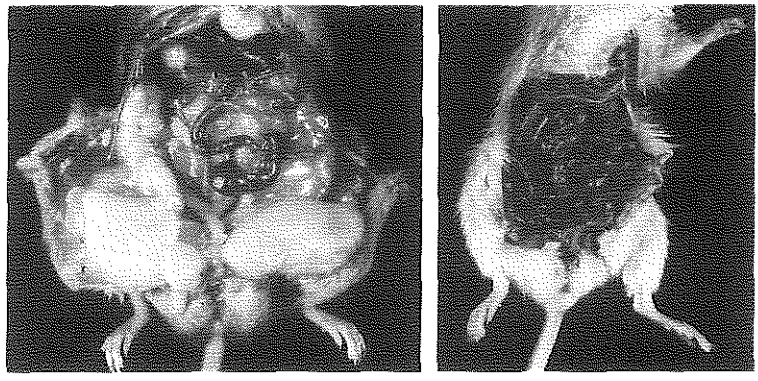

Figure 5. Fatty tissue hypoplasia in TTD mice. Photograph of the abdominal cavity of an 18 months old wild-type (left) mouse shows excessive fat compared to the TTD littermate (right).

DISCUSSION

Human aging syndromes

Aging is a very complex and multi-factorial process. Despite intense speculations for many decades, the molecular basis for aging is unresolved. One line of aging research involves the human segmental progeroid syndromes for which the causative genes have been identified. These include Werner syndrome, Bloom syndrome, ataxia telangiectasia, xeroderma pigmentosum and Cockayne syndrome [5,25-27] and also *ERCC1*- and telomerase-deficient mice display features of accelerated aging [28-30]. Despite the fact that the clinical picture in each of these syndromes resembles aging incompletely and some symptoms are even congenital, they may shed light on the multifactorial molecular mechanism underlying the complex pathology of aging. Interestingly, all mentioned progeroid syndromes are associated with abberant DNA metabolism and a number point to the involvement of DNA damage. Photosensitive TTD is mostly caused by mutations in the *XPD* gene [12], which is involved in NER and basal transcription and therefore complies with the rule. Interestingly, TTD mice display a range of pleiotropic premature aging symptoms including cachexia, reduced life span, kyphosis, osteoporosis, reduced female fertility, sebaceous gland hyperplasia and early graying. Some of these features (growth failure, reduced life span and skeletal abnormalities) are also observed in Cockayne syndrome [6]. Considering this report on TTD mice , the clinical description of photosensitive TTD patients [11] and the many parallels with CS [15], trichothiodystrophy may also be considered as a segmental progeroid syndrome.

Features of premature aging in TTD mice

TTD mice have a generally aged appearance and die prematurely. Adipose tissue hypoplasia, mild anemia and reduction of the branched-chain amino acids in blood of TTD mice demonstrate that the cachectic appearance and early death are caused by starvation. Similarly, 4 out of 5 TTD patients harboring the XPD^{R722W} allele, mimicked in the mouse germline, died before age 4 from dehydration, cachexia and respiratory infection [31].

In TTD mice we detected increased plasma levels of 1-methyl histidine, an endproduct in anserine degradation, a muscle tissue-specific protein [32]. Its accumulation indicates that muscle degradation occurs, probably to serve as carbon source in gluconeogenesis. Osteoporosis in TTD mice and patients [23] could be a secondary effect of starvation, because in human aging osteoporosis is associated with muscle weakening and inactivity. However, no strict correlation between severeness of cachexia and osteoporosis is found in TTD mice or patients. Possibly, aberrant vitamin or hormone levels or autonomous effects of the TTD mutation on

cells involved in bone homeostasis underlie demineralization. Similarly, the heterogeneous picture of infertility in TTD females may be a result of starvation-induced hormonal changes. Fertility in mammals requires adequate nutrition and reserves of metabolic fuel. Failure of the hypothalamus-pituitary-gonadal axis occurs in both sexes after starvation, although the female appears to be more sensitive to the effect of undernourishment [33]. Body weight and, more specifically, body fat mass has been implicated to influence the timing of menarche [34] and low body fat mass can cause secondary amenorrhea [35]. It has become clear that the cytokine leptin is produced by adipocytes and is required for fertility in the mouse [36] as it affects luteinizing hormone (LH) secretion [37]. Therefore, the broad range of ovarian dysfunction in the TTD mice may be caused by a decrease in the level of LH and/or mistiming of the ovulatory LH peak resulting from an effect of malnutrition on the hypothalamus or pituitary. The fact that starvation in TTD mice cannot be appointed to abnormalities in a certain target organ suggests that the defect in TTD is a general cellular defect. Unfortunately the phenotype leaves but little clues about the molecular defect underlying this very pleiotropic and heterogeneous syndrome.

Molecular basis of aging

Genetic evidence from yeast to mouse suggests that the rate of cellular metabolism and the oxidative damage load in particular underlie at least part of the pathogenic mechanism of aging [1]. Two TTD mouse phenotypes *i.e.* early graying (also observed in Werner syndrome) and sebaceous gland hyperplasia are correlated to oxidative damage. First, melanogenesis generates free radicals and melanocytes may be vulnerable to free radical attack by melanin products because they have

Table 2. Amino acid values in blood of wild-type and TTD mice

amino acid [μMol]	wild-type mice	TTD	p-value
valine	222	169	0.04
isoleucine	103	69	< 0.01
leucine	164	118	0.03
1-me-histidine	15.7	21.2	0.05
phenylalanine	78	60	0.035
proline	166	139	>0.05
arginine	130	120	>0.05
asparagine	25	27	>0.05
albumine	0.40	0.48	>0.05
glucose	1.34	1.04	>0.05
creatinine	63.4	54.6	>0.05
urea	11.4	14.7	>0.07

lower levels of the anti-oxidants superoxide dismutase and peroxidase [38]. In accordance, homozygous bcl-2 knockout mice, an oncogene that regulates an anti-oxidant pathway, turn gray with the second hair follicle cycle due to depletion of melanocytes [39, 40]. Similarly, increased sensitivity of TTD melanoctyes to oxidative damage could explain depletion of melanocytes and premature graying in TTD mice. Second, sebaceous gland hyperplasia is frequently observed in males past middle age [41] and also in TTD mice. In a certain type of male baldness, sebaceous gland hyperplasia is associated with increased levels of GST, the key enzyme in biosynthesis of the radical scavenger glutathion [42]. A similar association between oxidative stress and sebaceous gland hyperplasia may exist in TTD mice. Although systemic effects on melanocytes and sebocytes (e.g. hormonal differences) are not excluded, the correlation with oxidative stress suggests that at least part of the clinical symptoms of TTD are caused by sensitivity to endogenous (oxidative) DNA damage. In addition, accumulating evidence shows that oxidative lesions play a role in the onset of CS symptoms. Considering the broad overlap between CS and TTD this could apply to TTD as well. Deficient repair of endogenous lesions alone cannot explain the TTD symptoms because TTD mice have a partial repair defect while completely NER deficient XPA mice are phenotypically normal [43,44]. Previously, we proposed the repair/transcription syndrome hypothesis to explain photosensitive TTD as a disease where deficient repair of UV-induced lesions causes UV-sensitivity whereas the other clinical symptoms result from an intrinsic transcription defect [18,19]. Theoretically however, a combined defect in transcription and repair cannot be excluded. In this respect, it is of interest to note that TTD is mostly associated with mild sensitivity to genotoxic agents and a partial NER defect.

Endogenous lesions involved in TTD symptoms

To investigate the proposed role of defective DNA repair in the onset of transcription-related symptoms, we crossed TTD mice with a partial NER defect into a completely NER-deficient XPA background. XPA/TTD double mutant mice display growth retardation and kyphosis much more severe and earlier in onset, causing death before weaning (unpublished data). Aparrantly, certain endogenous DNA lesions, which are substrates for NER, are involved in transcription-related symptoms of TTD. We suggest that the crippled transcription apparatus of TTD cells is particularly sensitive to these endogenous lesions. Thus, a complete NER deficiency in XPA/TTD mice results in a higher level of lesions, a more severe depletion of transcription and as a consequence more prominent clinical symptoms. It would be very interesting to examine whether this principle of lesion-induced transcription deficiency also applies to certain facets of normal aging. TTD mice provide an excellent model to study the possible relationship between repair of endogenous lesion, transcription capacity and progeroid symptoms in TTD and may provide us insight into the molecular mechanism of aging.

EXPERIMENTAL PROCEDURES

Mice

The generation of TTD mice by gene targeting of the XPD^{R722W} allele in embryonic stem cells has been described previously [20]. $XPD^{+/+}$ and $XPD^{+/R722W}$ mice are referred to as wild-type mice and $XPD^{R722W/R722W}$ mice are referred to as TTD mice. All mice used in these experiments were in a mixed 129/C57Bl/6 background.

Measurement of blood parameters

Blood cell values were analyzed using a Sysmen F800 apparatus (Toa Medical Electronics) and general blood content values via spectrophotometry on an Elan Autoanalyzer (Eppendorf Merck).

Amino acids in plasma were measured by ion exchange chromatography on a Pharmacia Biochrome 20 amino acid analyzer with ninhydrin detection. Organic acids in urine were extracted with ethylacetate, dried and converted into methylesters by diazomethane. Measurements were performed by gaschromatography-mass spectrometry (Fisons MD-800).

Radiology and histology

Radiographs with a two-fold magnification were taken in dorso-frontal and lateral direction. A special X-ray system, developed for human mammography (CGR Senograph 500T) was used at 30 kV and 32 mAS. A molybdeen focus (0.1 mm) was used, with focus-film distance 65 cm and focus-object distance 32.5 cm. Kodak X-ray films (MIN-R MA 18x24 cm) were used in combination with a Dupont Cronex low-dose mammography-intensifying screen. Mice were sedated during the radiopgraphic procedure. Mineral density was quantified by scanning the radiographs (DuoScan Agfa). Using Imagequant the number of pixels in a defined area of the skull or of the complete sixth tail vertebra was determined.

For histological examination, dissected tissues fixed in 10% formal saline were processed and embedded in paraffin and stained with hematoxylin and eosin using routine procedures.

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SUMMARY AND CONCLUSIONS

DNA damage is implicated in cancer and aging, and several DNA repair mechanisms exist that safeguard the genome from these deleterious consequences. Nucleotide excision repair (NER) removes a wide diversity of lesions, among which the main UV-induced lesions, bulky chemical adducts and some forms of oxidative damage. The NER process involves the action of at least 30 proteins in subsequent steps of damage recognition, local opening of the DNA double helix around the injury, and incision of the damaged strand on either side of the lesion. After excision of the damage-containing oligonucleotide, the resulting gap is filled by DNA repair synthesis followed by strand ligation. The consequences of a defect in one of the NER proteins are apparent from three rare recessive syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and the photosensitive form of the brittle hair disorder trichothiodystrophy (TTD). Sun-sensitive skin is associated with skin cancer predisposition in the case of XP, but remarkably not in CS and TTD. Moreover, the spectrum of clinical symptoms differs considerably between the three syndromes. CS and TTD patients exhibit a spectrum of neurodevelopmental abnormalities and in addition, TTD is associated with ichthyosis and brittle hair. These typical CS and TTD abnormalities are difficult to comprehend as a consequence of defective NER. The involvement of certain NER proteins in the process of transcription suggests that impaired transcription may underlie the non-NER features of CS and TTD.

Chapter 1 provides a review of the molecular mechanism of NER, the genes encoding the different repair proteins, and the clinical consequences of a defect in NER. Several transgenic NER-deficient mouse models have been generated in recent years, and **Chapter 2** summarizes the literature on mouse experiments focusing on the role of NER in mutagenesis and carcinogenesis. Importantly, several NER proteins have a dual involvement in NER and other cellular processes like transcription or mitotic recombination. The phenotypic consequence of a defect in these other processes in the mouse is discussed.

This thesis describes the biological characterization of the mouse *XPD* gene, involved in the DNA helix-unwinding step in NER and also in basal transcription initiation. We generated an *XPD* knockout mouse and a mouse model for the DNA repair/basal transcription disorder trichothiodystrophy (TTD), which was accomplished by mimicking a point mutation as found in the *XPD* gene of a photosensitive TTD patient. The unique spectrum of phenotypes of the TTD mouse and the molecular defect suspected to underlie it allowed us to investigate fundamental processes like carcinogenesis, the transcription syndrome and aging *in vivo*.

CARCINOGENESIS

Previously, the NER process and its role in preventing cancer have been investigated in great detail in fibroblasts of XP, CS and TTD patients. More recently similar studies were done using mouse embryonic fibroblasts of the respective mouse models. Parameters of NER such as UV-induced cell killing, recovery of RNA synthesis after UV irradiation, and UDS may vary considerably between different cell types and in specific cellular environments. Therefore, precaution is required when data obtained *in vitro* are extrapolated to the organismal level. NER-deficient mouse models provide valuable tools to acquire a more holistic view of UV-induced skin carcinogenesis. **Chapter 5** of this thesis describes that TTD mouse cells, like the fibroblasts from the patient, exhibit a partial NER defect as evident from the UV-induced DNA repair synthesis (residual repair capacity ~25%) and limited recovery of RNA synthesis after UV exposure. TTD cells display a relatively mild cytotoxic sensitivity to UV or DMBA. In accordance with the cellular studies, TTD mice exhibit a modestly increased sensitivity to UV-induced inflammation and hyperplasia of the skin. In striking contrast to the human syndrome, TTD mice manifest a clear susceptibility to UV- and DMBA-induced skin carcinogenesis, albeit not as pronounced as the totally NER-deficient XPA mice. This indicates that cytotoxic sensitivity is not necessarily linearly correlated with cancer susceptibility. Possibly, the more cytotoxic 6-4PPs are repaired proficiently in TTD cells but the remaining, less cytotoxic CPDs induce mutations. To explain the discrepancy in cancer proneness between TTD mice and patients, the difference in genotoxic dose to which either is exposed should be considered. TTD mice obtain much higher genotoxic doses in skin carcinogenesis protocols than TTD patients normally would. The capacity of the crippled TTD NER machinery may be insufficient to repair all lesions in chronically exposed TTD mice, and thus mutagenesis and carcinogenesis occurs. Other parameters that may influence tumorigenesis are the thick hyperkeratotic epidermis and frequent hospitalization of TTD patients, which may reduce UV-exposure. To get a better understanding of carcinogenesis in TTD, other UV-induced processes like apoptosis, immune suppression and hyperplasia of the skin, which may influence carcinogenesis should be included for a multi-facetted examination of UV-induced skin cancer in TTD mice. This will not only provide insight into the etiology of TTD, but will also provide clues to fundamental principles of the multi-step process of skin carcinogenesis.

TRANSCRIPTION

The repair/transcription hypothesis was put forward to explain the clinical heterogeneity associated with defects in the *XPD* and *XPB* genes of the dual functional DNA repair/basal transcription complex TFIIH subunits. It was hypothesized that non-XP features in TTD and XP patient with combined CS

features are due to an impairment of the transcription function of *XPD* or *XPB*, whereas the photosensitivity is a consequence of affecting the repair function of *XPD* or *XPB*. The repair/transcription syndrome hypothesis provided a guiding-principle to study the molecular defect underlying the non-NER symptoms of TTD and CS. Although the concept is consistent with all observations, unequivocal proof for it has yet to be obtained. Early embryonic death of XPD knockout mice was consistent with the essential role of XPD in transcription (**Chapter 3**). *In vitro* growth experiments with preimplantation stage embryos obtained from heterozygous *XPD* knockout intercrosses showed a significant higher fraction of embryos that died at the 2-cell stage, compared to wild-type embryos. These results establish the essential function of the XPD protein in mammals and in cellular viability and are consistent with the notion that mostly single point mutations are found in the *XPD* gene of XP, XP/CS and TTD patients.

By establishing a mouse model for the photosensitive form of TTD (**Chapter 4**), we have unequivocally demonstrated that the full clinical spectrum of the disease is caused by a single point mutation in the *XPD* gene. TTD is a very pleiotropic and probably partially systemic disease. Osteoporosis and female infertility may be secondary effects of starvation. In TTD mice, the skin is the only tissue where it is almost certain that the observed symptoms (hyperkeratosis and reduction of cysteine-rich matrix proteins in the hairs) are caused by a cell autonomous defect (probably at the level of transcription). Therefore, the skin is of first choice to study the role of transcription insufficiency in the onset of TTD symptoms. In Chapter 4, we describe that the skin abnormalities are associated with reduced expression of the late-terminal differentiation gene *SPRR2*. An unsolved but important biochemical issue is the consequence of TTD and XP/CS mutations in *XPD* on the properties of the TFIIH complex: is it an effect on stability of the complex or on the enzymatic properties alone? In a recent paper, Coin *et al.* (Nat. Gen. 20:184-188, 1998) demonstrated that certain patient-specific *XPD* mutations alter the protein-protein interaction between XPD and the TFIIH subunit p44. This may affect stability of the complex and consequently results in reduced transcription capacity of the cell, thereby causing TTD. TTD mice provide a valuable experimental model to test these predictions.

A breakthrough in the molecular TTD puzzle is expected from the identification of genes involved in NER-proficient TTD. A candidate approach was performed in our lab by sequencing *XPD* and other genes encoding TFIIH subunits of non-photosensitive TTD patients but unfortunately no mutations were identified. The rarity and heterogeneity of the disease precludes identification of candidate genes via positional cloning. Perhaps *Drosophila* could be used as a more versatile genetic tool to search for genes causing non-UV sensitive TTD.

AGING

The complex process of aging involves age-related decline of physiological functions necessary for fertility and survival of an organism. The molecular basis of aging remains largely obscure but a role for oxidative stress (the oxidative damage theory of aging) is anticipated. Furthermore, an essential role of maintenance of genomic integrity in aging is demonstrated by several human segmental progeroid syndromes and mouse models exhibiting aspects of aging. These include Werner and Bloom syndromes, ataxia telangiectasia and knockout mouse models for the *mTR* and *ERCC1* genes, all of which have a defect in genes involved in DNA metabolism.

TTD mice display several premature aging features and may provide a valuable model to find a molecular clue to certain aspects of aging (**Chapter 6**). Reduced life span in TTD mice is associated with physical decline later in life due to starvation. Cutaneous symptoms of aging include early depigmentation and sebaceous gland hyperplasia and TTD mice display spinal kyphosis, osteoporosis and atrophy of female gonads. According to the repair/transcription syndrome hypothesis, the repair defect of TTD mice cannot explain these aging features, because they are not apparent in XPA mice, which display an even more severe NER defect. Consequently, a transcriptional defect is more likely to be involved, although a combined defect in transcription and repair cannot be excluded. In this respect, it is of interest to note that TTD is mostly associated with mild sensitivity to genotoxic agents and a partial NER defect. To investigate the relation between repair and the presumed transcription-associated cutaneous and progeroid symptoms, we crossed TTD mice into a completely NER-deficient *XPA* background. Strikingly, completely NER-deficient XPA/TTD mice display TTD features like growth impairment, spinal kyphosis, hyperkeratosis and starvation much earlier in life and/or to a much more severe extent. This suggests a relation between impairment of repair of (endogenously generated) DNA damage and the onset of premature aging symptoms in TTD. We postulate the following hypothesis. In every cell, low steady state levels of endogenous lesions are induced which can be repaired by NER, having limited consequence for the organism. However, these low levels of lesions somehow hamper the crippled transcription apparatus of the TTD cell, causing a reduced transcription capacity. It is not unlikely that TFIIH is actively involved in repair of these lesions, and that depletion of transcription is somehow related to this involvement. We postulate that a complete NER deficiency in XPA/TTD mice results in a higher level of lesions, a more severe depletion of transcription and as a consequence more prominent clinical symptoms. Similarly, the non-NER features of the TCR-deficient CSB mouse model are strongly enhanced in completely NER-deficient XPA/CSB double mutant mice (van der Horst, unpublished data). Considering the involvement of CSB with transcription, this implies that also these CS features are due to lesion-induced transcription deficiency. In this rationale, transcription deficiency may also underlie certain

symptoms in normal aging. Oxidative damages have previously been implicated in aging and the "oxidative damage" theory of aging complies very well with our postulated "transcription capacity" theory of aging. Possibly, the transcription blocking rather than the mutagenic potential of oxidative lesions determines the rate of the aging process. This is in particular reasonable for the non-dividing, post-mitotic cells of aging organisms, because mutation fixation mainly occurs upon DNA replication. To test this model, we would like to know the nature of the lesion(s) and the effect of these types of DNA damage on transcription in TTD cells. Once the causative lesion(s) has been identified, it should be possible to modify the severity of symptoms in the TTD mouse by either increasing or attenuating the genotoxic pressure *in vivo*, as we did already by genetically modifying the repair capacity. Testing of lesion-scavenging chemicals in the TTD mouse may even have therapeutic implications, because they may also alleviate the severe, life-threatening growth retardation of TTD patients.

SAMENVATTING VOOR NIET-BIOLOGEN

Dit proefschrift beschrijft hoe we, met behulp van genetisch veranderde muizenstammen, fundamentele processen als kanker en veroudering hebben bestudeerd. Het eerste deel van de samenvatting bevat achtergrondinformatie, die nodig is om het praktische werk te begrijpen dat beschreven is in het tweede deel van de samenvatting.

DE GENETISCHE CODE

Elk levend wezen is opgebouwd uit cellen. Er zijn 1-cellige organismen zoals bakkersgist, terwijl een mens opgebouwd is uit miljarden cellen. Elke cel heeft een apart compartiment, de kern genaamd, waarin de volledige set aan blauwdrukken ligt voor de ontwikkeling van een organisme vanuit een bevruchte eicel, voor het functioneren van een organisme en ook voor de voortplanting. De blauwdrukken liggen er in de vorm van een vier-letterige code, in strengen van DNA moleculen. De kern is het makkelijkst te bevatten als een enorme bibliotheek. In de bibliotheek staan boeken (de genen), en elk gen codeert voor een bepaald eiwit. Er zijn naar schatting enkele tienduizenden verschillende eiwitten en sommige zijn essentieel als bouwstenen van de cel, terwijl andere betrokken zijn bij biologische processen *in* de cel. Het proces waarbij de DNA code vertaald wordt (van gen naar eiwit) gebeurt in twee stappen. Eerst wordt in de kern de DNA code afgeschreven (er wordt als het ware een copie van het boek gemaakt) waarna de copie buiten de kern wordt vertaald naar een eiwit. Elk celtype (zoals huidcellen, rode bloedcellen, geslachtscellen) schrijft een selectie van alle genen af, wat de cel zijn specifieke eigenschappen geeft. Een voorbeeld hiervan is het β-globine gen dat codeert voor een eiwit dat zuurstof kan binden en alleen in rode bloedcellen wordt afgeschreven en vertaald.

DNA SCHADES EN HUN GEVOLGEN

Net zoals een boek langzaam in kwaliteit achteruit gaat als het vaak gebruikt wordt, en zelfs als het niet gebruikt wordt, zo is DNA aan slijtage onderhevig. Zonlicht, stoffen in sigarettenrook maar ook schadelijke stoffen die vrij komen bij chemische processen in de cel beschadigen het DNA wat ondermeer kan leiden tot het verhinderen van het afleesproces, de celdeling en tot veranderingen in de DNA code (mutaties). Mutaties kunnen de eigenschappen van eiwitten veranderen zodat ze onwerkzaam of juist overactief worden, wat ziekte tot gevolg kan hebben. Als de verkeerde code wordt doorgegeven aan het nageslacht via de geslachtscellen, spreekt men van een erfelijke ziekte. Een voorbeeld hiervan is een mutatie van het

β-globine gen dat codeert voor een gammel eiwit waardoor het normale zuurstoftransport verstoord wordt. Deze ziekte staat bekend als sikkelcel anemie. Ook veel vormen van kanker onstaan door mutaties in celdeling-regulerende eiwitten waardoor cellen ongecontroleerd kunnen gaan groeien. Bij het proces van veroudering bestaat het vermoeden dat schade die ontstaat door stoffen, die vrijkomen bij chemische processen in de cel zelf, een rol speelt. Het is niet helemaal duidelijk of dit komt doordat deze schades mutaties veroorzaken, of doordat ze bepaalde processen in de cel verstoren, maar een feit is dat het opruimen van DNA schades van levensbelang is. Elke cel heeft daarom een aantal reparatiemechanismen, waarvan elk in staat is om een aantal van de vele verschillende soorten DNA schades te herstellen. Zonlicht-schade wordt verwijderd door het nucleotide excisie herstel (NEH) mechanisme waarbij een tiental eiwitten samen werken om de schades op te sporen, en ze uit het DNA weg te knippen om zo de genetische code in stand houden. Het XPD eiwit is één van de componenten van het NEH en dit proefschrift gaat over de gevolgen van een defect in het XPD eiwit.

TWEE ZIEKTEN, TWEE FUNCTIES

Xeroderma pigmentosum is een erfelijke ziekte waarbij de patienten een defect hebben in het NEH met als belangrijkste kenmerk dat ze zeer gevoelig zijn voor zonlicht en een sterk verhoogde kans op huidkanker hebben. Een deel van de patienten heeft een mutatie in het *XPD* gen.

Een tweede ziekte waarbij mutaties in het XPD eiwit een NEH defect en zonlicht gevoeligheid veroorzaken is trichothiodystrophy (TTD). TTD patienten zijn bijzonder omdat ze ondanks het herstel defect geen verhoogde kans op huidkanker hebben. Minstens zo wonderbaarlijk is het feit dat ze, naast gevoeligheid voor zonlicht ook een aantal bijzondere groeiafwijkingen hebben, waaronder breekbare haren en nagels, dwerggroei, botafwijkingen en problemen met het zenuwstelsel. Deze typische TTD symptomen zijn niet te verklaren als een gevolg van een defect in schade herstel. Een verklaring wordt gezocht in het feit dat XPD behalve voor NEH ook nodig is voor het aflezen van de genetische code (de copïeer stap). Als het XPD eiwit dus niet goed functioneert kan dat beide processen beïnvloeden en in het geval van een afleesproces zou dat de aanmaak van bepaalde eiwitten in b.v. het zenuwstelsel of in de haren kunnen verstoren. Onze hypothese is dat in XP patienten, het XPD eiwit dusdanig gemuteerd is dat alleen NEH is aangedaan. Anderzijds zou TTD verklaart kunnen worden door een mutatie die XPD enerzijds beïnvloedt in de NEH functie maar daarnaast ook gedeeltelijk het aflezen van de code inactiveert. Gedeeltelijk, want het aflezen van de genetische code is zo belangrijk voor het functioneren van de cel, dat een volledig defect waarschijnlijk niet levensvatbaar is. Het onderzoek in dit proefschrift gaat enerzijds in op de tegenstrijdige afwezigheid van huid kanker in TTD patienten en anderzijds op de vermeende rol van een defect in het afschrijfmechanisme in het ontstaan van de

typische TTD symptomen als breekbare haren en dwerggroei. Als onderzoeksmodel is er voor gekozen om de ziekten na te bootsen in de muis door het muizen *XPD* gen te veranderen.

HET MAKEN VAN EEN GENETISCH VERANDERDE MUIZENSTAM

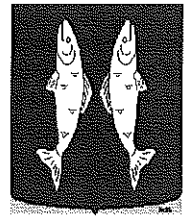
Het aanbrengen van genetische veranderingen in de muis gebeurt met de embryonale stamceltechniek. Enerzijds is er DNA van het muizengen nodig dat je wilt inactiveren of veranderen en anderzijds heb je embryonale stam (ES) cellen nodig. In een reageerbuis wordt het DNA van het gewenste gen veranderd waarna het in ES cellen wordt ingebracht. Via een moleculair biologische truc kunnen cellen geselecteerd worden, waarbij het ingebrachte DNA het gen in de cel als het ware opzoekt en uitschakelt. Het unieke van embryonale stamcellen is dat ze, in tegenstelling tot andere gekweekte cellen (b.v. huidcellen of kraakbeencellen), nog alle celtypen kunnen vormen. Ze zijn uit 3,5 dagen oude embryos geïsoleerd waarbij nog geen keuze is gemaakt welke cel welk gedeelte van het lichaam gaat vormen. De genetisch veranderde ES cellen worden met een glazen naald in een normaal 3,5 dag oud embryo ingebracht en hier onstaat een chimere muis die gedeeltelijk uit normale cellen bestaat en gedeeltelijk uit cellen die afkomstig zijn van de genetisch veranderde ES cellen. Als de ES cellen hebben bijgedragen aan de geslachtscellen zullen de nakomelingen voor 100% de genetische verandering hebben, en is er sprake van een genetisch veranderde muizenstam.

DIT PROEFSCHRIFT

Hoofdstuk 1 is een algemene inleiding over het mechanisme van NEH en over de dubbele functie van het XPD eiwit. Verder worden de erfelijke ziektes beschreven die veroorzaakt worden door mutaties in eiwitten die betrokken zijn bij NEH. Hoofdstuk 2 is een literatuursamenvatting van experimenten waarbij gebruik is gemaakt van muizenstammen met genetische veranderingen in NEH genen. Met behulp van deze muizenstammen kunnen bepaalde onderzoeksvraagstellingen beantwoord worden die niet in patienten of met gekweekte cellen van patienten onderzocht kunnen worden. In Hoofdstuk 3 beschrijven we het isoleren van het muizen XPD gen en het via de ES-cel techniek volledig inactiveren van het XPD gen. Zoals verwacht is dit niet levensvatbaar vanwege de rol van XPD in het afschrijfproces. Om toch een levensvatbaar muizenmodel te verkrijgen hebben we het subtiele genetische defect van een TTD patient nagebootst in het muizen XPD gen. In Hoofdstuk 4 beschrijven we hoe deze muizenstam via een aanpassing van de ES cel techniek hebben ontwikkeld. TTD muizen vertonen identieke symptomen als de patient waaronder dwergroei, onvruchtbaarheid van de vrouwtjes, en breekbare haren. De huid vertoont schilfering en moleculair biologische analyse wijst er op dat dit mogelijk door een afleesdefect veroorzaakt kon worden.

In Hoofdstuk 5 worden experimenten beschreven om de gevoeligheid van TTD huid en gekweekte TTD cellen voor UV-licht en andere DNA beschadigende stoffen te onderzoeken. Het blijkt dat TTD muizen een gedeeltelijk schade herstel defect hebben en maar matig gevoelig zijn voor DNA beschadigende stoffen. Desondanks, en in tegenstelling tot patienten, krijgen TTD muizen eerder huidkanker dan normale muizen maar niet zo snel als muizen met een volledig NEH defect. Dit toont aan dat TTD patienten in principe ook gevoelig zijn voor huidkanker die veroorzaakt wordt door zonlicht maar dat het herstel defect van XP en TTD patienten wel van elkaar verschilt. Hoofdstuk 6 beschrijft in meer detail een aantal aspecten van de TTD muis die overeenkomsten vertonen met verouderingsverschijnselen. TTD muizen sterven vroegtijdig en het lichaamsgewicht van oudere TTD muizen neemt af. TTD muizen vertonen een aantal aspecten van verhongering en sterven door aftakeling van de fysieke toestand. Daarnaast worden TTD muizen grijs, ontwikkelen botafwijkingen en zijn vrouwtjes verminderd vruchtbaar. Vanwege deze verschijnselen zijn TTD muizen een waardevol model om te onderzoeken wat de relatie tussen NEH, het afschrijfproces en het proces van veroudering is. We hebben redenen om te vermoeden dat DNA schades die ontstaan door schadelijke stoffen in de cel zelf, op nog onbekende wijze het afschrijfproces in TTD muizen verstoren, wat dan leidt tot de TTD symptomen die zo sterk lijken op veroudering in de mens. Het is niet uitgesloten dat die schades bij normale veroudering ook het afleesproces verstoren. TTD muizen zijn een interessant nieuw proefdiermodel voor onderzoek naar het moleculair-biologische mechanisme van veroudering.

CURRICULUM VITAE



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