Genome Caretaking and Differentiation

Genoomonderhoud en differentiatie

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Hoe talrijk zijn uw werken, Heer.
Alles hebt u met wijsheid gemaakt,
vol van uw schepselen is de aarde.

Psalm 104:24 (NBV)

Voor Anita

Contents

Chapter 1

Genome stability and genome caretaking mechanisms

Manuscript in preparation

Genome stability and genome caretaking mechanisms

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1.1 Genome instability

DNA contains the genetic information for the proper functioning of all cell types that make up an organism. Although this information should be error-free passed from generation to generation, DNA is prone to deterioration and modifications, originating from environmental and endogenous produced physical and chemical agents. For example, the UV-component of sunlight causes formation of helix distorting cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone adducts (6-4PPs). Ionizing radiation can cause single and double strand breaks in the DNA, and in addition intracellular reactive oxygen species (ROS) that subsequently can induce oxidative DNA lesions. Similarly, chemotherapeutics (e.g. cis-platinum and mitomycin C) and other environmental chemical agents (as present in inhaled smoke or polluted air) underlie a plethora of different DNA lesions, including oxidative DNA base damages, intra- and inter-strand cross-links, as well as monoadducts. Importantly, also endogenous chemicals and physical agents cause a wide variety of DNA lesions. Metabolic processes will lead to reactive oxygen species in the cell, which will react with proteins, lipids, but also with DNA, and as such underlying a broad spectrum of oxidative DNA lesions, including 8-oxo-2'-deoxyguanosine (8-oxodG), thymine glycols, cyclopurines, etc, as well as single and double strand breaks (31). Finally, spontaneous hydrolysis or modifications of nucleotides is common in cells, which leave non-informative a-basic sites or altered, miscoding nucleotides (106).

1.1.1 Consequences of genome instability

An immediate effect of DNA lesions is interference with transcription and replication (100, 118), causing cellular dysfunctioning and leading to a cell cycle arrest or programmed cell death (apoptosis). In addition, persistent DNA damage can be misinterpreted by the replication machinery, which results in the induction of mutations. These mutations, as well as other genetic changes resulting from chromosome instability and mis-segregation (rearrangements, deletions, insertions, and numerical aberrations), can on the long term result in cancer and inborn diseases. Moreover, cellular dysfunctioning, depletion of proliferative capacity of cells by senescence (a permanent cell cycle arrest) and apoptosis can cause aging (reviewed by (77)).

1.1.2 Cellular defense against genome instability

To counteract the deleterious effects of DNA damage, the cell is equipped with a wide variety of genome caretaking mechanisms. Various DNA repair machineries, with partially overlapping substrate specificity, are capable of repairing DNA damage (Figure 1, reviewed by (67, 81)). Known repair processes are nucleotide excision repair (NER), base excision repair (BER), direct damage reversal, double strand break repair, cross-link repair and mismatch repair. The specificity and function of these repair processes is discussed below. To provide cells with an extended time window for repair, cells are able to transiently block cell cycle progression (reviewed in (16)). When repair fails, cells may abort their proliferative capacity by executing a permanent cell cycle block (senescence) (32) or apoptosis (19). Cells lost via apoptosis might be replaced by progenitors. In time, this may lead to a situation where the regenerative capacity of that particular tissue may be exhausted. This phenomenon probably underlies the process of aging (reviewed by (77, 131)). Therefore,

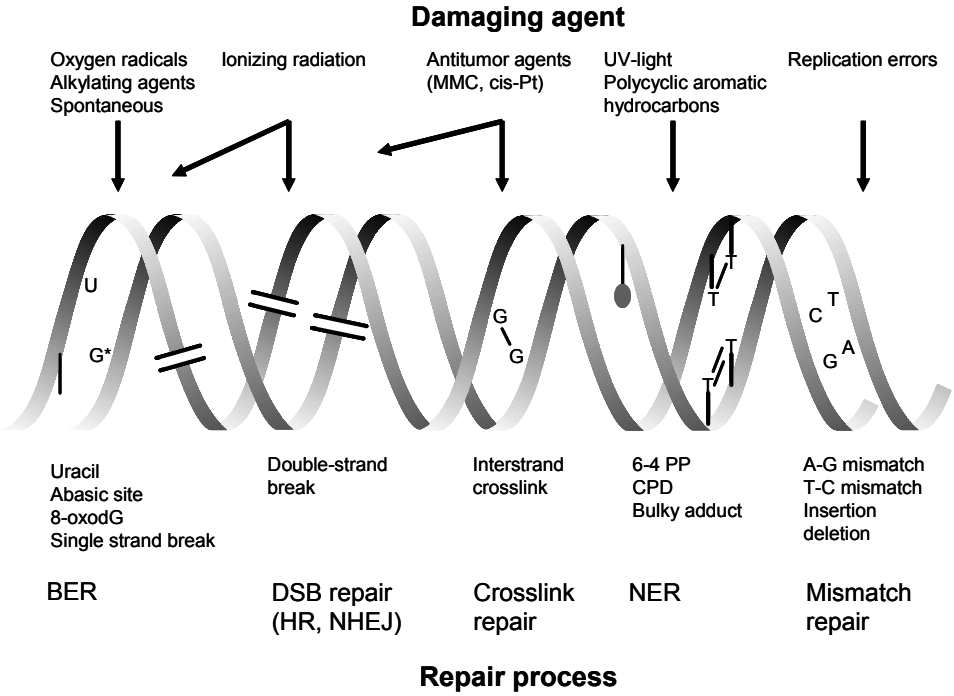

Figure 1
DNA lesions and repair mechanisms
At the top of the figure, examples of common DNA damaging agents are depicted. As indicated by the arrows, it is important to realize that many DNA damage inducing agents rather than inducing one specific type of lesion, produce a spectrum of different (classes of) lesions. Oppositely, different DNA damaging agents can cause similar DNA lesions. The middle part of the figure shows the DNA helix, with several DNA lesions, as depicted under the figure. The lower part of the figure shows the various repair pathways that cells use to remove these lesions. Note that these pathways overlap in damage spectrum (adapted from (44)).

apoptosis might not always be desirable and cells with persisting DNA damage, instead of executing apoptosis, may bypass DNA damage during replication at the risk of mutation induction. The various repair pathways, apoptosis, cell cycle regulation, damage bypass and their relationship will be discussed in more detail in the following paragraphs.

1.2 Repair mechanisms

1.2.1 Nucleotide excision repair

Nucleotide excision repair is a versatile DNA repair mechanism, capable of removing numerous types of helix-distorting lesions, like UV induced photoproducts. Other substrates for NER include reactive oxygen species (ROS) induced 5',8-purine cyclodeoxynucleotides (28, 93) and bulky lesions, which could for example be caused by polycylclic aromatic hydrocarbons (as present in tobacco smoke and air pollution). NER functions by a "cut and patch"-like mechanism, in which damage recognition, local opening of the DNA helix around the lesion, damage excision and gap-filling are the successive steps (Figure 2, reviewed by (1, 46, 81)). NER is composed of two subpathways that differ in the way lesions are recognized: global genome NER (GG-NER) and transcription-coupled NER (TC-NER).

1.2.2 Global genome repair

The first step in GG-NER is damage recognition by the heterodimer XPC/hHR23B/Cen2 (9, 170, 197), which binds with higher affinity to helix-distorting DNA lesions than to non-damaged double stranded DNA (dsDNA) (151, 171). Since damage recognition is highly dependent on the degree of DNA helix distortion, DNA lesions that only mildly disturb the helical structure are poorly recognized by XPC/hHR23B and as a consequence are inefficiently repaired by GG-NER. One such lesion is the UV-induced CPD. GG-NER of this photolesion is greatly enhanced by the damaged DNA binding complex (DDB) (82, 173, 175). The DDB complex is composed of the DDB1 (damaged DNA binding protein 1, p125) and DDB2 protein (damaged DNA binding protein 2, p48, XPE). In rodents, in contrast to other mammals, expression of the p48 subunit of DDB is not up regulated upon UV, probably due to lack of a p53 responsive element in the *p48* promoter (173). Therefore, rodents poorly repair CPDs by GG-NER. Similarly, XPC is up regulated by UV in a p53-dependent manner, as determined both on the RNA and the protein level (3, 7). Thus the damage recognition step is likely the rate-limiting factor in the GG-NER reaction.
Subsequent to damage recognition, the multisubunit transcription factor TFIIH and the structure-specific endonuclease XPG are recruited to the lesion (197, 207). TFIIH contains the XPB and XPD proteins that act as 3'-5' and 5'-3' helicases, respectively, and function in local unwinding of the DNA around the lesion (55, 64, 156, 157, 203). Initial stability of the open structure is guaranteed by the presence of the XPG protein (64, 132). After verification of the damage by the XPA protein (197), this open structure is further stabilized

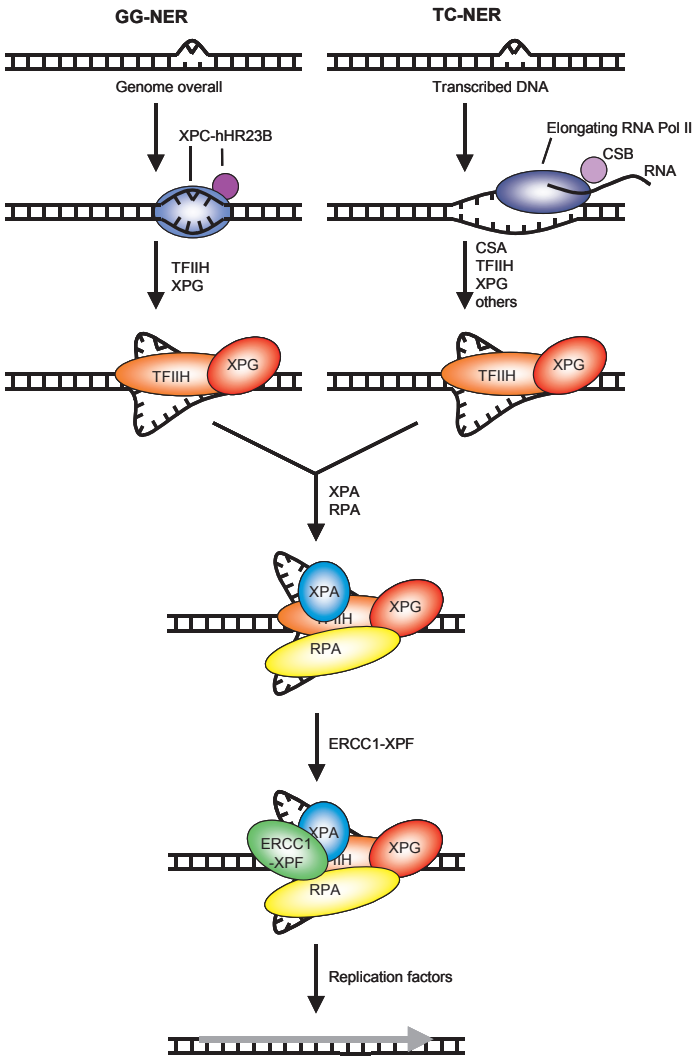

Figure 2
Mechanism of nucleotide excision repair
This figure shows the principle of nucleotide excision repair (NER) and its two subpathways, global genome NER (GG-NER) and transcription-coupled NER (TC-NER). In GG-NER, the XPC/hHR23B protein complex recognizes the helix distorting lesion. In contrast, TC-NER is initiated when RNA polymerase II is stalled upon a lesion, a step that requires the function of the CSA and CSB protein. After damage recognition, GG-NER and TC-NER both use the XPB and XPD helicases from the TFIIH complex to unwind the DNA around the lesion. The initial open complex is stabilized by XPG. Next, XPA verifies the lesion and RPA stabilizes the open intermediate by binding single stranded DNA. The structure specific endonucleases ERCC1/XPF and XPG cleave 5' and 3' of the lesion, respectively. The resulting 24-32 nucleotide fragment, containing the lesion, is subsequently removed and the remaining single strand gap is filled in by the regular replication machinery (adapted from (81)).

by XPA and replication protein A (RPA) (45, 64, 105, 168). Next, the endonucleases XPG and ERCC1/XPF cleave 3' and 5' of the lesion, respectively, thereby excising a 24-32 nt single stranded DNA (ssDNA) fragment containing the DNA damage (132, 137, 163). Using the undamaged strand as a template, filling of the ssDNA gap is performed by the regular DNA replication machinery, consisting of RPA, proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and DNA polymerase δ and ε (1, 30, 162). Finally, the resulting nick is sealed by DNA ligase I (10, 15).

1.2.3 Transcription-coupled repair

Damage in the transcribed strand of active genes can arrest the transcription machinery, which can cause a temporary block of RNA synthesis. This particularly holds for lesions that are slowly (or even not) repaired by GG-NER. To quickly remove damage from the transcribed strand of active genes and to resume transcription, the cell is equipped with the transcription-coupled NER system (22, 99, 126, 127). This mechanism is initiated by stalling of an elongating RNA polymerase II upon a lesion (181). TFIIH and XPG are recruited to the site of damage and as in GG-NER, XPA is needed for the damage verification. The remaining repair reaction is executed by the same mechanism, used by GG-NER. The cross-talk between a blocked RNA polymerase and the actual repair reaction, as well as the way in which the damage is made accessible, is still matter of debate. However, it is clear that somehow *CSA*- (78), *CSB*- (182) and *XAB2*-gene products (133) are involved in these steps. It has been shown that cell lines mutated for the *CSA* or the *CSB* gene or with an inactivated XAB2 protein are deficient in performing TC-NER. As a consequence, these cells fail to recover RNA synthesis after induction of UV damage, which indicates that the transcription block is not released (118, 133).
Since damage recognition in TC-NER does not depend on helix distortion, but instead on blockage of RNA polymerase II, the spectrum of lesions recognized by TC-NER and GG-NER differs. For example, whereas CPDs are poorly (and in rodents almost not at all) recognized and repaired by GG-NER, these lesions efficiently block transcription and accordingly are efficiently repaired by TC-NER (22). Moreover, also some lesions that were previously thought to be repaired by base excision repair only, might cause a (transient) block of the transcription machinery and might therefore be repaired in a transcription-coupled manner. Evidence for transcription-coupled repair of BER lesions is given by the slight hypersensitivity of human *CSA* and *CSB* fibroblasts for ionizing radiation (98) and by deficient repair of 8-oxodG in the transcribed strand of a gene, introduced to the cell by transfection (97). Although TCR of oxidative DNA lesions has been suggested, these lesions do not very efficiently block RNA polymerase. It is possible that TCR is needed for the subset of oxidative DNA lesion, which efficiently block RNA polymerase, or that a transient pausing of the RNA polymerase on the lesion is already sufficient to recruit the repair machinery. Whether, damage recognition of oxidative lesions is followed by repair via NER or BER is unknown. We will refer to TC-NER when clearly the transcription-coupled repair pathway of NER is meant and to TCR in cases where it is not per sé known whether NER or BER is involved.

1.2.4 Other repair mechanism

Base excision repair

Base excision repair (BER) is capable of removing a wide variety of nucleotide modifications, which are mostly not helix-distorting (reviewed by (66, 91)). This repair mechanism is considered as the main guardian against DNA lesions caused by cellular metabolism, including base adducts resulting from ROS, methylation, deamination and hydroxylation. The BER reaction starts by recognition of the lesion by a battery of glycosylases with overlapping lesion specificity. These glycosylases act by flipping out the damaged base of the helix and subsequent cleavage of this base from the DNA sugar backbone, leaving an abasic site (54). These abasic sites can also arise spontaneously in the DNA by hydrolysis (106). Next, these abasic sites are converted into a single strand break by the action of either endonuclease APE1 (48), or by the intrinsic apurinc/apyrimidinic (AP) lyase activity that certain glycosylases posses (154). These breaks might as well occur from other sources, like γ-ray irradiation, and funnel into the BER reaction. Probably poly(ADP-ribose) polymerase (PARP) and polynucleotide kinase (PNK) are needed to protect these single strand nicks and to trim the ends for repair synthesis (202).
The nicked DNA is further processed by either short-patch or long-patch repair. During short-patch repair, the dominant repair mechanism in mammals, DNA polymerase β (DNApolβ) performs a one-nucleotide gap-filling reaction and removes the 5'-terminal baseless sugar via its lyase activity (116, 166). Finally, the XRCC1-ligase3 complex seals the remaining nick (33). Long patch DNA repair involves DNApolβ, polδ/ε, PCNA and replication factor-C (RFC) for repair synthesis of a 2-10 nt patch, thereby replacing a small DNA flap. This flap is cleaved of by the FEN1 endonuclease and finally the nick is sealed by DNA ligase I (89, 117, 140, 143).

Mismatch repair

Erroneous base incorporation, as well as slippage of DNA polymerases during replication and recombination can cause single base-base mismatches and small insertion/deletion loops. Such lesions are dealt with by the mismatch repair (MMR) pathway, which is capable of repairing both classes of mismatches, hereby preventing the accumulation of these mutagenic lesions (reviewed by (113)).
Damage recognition is performed by the hMutSα en hMutSβ complexes. hMutSα (hMSH2-hMSH6 heterodimer) recognizes base-base mismatches, 1 nucleotide insertion/deletion loops and 2-8 nucleotide insertion/deletion loops. In contrast, the less abundant present hMutSβ (hMSH2-hMSH3 heterodimer) only recognizes 2-8 nt insertion/deletion loops (72). Subsequently, MLH1-PMS2 (or to a lesser extent the MLH1-PMS1, or MLH1-MLH3) binds to these MutS-DNA complexes. The misinserted base(s) is distinguished from the original template and removed by exonuclease I (71). The eliminated strand is resynthesized by polδ, using the original DNA template (110).

Direct damage reversal

In contrast to genome care-taking systems that remove damage via a "cut and patch"-like mechanism (i.e. NER, BER and MMR), cells can also possess repair enzymes that directly revert specific DNA lesions to the undamaged bases. For example, alkyltransferases repair methylated nucleotides (like the highly mutagenic and cytotoxic O^6-methylguanine) by transferring the methyl group of a damaged base to an internal cysteine. As a consequence of this reaction, the protein irreversible inactivates itself, showing an example of the use of one entire protein to repair only one DNA lesion (reviewed by (141)).
Another example of direct damage reversal is given by photoreactivation, an enzymatic reaction in which photolyases cleave the UV-induced bond between two adjacent pyrimidines, using visible light as an energy source. Since damage recognition and repair is highly substrate specific for these enzymes, both CPD- and 6-4PP-photolyases exists. Photoreactivation occurs in prokaryotes as well as in eukaryotes, but has not been observed in placental mammals (206).

Double strand break repair

Double strand breaks (DSBs) arise from ionizing radiation, free radicals and chemicals. In addition they are formed during replication of single strand breaks. To repair these deleterious lesions, the cell is equipped with two different repair mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) (reviewed by (187, 189)).
Homologous recombination uses the homologous sequences of the sister chromatids or homologous chromosomes to precisely and error-free repair the DSB. Contrarily, NHEJ simply links two DNA ends together with little or no use of homologous sequences present in these ends. Use of microhomology close to the DNA ends may result in loss of small pieces of DNA. These deletions, as well as processing of the DNA ends, cause NHEJ to be an error-prone repair pathway. NHEJ is probably employed when sister chromatids are not present or when homologous recombination between chromosomes is undesirable because of the risk of loss of heterozygosity.

Cross-link repair

Cross-link repair, is capable of removing inter-strand cross-links, but of all repair process studied, is the poorest understood. This is probably due to the fact that it likely depends on many factors involved in other repair processes and apparently is the result of a combination of interwoven repair pathways (reviewed by (57)).
While in yeast NER enzymes generate most of the incisions during interstrand cross-link repair, their function is less clear in mammals. Thus far, only mutant cell lines with defects in the NER endonuclease ERCC1/XPF are known to display severe cross-link sensitivity (40). Whether this sensitivity can be attributed to an endonucleolitic incision adjacent to the cross-link, or by another function of the ERCC1/XPF complex later in the reaction is still not clear. Although it is not completely sure whether one of the first repair intermediates in repair in removal of cross-links is a double strand break, homologous recombination

proteins play an important role in the repair reaction, as illustrated by the severe cross-link sensitivity of cells with defective *Rad51, Rad54, BRCA1* or *BRCA2* genes (20, 40, 63, 172, 209). Some reports suggest that translesion repair might be an alternative pathway in cross-link repair (129, 211). Defects in other factors with mainly unknown function, like SNM1 and FA genes (mutated in Fanconi anemia) also cause sensitivity to cross-linking agents (29, 56). Therefore, these factors at least perform a function in the cellular cross-link response, or even may be directly involved in the cross-link repair reaction.

1.3 Repair-related disorders

1.3.1 Diseases associated with deficiencies in genome caretaking processes

The importance of DNA repair and other vital genome caretaking processes is best demonstrated by a variety of rare autosomal recessive disorders. Most of the DNA repair associated diseases show an elevated cancer risk, underscoring the prime function of DNA repair in preventing cancer.
For instance, mutations in mismatch repair genes are known to cause hereditary non-polyposis colorectal cancer (HNPCC) (142). In the case of Fanconi anemia, a severe hypersensitivity for cross-link producing agents is observed. Clinical symptoms of Fanconi anemia include progressive bone marrow failure, developmental abnormalities, growth retardation and a predisposition to cancer (11). Failure to respond to double strand breaks underlie ataxia telangiectasia (mutations in ATM, a key player in cellular response to DSBs; (152, 155)), ataxia telangiectasia-like disorder (mutations in Mre11; (167)) and Nijmegen breakage syndrome (mutations in NBS1; (53)). All three DSB-repair related disorders display cancer predisposition, hypersensitivity to ionizing radiation and chromosomal instability (179).

Table 1: Main clinical symptoms of XP, CS and TTD

Clinical Symptoms	XP	CS	TTD
Photosensitivity	++	+	+
Abnormal pigmentation	++	-	-
Skin cancer	++	-	-
Progressive mental degeneration	-/+ *	+	+
Neuronal loss	-/+ *	-	-
Neurodysmyelination	-	+	+
Bird-like face	-	+	+
Growth defect	+/- *	+	+
Hypogonadism	-/+	+	+
Brittle hair and nails	-	-	+
Ichthyosis	-	-	+

* These neurological and growth defects are characteristic features of XP patients with the DeSanctis-Cacchione syndrome
(Adapted from (24))

Diseases specifically associated with mutations in BER enzymes have only recently been described. Patients with a form of autosomal recessive adenomatous polyposis were shown to carry biallelic mutations in MYH (a glycosylase) (6, 37).
Mutations in genes involved in NER underlie three different disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (reviewed by (24, 44)). Clinical characteristics of XP, CS and TTD are summarized in table 1. Specific features of these syndromes will be discussed in more detail in the following paragraphs. Interestingly, mutations in the *XPV* gene, coding for translesion polymerase η, underlie the cancer prone variant form of xeroderma pigmentosum (85, 114). Since *XPV* is caused by a defect in translesion synthesis rather than in NER, we further consider XPV as a separate disease. Therefore in the following paragraphs we refer to XP as the NER deficient type of XP.

1.3.2 Xeroderma pigmentosum

Xeroderma pigmentosum can be considered as the "classical" NER-disorder. Although symptoms are heterogeneous in occurrence as well as in severity amongst the different XP patients, the diagnostic features of XP are a dry scaly skin (xeroderma), abnormal pigmentation in sun-exposed skin-areas (pigmentosum), photosensitivity, and a 1000 fold increased risk of developing UV-induced skin cancer. Besides this skin cancer predisposition, a 10-20 fold increased risk of developing several types of internal cancers before the age of 20 has been described (24).
Complementation studies have shown the involvement of 7 genes in XP (*XPA* through *XPG*). While XPC and XPE are specifically deficient in the global genome repair pathway (82, 175, 195, 196), the other five complementation groups display defects in both subpathways. The generally mild XP features (except for the cancer risk) in XPC and XPE patients can be explained by the fact that the TC-NER pathway in these complementation groups is still functional. Moreover, many mutations in *XP* genes do not cause a complete inactivation of proteins, thereby providing the cell with residual repair capacity. This probably causes the milder forms of XP (reviewed by (24)).
Most XP patients develop almost normally but die of neoplasia, which reduces their average life span by approximately 30 years (90). A fraction of XP patients (18 %) display accelerated mental retardation, likely caused by enhanced neuronal degeneration due to loss of neurons throughout the brain, spinal cord and peripheral nervous system. Symptoms associated with XP neurological disease include peripheral neuropathy, sensorineural deafness, and loss of reflexes, followed by ataxia, EEG chances, and dementia. Neurological symptoms only occur in XP patients belonging to complementation groups A, C and D and are associated with a severe or total loss of repair capacity ((150), reviewed by (27)). The most severe form of XP is seen in patients displaying the clinical spectrum of DeSanctis-Cachione syndrome, with symptoms including immature sexual development, growth retardation, mental retardation, microcephaly and sensorineural deafness (reviewed by (24)).

1.3.3 Cockayne Syndrome

Similar to XP, Cockayne syndrome (CS) is characterized by photosensitivity of the skin. Somewhat surprisingly for a DNA repair disorder, CS is not associated with an increased skin cancer risk. This low cancer predisposition is probably associated with functional GG-NER in CS cells and an increased apoptosis rate, that likely removes premutagenic cells. In contrast to classical XP, Cockayne syndrome attributes pleiotropic features with physical and mental retardation (reviewed by (24, 134)). In general, CS patients display skeletal abnormalities like kyphosis, bird-like face, and in older patients osteoporosis. Other CS characteristics include impaired sexual development, caries, cachexia, progressive neurological degeneration, as evidenced by delayed psychomotor development, sensorineural hearing loss and mental retardation. These neurological symptoms might be associated with microcephaly, calcification of basal ganglia of the brain and progressive "patchy" neurodysmyelination. Other symptoms associated with Cockayne syndrome are pigmentary retinopathy, thin hair and cataracts. The mean age of death is 12.5 years, mainly caused by respiratory infections, resulting from an overall poor condition of patients. Many of these symptoms classify CS as a progeroid disease.
CS, like XP, is a heterogeneous disease and several forms have been described: mild CS, classical CS (CS I) and severe CS (CS II). As a hallmark of CS, cellular studies must show impaired UV survival and lack of RNA synthesis recovery. Patients with mild CS have a late onset of symptoms, and in some cases show normal growth, intelligence and reproductive capacity. In addition to the cellular phenotype, classical CS is diagnosed by the presence of at least two of the following characteristics: growth failure, neurodevelopmental problems, photosensitivity of the skin, pigmentary retinopathy and cataracts, sensorineural hearing loss, dental caries and cachectic dwarfism. Finally, CS II patients are characterized by a more severe and early onset of symptoms, causing a mean age of death around 6-7 years (134).
Complementation studies revealed the involvement of two genes in the onset of CS, *CSA* and *CSB* (174). A correlation between complementation group and severity of CS has not been observed. A subset of mutations in *XPB*, *XPD* and *XPG* can lead to the combined phenotype of XP and CS. In contrast to classical CS-patients these patients are cancer prone (24).

1.3.4 Trichothiodystrophy

The hallmark of TTD is the occurrence of brittle hair that is dry, sparse and easily broken, as a result of the absence of sulfur-rich proteins (145). Many, but not all TTD patients, also present a scaly skin, known as ichtyosis. In addition to these specific TTD characteristics, TTD shares many features with CS, and is thereby categorized as a premature aging syndrome. Although most TTD patients are photosensitive, few cases of non-photosensitive TTD have been reported. TTD exhibits a pattern of mental retardation, including: low IQ, spasticity, hyperreflexia, tremor and ataxia. Microcephaly and hypomyelination of the cerebellar white matter have been shown, as well as growth retardation and cachexia. Also skeletal abnormalities, like a bird-like face, axial

osteosclerosis, peripheral osteoporosis and kyphosis have been described. Like CS, TTD is not associated with an increased cancer risk.
Genetically, TTD has been associated with mutations in XPB, XPD or TTDA. Hereby TTD is the third disease associated with XPB and XPD, clearly showing that specific mutations in these proteins can cause different diseases.

1.3.5 XP, CS and TTD; defective NER and what else?

As described in the previous paragraphs, XP, CS and TTD are associated with a NER-defect. Photosensitivity, as observed in all three syndromes, as well as the increased cancer risk and hyperpigmentation in XP, can easily be explained by a defect in repair of UV induced DNA damage. The absence of cancer in CS can be attributed to proficient global genome repair in CS. Also other symptoms of XP, CS and TTD could possibly be connected to a repair defect of endogenous occurring NER-lesions (like 5',8-purine cyclodeoxynucleotides). For example, the severity of neurological symptoms in XP patients correlates with the UV-sensitivity of fibroblasts derived from these patients (8). However, for many CS and TTD symptoms it is difficult to imagine a connection to defective NER and therefore these symptoms should probably be attributed to defects in other processes. For example, (partial) reduction in GG-NER- or TC-NER-activity can not explain the occurrence of specific TTD-characteristics, since these symptoms are not found in completely NER-deficient XPA patients. Since, mutations causing TTD are all found in components of the TFIIH complex, a link between defective transcription and the clinical onset of some TTD symptoms has been made (18, 23, 25, 43, 58).
Another example is the notion that CS patients attribute in general more severe symptoms than XP patients, while in contrast to mutations in *XP* genes, mutations in *CSA* or *CSB* are associated with a TC-NER defect only (191, 194). To explain the CS features, additional roles for the CS proteins - outside the context of NER - have been suggested. Some of these functions are discussed below.
CSB possesses chromatin remodeling activity and has affinity for histone tails (39). This activity can have a function in repair, but may also be required during transcription. Other indications that CSB might be involved in transcription are given by the interaction of CSB with TFIIH, and the fact that CSB is present in an RNA polymerase II-containing elongation complex (84, 176, 177, 190). Furthermore, CSB has been shown to function as a non-essential transcription elongation factor, required for bypass of pause-sites and of transcription of genes encoding structured RNAs (12, 51, 160, 208). Surprisingly, although CSA might as well interact with TFIIH (78), it does not reside in the same complex as CSB (190). A connection with transcription has not been shown for CSA, suggesting that although CSB might be involved in transcription, a transcription deficiency alone can not be held responsible for the onset of CS features.
In addition, as discussed above, indications have been obtained for involvement of CSA and CSB proteins in repair of non-NER lesions, which suggests a wider action spectrum of transcription-coupled repair than NER lesions only (47, 97, 98). Interestingly, also cell lines from XP/CS patients with mutations in the *XPB*, *XPD* and *XPG* genes show a transcription-coupled repair defect for oxidative lesions (42, 97). In addition, CSB cell lines

have been reported to display a reduction of global genome repair of oxidative DNA damage, which may also contribute to the higher ionizing radiation sensitivity of these cells (139, 201). Also in XPG-CS patients, the repair of oxidative lesions may be further compromised by an additional reduction in global genome base excision repair (BER) (52, 88, 201), contributing to the observed Cockayne syndrome phenotype. In chapter 2, 3 and 4 we further investigate the link between non-classical NER lesions and Cockayne syndrome in *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice and cells derived thereof.

1.4 When repair is incomplete

Besides these various repair machineries, the cell is equipped with other defense-mechanisms. To extend the time-window for repair, cells are able to arrest their cell cycle and thereby prevent induction of mutations during replication. However, when even this extra time is not enough to remove all lesions, or when conditions are encountered where rapid division is desired, cells can tolerate persisting DNA damage and continue replication. Since DNA polymerases are stalled by a variety of DNA lesions, specific damage tolerance mechanisms are needed for replication progression. However, these pathways can be error-prone and thereby cause mutations. Alternatively, heavily damaged cells can be removed by senescence or apoptosis, to prevent genome instability. These pathways might be used if extra time is not enough to attenuate the damage load or if the risk of inappropriate replication is undesirable. The drawback of this strategy could be that cells have to be replaced, which ultimately might lead to exhaustion of the regenerative capacity of that particular cell compartment. In scope of this thesis we will discuss damage tolerance during replication, and the relation between NER and cell cycle arrest or apoptosis.

1.4.1 Damage tolerance

In order to prevent replication blockage when repair is too slow or even fails, the cell is equipped with two main mechanisms to bypass the damage during replication: damage avoidance and translesion synthesis (TLS) (reviewed by (17, 68, 119)).
Damage avoidance is achieved by daughter strand gap repair or by DNA template switching. Daughter strand gap repair of newly synthesized DNA involves homologous recombination for repair of the gap that is formed as a result of dissociation and restart of the polymerase in the vicinity of the DNA lesion. During DNA template switching, the DNA polymerase transiently uses the newly synthesized DNA of the daughter chromatid to detour the DNA lesion. Since both mechanisms do not use the damaged template, these processes can be considered as error-free.
In marked contrast, translesion synthesis is a process in which a collection of specific low-fidelity translesion polymerases with a less stringent template usage (polζ-ϰ), put a nucleotide opposite the damage. TLS is initiated when the regular DNA polymerase gets stalled at a DNA lesion. Few bases after the damage, the normal processive DNA polymerase again takes over from the TLS polymerase. Since the chance of incorporation of a wrong nucleotide opposite the damaged base is rather high, these translesion polymerases can cause replication errors, which ultimately might cause cancer. However, since for

example polymerase η has a tendency to incorporate adenine opposite the damaged nucleotide, bypass of TT-photoproducts is error-free. The increased UV-induced cancer risk in XPV patients (which have mutations in polymerase η) forms an example of the protective activity of a translesion polymerase.

1.4.2 Cell cycle arrest

The cell is equipped with a set of checkpoints acting at different phases during the cell cycle. These checkpoints are meant to (1) prevent mitosis when the DNA is not yet fully replicated (G2/M checkpoint), (2) delay replication when DNA perturbations are signaled (intra-S-phase-checkpoint) and (3) prevent a premature start of replication (G1-checkpoint) (for recent reviews on cell cycle control see (2, 34, 161)).
The first step in the cell cycle control process is damage sensing, leading to activation of transducers. This, in turn, can activate effectors that evoke the cell cycle block. Central transducers in the DNA damage checkpoint response are the protein kinases ATM and ATR. Both proteins are members of the PI3-kinase related protein kinase family and are activated upon DNA damage induction. While ATM is mainly activated by ionizing radiation, ATR is activated by exposure of cells to UV and ionizing radiation. Upon activation, both proteins phosphorylate numerous other effectors as well as primary sensors. Integration of the different signals of this effector-network and modification of the ATM- and ATR-activity by feedback loops, drive the decisions whether a cell should arrest in a specific phase of the cell cycle. The substrate specificity for ATR and ATM differs, which underlies the different cell cycle response upon ATM or ATR activation (2, 161).
Although the core machinery of cell cycle control is known, it is less well understood how the DNA damage sensing process that leads to a cell cycle block, functions. Recently, it has been shown that ATR binds preferentially to UV damaged DNA (186), and that ATR -together with is partner ATRIP- is recruited to the site of damage by its interaction with RPA (214). RPA binds single stranded DNA, which in many repair pathways (e.g. NER) is an intermediate. Therefore, this mechanism could provide the cell with a single activity to sense different classes of DNA damage. Whether, NER activity is needed for checkpoint activation remains under debate. In S. cerevisiae, Zhang et al. showed that checkpoint signaling is independent of NER (210). However, others show that NER activity is indispensable for proper G1 and G2 checkpoint response upon UV (74). Also for mammalian cells, it is not clear whether NER activity acts as one of the pathways that cause a cell cycle block. Alternatively, sensing of DNA damage (and in particular for NER-type lesions) can be due to blockage of the transcription- or replication-machinery. It has been shown that UV light and other NER-type lesions block RNA polymerase II transcription (118, 159), and that the stalled RNA polymerase can initiate a cell cycle arrest in a p53 dependent or independent manner (21, 35, 73, 83, 204). When induced during the S-phase, UV lesions might block the replication machinery and cause a cell cycle arrest (192). In hamster cells, photoproducts have been shown the underlying cause of this S-phase delay (138). Since the cells in that study were p53-deficient, this response is probably p53 independent. In conclusion, upon UV treatment (and other genotoxic assaults) the cell is able to stop the cell cycle at different moments during the cell cycle.

1.4.3 Apoptosis

As mentioned in paragraph 1.4, it may happen that the damage load exceeds the repair capacity of a cell, even after a prolonged repair period provided by a cell cycle block. These cells form a risk factor for the organism since there is a chance that these cells no longer properly perform their function. Even more important, re-entering into the cell cycle when the damage is still present can cause mutations, which might lead to loss of growth control and, as a consequence, initiate carcinogenesis. Therefore, it is often beneficial for the organism to eliminate such heavily damaged cells, rather than maintaining them as a potential risk factor. Two distinct pathways of cell death exist: apoptosis and necrosis (reviewed by (96, 158)). Necrosis is a passive, traumatic fate of cell death in which ion pumps fail, the cell swells, and then undergoes lysis, which will lead to an inflammatory response. In contrast, apoptosis is a controlled form of cell death, characterized by cell shrinkage, dehydration, fragmentation of the nucleus and phagocytosis of cell remnants. The benefit of apoptosis over necrosis is the prevention of inflammation. In some cases however, the cell can just not perform apoptosis and is therefore eliminated by necrosis (101). For example, this occurs when the cellular energy level is too low to fuel the apoptosis process.

Apoptosis execution, like the mechanism of cell cycle arrest, depends on sensing of the DNA damage. UV irradiation causes elevated p53 levels (26, 204). Several reports show that indeed induction of apoptosis by UV irradiation is p53 dependent (102, 183, 213). In line with these observations, it has been shown that p53 deficient cells are less sensitive to UV-exposure than their p53 proficient counterparts (14, 65, 86). Contrarily, other investigators show UV induced apoptosis to be p53 independent (146, 183, 185) and do not observe an effect of a p53 deficiency on UV sensitivity (83). In sharp contrast to its role as apoptosis promoting factor, p53 has been reported to protect cells from UV-induced apoptosis (61, 94, 95, 120, 123, 200). A protective role of p53 against UV induced apoptosis is further illustrated by an increase in UV sensitivity in p53 deficient cell lines (61, 65, 94, 164, 200).

Probably, most of the differences in p53 dependence of UV-induced apoptosis can be attributed to the fact that different cell types as well as different p53 mutant models have been studied. For example, Tron et al show apoptosis in differentiated keratinocytes to be p53-dependent, while in undifferentiated keratinocytes p53 appears not to be required for apoptosis induction (183). However, mouse dermal fibroblasts have been reported to posses p53-dependent apoptosis on the one hand (102), and p53 protection against apoptosis on the other hand (94, 95). Strikingly, in the first study UV-B light was used to induce damage, while the latter two studies were performed with a UV-C source. Given the reported difference in p53 response upon UV-B and UV-C exposure (115), it could well be that the effect of p53 on apoptosis partly depends on the wavelength used. Although UV-B and UV-C light do not induce different photoproducts, the wavelength might well be a major determinant on the UV-response of cells, which may (in part) originate from other factors than DNA damage. For example, the activation of mitogen-activated protein kinases is more efficient with UV-C, rather than UV-B light (4, 50, 125). This could be due to the fact that UV-B, to a far greater extent than UV-C, is able to alter the redox state of the cell

through production of reactive oxygen species (ROS) (184). ROS have also been shown to contribute to UVB induced apoptosis (92). Like the wavelength of UV used, the cell environment (such as the substrate on which cells are grown) may also have an impact on the p53 dependency of apoptosis (183). Using a human cell line expressing a murine temperature-sensitive p53 mutant protein (allowing tightly and reversibly regulation of p53 function), it was shown that p53 induces distinct pro-apoptotic and anti-apoptotic signals, which could be separated both temporally and by the requirement for de novo protein synthesis (122). Taken together, UV induced apoptosis can be executed via p53-dependent and -independent pathways. Moreover p53 possesses activities that counteract its pro-apoptotic function.

The role of p53 in protection against apoptosis is probably linked to its role in repair and RNA synthesis recovery upon induction of UV damage (and other NER damages). For example, it has been shown that attenuation of p53 function causes only a modest increase in UV induced apoptosis in normal human fibroblasts. However in p53/XPC deficient human fibroblasts (120) a strong UV induced hyperapoptotic response is observed which strongly correlates with the strength of the RNA synthesis block. This suggests a protective role of p53 against apoptosis by promoting RNA synthesis recovery (120). A function of p53 in RNA synthesis recovery upon UV treatment has been suggested by several other reports (14, 122-124, 128). Moreover p53-dependent stimulation of NER has been suggested (14, 61, 65, 82, 104, 115, 124, 128, 153, 164, 165, 173, 178, 198-200, 212).

Cells with a TC-NER- or with a total NER-deficiency are not able to repair damage in the transcribed strand and are unable to restore a transcription arrest after genotoxic treatment (118). Several reports show that TC-NER deficient cells are hyperapoptotic, both in vitro and in vivo (13, 26, 41, 70, 108, 124, 192). Although, RNA polymerase II, when stalled on DNA damage, is an efficient inducer of p53 (26, 41, 108, 109, 204) and p53 induction strongly correlates with execution of apoptosis, the latter process can be at least partially p53 independent in TC-NER deficient cell lines and mice (111, 120, 146, 147). Evidence is accumulating that the apoptotic response of TC-NER deficient cell lines vanishes at high UV doses (121, 149). Since, the attenuation of the apoptotic response in TC-NER deficient cells coincides with their inability to enter S-phase, it seems that replication is a prerequisite for apoptosis induction (121). In line with these findings, it has been shown that apoptosis in ERCC1 and ERCC3 (XPB) mutant Chinese hamster fibroblasts is predominantly occurring in the second cell cycle after UV treatment (59, 60). This coincides with replication-induced double strand breaks which probably induce the apoptotic response (60, 86). Proietti De Santis et al. showed that in *CSB*-deficient Chinese hamster's fibroblasts part of the apoptosis is related to entry into the S-phase, and part can occur during G1 phase. This suggests the existence of different mechanisms of apoptosis induction upon transcription stalling (147, 148).

Beside induction of apoptosis by arrested RNA polymerases, other sensing mechanisms for NER type of lesions might exist. For example, prolonged S-phase arrest (138) and also damage in the non-transcribed areas of the genome can contribute to apoptosis (this study, chapter 5).

1.4.4 Link between repair, cell cycle and apoptosis

Repair, cell cycle regulation, and apoptosis are not separate processes that function independently. As described earlier, some repair factors or repair intermediates are signaling to the cell cycle machinery. Moreover, many factors are functional in more than one process (for reviews see (5, 19)). For example, whereas the p53 protein is already known for its function in regulation of the cell cycle, as well as in apoptosis-execution, it recently also has been shown to stimulate NER (65, 115, 124, 153, 164, 165, 178, 198, 199, 212). Use of a particular factor in multiple pathways provides the cell with the possibility to decide - depending on the situation - which pathway it is going to use. For instance, only low dose UV irradiation stimulates NER in a p53 dependent fashion, whereas at higher UV doses a p53 dependent apoptotic response is activated (102). Also, the balance between cell cycle arrest and apoptosis is partly influenced by the cellular level of p53 and partly requires separate p53 functions (38).
In conclusion, it seems that repair, cell cycle arrest and apoptosis are inter-regulated and that a specific balance between these different genome caretaking processes exists.

1.5 The importance of different genome caretaking processes in a variety of cell types

Vertebrates are built up of a wide spectrum of different cell types, all with their specific functions and demands. All cells are somewhere in the spectrum between terminally differentiated, without any possibility to be replaced, till totally undifferentiated with a good chance to be replaced. Since all these cells have different needs and functions, it appears logical that they may need a different balance between the interwoven processes of repair, cell cycle control and apoptosis that together are responsible for genome caretaking. For example, persisting DNA damage can cause mutations, when replicated, which ultimately will cause cancer. To counteract the chance of cancer induction, cells can be eliminated by apoptosis. However, when apoptotic cells are not replenished in time, this can lead to organ dysfunctioning. Moreover, even when a high apoptotic rate is sufficiently compensated by new cells, the organism can still suffer from the effect of apoptosis, as elevated levels of apoptosis and tissue regeneration can lead to depletion of the specific stem cell compartment. This process has been suggested to contribute to aging and likely forms the connection between aging and impaired repair (reviewed in (131)). Therefore, not only the need of the specific cell, but also the balance between cancer and aging demands for a specific cell-type-dependent response upon DNA damage. In the following paragraphs, some examples of a specific DNA damage response in defined cell types will be discussed.

1.5.1 Genome caretaking in neurons and other post-mitotic cell types

Neurons are probably the epitome of terminally differentiated cells. A given number of neurons should perform their task during the entire life time of an organism. Since neurons do not proliferate and are generally not replaced when lost, it is important that such cells stay in good condition over a long time. In the absence of replication, the chance of gaining

mutations and development of cancer is negligible. This suggestion is supported by the rarity of neuronal tumors. Because of their terminally differentiated status, there is probably little variation in the transcriptional program of neurons. Together with the notion that neurons do not divide, this suggests that removing DNA damage from non-transcribed DNA (representing the bulk of their genome) is dispensable in these cells. Indeed, a low level of global genome NER in neurons has been reported (87, 136, 169). Over time, this reduced GG-NER activity probably causes an elevated damage load in the entire genome. Under extraordinary conditions, as is the case when non-cycling neurons are forced to re-enter the cell cycle, this increased damage load may cause a replication catastrophe. Interestingly, it has been shown that neuron death in Alzheimers disease happens after re-entry into the cell cycle (205).

In contrast to GG-NER, TC-NER is active in neurons, allowing efficient repair of the transcribed strand of active genes. Moreover, it has been shown that in certain cells also the non-transcribed strand of transcribed genes is repaired more efficiently than nontranscribed DNA (76, 135, 136). The latter mechanism, associated with repair of active regions (but not per sé with repair of the transcribed strand) is named differentiation associated repair (DAR). Repair of the non-transcribed strand of active genes is needed for maintaining an error-free template for repair of the transcribed strand of active genes by TC-NER.

Nouspikel et al. suggest that DAR is active in a range of different cell types (135), although these cells differ from neurons in the sense that they can be replaced. For example, Ho and Hanawalt show that upon differentiation of L8 myoblasts to myocytes, global genome repair is largely lost while gene specific repair is improved. They do not show a strand bias in repair of active genes, suggesting the existence of DAR in myocytes (80). Recently, this finding was disputed since differences in global genome repair between cardiac myocytes and fibroblasts could not be detected. Moreover, TC-NER is similar in fibroblasts and myocytes (188). The existence of DAR, at least in some cell types, is a fine example of an adaptation of a repair mechanism to the demands of a specific cell type (for review see (135)).

1.5.2 Genome caretaking in differentiating keratinocytes

It is well known that keratinocytes do not form a homogenous cell population within the epidermis. New keratinocytes are continuously generated from cells in the basal layer of the epithelium, and subsequently differentiate progressively while migrating towards the surface where these cells ultimately shed off (69). The spectrum of differences in proliferative capacity and differentiation status in keratinocytes, together with the fact that these cells are severely challenged by UV light in sun-exposed areas of the human body, make these cells an interesting example to study how different genome caretaking processes interact.

Keratinocytes are more resistant to UV irradiation than fibroblasts, present in the underlying dermis (49). Despite their reduced UV sensitivity, keratinocytes are more susceptible to UV induced apoptosis than fibroblasts. This seeming discrepancy is probably caused by the fact that sensitivity not only reflects the apoptotic response of a cell, but also other pathways of cell death and persistent cell cycle blocks. In contrast to keratinocytes, fibroblasts attribute a prominent G1-S phase arrest after UV irradiation. It has also been

shown that repair of CPDs in the global genome is more efficient in keratinocytes compared to fibroblasts (49). These findings are indicative for a clear divergence in the use of different genome caretaking processes in two distinct cell types. As mentioned, keratinocytes do not form a homogeneous cell population and therefore differences might exist between keratinocytes at various stages in differentiation.
Several lines of evidence show that DNA repair in differentiated keratinocytes is less efficient than in their undifferentiated proliferating counterparts (103, 107, 130). Moreover, whereas in undifferentiated keratinocytes NER is p53 regulated, this regulation fades in differentiated keratinocytes (103). Tron et al. show that in contrast to NER, apoptosis is more prominent in differentiated mouse keratinocytes (183). This finding is in line with the observation that sunburn cells, which are in fact apoptotic cells, are predominately located in the suprabasal, differentiated keratinocyte compartment of the human skin (75). Mirroring the NER situation, apoptosis in undifferentiated keratinocytes is p53 independent and becomes p53 dependent upon differentiation (183). Interestingly, also formation of sunburn cell is p53 dependent in the mouse skin (213). These findings are in contrast with the reported decline in apoptosis upon differentiation in human keratinocytes (36). This discrepancy might be due to a difference between mouse or human keratinocytes, or to differences in culture conditions, which can drastically influence apoptosis (36). Notwithstanding this discrepancy, it is clear that differentiation status of the cell is a major determinant in genome caretaking decisions in keratinocytes. Evidently, this balance in the use of different genome caretaking processes in a certain cell is logically influenced by the need of that particular cell and the organ it is part of. For instance, loss of the epidermis would resemble a third-degree burn, probably resulting in death. Therefore DNA repair is the preferred method of managing damaged DNA in basal keratinocytes, including the cell population they originate from. In the differentiated compartment of the epidermis, apoptosis may be an acceptable manner for eliminating damaged cells, as differentiating keratinocytes have a limited life time anyway.

1.5.3 Genome caretaking in undifferentiated cells

Embryonic stem cells are the precursors of all different cell types present in the body. Therefore, mutations in these cells could have detrimental consequences to the whole organism. Early phases of embryonic development are associated with a dramatic rate of proliferation of undifferentiated cells. During this developmental stage, the cell cycle can even be as short as 3.5 to 7.5 hours (112). Since proliferation and differentiation are tightly time and space regulated, it is not difficult to envisage that a transient cell cycle block after DNA damage will dramatically interfere with the developmental program, and therefore is a non-favorable option. Instead, Heyer et al. show that upon *in utero* irradiation with ionizing radiation at a dose, that does not cause any effect in somatic cells, early embryonic cells show a hyper-apoptotic response (79). This apoptotic response is most prominent in 5.5 up to 8.5 days old embryos, appears clearly restricted to cells of the embryonic lineage, and is not observed in the extra embryonic regions. Moreover, within the embryonic region most apoptosis is observed in the ectoderm, indicative for a lineage specific DNA damage response, and resulting in a 50 % reduction in number of animals born. Notwithstanding

this high level of intra-uterine death after in-utero radiation, all animals born are healthy, fertile and do not develop any abnormalities, as determined up to 10 months after birth. This suggest that apoptosis apparently is an effective way to protect the embryo from developing large malformation, however at the cost of death of a subpopulation of embryos.

Also ES cells, when challenged with DNA damaging compounds, are hyper-apoptotic (62, 180, 193). This hyper-apoptotic response, as observed in (relative) undifferentiated embryonic cells, is probably reflected in other undifferentiated cells. For example, it has been shown that ionizing radiation exposure of the small intestine causes apoptosis in stem cells of the crypt, but not in the more differentiated cells in the villi (144).

Although apoptosis plays a prominent role in protecting stem cells against genotoxic insults, also a role for DNA repair exist. This is evident from the sensitivity of ES cells that lack certain DNA repair pathways to DNA damaging agents (62, 180, 193). Also, a differential use of repair pathways with overlapping substrate specificity has been observed. Essers et al. showed that while double strand break repair in differentiated cells largely depends on the error prone non homologous end joining, in ES cells the error free repair of double strand breaks by homologous recombination is an alternative (62). Van Sloun et al. show that NER is functional in ES cells, however, repair capacity seems to be saturated a low dose (193). So far it is unknown how processes like TC-NER and GG-NER are regulated in ES cells, and whether, as shown for neurons, these processes are differentially regulated compared to other cell types. In this study we examined the role of TC-NER and GG-NER in protection of ES cells and more differentiated cells against various genotoxic agents. We also relate deficiencies in either TC-NER, GG-NER or in total NER to the apoptotic and cell cycle response of ES cells.

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Chapter 2

Cell type-specific hypersensitivity to oxidative damage in *Csb* and *Xpa* mice

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Cell type-specific hypersensitivity to oxidative damage in *Csb* and *Xpa* mice

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Abstract

Mutations in the *CSB* gene cause Cockayne syndrome (CS), a rare inherited disorder, characterized by UV sensitivity, severe neurodevelopmental and progeroid symptoms. CSB functions in the transcription-coupled repair (TCR) subpathway of nucleotide excision repair (NER), responsible for the removal of UV-induced and other helix-distorting lesions from the transcribed strand of active genes. Several lines of evidence support the notion that the CSB TCR defect extends to other non-NER type transcription-blocking lesions, notably various kinds of oxidative damage, which may provide an explanation for part of the severe CS phenotype. We used genetically defined mouse models to examine the relationship between the CSB defect and sensitivity to oxidative damage in different cell types and at the level of the intact organism. The main conclusions are: (1) $Csb^{-/-}$ mouse embryo fibroblasts (MEFs) exhibit a clear hypersensitivity to ionizing radiation, extending the findings in genetically heterogeneous human *CSB* fibroblasts to another species. (2) $Csb^{-/-}$ MEFs are highly sensitive to paraquat, strongly indicating that the increased cytotoxicity is due to oxidative damage. (3) The hypersensitivity is independent of genetic background and directly related to the *Csb* defect and is not observed in totally NER-deficient $Xpa^{-/-}$ MEFs. (4) Wild type embryonic stem (ES) cells display an increased sensitivity to ionizing radiation compared to fibroblasts. Surprisingly, the *Csb* deficiency has only a very minor additional effect on ES cell sensitivity to oxidative damage and is comparable to that of an *Xpa* defect, indicating cell-type-specific differences in the contribution of TCR and NER to cellular survival. (5) Similar to ES cells, *Csb* and *Xpa* mice

both display a minor sensitivity to whole body X-ray exposure. This suggests that the response of an intact organism to radiation is largely determined by the sensitivity of stem cells, rather than differentiated cells. These findings establish the role of transcription-coupled repair in resistance to oxidative damage and reveal a cell- and organ-specific impact of this repair pathway to the clinical phenotype of CS and XP.

2.1 Introduction

Cockayne syndrome (CS) is a rare inherited DNA repair disorder, with a wide range of physical and mental manifestations, including: UV-sensitivity, severe postnatal growth failure, cachectic dwarfism, mental retardation, retinal degeneration, deafness, associated with neurodemyelination and skeletal abnormalities including osteoporosis and a bird-like face. Various symptoms point to premature aging, classifying Cockayne syndrome as a progeroid condition. The mean age of death is 12.5 years, mainly caused by respiratory infections, associated with neurological problems, and overall poor condition of patients. Complementation studies revealed the involvement of two genes in the onset of CS, *CSA* and *CSB* (for review see (3, 33)).

In mammals, helix-distorting DNA lesions induced by UV-light and numerous chemicals are removed by the nucleotide excision repair (NER) pathway. NER functions by a 'cut and patch'-like mechanism in which the damaged nucleotide is removed together with some flanking sequences. The resulting single-stranded gap is filled in by DNA polymerase and ligase (for review see (9, 16, 25)). Two NER sub-pathways exist: global genome NER (GG-NER) repairs helix-distorting base damages in the entire genome, while transcription-coupled NER (TC-NER) specifically repairs transcription-blocking lesions in the transcribed strand of active genes. Some transcription-blocking lesions such as UV-induced cyclobutanepyrimidines (CPDs) are repaired inefficiently by GG-NER: thus repair in the transcribed strand mainly depends on TC-NER. *CSA*- and *CSB*-deficient cells are specifically impaired in this TC-NER pathway (49).

The prototype NER disorder is xeroderma pigmentosum (XP), which shares with CS the pronounced UV sensitivity. Otherwise the diseases are remarkably dissimilar: XP features include pigmentation abnormalities in sun-exposed skin-areas and a more than 1000-fold increased risk of skin cancer, which is strikingly absent in CS. Many XP patients develop quite normally but die of neoplasia, reducing their life span by an average of 30 years (3). Accelerated mental retardation has been noted in a fraction of XP patients, likely caused by enhanced neuronal degeneration due to loss of neurons (35), contrasting with the much more severe progressive mental deterioration in CS due to neurodemyelination (35). Complementation studies have shown the involvement of 7 genes in XP (*XPA* through *XPG*) (3). Defects in both NER sub-pathways are observed in 5 out of 7 XP complementation groups: *XPC* and *XPE* are specifically deficient in the global genome repair pathway (17, 42, 50, 51). A subset of mutations in *XPB*, *XPD* and *XPG* can lead to a combined phenotype of XP and CS (3).

The notion that mutations in *CSA* and *CSB* are associated with defects in only one pathway, TC-NER, is difficult to reconcile with the more severe symptoms observed in CS compared to XP patients, which frequently carry defects in both GG-NER and TC-NER. Additional

roles for the CS proteins outside the context of NER have been suggested. It has been shown that CSB can remodel chromatin and has affinity for histone tails (5). CS proteins may have an auxiliary function in transcription. Moreover CSA and CSB have been reported to physically interact with TFIIH, a protein complex involved in NER and transcription initiation (15, 38, 43) and a fraction of CSB resides in an RNA polymerase II-containing elongation complex (43, 48). Along the same lines, evidence has been collected for a role of CSB as a non-essential transcription elongation factor, required for bypass of pause-sites and of structured RNA (2, 11, 37, 56). In addition, indications have been obtained for involvement of CSA and CSB proteins in repair of non-NER lesions. TC-NER deficient human *CSA* and *CSB* fibroblasts display a slight hypersensitivity to ionizing radiation, which is not observed with completely NER-deficient *XPA* patient fibroblasts (23) and is attributed to defective transcription-coupled repair of oxidative lesions (23), suggesting a wider action spectrum of transcription-coupled repair than only NER lesions. In view of the ubiquitous occurrence of oxidative damage, the biological impact of this lesion may be significant. Interestingly, also cell lines from XP/CS patients with mutations in *XPB*, *XPD* and *XPG* show a transcription-coupled repair defect for oxidative lesions (7, 22). In XPG-CS patients the repair of oxidative lesions may be further compromised by an additional reduction in global genome base excision repair (BER), contributing to the observed Cockayne syndrome phenotype (7, 12, 54).
In recent years, mouse models for XPA and CSB have been generated (10, 32, 47). *Xpa*-deficient mice resemble to a considerable extent the human phenotype. The *Csb* mutant mouse mimics the human phenotype in terms of the repair defect and manifestation of UV-sensitivity of skin and eyes and retinal degeneration. In contrast to *CSB* patients, UV- or DMBA-induced cancer predisposition and only mild neurological problems have been observed in *CSB* mutant mice (47).
These mouse models provide an excellent system to analyze genotype-phenotype relations in a defined and homogenous genetic context and not only allow studies in a variety of cell types and tissues, but, importantly, also in the intact organism. Here we present a systematic analysis of the spectrum of sensitivities caused by the *Csb* and *Xpa* defects.

2.2 Materials and methods

2.2.1 Cell lines

Isolation of primary *Csb*$^{-/-}$ (FVB/129Ola) and *Xpa*$^{-/-}$ (C57Bl6J/129Ola) MEFs and corresponding wt cell lines has been described (10, 47). Cells were cultured in F10/DMEM (1:1) (Gibco) medium, supplemented with 10 % fetal calf serum and 50 μg/ml penicillin and streptomycin (Gibco). Spontaneously immortalized cell lines were obtained by continuous subculturing of primary MEFs.
Wild type, *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ embryonic stem (ES) cells were isolated from blastocysts (3.5 days *post coitum*) obtained from wt, *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ mice intercrosses respectively (all in a C57Bl6J genetic background). Day 3.5 embryos were isolated and individually placed into a gelatinized well of a 24-well plate. After incubation for 7 days, without medium replacement, the wells were washed with PBS and single inner cell mass outgrowths were

carefully dislodged. After incubation for 5 min at 37°C in 100 µl trypsin/EDTA, they were mechanically dissociated in small cell clumps (2-5 cells). Finally, they were transferred to a 12-well plate with a feeder-layer. Feeder-layers were obtained by lethal irradiation from confluent primary MEF cultures irradiated with 20 Gy from a ^{137}Cs-source. Medium was changed every other day and colonies were picked 5-14 days after plating the dissociated inner cell mass. Colonies were dissociated by trypsin/EDTA and plated on gelatinized plates. Alternatively, in stead of picking colonies the whole well was trypsinized and plated on gelatinized plates. ES cells were maintained on gelatin-coated dishes in 50% buffalo rat liver cell conditioned DMEM / 50 % fresh DMEM supplemented with 15 % fetal calf serum, 0.1 mM non-essential amino acids (Gibco), 50 µg/ml penicillin and streptomycin (Gibco), 1000 U/ml leukemia inhibitory factor (Chemicon) and 0.1 mM 2-mercaptoethanol (Sigma) .

2.2.2 Cellular sensitivity studies

For determination of the γ-ray-sensitivity of immortalized MEFs and ES cells, cells were plated in 6cm dishes, at various dilutions. After 12-16 h, cells were irradiated with a single dose in the range of 0-8 Gy using a ^{137}Cs source. Cells were grown for 5 to 14 days, fixed, stained and counted to asses the colony-forming ability. All experiments were performed in triplicate.
UV sensitivity was determined as described (39). Briefly, MEFs were exposed to different doses of UV (254 nm, Philips TUV lamp) and allowed to grow for another 3-5 days, before reaching confluency. The number of proliferating cells was estimated by scintillation counting of the radioactivity incorporated during a 3 hr pulse with [^{3}H]Thymidine (5µCi/ml, s.a. 40-60 Ci/mmole; Amersham). Cell survival was expressed as the ratio of ^{3}H-incorporation in treated and non-treated cells. This protocol was adapted for paraquat survival by growing MEF cultures for 3-5 days in medium containing different concentrations of paraquat, followed by determination of the amount of proliferating cells as described above.
RNA synthesis recovery after UV treatment was measured according to Mayne et al. (1982) (29). Cells were exposed to 10 J/m^2 of UV (254 nm, Philips TUV lamp) allowed to recover for 16 hr, labeled 1 hour with [5,6-^{3}H]uridine (10 µCi/ml, s.a. 50 Ci/mmole; Amersham), and processed for autoradiography. The relative rate of RNA synthesis was expressed as the quotient of the number of autoradiographic grains over the UV-exposed nuclei and the number of grains over the nuclei of non-irradiated cells (average of 50 nuclei per cell line).

2.2.3 X-ray irradiation of mice

Wild type (n=45), *Csb*$^{-/-}$ (n=38) and *Xpa*$^{-/-}$ (n=37) mice in a C57Bl6 background received total body irradiations of 2.2 Gy for 5 consecutive days (cumulative dose 11 Gy) using a 200kV X-ray machine operating at 20 mA. Animals (3-8 mice/cage) were irradiated in a filter-top containing polyethylene cage fitting in the 18x24cm field of the applicator. At a source to target distance of 74cm, a dose rate of 0.31 Gy/min was obtained. Dosimetry indicated a dose variation of less than 5% over the irradiation field. Except for the

irradiation procedure, animals were housed in sterile micro-isolators to minimize the risk of infections and followed for 30 days after the last irradiation. Animals were daily screened for discomfort. Differences in radiosensitivity of wt and mutant mice were assessed on the basis of the time interval between the last dose and death (latency period). Mice that died during the experiment as well as surviving animals (euthanized at day 30) were fixed in 1 % formalin for pathology. Animal experiments were approved by the local animal ethical committee of the Erasmus University Rotterdam.

2.3 Results

2.3.1 Csb$^{-/-}$ mouse embryonic fibroblasts are hypersensitive to γ-ray irradiation

To critically investigate whether cells from *Csb*$^{-/-}$ mice are more sensitive to ionizing radiation than cells from wild type (wt) and *Xpa*$^{-/-}$ mice, we performed clonogenic γ-ray survival experiments on spontaneously immortalized *Csb*$^{-/-}$, *Xpa*$^{-/-}$ and wt mouse embryonic fibroblasts (MEFs). Figure 1a shows that *Csb*$^{-/-}$ MEFs are approximately two times more sensitive to a single dose of γ-ray irradiation than MEFs derived from wt littermates. *Xpa*$^{-/-}$ MEFs, which are completely deficient in both NER sub-pathways show similar γ-ray survival as wt MEFs (Figure 1a). The observed difference in γ-ray survival rates of *Csb*$^{-/-}$ and X*pa*$^{-/-}$ MEFs is in contrast to UV-survival characteristics. As shown in figure 1b, *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs are both hypersensitive for UV-irradiation, although *Csb*$^{-/-}$ MEFs appear slightly more resistant than *Xpa*$^{-/-}$ MEFs. The difference presumably reflects the relatively minor contribution of global genome NER to UV survival. Thus, we observe a specific

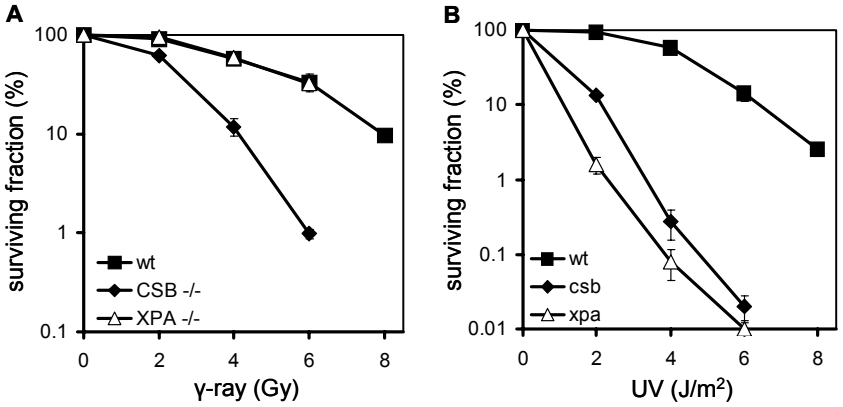

Figure 1

Survival of wild type, *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ mouse embryonic fibroblasts after exposure to γ-ray or UV-light.

Panel A: Survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Xpa*$^{-/-}$ (open triangles) and wild type (squares) MEFs after exposure to increasing doses of γ-rays, as determined by the colony survival assay. Panel B: Survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Xpa*$^{-/-}$ (open triangles) and wild type (squares) MEFs after exposure to increasing doses of UV-light (254 nm), as determined by the [^{3}H]-thymidine incorporation assay. For each cell line, experiments were performed at least 3 times and for each genotype identical results were obtained with at least two independent cell-lines (data not shown). Representative examples are shown. Bars indicate the standard error of the mean.

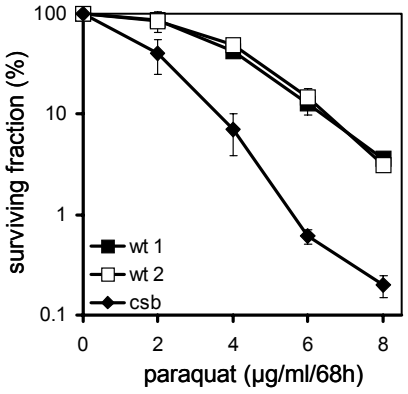

Figure 2
Sensitivity of *Csb*$^{-/-}$ mouse embryonic fibroblasts to paraquat.
Paraquat survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds) and wild type (two independent lines; closed and open squares) mouse embryonic fibroblasts. Experiments were performed at least 3 times per cell line, as determined by the [^{3}H]-thymidine incorporation assay. Shown are representative curves. Bars indicate the standard error of the mean.

γ-ray sensitivity of *Csb*$^{-/-}$ MEFs, which differs from the UV sensitivity, indicating that this sensitivity is not caused by a classical NER-deficiency.
To investigate the effect of variations in genetic background on the observed γ-ray sensitivities, we determined the γ-ray survival of wt MEFs from genetically different mouse strains (FVB/129OLA, C57Bl6/129OLA, C57Bl6). No difference in γ-ray-sensitivity was detected (data not shown); indicating that influence of genetic background is negligible.

2.3.2 Sensitivity of Csb$^{-/-}$ *MEFs to γ-ray-irradiation is probably due to oxidative lesions.*

Treatment of cells with γ-rays causes different classes of DNA lesions, including single and double strand DNA breaks and various types of oxidative base damages. To determine whether γ-ray sensitivity in *Csb*$^{-/-}$ MEFs originated from oxidative DNA lesions, we treated these cells with paraquat. Enzymatic reduction of paraquat produces paraquat radicals, which react with molecular oxygen, thereby generating a superoxide anion that is converted into hydrogenperoxide. This inflicts oxidative stress to cells and thereby causes oxidative DNA injury (1). Figure 2 shows that *Csb*$^{-/-}$ MEFs are approximately two times more sensitive to paraquat treatment than wt MEFs, which is strikingly similar to the γ-ray-sensitivity. In conclusion these data strongly suggest that the sensitivity of *Csb*$^{-/-}$ MEFs to both γ-ray and paraquat treatment is caused by oxidative DNA lesions.

2.3.3 Hypersensitivity of Csb$^{-/-}$ *MEFs for paraquat and γ-rays is due to a CSB defect*

To find out whether the observed paraquat sensitivity of *Csb*$^{-/-}$ MEFs is indeed due to the *Csb* defect, we transfected a human *CSB* cDNA into these cells and selected clones which stably express the exogenous *CSB*. In line with previous findings with CSB-deficient CHO mutants (45) introduction of the human *CSB* cDNA induces a detectable albeit incomplete rescue of UV sensitivity and RNA synthesis recovery after UV-treatment (Figure 3a and 3b). Thus, expression of the human *CSB* cDNA partly corrects the UV-phenotype of *Csb*$^{-/-}$ MEFs. Concomitantly, transfected MEFs have acquired increased resistance to paraquat treatment (Figure 3c). Similar results are obtained with γ-ray irradiation (data not shown). Therefore, we conclude that the observed sensitivity of C*sb*$^{-/-}$ MEFs to paraquat treatment and γ-ray-irradiation is caused by the *Csb* defect and not by other genetic factors. The partial

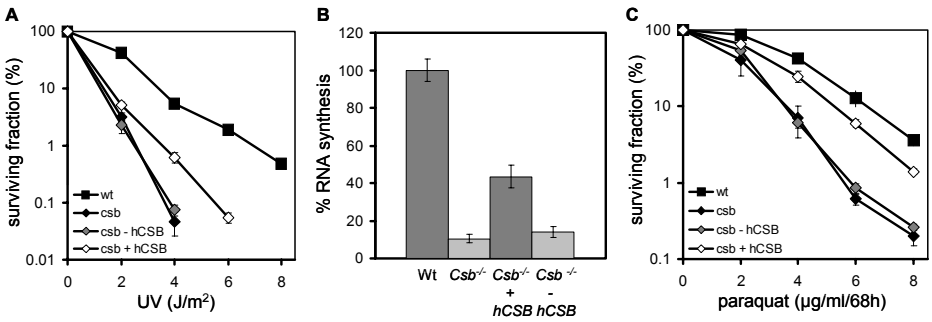

Figure 3

Correction of the *Csb* sensitivity by h*CSB* cDNA.

Panel A: Survival of spontaneously immortalized *Csb*$^{-/-}$ (closed diamonds), h*CSB* cDNA corrected *Csb*$^{-/-}$ (open diamonds), an uncorrected *Csb*$^{-/-}$ subclone derived from the same transfection experiment (gray diamonds) and wild type (closed squares) MEFs after exposure to increasing doses of UV-light (254 nm), as determined by the [^{3}H]-thymidine incorporation assay. Panel B: RNA synthesis recovery of wild type and *Csb*$^{-/-}$ MEFs as well as h*CSB* cDNA corrected and uncorrected *Csb*$^{-/-}$ MEFs, 16 hr after exposure to 10 J/m^2 254 nm UV. Panel C: Paraquat survival of spontaneously immortalized *Csb*$^{-/-}$ (closed diamonds), h*CSB* cDNA corrected *Csb*$^{-/-}$ (open diamonds), an uncorrected *Csb*$^{-/-}$ subclone derived from the same transfection experiment (gray diamonds) and wild type (closed squares) MEFs, as determined by the [^{3}H]-thymidine incorporation assay. For each cell line, experiments were performed at least 3 times and for each genotype identical results were obtained with at least two independent cell-lines (data not shown). Representative examples are shown. Bars indicate the standard error of the mean.

nature of the correction may be due to interspecies (human-rodent) type of complementation or to suboptimal expression properties of the transfected cDNA vector construct compared to the endogenous gene.

2.3.4 Cell-type specific differences in γ-ray sensitivity associated with the Csb$^{-/-}$ *defect*

To determine whether the sensitivity of *Csb*$^{-/-}$ MEFs to oxidative DNA lesions is a general feature, we extended our study to another cell type. To this end, an isogenic set of pluripotent, undifferentiated mouse embryonic stem cells (ES cells) was established from wt, *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ blastocysts. Figure 4 shows clonogenic γ-ray survival curves for *Csb*$^{-/-}$, *Xpa*$^{-/-}$ and wt ES cells. In comparison to the MEF data, three important differences are observed. Firstly, wt ES cells are clearly more sensitive to γ-rays when compared with the survival of wt MEFs at the same γ-ray-dose. Secondly, *Csb*$^{-/-}$ ES cells display a slight increase in γ-ray sensitivity compared to wt ES cells, however this hypersensitivity is very mild in comparison with the hypersensitivity caused by *Csb* deficiency in MEFs. Thirdly, the *Xpa* mutation in ES cells -in striking contrast with MEFs- gives rise to similar γ-ray sensitivity as a *Csb* defect. We conclude that a *Csb* deficiency causes cellular sensitivity to γ-ray irradiation in various cell types, but with significant variation in the magnitude of the response: fibroblasts being significantly more ionizing radiation sensitive than ES cells. The opposite is observed for a total NER defect: no hypersensitivity is registered in *Xpa*$^{-/-}$ fibroblasts whereas *Xpa*$^{-/-}$ ES cells exhibit a (albeit minor) γ-ray sensitivity. Thus, defects in TCR as well as total NER cause cell-type specific (differences in) responses to γ-rays.

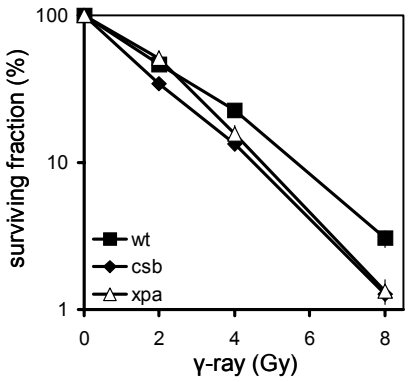

Figure 4
γ-ray sensitivity of *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ embryonic stem cells.
Survival of *Csb*$^{-/-}$ (diamonds), *Xpa*$^{-/-}$ (open triangles) and wild type (squares) mouse embryonic stem cells after exposure to increasing doses of γ-rays, as determined by the colony survival assay. Shown is the average of at least three experiments, with for each genotype at least two independent cell lines. Bars indicate the standard error of the mean.

2.3.5 Whole body X-ray irradiation of wt, Csb$^{-/-}$ and Xpa$^{-/-}$ mice

The cellular findings above raise the clinically relevant question how *Csb* and *Xpa* defects translate into γ-ray response upon whole-body exposure to ionizing radiation and if oxidative damage sensitivity of MEFs and/or ES cells has a predictive value for ionizing radiation sensitivity of the animal. To compare the ionizing radiation sensitivity of wild type and *Csb*$^{-/-}$ mice, we considered to use a split-dose protocol (as frequently used in the clinic in radiotherapy treatment) to enlarge potential minor differences in survival of repair proficient and deficient cells. To explore this approach *in vitro,* we first irradiated wt and *Csb*$^{-/-}$ MEFs with two doses of γ-rays at a 6h interval and compared this with a single dose, equal to the cumulative dose used in the 'split dose protocol'. Indeed, we observed a significant increase in resistance when using a split dose instead of a single dose. This increase is larger in wt MEFs compared to *Csb*$^{-/-}$ MEFs, indicating a poorer recovery from irradiation in *Csb*$^{-/-}$ MEFs during the 6 hours time interval between the exposures (Figure 5a).
On this basis, we decided to use a split dose whole body irradiation protocol and exposed wt (n=45), *Csb*$^{-/-}$ (n=38) and *Xpa*$^{-/-}$ (n=37) mice on 5 consecutive days to 2.2 Gy X-rays per day. Animals were housed in micro-isolators to minimize the risk of infections during the experiment. Mice were scored as dead either when found dead, or when they had to be sacrificed, according to the local bio-ethical standards (criteria applied: severe weight loss (>20%), no food-intake, no movement, no response to external stimuli and hunchbacked appearance). Figure 5b shows the percentage of surviving mice up to 30 days after the last day of irradiation. There is a tendency for *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ animals to have a shorter latency time and a lower chance to survive, resembling the ES cell response. However, statistical analysis revealed that the differences are below the level of confidence (Kaplan-Maier, p=0.3). Pathological examinations of non-surviving animals revealed bleedings and anemia in almost all wt and mutant animals (Table 1). In accordance with these findings, histological examination of the bone marrow showed a virtual complete deprivation of blood-forming cells. However, in comparison to wt animals *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ deficient mice seemed more prone to infections, resulting in sepsis as scored by the presence of bacterial

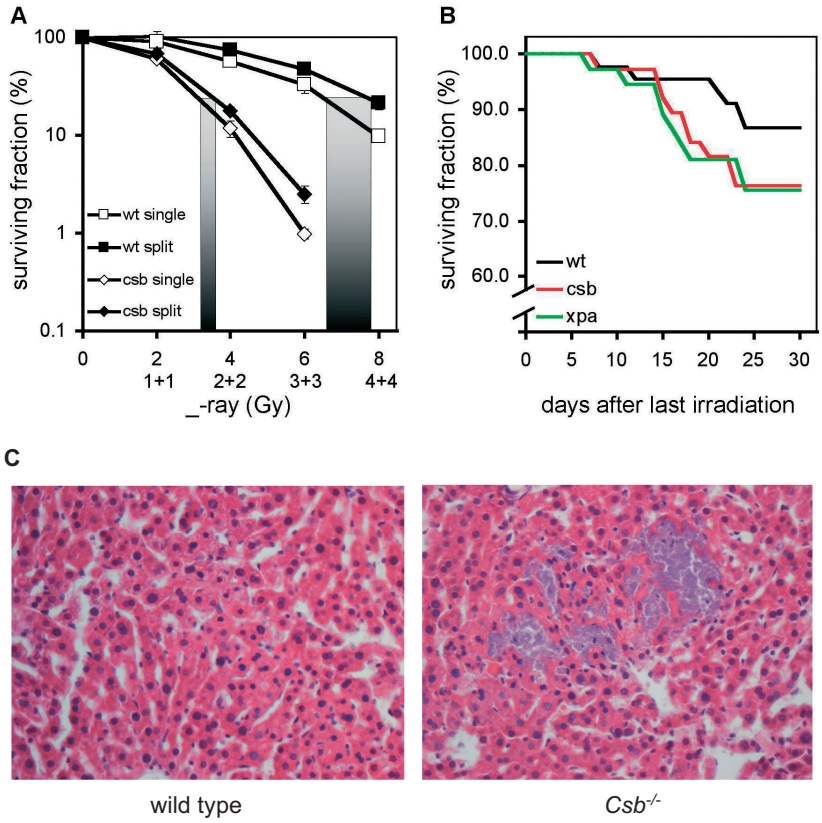

Figure 5
X-ray sensitivity of *Csb*$^{-/-}$ mice.
Panel A: γ-ray survival curves of spontaneously immortalized *Csb*$^{-/-}$ (diamonds) and wild type (squares) MEFs. Radiation has been performed on the basis of a split dose (2 exposures with a 6h interval, closed symbols) or a single dose protocol (open symbols). The gray bars indicate the difference in dose needed to kill 37 % of cells by a split dose or a single dose protocol. Panel B: Surviving fraction of *CSB*$^{-/-}$ (n=38; red line), *Xpa*$^{-/-}$ (n=37; green line) and wild type (n=45; black line) mice after a fractionated whole body exposure of 11 Gy (2.2 Gy/day for 5 consecutive days). Panel C: Representative examples of HE stained livers of X-ray exposed wild type and *Csb*$^{-/-}$ mice (taken at day 23). Note the presence of bacterial foci (stained purple) in the liver of the *Csb*$^{-/-}$ mouse.

foci in various organs. Figure 5c shows representative examples of liver sections of non-surviving *CSB*$^{-/-}$ and wt mice.
A pilot experiment (20 wt, 12 *Csb*$^{-/-}$ mice, *Xpa*$^{-/-}$ not included), performed under conditions in which the animals were not housed in microisolators, showed a comparable result. In this experiment *CSB*$^{-/-}$ mice also tend to have a shorter latency period and increased sensitivity to X-rays. We conclude that C*sb*$^{-/-}$ (and *Xpa*$^{-/-}$) mice exhibit a tendency to increased X-ray sensitivity compared to wt mice.

2. 4 Discussion

2.4.1 *NER-independent sensitivity of Csb$^{-/-}$ cells to ionizing radiation and paraquat*

We have exploited the genetically defined mouse system for a detailed analysis of the effect of CSB transcription-coupled repair defect to oxidative DNA damage sensitivity at the cellular level and at the level of an intact organism. We have demonstrated that *Csb*-deficient MEFs are hypersensitive to both γ-ray irradiation and paraquat, indicating that ionizing radiation sensitivity of *Csb*$^{-/-}$ cells is caused by oxidative lesions. Importantly, this finding unequivocally proofs that the reported ionizing radiation sensitivity of human *CSB* fibroblasts (23) originates from the *CSB* gene defect rather than potential differences in genetic background, and extends to other mammalian species. Furthermore, we find that the *CSB* sensitivity extends to other cell types, including keratinocytes (unpublished results) and ES cells. However, the latter cells are only marginally sensitive compared to wt ES cells, pointing to cell type-specific differences in dependence on TCR.

2.4.2 Oxidative DNA damage as cause of the γ-ray and paraquat sensitivity of Csb$^{-/-}$ cells

γ-Rays cause a wide diversity of DNA damages, notably single and double strand DNA breaks and various kinds of oxidative base modifications. The latter type of lesion is induced by reactive free radicals originating from the radiolysis of water in the vicinity of DNA (53). Double strand breaks, the most cytotoxic type of damage induced by γ-rays, are dealt with by homologous recombination repair or non-homologous end-joining (34). Despite their cytotoxic effect, it is unlikely that these are responsible for the ionizing radiation hypersensitivity of human and mouse *CSB*$^{-/-}$ cell lines. This is consistent with the similar sensitivity of *Csb*-deficient cells for paraquat and γ-rays, indicating that the critical lesions causing increased cytotoxicity are oxidative damage. Therefore, oxidative lesions such as thymine glycols and 8-oxo-2'-deoxyguanosines appear the prime candidates causing ionizing radiation sensitivity in *Csb*-deficient cells. This is further underlined by the finding that ionizing radiation-exposed human *CSB* fibroblasts accumulate more 8-oxo-2'-deoxyguanosine than wild type cells (46).
Oxidative DNA damage is primarily repaired via base excision repair (24, 30), although a minor fraction (e.g. cyclo-purines) is eliminated by the NER machinery (4, 20). The finding that completely NER-deficient *Xpa*$^{-/-}$ MEFs are not sensitive to γ-ray irradiation suggests that the latter type of DNA lesions is not responsible for the hypersensitivity of *Csb*$^{-/-}$ MEFs and excludes cyclo-purines as the causative lesions of the *Csb* sensitivity.
The link between UV-sensitivity and inability to handle transcription-blocking UV-induced lesions suggests that the γ-ray and paraquat sensitivities of *Csb*$^{-/-}$ cells originate from transcription-blocking oxidative DNA lesions. Yet, we failed to observe a strong RNA synthesis block in wt and *Csb*$^{-/-}$ cells after γ-ray-irradiation (data not shown). However, controversy exists with respect to the ability of oxidative damage to block transcription by viral and bacterial RNA polymerases (13, 44, 52), whereas relatively little is known about this aspect for mammalian RNA polymerase II. Thymine glycols appear not to affect

transcription by RNA polymerase II *in vitro* (44). On the other hand, 8-oxo-2'-deoxyguanosine has been found to block RNA polymerase II mediated transcription of a shuttle vector *in vivo* in the absence of the CSB protein (22). Nevertheless, both lesions constitute targets for TCR, as evident from the inability of human CSB as well as XP/CS fibroblasts to remove them from the transcribed strand (7, 22).

2.4.3 A universal transcription-coupled repair model

It is unclear whether transcription-coupled repair of oxidative lesions works via a presumed transcription-coupled BER mechanism or via transcription-coupled NER, documented for UV damage. Figure 6 puts the TCR process in a broader context. Oxidative DNA lesions block RNA polymerase II, which in turn can trigger p53-dependent and -independent apoptotic responses, as shown for UV-lesions (6, 27, 28). To enable repair CSB protein is required to make the lesion accessible, either by back-tracking or dissociation of RNA polymerase II. In a recent study, Woudstra et al. reported that in *Saccharomyces cerevisiae* a stalled RNA polymerase II triggers a coordinated rescue mechanism, requiring the Rad26 (the yeast counterpart of CSB) and DEF1 proteins (55). DEF1 was suggested to enable proteolysis of RNA polymerase II in case the lesion cannot be rapidly removed via RAD26 (55). Although the considerable differences between yeast and mammalian TCR as well as CSB/Rad26 function hamper a proper extrapolation of data, this observation underlines the importance of damage clearance. CSB-mediated clearance of the lesion can be directly coupled to a BER-type repair reaction involving DNA glycosylases. Recent findings that *Ogg1*$^{-/-}$ cells only lack repair of 8-oxo-2'-deoxyguanosine in the non-transcribed strand (21) indicate that transcription-coupled repair of this oxidative lesion does not depend on the OGG1 glycosylase. These results are consistent with either the removal of the lesion by classical TC-NER or by special DNA glycosylases, such as the recently identified NEI1/NEH1 glycosylase (14), as well as two novel glycosylases (TGG1 and TGG2) which might serve as specific transcription-coupled glycosylases or as backup repair proteins for oxidative lesions (41). Alternatively, or in addition, it is possible that transcription-coupled repair of oxidative damage is linked to a mechanism similar as the recently in *E. coli* discovered nucleotide incision repair. In this reaction the nfo protein incises the DNA at the

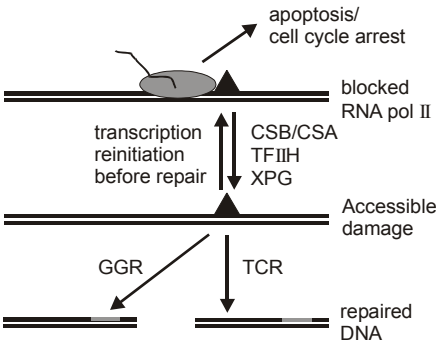

Figure 6
Hypothetic model of the fate of transcription blocking lesions.
Oxidative DNA lesions block RNA polymerase II, which in turn can trigger p53-dependent and -independent apoptotic responses. To enable repair of the lesion -and thus prevent apoptosis or cell cycle arrest- CSB protein is required to make the lesion accessible by either back-tracking or dissociating of RNA polymerase II. This action can be directly linked to transcription-coupled repair. Alternatively, when TCR is unable to cope with the damage, the lesion is made accessible for global genome repair. TCR can be mediated by the documented TC-NER reaction or by a presumed TC-BER pathway.

5' side of oxidatively damaged bases, thus circumventing the generation of a toxic abasic site (18). The mammalian genome, in contrast to the yeast genome, lacks an nfo homologue but the incision activity might be provided by a yet unidentified enzyme. The dangling damaged base could subsequently be excised via FEN1 nuclease. Lastly, unmasking of the lesion by the action of the CSB protein in principle also allows global genome repair (NER or BER) to function as a backup mechanism for removal of damage in the transcribed strand of active genes. In conclusion, the hypersensitivity of *Csb*$^{-/-}$ cells to ionizing radiation and paraquat may be caused by the inability to remove the stalled polymerase from the lesion in the absence of the CSB protein, hereby preventing transcription-coupled repair as well as the possibility of repair of the lesion by the global genome BER system. CSB (and also XPG) proteins have been proposed to stimulate global genome repair of oxidative DNA damage (7, 12, 40, 46, 54). Although this might contribute to the observed ionizing radiation sensitivity of CSB cells, it is unlikely the only underlying mechanism, since for example cell lines defective for mNTH do not show any sensitivity to oxidative stress ((41) and our own unpublished results).
The observed wt γ-ray resistance of *Xpa*$^{-/-}$ MEFs is not inconsistent with a possible involvement of TC-NER in removal of oxidative lesions. In *Xpa*$^{-/-}$ MEFs, in contrast to *Csb*$^{-/-}$ cells, the RNA polymerase II can be removed from the lesion. This makes the lesion accessible for global genome BER, thereby preventing prolonged stalling of RNA polymerase II and subsequent triggering of the apoptotic response, resulting in wt cellular survival.

2.4.4 Specific stem cell response

Csb-deficient MEFs, keratinocytes (results not shown) and ES cells all displayed significant γ-ray hypersensitivity. Interestingly, however, we observed a marked difference between cell types in the intrinsic γ-ray sensitivity as well as in the consequence of a *Csb* defect. ES cells appear the most sensitive, but the influence of a *Csb* defect is relatively small. The same is found for other types of damages, such as UV injury (our own unpublished data). Interestingly, *Xpa*$^{-/-}$ ES cells (in contrast to *Xpa*$^{-/-}$ MEFs) display γ-ray sensitivity comparable to that of *Csb*$^{-/-}$ ES cells. The use of multiple independent cell lines of the same mutant (all in a C57Bl6 genetic background) excludes that this result is due to inter cell line variation. A possible explanation for the intrinsic sensitivity of ES cells and the similar behavior of *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ ES cells to γ-ray irradiation can be inefficient back up repair and/or a lower threshold for the apoptotic response in undifferentiated cells, causing ES cells to die before back-up mechanisms can perform their repair activity. This may also explain why a number of XP lymphoblastoid cell lines are ionizing radiation sensitive while fibroblast cell lines derived from the same or similar patients are not sensitive to oxidative lesions (19, 26, 36).

2.4.5 X-ray effects on Csb$^{-/-}$*, Xpa*$^{-/-}$ *and wt mice*

The differences in survival between X-ray exposed wt, *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ mice were not statistically significant. Nevertheless, our data point to a tendency for *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ mice to have a shorter latency time as well as a minor increase in sensitivity. A similar difference

between wt and *Csb*$^{-/-}$ mice was also noted in a pilot experiment (involving 20 wt and 12 *Csb*$^{-/-}$ animals), which constituted the basis for the more extensive study described above. Pathological examination of deceased animals points to hematopoietic failure as the cause of death: almost all animals demonstrated bleedings and a virtual complete deprivation of blood-forming cells resulting in anemia (data not shown).
However, in comparison to wt animals, *Csb*- and *Xpa*-deficient mice appear more prone to infections, resulting in sepsis. This suggests that *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ mice might be slightly more sensitive to X-ray irradiation than wt animals, due to a less effective immunological response. The response of *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ mice after exposure to X-rays might be explained by a higher ionizing radiation sensitivity of progenitor stem cells of the immune system, which would reflect the ES cells response. This observation is in accordance with the reported ionizing radiation sensitivity of some XP lymphoblastoid cell lines as discussed in the previous section. These observations clearly show that the ionizing radiation sensitivity of cultured cells as a predictive value for sensitivity of the whole organism largely depends on the cell type used. Extrapolating to the human situation one might expect CS patients to be clinically more sensitive to X-rays but the magnitude may vary between cells and tissues.
Recently, we have shown that inactivation of the *Xpa* gene (and thus abolishing NER) evokes acceleration of the premature aging phenotype in trichothiodystrophy (TTD) mice, which correlated with an increased cellular sensitivity to oxidative DNA damage, leading us to hypothesize that aging in the TTD mice is caused by unrepaired DNA damage that compromises transcription, leading to functional inactivation of critical genes and apoptosis (8). Interestingly, inactivation of the *Xpa* gene also has a profound effect on CSB mice: animals show early postnatal ataxia, abnormal cerebellar development and (like TTD/XPA mice) die around weaning (31). In view of the data presented in this paper, this aggravation of the TTD and CSB is likely to originate from a cell type-specific increase in oxidative damage sensitivity caused by the *Xpa* gene defect.

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Chapter 3

Different effect of *Csa* and *Csb* deficiency on sensitivity to oxidative DNA damage

Submitted

Different effect of *Csa* and *Csb* deficiency on sensitivity to oxidative DNA damage

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Abstract

Mutations in the *CSA* and *CSB* gene cause Cockayne Syndrome (CS), a rare inherited disorder characterized by UV sensitivity, severe neurological abnormalities and progeroid symptoms. Both gene products function in the transcription-coupled repair (TCR) subpathway of nucleotide excision repair (NER), providing the cell with a mechanism to remove transcription-blocking lesions from the transcribed strand of actively transcribed genes. Besides a function in TCR of NER lesions, a role of CSB in (transcription-coupled) repair of oxidative DNA damage has been suggested.
In this study we used mouse models to compare the effect of a *Csa* or a *Csb* defect on oxidative DNA damage sensitivity at the level of the cell and the intact organism. In contrast to $Csb^{-/-}$ mouse embryonic fibroblasts (MEFs), $Csa^{-/-}$ MEFs are not hypersensitive for γ-ray or paraquat treatment. Similar results were obtained in keratinocytes. In contrast, $Csb^{-/-}$ and $Csa^{-/-}$ ES cells both show slight γ-ray sensitivity. Finally, $Csb^{-/-}$, but not $Csa^{-/-}$ mice fed with DEHP containing food (causing elevated levels of oxidative DNA damage in the liver) show weight reduction. These findings not only uncover a clear difference in oxidative DNA damage sensitivity between *Csa*- and *Csb*-deficient cell lines and mice, but also show that sensitivity to oxidative DNA damage is not a uniform characteristic of Cockayne syndrome. This difference in the DNA damage response between *Csa*- and *Csb*-deficient cells is unexpected, since until now no consistent differences between CSA and CSB patients have been reported. We suggest that CSA and CSB proteins in part perform a separate role in different DNA damage response pathways.

3.1 Introduction

In order to cope with the continuous attack of endogenous and environmental genotoxic agents on the integrity of their genomes, cells are equipped with a battery of DNA repair systems with partly overlapping substrate specificity. In mammals, chemically and UV-induced helix-distorting lesions are removed through the versatile nucleotide excision repair (NER) pathway. NER functions by excision of the lesion, as a ±30 nt oligonucleotide, after which the resulting single stranded gap is filled in by DNA polymerase and ligase. Recognition of the lesion occurs via two subpathways. In global genome NER (GG-NER), repair of helix-distorting base damage in the entire genome is initiated by recognition of these lesions by the XPC-HR23B-Cen2 complex, facilitated by the XPE dimer (UV-DDB1/2). In transcription-coupled NER (TC-NER), repair of transcription blocking lesions is thought to be initiated by a RNA polymerase, unable to pass the lesion (for review see (11, 20, 31)).
Mutations in NER genes can lead to several rare inherited recessive disorders. The prototype NER syndrome is xeroderma pigmentosum (XP), characterized by pronounced UV sensitivity, pigmentation abnormalities in sun-exposed areas of the skin, and a more than 1000-fold risk of developing skin cancer, causing a 30 year life span reduction. In a subpopulation of XP patients accelerated neurodegeneration occurs, due to early loss of neurons (4, 39). A distinct NER-associated disorder is Cockayne syndrome (CS), which shares with XP the pronounced UV sensitivity, but in addition has a wide range of severe physical and mental manifestations. These include: postnatal growth failure, chachectic dwarfism, retinal degeneration, deafness, mental retardation associated with neurodemyelination, and skeletal abnormalities such as osteoporosis and a bird-like face (4, 37). Many of these symptoms, together with the average short lifespan of 12.5 years, point to premature aging.
Complementation analysis by cell hybridization studies have shown the involvement of seven genes in the NER-deficient form of XP (*XPA* through *XPG*) (4). Mutations in XP genes cause a combined defect in both TC-NER and GG-NER pathways in five out of seven XP complementation groups. In contrast, mutations in *XPC* and *XPE* cause a deficiency in GG-NER only (21, 47, 55, 56). Cockayne syndrome is associated with a specific TC-NER defect, caused by mutations in two genes, *CSA* or *CSB* (54). Interestingly, mutations in *XPB*, *XPD* or *XPG* can cause a combination of XP and CS (4).

The notion that mutations in *CSA* or *CSB* only affect the TC-NER pathway, while mutations causing XP frequently inflict both TC-NER and GG-NER, is difficult to reconcile with the more severe symptoms observed in CS compared to XP patients. To explain this phenomenon, a role of the CS proteins outside TC-NER has been suggested, such as an auxiliary function in transcription (3, 14, 42) and/or in (transcription-coupled) repair of oxidative DNA damage and other non-NER lesions (12, 13, 26, 28, 38, 46, 50). Similarly, cell lines from XP/CS patients with mutations in *XPB*, *XPD* or *XPG* show a defect in transcription-coupled repair (TCR) of oxidative DNA damage (9, 26), underscoring the possible involvement of unrepaired oxidative DNA lesions in the CS etiology.

Most studies on the role of CS proteins in other processes than classical TC-NER have been performed on *CSB*-deficient human cell lines. Since clinical differences have not been observed between patients belonging to CSA and CSB complementation groups, a similar response for *CSA*- and *CSB*-deficient cell lines is expected. Indeed, for NER-related assays there is no evidence for a *CSB*- or *CSA*-related difference. Therefore, findings obtained with non-NER-related assays in *CSB*-deficient systems have often been extrapolated to be general CS-characteristics. Yet, as clear biochemical differences between CSA and CSB exist (53), a minor variance in the cellular response to genotoxic stress may be present in *CSA* and *CSB* cells. In studying such potential subtle differences, isogenic NER deficient mouse models are highly valuable, as obtained results are not influenced by differences in genetic background. Previously, using a mouse model for CSB (52), we showed that *Csb*-deficient cells and animals are sensitive to oxidative DNA damage (12). Recently, we also have generated a mouse model for CSA (51) and have shown that both CS mouse models mimic the human phenotype in terms of the repair defect, retinal degeneration and manifestation of UV sensitivity of skin and eyes. To determine whether CSA and CSB are truly equivalent in their oxidative DNA damage response, we systematically compared the sensitivity to oxidative stress in a variety of cell types and in the intact organism in a *Csa*- and *Csb*-deficient background.

3.2 Material and Methods

3.2.1 Cell-lines

Isolation of primary *Csb*$^{-/-}$ (FVB/129Ola) and Csa$^{-/-}$ (C57BL6J/129Ola) MEFs and corresponding wild type cell lines has been described (51, 52). Cells were cultured in F10/DMEM (1:1) (Gibco) medium, supplemented with 10 % fetal calf serum and 50 μg/ml penicillin and streptomycin (Gibco). Spontaneously immortalized cell lines were obtained by continuous subculturing of primary MEFs.

Primary wild type, *Csa*$^{-/-}$ and Csb$^{-/-}$ keratinocytes from 2-day old mice (in a pure C57BL6 genetic background) were isolated as described (15, 19). Keratinocytes were cultured on collagen-fibronectin coated dishes in low calcium (0.05 mM) EMEM (BioWhittaker), supplemented with 8 % fetal calf serum (treated with chelex 100 (Biorad) to remove Ca^{2+} ions), 1 ng/ml keratinocyte growth factor (R&D systems) and 50 μg/ml penicillin and streptomycin (Gibco). Spontaneously immortalized cell lines were obtained by continuous subculturing of primary keratinocytes.

Isolation of *Csb*$^{-/-}$ and wild type embryonic stem cell lines, in a C57BL6 background has been described (12). *Csa*$^{-/-}$ ES cell lines are isolated following the same procedure (12). ES cells were maintained on gelatin-coated dishes in 50% buffalo rat liver cell conditioned DMEM / 50 % fresh DMEM supplemented with 15 % fetal calf serum, 0.1 mM non-essential amino acids (Gibco), 50 μg/ml penicillin and streptomycin (Gibco), 1000 U/ml leukemia inhibitory factor (Chemicon) and 0.1 mM 2-mercaptoethanol (Sigma).

3.2.2 Cellular sensitivity studies

For determination of the γ-ray-sensitivity of immortalized MEFs, keratinocytes and ES cells, cells were plated in 6cm dishes, at various dilutions. After 12-16 h, cells were irradiated with a single dose in the range of 0-8 Gy using a ^{137}Cs source. Cells were grown for 5 to 14 days, fixed, stained and counted to assess the colony-forming ability. All experiments were performed in triplicate. This protocol was adapted for determination of UV sensitivity of keratinocytes and ES cells by irradiating the cells with different doses of UV (254 nm, Philips TUV lamp) in stead of the γ-ray irradiation. UV sensitivity of MEFs was determined as described previously (43). Briefly, MEFs were exposed to different doses of UV (254 nm, Philips TUV lamp) and allowed to grow for another 3-5 days, before reaching confluency. The number of proliferating cells was estimated by scintillation counting of the radioactivity incorporated during a 3 hr pulse with [^{3}H]Thymidine (5μCi/ml, s.a. 40-60 Ci/mmole; Amersham). Cell survival was expressed as the ratio of ^{3}H incorporation in treated and non-treated cells. This protocol was adapted for paraquat survival by growing MEF cultures for 3-5 days in medium containing different concentrations of paraquat, followed by determination of the amount of proliferating cells as described above.

3.2.3 DEHP treatment of mice

Wild type, *Csb*$^{-/-}$ and *Csa*$^{-/-}$ female mice in a C57BL6 background were put on a DEHP (6000 ppm di(2-ethylhexyl)phthalate)(Sigma) containing diet or on a regular diet for four weeks (untreated: 5 wild type, 7 *Csb*$^{-/-}$ and 10 *Csa*$^{-/-}$ mice; treated 8 wild type, 7 *Csb*$^{-/-}$ and 13 *Csa*$^{-/-}$ mice). Animals were daily screened for discomfort. At the start and during the experiment animals were weekly weighed. The relative weight is calculated as the ratio between the weights of the mouse during the experiment versus the weight at the start of the experiment. Plotted is the ratio of these relative weights of treated versus untreated animals of the same genotype. Animal experiments were approved by the local animal ethical committee of the National Institute of Public Health and the Environment, Bilthoven, the Netherlands.

3.2.4 8-oxo-dG measurement in mouse liver

The 8-oxo-dG analyses were performed as previously described (41). In short, the DNA from approximately 200 mg liver or 160 mg kidney (1 kidney) was extracted and precipitated by a NaI-based procedure originally described by Nakae et al. (36) and Asami et al. (2). The DNA was resuspended in 10 mM Tris/0.1 mM desferrioxamine prior to enzymatic hydrolysis with nuclease P_1 and alkaline phosphatase (Boehringer Mannheim, Germany). The deoxyribonucleotides were then treated with DOWEX 1×8-400 ion-exchange resin (The Dow Chemical Company, Midland, MI) to remove I^- and finally filtered through a Micropure-EZ filter (Millipore, Bedford, MA). The levels of 8-oxo-dG and dGuo were measured using a HPLC system with electrochemical and UV detection. Peak areas were used for calculations. Calibrations curves were run together with each batch of samples.

3.3 Results

3.3.1 Csa$^{-/-}$ mouse embryonic fibroblasts lack hypersensitivity to γ-ray irradiation

Using [^{3}H]-thymidine incorporation assays, primary *Csa*$^{-/-}$ and *Csb* $^{-/-}$ mouse embryonic fibroblasts (MEFs) have been shown UV-sensitive (51, 52). Since γ-ray sensitivity can not be determined in this manner, but rather requires a clonogenic assay, and since primary cells are not suitable for performing clonogenic experiments, we first subcultured MEFs until spontaneous transformation resulted in formation of established cell lines. Similar to primary MEFs, immortalized *Csa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs are both UV sensitive (Figure 1A).
To critically investigate whether *Csa*$^{-/-}$ MEFs, like *Csb*$^{-/-}$ MEFs, display hypersensitivity to ionizing radiation, we performed clonogenic γ-ray survival experiments with spontaneously transformed wild type, *Csb*$^{-/-}$ and *Csa*$^{-/-}$ MEFs (at least 2 independent cell lines/genotype). In accordance with previous experiments (12), we observed that *Csb*$^{-/-}$ MEFs are approximately 2-fold more sensitive to γ-ray-irradiation than wild type MEFs. Surprisingly however, *Csa*$^{-/-}$ MEFs show a similar γ-ray survival as wild type MEFs (Figure 1B). The observed difference in γ-ray sensitivity between *Csa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs can not be attributed to differences in genetic backgrounds (FVB/129OLA and C57BL6/129OLA), since wild type MEFs from these different backgrounds attribute comparable γ-ray sensitivities (Figure 1C).

3.3.2 Csa$^{-/-}$ MEFs are not sensitive to paraquat

To confirm that *Csa*$^{-/-}$ MEFs are insensitive to oxidative DNA damage, we next tested the survival of these cell lines following treatment with the herbicide paraquat. Enzymatic reduction converts paraquat into radicals that react with molecular oxygen and thereby produce superoxide anions, giving rise to hydrogen peroxide (1). As shown previously, *Csb*$^{-/-}$ MEFs are sensitive to paraquat exposure (12). In marked contrast, *Csa*$^{-/-}$ MEFs possess paraquat sensitivity in the wild type range (Figure 1D). On the basis of the observed insensitivity of *Csa*$^{-/-}$ MEFs to both γ-rays and paraquat exposure, we conclude, that *Csa*$^{-/-}$ MEFs are not sensitive to oxidative DNA damage.

3.3.3 Cell type-specific differences in γ-ray sensitivity in Csa$^{-/-}$ and Csb$^{-/-}$ cells

To determine whether the lack of hypersensitivity to oxidative damage of the *Csa*$^{-/-}$ MEFs is a general feature, we extended our study to other cell types. To this end we isolated keratinocytes from wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ newborn mice, all in a genetically identical C57BL6 background to avoid any influence of genetic background. Similar to MEFs, spontaneously transformed *Csa*$^{-/-}$ and *Csb*$^{-/-}$ keratinocytes are UV sensitive, as determined by clonogenic assays (Figure 2A). Next, we performed clonogenic γ-ray survival experiments on wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ keratinocytes. In line with our observation in MEFs, we show that a *Csb* deficiency causes hypersensitivity of keratinocytes for γ-ray irradiation, whereas a deficiency for *Csa* does not make these cells more sensitive to γ-rays (Figure 2B).
Previously, we have demonstrated that *Csb*$^{-/-}$ embryonic stem cells (ES cells) attribute a slight γ-ray sensitivity (12). To extend this study to *Csa*$^{-/-}$ ES cells, we isolated pluripotent

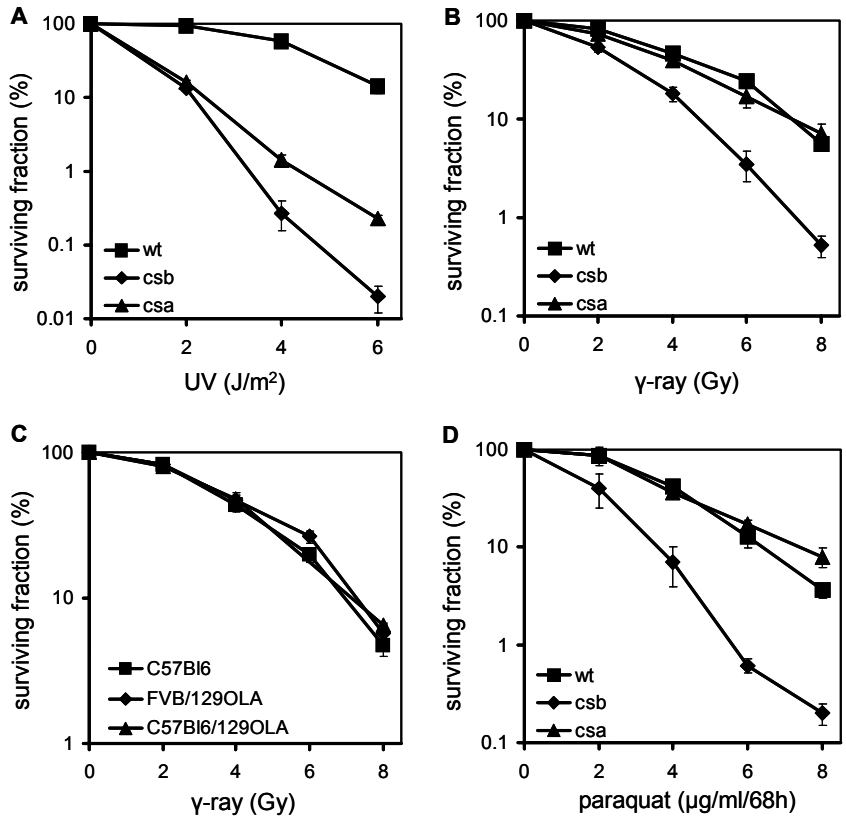

Figure 1

Survival of wild type, *Csb*$^{-/-}$ and *Csa*$^{-/-}$ mouse embryonic fibroblasts (MEFs) after exposure to UV, γ-rays or paraquat.

Panel A: UV survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles) and wild type (squares) MEFs. Experiments were performed at least two times per cell-line, with at least 2 cell lines per genotype as determined by the [^{3}H]-thymidine incorporation assay. Shown are representative curves. Bars indicate the standard error of the mean. Panel B: Survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles) and wild type (squares) MEFs after exposure to increasing doses of γ-rays, as determined by the colony assay. Shown is the average over at least 2 cell lines per genotype, as measured by at least three independent experiments. Bars indicate the standard error of the mean. Panel C: Survival of spontaneously immortalized wild type MEFs in different C57BL6 (squares), FVB/129OLA (diamonds) or C57BL6/129OLA (triangle) background, after exposure to increasing doses of γ-rays, as determined by the colony assay. Shown is the average of at least three independent experiments. Bars indicate the standard error of the mean. Panel D: Paraquat survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles) and wild type (squares) MEFs. Experiments were performed at least two times per cell-line, with at least 2 cell lines per genotype as determined by the [^{3}H]-thymidine incorporation assay. Shown are representative curves. Bars indicate the standard error of the mean.

ES cells from blastocysts derived from intercrosses between C*sa*$^{-/-}$ animals in a C57BL6 background. We first analyzed the UV sensitivity of these ES lines, and observed that *Csa*$^{-/-}$ and to a somewhat lesser extent *Csb*$^{-/-}$ ES lines are UV sensitive (Figure 2C). Subsequently, we performed a clonogenic γ-ray assay on wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ ES cells. Unexpectedly,

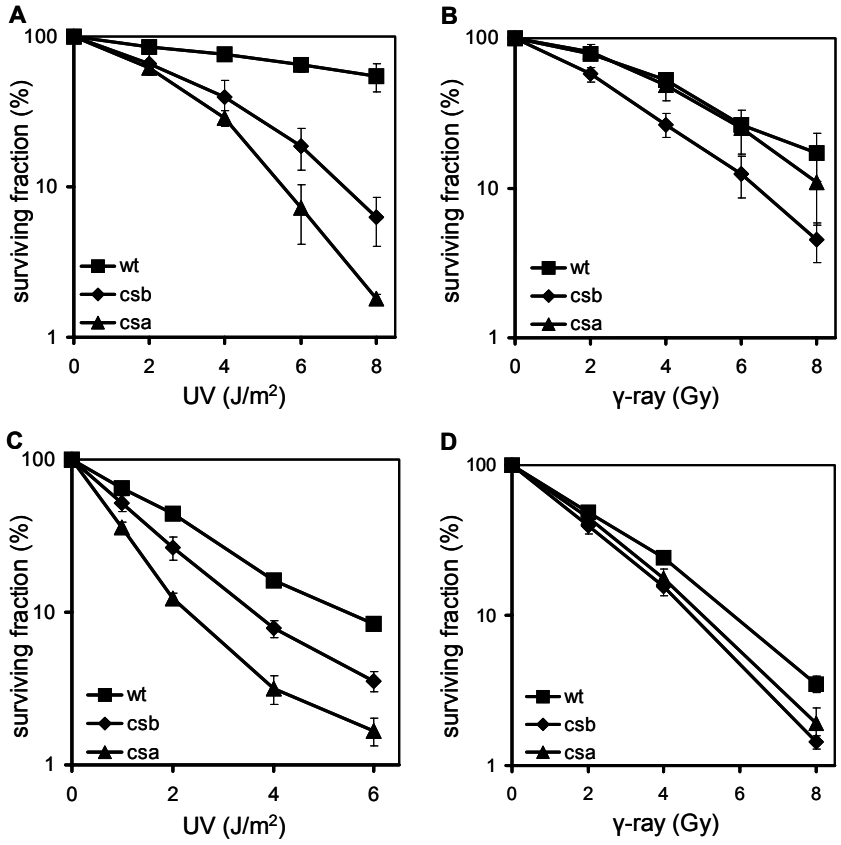

Figure 2

UV- and γ-ray-sensitivity of *Csb*$^{-/-}$, *Csa*$^{-/-}$ and wild type keratinocytes and embryonic stem cells.

Panel A and B: UV (A) and γ-ray (B) survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles) and wild type (squares) keratinocytes, as determined by the colony assay. The wild type curve represents the average of a wild type, *Csa*$^{+/-}$ and *Csb*$^{+/-}$ cell line, as determined in at least 3 experiments. The *Csb*$^{-/-}$ curve is the average over 2 cell lines, as measured by three independent experiments. The *Csa*$^{-/-}$ curve is the average of at least two independent experiments. Bars indicate the standard error of the mean. Panel C and D: UV (C) and γ-ray (D) survival of *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles) and wild type (squares) ES cells, as determined by the colony assay. Shown is the average over at least 2 cell lines per genotype, as measured by at least three independent experiments. Bars indicate the standard error of the mean.

since *Csa*$^{-/-}$ MEFs and keratinocytes display a wild type γ-ray sensitivity, we found a slight γ-ray sensitivity in three independent *Csa*$^{-/-}$ ES cell lines, comparable to the γ-ray sensitivity in *Csb*$^{-/-}$ ES cells (Figure 2D). A fourth *Csa*$^{-/-}$ ES cell line exhibited wild type sensitivity, which might be associated with loss of pluripotency.

We conclude that a *Csb*-deficiency causes cellular sensitivity to γ-ray-irradiation in MEFs, keratinocytes and ES cells, however with a significant difference in magnitude. A deficiency for *Csa* has no effect on cellular sensitivity for γ-rays in MEFs and keratinocytes, whereas in ES cells a *Csa* deficiency causes a slight hypersensitive phenotype.

To investigate whether a combined *Csa-Csb* deficiency would act either synergistically or epistatic, we generated double mutant *Csa*$^{-/-}$/*Csb*$^{-/-}$ mice. Double mutant animals appeared normal and do not display any overt phenotype up to an age of 18 months. A detailed comparative study of the phenotype of *Csa*$^{-/-}$, *Csb*$^{-/-}$ and *Csa*$^{-/-}$/*Csb*$^{-/-}$ mice is underway. Next, we generated established *Csa*$^{-/-}$/*Csb*$^{-/-}$ MEFs and showed that they do not display an increased sensitivity to UV- or γ-ray-irradiation, when compared to the most sensitive single mutant. These findings indicate that both proteins function in the same pathway and are epistatic (data not shown).

3.3.4 Different response of Csb$^{-/-}$ and Csa$^{-/-}$ mice to a DEHP-containing diet

It is not known how the observed cell-type and genotype specific γ-ray response in cultured wild type, *Csb*$^{-/-}$ and *Csa*$^{-/-}$ cells can be extrapolated to cells in the context of the whole animal. Therefore, we aimed at investigating the sensitivity of the intact animal to oxidative DNA damage. As a potential oxidative damage inducing agent we used the plasticizer di(2-ethylhexyl)phthalate (DEHP), which causes proliferation of peroxisomes in the liver by activation of peroxisome proliferator-activated receptor alpha (PPAR alpha) (58). This is believed to induce higher oxidative stress in the liver. To test whether indeed DEHP could be used as a proper method to induce oxidative stress in mice, we administrated wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ female mice ($n \geq 5$) with food containing 6000 ppm DEHP for four weeks. The control group received unmodified food. Induction of oxidative DNA damage was assessed by (double-blind) measurement of the 8-oxo-2'-deoxyguanosine (8-oxo-dG) damage level in the DNA of treated versus untreated animals. We observed that DEHP-treated mice showed a 2.5 times higher 8-oxo-dG content in the liver than untreated animals (Figure 3A), whereas no significant induction of 8-oxo-dG in kidneys is observed in any genotype tested (Figure 3B). This result confirms that DEHP is a liver specific toxic compound, inducing oxidative DNA damage. Moreover there seems to be no difference in accumulation of 8-oxo-dG between wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice.
As read-out of the DEHP sensitivity of the mouse, we used the overall condition, as determined by body weight. The weight of every mouse was compared to its original weight before treatment. Plotted in Figure 3C is the relative weight of treated animals divided by that of untreated animals (x100), showing a clear weight loss in *Csb*$^{-/-}$ mice compared to wild type and *Csa*$^{-/-}$ mice ($n \geq 5$) (Figure 3C). Surprisingly, wild type and *Csa*$^{-/-}$ (although to a somewhat lesser extent) animals fed with a DEHP containing diet gain more weight than untreated animals, from the same genotype. This phenomenon is probably due to an increased food intake in treated animals (Figure 3D). We suggest that *Csb*$^{-/-}$ mice at the organismal level are sensitive for oxidative DNA damage, reflecting the observed hypersensitivity for oxidative DNA damage in various cultured cell types. *Csa*$^{-/-}$ mice are far less sensitive for oxidative damage caused by DEHP treatment, which is in agreement with the observed lack of hypersensitivity of *Csa*$^{-/-}$ keratinocytes and MEFs for oxidative DNA damage.

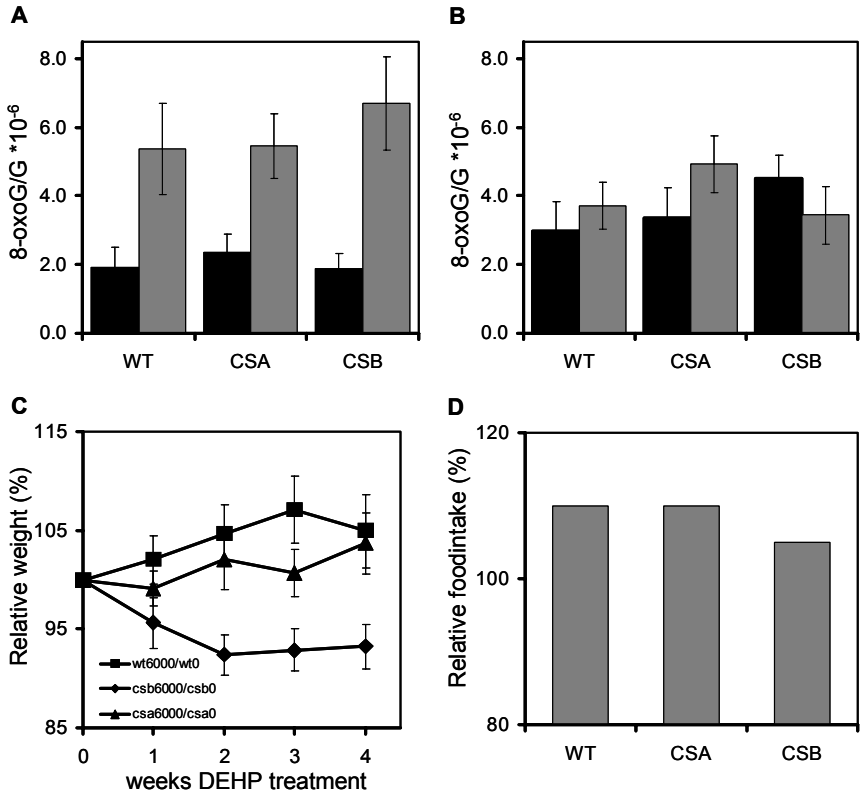

Figure 3
DEHP-sensitivity of *Csb*$^{-/-}$, *Csa*$^{-/-}$ and wild type mice.
Panel A and B: Average 8-oxo-dG/G ratio in livers (A) and kidneys (B) of animals after 4 weeks of DEHP-containing (grey) or control diet (black) in *Csb*$^{-/-}$, *Csa*$^{-/-}$ and wild type mice, as measured by using a HPLC system with electrochemical and UV detection. Shown is the average 8-oxo-dG/G ratio of at least 5 animals per group. Bars indicate the standard error of the mean. Panel C: Relative weight of *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles) and wild type (squares) mice fed with food containing 6000 ppm DEHP versus animals on a regular diet. Shown is the average weight-ratio of at least 5 animals per group. Panel D: Relative food intake of *Csb*$^{-/-}$, *Csa*$^{-/-}$ and wild type mice. Depicted is the ratio of average food intake per week over the whole treatment of mice fed with 6000 ppm DEHP-containing food versus regular diet.

3.4 Discussion

3.4.1 Sensitivity of Csb$^{-/-}$ *cells and mice to oxidative stress*

Using genetically homogeneous mouse models, we investigated the effect of a *Csb* deficiency on oxidative DNA damage sensitivity in various cell types and at the level of the intact organism. As shown previously (12), cellular γ-ray sensitivity in general markedly depends on cell type. For instance, wild type ES cells are significantly more sensitive to γ-ray irradiation than either MEFs or keratinocytes. In line with previous findings (12, 28), we show oxidative DNA damage-sensitivity in *Csb*-deficient fibroblasts, keratinocytes and ES

cells, although to different relative extent. Moreover, also at the level of the intact animal, we could demonstrate a significant effect of a *Csb* deficiency on sensitivity for oxidative DNA damage, as illustrated by the DEHP experiment. This finding is in line with the previously observed tendency of *Csb*$^{-/-}$ mice to be more sensitive for the toxic/killing effects of γ-rays than wild type mice (12). In light of the recent discussion about the validity experiment showing TCR of oxidative DNA lesions (10, 16, 27), these data clearly show that *Csb* deficiency causes sensitivity for oxidative DNA damage in various cell types and on the organismal level.

3.4.2 A cell-type-specific Csa effect on γ-ray response

Previously, one primary human fibroblast cell line derived from a CSA patient was investigated, and reported to display a slight γ-ray sensitivity (28), suggesting that CSB and CSA are similar in this respect. However, because of the large genetic variation in the human population on the one hand, and the only minor difference in sensitivity on the other hand, it is difficult to draw firm conclusions on the potential link between a *CSA* deficiency and γ-ray sensitivity on the basis of the human fibroblast studies. Surprisingly, we fail to show an increased sensitivity for oxidative DNA damage in *Csa*$^{-/-}$ MEFs and keratinocytes. DEHP-treated *CSA*$^{-/-}$ mice, in contrast to *Csb*$^{-/-}$ mice fail to show a pronounced reduction in body weight compared to untreated mice, indicating that *Csa*$^{-/-}$ animals are barely sensitive to the 2-3 fold higher levels of 8-oxo-dG lesions in the liver. Despite the absence of oxidative damage sensitivity in *Csa*$^{-/-}$ MEFs, keratinocytes and animals, we could still demonstrate a slight γ-ray sensitivity in *Csa*$^{-/-}$ ES cells, which compares well to that observed in *Csb*$^{-/-}$ ES cells.
These findings suggest that in a wide range of cell types, CSA is dispensable for the response to oxidative DNA damage. Yet, given the slight γ-ray sensitivity of *Csa*$^{-/-}$ ES cells and the tendency for somewhat less growth in DEHP treated *Csa*$^{-/-}$ mice compared to wild type mice, its function might be needed in specific cell types. To our knowledge, these data provide the first cell biological evidence that CSA and CSB proteins possess separable functions.

3.4.3 Different functions of CSA and CSB?

These observed biological differences between *Csa*- and *Csb*-deficient cells and mice, as uncovered by the divergence in oxidative damage sensitivity seem to be in contrast with the widely accepted notion that both CSA and CSB function in the same subpathway of transcription-coupled repair (26, 28, 54). However, biochemical analysis of the CSA and CSB protein has revealed marked differences that are suggestive for a potential difference in function. The CSA protein resides in a 420 kDa complex, whereas CSB is part of a >700 kDa complex (53). While CSA was found to be a constituent of a complex containing DDB1, cullin 4A, Roc1 and the COP9 signalosome (17), CSB was found to interact with RNA polymerase II (48, 53), XPA, XPG, TFIIE and TFIIH (23, 42) and with several splicing factors. Moreover, a role of CSB, but most likely not of CSA in RNA Pol I

transcription has been suggested (5). Also, a *CSB* rather than a *CSA* deficiency might cause metaphase fragility for genes encoding specific highly structured transcripts (59).
In contrast, arguments that CSA and CSB in some way might function together are provided by the reported *in vitro* interaction between CSA and CSB (18). Moreover, a recent study shows that genotoxic stress mediated translocation of CSA to the nuclear matrix is hampered in *CSB*-deficient cell lines (24). Most importantly, there is no evidence for a difference in the clinical appearance of CSA and CSB patients (37, 44). Similarly, the phenotype of *Csa* $^{-/-}$ and *Csb*$^{-/-}$ mice fails to reveal obvious differences, since both mouse models are UV-sensitive, lack TCR (51, 52), show photoreceptor loss upon aging (51)(T.G.M.F. Gorgels, personal communication) and die before weaning when combined with a *Xpa* or *Xpc* deficiency (35, 51)(I. van der Pluijm, personal communication). Finally, our *Csa*/*Csb* double mutant cells and mice provide genetic evidence for an epistatic relationship and involvement in the same pathway.
In conclusion, arguments both in favor and against differences between CSA and CSB functions exist.

3.4.4 Possible functions of CSA and CSB in different biological processes

To find an explanation for the observed differences in γ-ray sensitivity between *Csa*- and *Csb*-deficient cells, we systematically list the possible functions of CSA and CSB in response to oxidative DNA damage.
Transcriptional bypass
It has been shown that oxidative DNA lesions (like 8-oxo-dG) can block RNA polymerase II, although far less efficiently than UV-induced lesions do (25). *E. coli* RNA polymerase can bypass 8-oxo-dG by putting either adenine or cytosine opposite the 8-oxo-dG (6, 57). For the yeast counterpart of CSB, *Rad26*, strong indications for a role in transcriptional bypass of MMS-induced DNA damage have been found (30). Recent reports show that also human RNA pol II is able to bypass oxidative DNA lesions *in vitro* (25, 49). Since, CSB has already been associated with transcription elongation (especially of damaged templates, pause sites and highly structured RNAs), these data suggest a possible role of CSB in transcriptional bypass of some oxidative DNA lesions. This function may probably require the reported chromatin remodeling activity of the CSB protein (8). Transcriptional bypass might be CSA-independent, since for example CSA appears not to be involved in stimulation of transcription of genes for highly structured RNAs (59). This function of CSB may not be relevant to UV-induced damage as photolesions form strong RNA polymerase blocks and are therefore not subject to transcriptional bypass.
Transcription-coupled repair
Transcription-coupled repair of UV-induced lesions, as well as recovery of RNA synthesis after UV treatment, has been demonstrated to depend on both CSA and CSB (33, 54). Although bypass of 8-oxo-dG is possible, the absence of the *mfd* protein in *E. coli* (required for TCR) causes higher bypass rates, suggesting that TCR is acting on 8-oxo-dG (6). Also, several reports point to a function of mammalian CSB in TCR of oxidative DNA lesions ((12) and references therein). The influence of CSA on TCR of oxidative DNA lesions is unknown.

Ubiquitination of RNA polymerase II

Upon UV treatment, stalled RNA polymerase can be ubiquitinated in a CSA- and CSB-dependent manner (7, 29, 40). This ubiquitination might be needed for TCR and/or the degradation of the stalled polymerase. The latter event would allow access of the repair machinery to the lesion and subsequent recovery of RNA synthesis (34). The potential link between CSA and ubiquitination of RNA polymerase II may lie in the fact that CSA is known to regulate the ubiquitin ligase activity of the complex containing DDB1, cullin 4A, Roc1 and the COP9 signalosome (17), and that this complex could, in some way, be involved in the ubiquitination of the polymerase. However, CSA and CSB proteins are not prerequisite for the break down of RNA polymerase II, as *CSA* and *CSB* deficient cells can still degrade the polymerase (32). Ubiquitination of RNA polymerase II has been shown to occur also after exposure of cells to oxidative DNA damage (22). However, the mechanism of ubiquitination differs from that provoked by UV-light and is not CSA- or CSB-dependent (22).

Other repair pathways

Evidence for an indirect role of CSB in BER mediated global genome repair and mitochondrial DNA repair of oxidative DNA lesions has been reported (13, 38, 45, 46, 50).

3.4.5 Explanation of Csa and Csb related differences in oxidative damage response

Taking into account the possible functions of CSA and CSB, we discuss two scenarios that might explain our findings.

1. Oxidative DNA damage does not have a major impact on the onset of Cockayne syndrome features. Although the effect of CSA on the response to oxidative DNA damage is poorly investigated, we do not favor this explanation, since this would argue against a large body of evidence supporting a function of CSB in repair of oxidative DNA damage and the general importance of oxygen radicals (12, 13, 26, 28, 38, 45, 46, 50).
2. The observed differences in oxidative stress sensitivity between *Csb*$^{-/-}$ and *Csa*$^{-/-}$ cells and animals are the consequence of the acute response of a heavily challenged system. The conditions used in cellular survival are not representative for the processes in a patient that cause the CS manifestations. Although in acute (high dose) experiments *Csa*- and *Csb*-deficient cells differ in sensitivity, under physiological conditions a deficiency for *Csa* or *Csb* may cause a similar effect upon exposition to constitutive low levels of oxidative DNA damage. The hyper-sensitivity of *Csb*$^{-/-}$ cells and mice could be due to other functions of CSB. For example *Csb*$^{-/-}$ cells could be unable to perform adequate transcription bypass, which might be CSA-independent. In the presence of high amounts of oxidative DNA damage, inefficient bypass in *Csb*$^{-/-}$ cells might lead to cell death. The absence of a significant RNA synthesis block after high γ-ray doses suggests that the majority of oxidative DNA lesions are bypassed. In contrast, since the major UV lesions are probably not bypassed during transcription, UV sensitivity of *Csa*- and *Csb*-deficient cell lines mainly reflects the inability of these cells to perform TCR and RNA synthesis recovery. These processes depend on both CSA and CSB for removal of the stalled RNA polymerase.

The second explanation suggests that the γ-ray sensitivity in *Csb*$^{-/-}$ cells could be partly attributed to other processes than a TCR-defect. Consequently, the lack of γ-ray sensitivity in *Csa*$^{-/-}$ cells does not per sé contradict a role of CSA in TCR of oxidative DNA damages. Moreover, the observed γ-ray sensitivity in *Csa*$^{-/-}$ ES cells suggests some function of CSA in response to oxidative DNA damage. Therefore a deficiency in TCR in specific cell types could still underlie CS symptoms.

3.4.6 Concluding remarks

Comparison of the response of isogenic cells of different tissues to ionizing radiation has demonstrated significant variation in sensitivity and in dependence on CS proteins. This finding suggests the use of different genome-caretaking strategies by different cell types and tissues. In addition, our study reveals that the absence of CSA or CSB has a different impact on the response to oxidative DNA damage, and consequently that both proteins are not functionally equivalent. However, this appears not to be reflected in the CS phenotype of patients and mice. The increased sensitivity for ionizing radiation due to *Csb* inactivation is consistent with a role of CSB protein in cellular resistance to oxidative damage. The notion that the TCR defect for UV lesions in both *Csa* and *Csb* mutants is the same suggests that the sensitivity to ionizing radiation of *Csb* deficient cells is due to some extra function of the CSB protein. However, this latter function does not significantly influence the clinical outcome. The above findings also highlight a hitherto unanticipated functional dissimilarity of the main TCR factors CSA and CSB.

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Chapter 4

Mitomycin C sensitivity in Cockayne cells and mice

Manuscript in preparation

Mitomycin C sensitivity in Cockayne cells and mice

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Abstract

Mutations in the *CSA* or *CSB* gene cause Cockayne syndrome (CS), a rare inherited disorder, characterized by UV sensitivity, severe neurodevelopmental and progeroid symptoms. CS proteins function in the transcription-coupled repair pathway of nucleotide excision repair (NER), responsible for the removal of UV-induced and other helix-distorting lesions from the transcribed strand of active genes. Several lines of evidence support the notion that the CS-B transcription-coupled repair (TCR) defect extends to other non-NER type transcription-blocking lesions, like oxidative DNA damage. Surprisingly, a *Csa* defect does not inflict sensitivity for oxidative DNA damage in various mouse cell lines, indicating a difference in cellular response of *Csa*- and *Csb*-deficient cell lines. Whether *Csa* or *Csb* deficiency causes sensitivity for other types of non-classical-NER transcription blocking lesions, like DNA inter-strand cross-links is so far unknown. We used genetically defined CS mouse models to examine the relationship between the *Csa* or *Csb* defect and sensitivity to the cross-linking chemotherapeuticum MMC in different cell types and at the level of the intact organism. The main findings are:
We observed that whereas $Csb^{-/-}$ and $Xpa^{-/-}$ MEFs are only slightly MMC sensitive, $Csa^{-/-}$ MEFs are hypersensitive to MMC. This sensitivity is not associated with a block in RNA synthesis; instead $Csa^{-/-}$ MEFs fail to recover DNA synthesis after MMC treatment. In addition, we show that ES cells are intrinsically hypersensitive to MMC, when compared to MEFs, and that a *Csa, Csb* or *Xpa* defect not further enhances the MMC sensitivity of ES cells. Finally, $Csa^{-/-}$ and $Csb^{-/-}$ mice, and to a lesser extent $Xpa^{-/-}$ mice are MMC sensitive. These findings establish a cell-specific sensitivity to MMC and suggest a cell- and organ specific contribution of inter-strand cross-links (generated by cellular metabolites) in the onset of the clinical phenotype of CS.

4.1 Introduction

Cockayne syndrome (CS) is a rare inherited disorder, characterized by a plethora of physical and mental manifestations, including UV hypersensitivity, severe postnatal growth failure, chachectic dwarfism, progressive mental retardation, retinal degeneration, sensori-neural hearing loss, skeletal abnormalities and a bird-like face. Several of these symptoms classify CS in the category of premature aging disorders. Complementation studies revealed the involvement of two genes in the onset of CS, *CSA* and *CSB* (for review see (3, 24)). Mutations in these genes have been associated with a specific defect in the transcription-coupled subpathway of nucleotide excision repair (TC-NER), which is responsible for removal of transcription-blocking lesions from the transcribed strand of active genes (30). The other subpathway, responsible for repair of helix-distorting nucleotide damage in the entire genome and referred to as global genome NER (GG-NER) is unaffected in CS. Both subpathways differ in the way damage is recognized, but subsequently use the same proteins for the actual excision of DNA lesions as a ±30 nt oligonucleotide, and resynthesis of the resulting single stranded DNA gap. Defects in genes involved in either the recognition of DNA damage in the GG-NER subpathway, or with a function in the core reaction of NER lead to another NER syndrome: xeroderma pigmentosum (XP). A total of seven genes have been associated with NER-deficient XP, referred to as *XPA* through *XPG*. Although XP resembles CS in pronounced UV sensitivity, major differences are observed. XP, in contrast to CS, is characterized by pigmentation abnormalities in sun-exposed skin and a more than 1000-fold elevated skin cancer risk. Moreover, the pronounced premature aging features of CS are not observed in XP. Interestingly, a subset of mutations in *XPB, XPD* and *XPG* may give rise to a combined XP and CS phenotype (for review see (3, 12)).

The notion that mutations in *CSA* or *CSB* cause a defect in one subpathway of NER is difficult to reconcile with the more severe symptoms observed in CS patients compared to XP patients, of which many carry mutations in both NER subpathways. Additional roles for CS proteins have been put forward. Specifically for *CSB*, an auxiliary role in transcription has been suggested (2, 8, 26). Moreover, *CSB* cells display an increased sensitivity for oxidative DNA damage (7, 20). A defect in transcription-coupled repair (TCR) of non classical NER lesions (e.g. oxidative DNA damage) has been for long a paradigm to explain CS features as well as a possible cause of aging (18-20). Whether TCR of oxidative DNA damage functions via NER or BER is unknown, therefore we refer to TCR when not per se TC-NER is meant. However, recent observations have shown that several *Csa*-deficient cell lines and also $Csa^{-/-}$ mice are not hypersensitive to oxidative DNA damage, suggesting that oxidative DNA damage is probably not the only cause for the observed CS features and aging (see chapter 3).

Since, defective TC-NER is so far the only common molecular feature of *CSA*- and *CSB*-deficient cell lines, we wondered whether other endogenously occurring transcription-blocking DNA lesions could be implicated in the etiology of CS. Endogenous agents formed during lipid peroxidation, such as malondialdehyde, can lead to inter-strand cross-links (4). By definition DNA inter-strand cross-links are a physical block for polymerases, since strand separation is prevented. In this study we used the chemotherapeuticum mitomycin C (MMC) as a model substrate causing inter-strand cross-links. We exploited

mouse models for CSA, CSB and XPA (6, 28, 29) to investigate whether CS cells and animals are hypersensitive to MMC. Usage of these mouse models in an isogenic genetic background (C57BL6/J) allows a unique comparison of defined genetic defects without the confounding effect of unknown differences in genetic make-up.

4.2 Material and Methods

4.2.1 Cell lines

Isolation of primary *Xpa*$^{-/-}$ (C57Bl6J/129Ola) *Csb*$^{-/-}$ (FVB/129Ola) and Csa$^{-/-}$ (C57Bl6J/129Ola) MEFs and corresponding wild type cell lines has been described (6, 28, 29). Cells were cultured in F10/DMEM (1:1) (Gibco) medium, supplemented with 10 % fetal calf serum and 50 μg/ml penicillin and streptomycin (Gibco). Spontaneously immortalized cell lines were obtained by continuous subculturing of primary MEFs.
Isolation of *Xpa*$^{-/-}$, *Csb*$^{-/-}$, *Csa*$^{-/-}$ and wild type embryonic stem (ES) cell lines, in a C57Bl6 background have been described (7)(see also chapter 3). ES cells were maintained on gelatin-coated dishes in 50% buffalo rat liver cell conditioned DMEM / 50 % fresh DMEM supplemented with 15 % fetal calf serum, 0.1 mM non-essential amino acids (Gibco), 50 μg/ml penicillin and streptomycin (Gibco), 1000 U/ml leukemia inhibitory factor (Chemicon) and 0.1 mM 2-mercaptoethanol (Sigma).

4.2.2 *Cellular sensitivity studies*

For determination of the MMC sensitivity of immortalized MEFs and ES cells, cells were plated in 6 cm dishes, at various dilutions. After 12-16 h, cells were treated for 1 hour with mitomycin C (0.2-8 μg/ml, Kyowa Hakko Kogyo Co., Ltd. Tokyo). Cells were grown for 5 to 14 days, fixed, stained and counted to assess the colony-forming ability. All experiments were performed in triplicate.
RNA synthesis recovery after UV or MMC treatment was performed as described (22), with several modifications. Briefly, cell were seeded in 6 well plates and after 24 h labeled with [2-^{14}C]-thymidine (50 μCi/ml, s.a. 50 mCi/mmole; Amersham) for 16 h. Cells were irradiated with 10 J/m^2 UV (254nm, Philips TUV lamp) or treated for 1 h with 2 or 8 μg/ml MMC. Three and six hours after treatment with UV or MMC, RNA synthesis was determined by 1h labeling with [5,6-^{3}H]-uridine (10 μCi/ml, s.a. 50 Ci/mmole; Amersham). ^{3}H and ^{14}C ratios were measured by scintillation counting. The relative RNA synthesis was expressed as the quotient of these ^{3}H-^{14}C ratios of treated over untreated cells.
DNA synthesis after MMC treatment was performed as described (11), with several modifications. Briefly, cells were seeded in 6 well plates and after 24 h labeled with [2-^{14}C]-thymidine (50 μCi/ml, s.a. 50 mCi/mmole; Amersham) for 16 h. Cells were treated for 1 h with 2 μg/ml MMC. DNA synthesis was determined by 1h labeling with [*methyl*-^{3}H]-thymidine (10 μCi/ml, s.a. 50 Ci/mmole; Amersham) after 7, 16, 24 and 48 hours post-treatment. ^{3}H and ^{14}C ratios were measured by scintillation counting. The relative DNA synthesis was expressed as the quotient of these ^{3}H-^{14}C ratio of treated over untreated cells.

4.2.3 MMC treatment of mice

Wild type, *Csb*$^{-/-}$, *Csa*$^{-/-}$ and *Xpa*$^{-/-}$ mice (males; 2-4 months) in a C57Bl6 background were i.p. injected with increasing doses MMC; 1 mg MMC per kg bodyweight (wild type; 4, *Csb*$^{-/-}$; 7, *Csa*$^{-/-}$; 6 and *Xpa*$^{-/-}$; 6 animals); 2.5 mg MMC/kg (wild type; 0, *Csb*$^{-/-}$; 12, *Csa*$^{-/-}$; 9 and *Xpa*$^{-/-}$; 0 animals); 5 mg MMC/kg (wild type; 0, *Csb*$^{-/-}$; 14, *Csa*$^{-/-}$; 11 and *Xpa*$^{-/-}$; 5 animals); 7.5 mg MMC/kg (wild type; 12, *Csb*$^{-/-}$; 5, *Csa*$^{-/-}$; 4 and *Xpa*$^{-/-}$; 10 animals); 10 mg MMC/kg (wild type; 15, *Csb*$^{-/-}$; 10, *Csa*$^{-/-}$; 2 and *Xpa*$^{-/-}$; 4 animals). Animals were housed in isolators and followed for 15 days after the treatment. Animals were daily screened for discomfort. Differences in MMC sensitivity of wild type and mutant mice were assessed on the basis of the time interval between the last dose and death (latency period). For significance test Kaplan-Meier statistics with a log-rank testing was performed (wild type vs. *Csb*$^{-/-}$, *Csa*$^{-/-}$ and *Xpa*$^{-/-}$ based on 7.5 and 10 mg MMC/kg; *Xpa*$^{-/-}$ versus *Csb*$^{-/-}$ and *Csa*$^{-/-}$ based on 5, 7.5 and 10 mg MMC/kg; *Csb*$^{-/-}$ vs *Csa*$^{-/-}$ based on 1, 2.5, 5, 7.5 and 10 mg MMC/kg). Animal experiments were approved by the local animal ethical committee of the National Institute of Public Health and the Environment, Bilthoven, the Netherlands.

4.3 Results

4.3.1 Csa$^{-/-}$ *mouse embryonic fibroblasts are hypersensitive to mitomycin C*

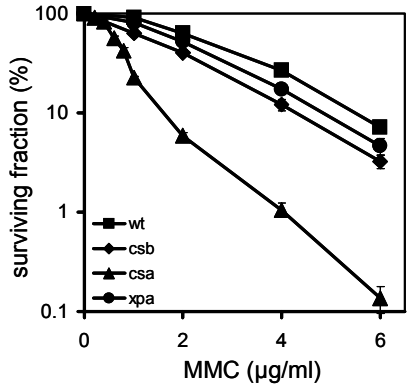

Figure 1
MMC survival of *Csb*$^{-/-}$, *Csa*$^{-/-}$, *Xpa*$^{-/-}$ and wild type ES cells.
Survival of spontaneously immortalized *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles), *Xpa*$^{-/-}$ (circles) and wild type (squares) MEFs after exposure to increasing doses of mitomycin C, as determined by the colony assay. Shown is the average of at least three experiments, with for each genotype at least two independent cell-lines. Bars indicate the standard error of the mean.

As part of a systematic characterization of the sensitivity spectrum of CS cells to different classes of genotoxic agents, we investigated whether *Csa*-deficient cell lines are hypersensitive to the cross-linking chemotherapeuticum mitomycin C (MMC). Clonogenic MMC survival assays on spontaneously transformed wild type and *Csa*$^{-/-}$ mouse embryonic fibroblasts (MEFs) revealed that *Csa*$^{-/-}$ MEFs are at least 4 times more sensitive to MMC than their wild type counterparts (Figure 1, D37). Since MMC sensitivity can be influenced by differences in proliferation rate, we checked at least two independent cell lines with different proliferation rate per genotype. We did not mark any correlation between proliferation rate and sensitivity, arguing that indeed the observed MMC sensitivity in *Csa*$^{-/-}$ MEFs is due to the *Csa*-defect.
Mitomycin C causes a spectrum of DNA lesions, including inter-strand cross-links, oxidative DNA damage and mono-adducts.

A defect in transcription-coupled repair of these mono-adducts could cause the observed increase in MMC sensitivity in *Csa*$^{-/-}$ MEFs, compared to wt MEFs. This hypothesis predicts that also *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ MEFs (that are both TC-NER deficient) would be hypersensitive for MMC. To test this model, we performed clonogenic MMC survival on *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ MEFs, showing a marginal increase in MMC sensitivity compared to wild type MEFs (Figure 1). Therefore we conclude that the observed MMC sensitivity in *Csa*$^{-/-}$ MEFs is not associated with a TC-NER defect of mono-adducts.

4.3.2 Severe MMC sensitivity in Csa$^{-/-}$ MEFs is not caused by a RNA synthesis block

Given the fact that CSA is involved in transcription-coupled repair, we determined transcription activity in *Csa*$^{-/-}$, *Csb*$^{-/-}$ and wild type MEFs after MMC treatment. We failed to show any block in RNA synthesis after MMC treatment at 2 or 8 μg/ml in wild type, *Csa*$^{-/-}$ or *Csb*$^{-/-}$ MEFs, while UV treatment (10 J/m²) caused a clear block in RNA synthesis (Figure 2A, 2 μg MMC/ml not shown). It is therefore unlikely, that a stalled RNA polymerase causes the observed MMC sensitivity in *Csa*$^{-/-}$ MEFs.
To further elucidate the mechanism causing the observed hypersensitivity to MMC in *Csa*$^{-/-}$ MEFs, we measured the induction of apoptosis after MMC treatment in wild type, *Csb*$^{-/-}$ and *Csa*$^{-/-}$ MEFs. Whereas we observed a clear induction of apoptosis 24 h after UV-treatment (10 J/m²), we fail to show any induction of apoptosis in wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs treated with 2 μg/ml MMC up to 72h after treatment (subG1-assay; data not shown). Also,

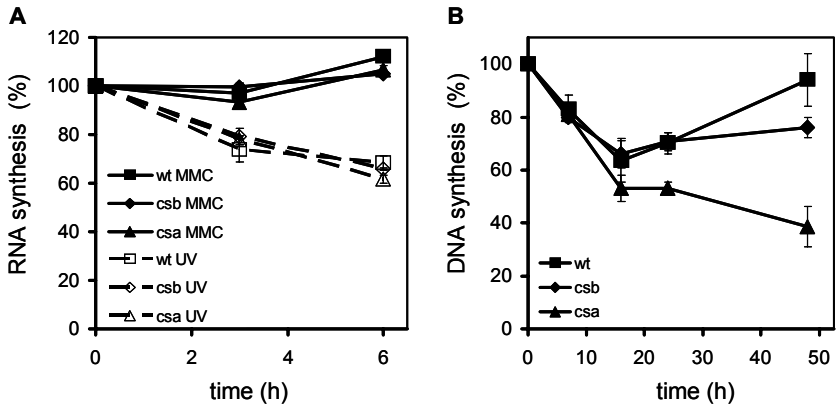

Figure 2
RNA and DNA synthesis after MMC treatment in wild type, *Csa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs after MMC treatment.
Panel A: RNA synthesis of spontaneously immortalized wild type (squares), *Csa*$^{-/-}$ (triangles) and *Csb*$^{-/-}$ (diamonds) MEFs 3 and 6 hours after treatment with 8 μg/ml MMC (closed symbols) or 10 J/m² UV (open symbols). Experiments were performed at least two times per cell-line, with at least 2 cell lines per genotype as determined by the [³H]-uridine incorporation assay. Shown are representative curves. Bars indicate the standard error of the mean. Panel B: DNA synthesis of spontaneously immortalized wild type (squares), *Csa*$^{-/-}$ (triangles) and *Csb*$^{-/-}$ (diamonds) MEFs 7, 16, 23 and 48 hours after treatment with 2 μg/ml MMC, as determined by [³H]-thymidine incorporation. Shown is the average of at least two experiments, with for each genotype at least two independent cell lines. Bars indicate the standard error of the mean.

treatment of *Csa*$^{-/-}$ MEFs with 8 μg/ml MMC does not result in a strong apoptotic response at early time points (24h), but induces apoptosis at 72h in one of the two *Csa*$^{-/-}$ MEF lines tested (data not shown). In contrast, the other *Csa*$^{-/-}$ MEF line showed accumulation of cells in the G2 phase of the cell cycle (data not shown). Therefore we conclude that apoptosis observed in MMC-treated *Csa*$^{-/-}$ MEFs is probably a secondary effect, rather than the primary cause of MMC sensitivity of these cells.

4.3.3 DNA synthesis block in MMC treated Csa$^{-/-}$ *MEFs*

Besides a defect in recovering from a damage-induced RNA synthesis arrest, also a defect in recovery from a damage-induced block in DNA synthesis has been demonstrated for CSA and CSB fibroblasts (21, 27). To determine whether MMC treatment causes a DNA synthesis block in MEFs, and if so, whether *Csa*$^{-/-}$ MEFs are defective in recovering from such an arrest, we measured DNA synthesis after MMC treatment by the [^{3}H]-thymidine incorporation assay. Upon treatment with 2 μg/ml MMC, we observe a clear drop in DNA synthesis in all genotypes tested. Moreover, after 24 and 48 h a significant recovery of the DNA synthesis arrest is observed in wild type and *Csb*$^{-/-}$ MEFs, while *Csa*$^{-/-}$ MEFs fail to recover DNA synthesis up to 48 h after treatment (Figure 2B). Similar results were obtained when cells were exposed to 4 and 8 μg/ml MMC (data not shown). This inability to restore DNA synthesis might significantly contribute to the observed MMC sensitivity of *Csa*$^{-/-}$ MEFs.

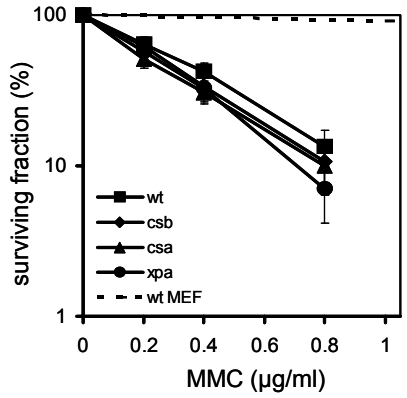

Figure 3
MMC survival of *Csb*$^{-/-}$, *Csa*$^{-/-}$, *Xpa*$^{-/-}$ and wild type ES cells.
Survival of *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles), *Xpa*$^{-/-}$ (circles) and wild type (squares) ES cells after exposure to increasing doses of mitomycin C, as determined by the colony assay. Shown is the average of at least three experiments, with for each genotype at least two independent cell-lines. Bars indicate the standard error of the mean. For comparison, the survival of wild type MEFs following MMC trewatment is indicated by the dashed line.

4.3.4 Cell type-specific differences in MMC sensitivity, associated with the Csa$^{-/-}$ *defect*

To determine whether the sensitivity of *Csa*$^{-/-}$ MEFs to MMC is a general feature, we extended our study to another very different cell type. In contrast to data obtained in MEFs, clonogenic MMC survival experiments on wild type, *Csa*$^{-/-}$, *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ embryonic stem cells (ES cells) show similar MMC sensitivity for all genotypes tested (Figure 3). Strikingly, wild type ES cells are over 5 times more sensitive to MMC than wild type MEFs. This intrinsic hypersensitivity of ES cells is more pronounced for MMC-treatment, than for γ-ray- or UV-irradiation ((7), chapter 5).

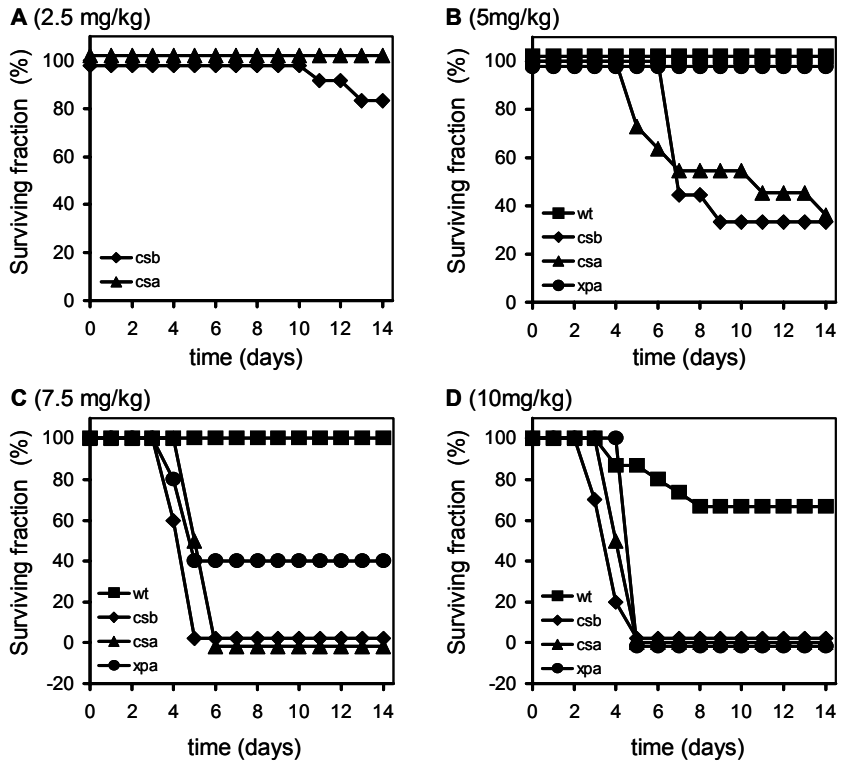

Figure 4

MMC survival of *Csb*$^{-/-}$, *Csa*$^{-/-}$, *Xpa*$^{-/-}$ and wild type mice.

Surviving fraction of *Csb*$^{-/-}$ (diamonds), *Csa*$^{-/-}$ (triangles), *Xpa*$^{-/-}$ (circles) and wild type (squares) mice after different doses of MMC. Panel A: 2.5 mg MMC/kg bodyweight, *Csb*$^{-/-}$ (n=12), *Csa*$^{-/-}$ (n=9). Panel B: 5 mg MMC/kg bodyweight, *Csb*$^{-/-}$ (n=14), *Csa*$^{-/-}$ (n=11), *Xpa*$^{-/-}$ (n=5). Panel C: 7.5 mg MMC/kg bodyweight, *Csb*$^{-/-}$ (n=5), *Csa*$^{-/-}$ (n=4), *Xpa*$^{-/-}$ (n=10) wild type (n=12). Panel D: 10 mg MMC/kg bodyweight, *Csb*$^{-/-}$ (n=10), *Csa*$^{-/-}$ (n=2) *Xpa*$^{-/-}$ (n=4) wild type (n=15).

4.3.5 MMC-toxicity in wild type, Csa$^{-/-}$, Csb$^{-/-}$ and Xpa$^{-/-}$ mice

The cell specific response to MMC observed above raises the clinical relevant question how the MMC response at the level of the whole animal is influenced by *Csa*, *Csb* or *Xpa* defects. To study the MMC response at the level of the whole animal, we injected mice intraperitoneally with a single dose of 1, 2.5, 5, 7.5 or 10 mg/kg MMC. Animals were housed in isolators throughout the experiment to minimize the risk of infections. Mice were scored as death either when found death, or when they had to be sacrificed, according to the local bio-ethical standards (criteria applied: severe weight loss (>20%), no food-intake, no movement, no response to external stimuli and hunchbacked appearance). Survival curves are shown in figure 4. Surprisingly, we observed a hypersensitive response to MMC treatment in both *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice, compared to wild type mice. *Xpa*$^{-/-}$ mice are also

Table 1: Kaplan-Maier statitistics on survival curves of MMC treated animals

p =	*Xpa*	*Csb*	*Csa*
wt	0.0002	0.0000	0.0000
Xpa		0.0218	0.0287
Csb			0.4297

hypersensitive to MMC, although to a significant lesser extent than *Csa*$^{-/-}$ and *Csb*$^{-/-}$ animals (Table 1). Therefore, we conclude that mice defective in TCR only (*Csa*$^{-/-}$ and *Csb*$^{-/-}$) are significantly more sensitive to MMC treatment than total NER deficient *Xpa*$^{-/-}$ mice, suggesting a NER-independent role of CSA and CSB in the response to MMC. Moreover, we show that MMC sensitivity in either MEFs or ES cells is not predictive for MMC sensitivity of the mouse.

4.4 Discussion

4.4.1 NER-independent sensitivity of Csa$^{-/-}$ *MEFs to MMC*

We have exploited genetically defined mouse models for a detailed analysis of the effect of a *Csa* or *Csb* deficiency on MMC sensitivity at the level of cells and the whole organism. Mitomycin C produces a wide range of different DNA lesions. Besides mono-adducts and oxidative DNA damage, MMC causes a low percentage of DNA inter-strand cross-links. In general, these cross-links are regarded as the most cytotoxic lesions, being largely responsible for the extreme toxicity of MMC, particularly for proliferating cells (9). In line with previous observations in hamster cell lines (5, 13), we demonstrate that *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs are slightly sensitive to MMC. This slight MMC sensitivity might be explained by a repair deficiency of mono-adducts and intra-stand cross-links. However, also a minor function of nucleotide excision repair in inter-strand cross-link repair has been suggested (23, 32-35). Surprisingly, *Csa*$^{-/-}$ MEFs appeared to be hypersensitive to MMC. This finding contrasts the observed induction of apoptosis in *CSA, CSB, XPG* and *XPD* human fibroblasts (all showing comparable induction) after continuous MMC treatment (1). However, clonogenic sensitivity studies do not per se reflect induction of apoptosis.
The observed MMC sensitivity in *Csa*$^{-/-}$ MEFs is unlikely to be attributed to a TC-NER deficiency in these cell lines, since totally NER-deficient *Xpa*$^{-/-}$ MEFs are only slightly sensitive to MMC. Moreover, although cross-links might be repaired more efficient in transcribed areas (10, 14-17, 25, 31) and TCR might be involved in repair of a minor fraction of cross-links (35), also a deficiency in TCR does not seem to explain the MMC sensitivity in *Csa*$^{-/-}$ MEFs, since *Csb*$^{-/-}$ MEFs are less MMC sensitive than *Csa*$^{-/-}$ MEFs. Therefore, a deficiency in a CSB-independent TCR mechanism might explain the MMC sensitivity in *Csa*$^{-/-}$ MEFs. However, no significant blockage of RNA synthesis after MMC treatment is observed. Finally, also defective cross-link repair by homologous recombination is an unlikely explanation, since no obvious deficiency in Rad51 foci formation after MMC treatment is observed in *Csa*$^{-/-}$ MEFs (data not shown). We conclude that *Csa*$^{-/-}$ MEFs are hypersensitive to MMC and that this hypersensitivity is not clearly associated with a deficiency in cross-link repair by NER, TCR or homologous recombination.

4.4.2 CSA is needed for restoration of DNA synthesis after MMC treatment

Since MMC treatment did not cause a RNA synthesis block or a rapid induction of apoptosis, we wondered what could be the underlying cause of the observed MMC sensitivity in *Csa*$^{-/-}$ MEFs. DNA inter-strand cross-links by definition prevent strand separation and thereby block replication. We checked whether DNA synthesis is hampered in *Csa*$^{-/-}$ MEFs after MMC treatment and show that indeed MMC treated wild type and *Csb*$^{-/-}$ MEFs undergo a transient DNA synthesis block, but are able to recover, while *Csa*$^{-/-}$ MEFs fail to recover. This DNA synthesis block is probably not caused by the direct effect of a low number of DNA inter-strand cross-links that obstruct DNA polymerases, but might originate from an S-phase block caused by the inter-strand cross-links. Moreover, the function of CSA in the restoration of a DNA synthesis block remains to be elucidated.

4.4.3 MMC sensitivity is cell type specific

Interestingly, we observed a marked difference in MMC sensitivity between MEFs and ES cells. ES cells appear to be over 5 times more sensitive to MMC than MEFs. Since the majority of ES cells are in the S-phase of the cell cycle, it is tempting to speculate that this causes the hypersensitivity of ES cells. Although also lower thresholds for apoptosis induction or lower repair efficiency might contribute to this phenotype. Moreover, in ES cells a deficiency of CSA does not contribute to the MMC response. Whether the increased MMC sensitivity associated with a *Csa* deficiency is masked in ES cells by the intrinsic hypersensitivity of this cell type for this agent, or whether ES cells do not depend on the CSA protein in their response to MMC, is unclear.

4.4.4 MMC effect on Csa$^{-/-}$, Csb$^{-/-}$, Xpa$^{-/-}$ and wild type mice

Surprisingly, we show that *Csa*$^{-/-}$, *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ mice, in contrast to wild type animals, are hypersensitive to MMC treatment. Both *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice are significantly more MMC sensitive, than Xpa$^{-/-}$ mice. This observation indicates that MMC sensitivity in Cockayne mice might be partly due to a NER defect. However, significant increased MMC sensitivity in *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice compared to *Xpa*$^{-/-}$ animals, indicates that besides NER also another mechanism contributes to the cellular MMC sensitivity. Whether this mechanism is transcription-coupled repair, still needs to be elucidated. Strikingly, while γ-ray sensitivity of ES cells reflects the X-ray sensitivity of mice (7), the MMC sensitivity of mice is not reflected by MMC sensitivities in any cell system tested. The fact that cell lines do not have a predictive value for MMC sensitivity of the whole organism, asks for great care in MMC treatment of patients, especially of patients with an XP defect.
Finally, although MMC sensitivity is cell type specific, it is tempting to speculate that cross-links originating from endogenously produced agents in specific cell types, might contribute to the CS phenotype in the patient.

Acknowledgements

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Chapter 5

Repair or die; mutation prevention in embryonic stem cells

Manuscript in preparation

Repair or die; mutation prevention in embryonic stem cells

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Abstract

Pluripotent embryonic stem cells (ES cells) are the precursors of all different cell types comprising the organism. Since persistent DNA damage, which might lead to mutations, can cause huge malformations in the organism, genome caretaking is of prime importance to this cell type. We compared the sensitivity of mouse embryonic fibroblasts (MEFs) and ES cells for various genotoxic agents and show that ES cells are more sensitive to treatment with UV-light, γ-rays and mitomycin C.

In this study, we further investigated the contribution of the transcription-coupled (TC-NER) and global genome (GG-NER) sub-pathways of nucleotide excision repair (NER) in protection of ES cells. Therefore, we isolated wild type, *Csb*$^{-/-}$ (TC-NER-deficient), *Xpc*$^{-/-}$ (GG-NER-deficient) and *Xpa*$^{-/-}$ (TC- and GG-NER-deficient) ES cells and compared the UV response of these cells and MEFs. TC-NER deficient *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ MEFs are hypersensitive to UV, whereas *Xpc*$^{-/-}$ MEFs attribute intermediate UV sensitivity. In contrast, the global genome repair deficient *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES are highly UV-sensitive, while a *Csb* deficiency only causes a mild increase in UV-sensitivity in ES cells. The observed hypersensitivity in *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ MEFs correlates with increased apoptosis upon UV irradiation. Surprisingly, a hyperapoptotic response upon UV irradiation is mainly observed in *Xpa*$^{-/-}$ ES cells, suggesting a different mechanism of apoptosis induction in ES cells, mainly relying on damage in the global genome rather than in transcribed genes. Moreover, we show a pronounced S-phase delay in *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES cells. This mechanism might well function as a safeguard in heavily damaged cells in case apoptotic response fails. Our finding that although *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES are totally NER-defective or GG-NER-deficient respectively, the mutation induction upon UV is similar compared to wild type ES cells suggests indeed that the observed apoptotic and cell cycle response is sufficient to protect against progression of mutated cells. In conclusion, we show a double safeguard mechanism in ES cells against NER-type of damages, which mainly relies on damage detection in the global genome.

5.1 Introduction

Preserving the genome is of prime importance for all living organisms since endogenous and exogenous agents (e.g. UV light, X-rays, oxidative stress and many chemicals) continuously induce a wide variety of DNA lesions. Replication of the damaged template may lead to mutations, potentially resulting in cancer. Alternatively, persistent DNA damage can hinder cellular key processes like transcription and replication, which may cause cell malfunctioning, ultimately leading to cell death. Evidence is accumulating that this overall functional decline of the genome may contribute to aging (reviewed in (31)). To counteract genotoxic stress, cells are equipped with an elaborate genome care-taking network, among which different DNA repair mechanisms with partly overlapping substrate specificity: (i) Non-homologous end joining and homologous recombination, for repair of double strand breaks. (ii) A variety of base excision repair (BER) enzymes, coping with small base modifications, like methylation and oxidation. (iii) Nucleotide excision repair (NER), for removal of UV-induced and other helix-distorting lesions (for recent review see (13, 17)).
The NER machinery consists of two sub-pathways, namely global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER removes DNA damage from the entire genome. TC-NER specifically and efficiently removes DNA damage from the transcribed strand of active genes, thereby releasing transcription arrest, caused by RNA polymerase II stalled at lesions. For some lesions that are repaired by the BER machinery, it has been shown that they can also be removed in a transcription-coupled manner (20, 21). Whether this process is performed by TC-NER or via transcription-coupled BER (TC-BER) is disputed. In cases that it is unknown whether TC-NER or TC-BER is functional, we refer to transcription-coupled repair (TCR). Defects in TCR are associated with the rare inherited disorder Cockayne syndrome (CS), while GG-NER defects or total NER deficiencies are interrelated with xeroderma pigmentosum (XP) (for review see (4)). In addition to repair, transient cell cycle arrest provides the cell with a time window to fix damaged DNA before lesions are converted into permanent genetic changes (for review on DNA damage induced cell cycle regulation see (5)). When repair fails or takes too long, cells carrying too much genetic damage can be eliminated via apoptosis or become senescent (reviewed by (3, 19)).
It has been suggested that, depending on cell type, differentiation-stage and age, cells may have a different need to withstand genotoxic stress and therefore might have different priorities in the use of the various genome-caretaking processes. For example, exposure of pregnant mice to very low doses of ionizing radiation causes severe apoptosis in the embryo, while apoptosis is not observed in extra-embryonic tissue (16). A similar observation has been made for the small intestine, where ionizing radiation-induced apoptosis is restricted to stem cells in the crypt and is absent in the more differentiated cells in the villi (35). An example of cell-type-specific usage of various repair pathways is the preference for error free repair of double strand breaks by homologous recombination in ES cells, while double strand break repair in more differentiated cells largely depend on the error prone non homologous end joining (12). It is even possible that certain repair mechanisms are only present in, and adapted to specific cell types. For example, neurons are postmitotic cells that do not divide and as a consequence will not use the gross of their

genome anymore. Therefore, it has been suggested that these cells do not need to keep their overall genome error-free, but instead only have to properly maintain their transcribed genes. Indeed, it has been shown that neurons have low levels of GG-NER, while TC-NER is normal (33). Since in the absence of GG-NER, damage could accumulate on the non-transcribed strand of active genes (serving as a template for the TCR reaction) also the non-transcribed strand of active genes should be kept lesion-free. A specific mechanism, differentiation associated repair (DAR), has been described to complement TC-NER by repair of damage in the non transcribed strand of active genes in neurons (32, 33).
It is currently unknown whether, as in terminally differentiated cells, stem-cell-specific damage response pathways exist. Embryonic stem cells (ES cells) are the ultimate stem cells, and therefore can be considered at the far end of the spectrum ranging from undifferentiated to differentiated cells. Tolerance of DNA damage in pluripotent ES cells could have detrimental consequences. For example clonal expansion of mutated cells to considerable parts of the organism can lead to huge malformations. As part of the genome-caretaking network, a hyperapoptotic response of ES cells to various genotoxic agents has been shown (1, 8, 38, 45). NER seems to contribute to some extent in protection against DNA damage (45). To get more insight in the contribution of TC-NER and GG-NER in the total genome care-taking machinery in pluripotent stem cells and in (partially) differentiated somatic cells, we established mouse ES cells and embryonic fibroblasts (MEFs) with defects in TC-NER (*Csb*$^{-/-}$), GG-NER (*Xpc*$^{-/-}$) or both pathways (*Xpa*$^{-/-}$), as well as wild type cells. We show that, in comparison to MEFs, ES cells are hypersensitive to a wide variety of genotoxic agents. Interestingly, inactivation of specific repair genes has different effects on the damage response in ES cells and MEFs. Our data suggest that the contribution of damage in the global genome is the major determinant for the ES cell response to helix distorting lesions. Therefore, in contrast to the situation in MEFs, the main contributor to apoptosis induction in *Xpa*$^{-/-}$ ES cells is the deficiency for GG-NER, rather than the TC-NER-defect. Finally, our study provides evidence for a double safeguard mechanism against mutations in ES cells.

5.2 Materials and Methods

5.2.1 Cell lines

Isolation of primary *Csb*$^{-/-}$ (FVB/129Ola), *Xpa*$^{-/-}$ (C57Bl6J/129Ola) MEFs and corresponding wild type cell lines has been described (10, 44). *Xpc*$^{-/-}$ (C57Bl6/129Ola) MEFs were isolated in a similar manner. Cells were cultured in F10/DMEM (1:1) (Gibco) medium, supplemented with 10 % fetal calf serum and 50 μg/ml penicillin and streptomycin (Gibco). Spontaneously immortalized cell lines were obtained by continuous subculturing of primary MEFs. Cellular sensitivity studies and RNA synthesis recovery experiments were performed on transformed MEFs. Cell cycle analysis and apoptosis induction was measured in primary MEFs.
Wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ embryonic stem (ES) cells were isolated from blastocysts (3.5 days *post coitum*) obtained from wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ mice intercrosses respectively (all in a C57Bl6 genetic background) as described previously (11). ES cells were

maintained on gelatin-coated dishes or on lethally irradiated MEF feeder layers (20 Gy from a ^{137}Cs source) in 50 % buffalo rat liver cell conditioned DMEM/50 % fresh DMEM supplemented with 15 % fetal calf serum, 0.1 mM non-essential amino acids (Gibco), 50 μg/ml penicillin and streptomycin (Gibco), 1000 U/ml leukemia inhibitory factor (Chemicon) and 0.1 mM 2-mercaptoethanol (Sigma).

5.2.2 Cellular sensitivity studies

Determination of cellular survival of ES cells and MEFs was performed using a colony assay. UV survival of MEFs was established using a ^{3}H-thymidine incorporation assay (41), however no difference in survival was observed for wild type MEFs using the two different assays (data not shown).
Colony assays were performed using the following protocol. Cells were plated in 6 cm dishes, at various dilutions. After 12-16 h, cells were exposed to different doses of UV (254 nm, Philips TUV lamp), irradiated with ionizing radiation, using a ^{137}Cs source, treated for one hour with various concentrations of mitomycin C (Kyowa) diluted in growth medium, or exposed to increasing doses of Illudin S (isolated from O. illudins as described (2)) for 72h diluted in growth medium. Cells were grown for 5 to 14 days, fixed, stained and counted to assess the colony-forming ability. All experiments were performed in triplicate.
^{3}H-thymidine incorporation assay was determined as described (41). Briefly, MEFs were exposed to different doses of UV (254 nm, Philips TUV lamp) and allowed to grow for another 3-5 days, before reaching confluency. The number of proliferating cells was estimated by scintillation counting of the radioactivity incorporated during a 3 hr pulse with ^{3}H-Thymidine (5μCi/ml, s.a. 40-60 Ci/mmole; Amersham). Cell survival was expressed as the ratio of ^{3}H incorporation in treated and non-treated cells.

5.2.3 RNA synthesis recovery

RNA synthesis recovery after UV or MMC treatment was performed as described (29), with several modifications. Briefly, cell were seeded in 6 well plates and after 24 h labeled with [2-^{14}C]-thymidine (50 μCi/ml, s.a. 50 mCi/mmole; Amersham) for 16 h. Cells were irradiated with 10 J/m^2 UV (254nm, Philips TUV lamp). Two and four hours after treatment with UV, RNA synthesis was determined by 1h labeling with [5,6-^{3}H]-uridine (10 μCi/ml, s.a. 50 Ci/mmole; Amersham). ^{3}H and ^{14}C ratios were measured by scintillation counting. The relative RNA synthesis was expressed as the quotient of these ^{3}H-^{14}C ratio of treated over untreated cells.

5.2.4 Apoptosis analysis

Determination of apoptosis by subG1 is performed as described (9). ES cells and MEFs are grown and UV irradiated (254 nm, Philips TUV lamp). After 24-48 h cells were washed and harvested by trypsinization. Growth medium, the washes and the harvested cells were pooled and fixed by ethanol fixation. From fixed cells small DNA fragments were extracted by a 0.2 M phosphate citrate buffer (pH 7.8). Subsequently cells were stained by PI and

DNA content was analyzed on a BD Facscan or a BD Facscalibur using Cellquest (pro) software.
For measurement of apoptosis by the Annexin V assay, cells were grown and UV irradiated (254 nm, Philips TUV lamp). After 24-48 h cells were washed and harvested by trypsinization. Growth medium, the washes and the harvested cells were pooled. Harvested cells were collected by centrifugation, resuspended in 1 ml of culture-medium in an eppendorf-tube and allowed to recover for 30 minutes at 37°C. Cells were shortly centrifuged, resuspended in 95µl AnnexinV-solution (0.8 µl FITC-labeled Annexin V (Pharmingen) in PBS) and incubated for 15 minutes on ice. 300 µl PI-stain (3.3 µM PI in PBS) was added to the sample, incubated for 1 minute on ice and analyzed on a BD Facscan, using Cellquest software. AnnexinV$^-$/PI$^-$ cells are viable, AnnexinV$^+$/PI$^-$ are early apoptotic cells and AnnexinV$^+$/PI$^+$ are late apoptotic and necrotic cells.

5.2.5 Cell cycle analysis

Determination of cell cycle profile is performed as described (9). Shortly, ES cells are grown and UV irradiated (254 nm, Philips TUV lamp). After 4 -24 h cells were washed and harvested by trypsinization and fixed by ethanol fixation. Fixed cells were stained by PI staining and analyzed on a (BD Facscan or a BD Facscalibur) using Cellquest (pro) software.

5.2.6 Mutation analysis

Mutation analysis at the *Hprt* gene is performed as described (45). Briefly, ES cells are seeded on gelatin-coated plates and UV irradiated (254 nm, Philips TUV lamp). After UV irradiation, cells were trypsinized and seeded on MEF feeder layers. Cells were propagated for 6 days by passaging every 2 days. At least 9 x 10^6 cells were plated per dose after each passage. After the 6-day expression period 0.2-1 x 10^7 cells per dose were plated for selection with 2.5 µg/ml 6-thioguanine (Sigma, St Louis, MO) at a density of 2 x 10^5 cells per 100 mm dish. Additionally, the cloning efficiency was determined by seeding 750-2500 cells per dish (five dishes per dose) in medium without 6-thioguanine. Colonies were fixed, stained and counted 6-7 days after seeding of the cells.
6-Thioguanine-resistant clones were subcultered and frozen at -80°C. For RNA isolation, cell pellets were lysed in 100 µl of TRIzol (Life Technologies) and RNA was extracted, according to the manufacturer's protocol. RNA was resuspended in 18 µl of annealing buffer (250 mM KCl, 10 mM Tris.HCl pH 8.3, 1 mM EDTA), mixed with 40 pmol of *Hprt*-cDNA primer (GCAGCAACTGACATTTCTAAA) and incubated at 65°C for 3 minutes to allow annealing of the primer to the *Hprt* mRNA strand. After this, the sample was split in two and cDNA synthesis was performed as described (47).
3 µl of synthesized cDNA was used to amplify the coding region of the *Hprt* gene in a total volume of 100 µl containing 20 µl of a 5x PCR buffer (500 mM KCl, 100 mM Tris.HCl pH 8.3, 15 mM $MgCl_2$), 4 µl of a dNTP mix (2.5 mM), 1 unit of Amplitaq polymerase (Perkin-Elmer) and 20 pmol of each of the PCR primers. For the first round of amplification the PCR primers were hprt-mus2 (AAAAAGCTTTACTAGGCAGATGG) and zee1-mus

(GGCTTCCTCC TCAGAC CGT). After an initial denaturation step for 5 min at 93°C, 35 cycles of 1 min at 93°C, 1 min at 50°C, and 3 min at 72°C were performed followed by a final extension step of 8 min at 72°C. 1 µl of amplified DNA was used in a reamplification reaction of 25 cycles with an annealing temperature of 55°C using primers hprt-mus1 (TTTTTGCCGCGAGCCGACC) and san2m13 (CGACGTTGTAAAACGACGGCCAGT GCAGATTCAACTTGCGCTC). 10 µl of amplified DNA was used for sequence analysis with the Thermo Sequenase fluorescent labelled primer cycle sequencing kit containing 7-deaza-dGTP (Amersham/Pharmacia/Biotech) on an automatic ALF sequencer (Amersham).

5.3 Results

5.3.1 ES cells are hypersensitive to a variety of genotoxic treatments

To investigate whether ES cells are more sensitive to genotoxic stress than other cell types, we compared sensitivities of ES cells and spontaneously transformed mouse embryonic fibroblasts (MEFs) to various genotoxic agents. Although, we never observed effects of the genetic background in MEFs (C57Bl6/129Ola, FVB/129Ola and C57Bl6) on UV-sensitivity, we isolated for this study ES cells in a pure C57Bl6 background to completely rule out the influence of differences in genetic background. As shown in figure 1A, ES cells attribute a modest increase in sensitivity to γ-ray irradiation, causing oxidative lesions and DNA breaks. Furthermore, we observe a strong hypersensitivity to the chemotherapeutic mitomycin C, which induces the very cytotoxic DNA inter-strand cross-links, besides a variety of other lesion, like oxidative DNA damage and mono-adducts. Finally, ES cells are more sensitive to UV-irradiation, than MEFs. Van Sloun et al reported that the same dose of UV causes half the amount of UV photoproduct in ES cells, compared to MEFs (45), indicating that the observed difference in UV sensitivity between ES-cells and MEFs is an underestimation. Taken together, we show that ES cells are more sensitive for a variety of different genotoxic agents than MEFs.

5.3.2 Deficiency in global genome NER has more influence on survival, than deficiency in transcription-coupled repair

Since the UV response is well-studied in human and mouse fibroblasts, but not in stem cells, we further focus on the UV response of ES cells. To investigate the contribution of GG-NER and TC-NER to cellular survival after UV treatment, we isolated wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells in an isogenic C57Bl6 background. Prominent differences in growth characteristics or morphology between these cells are not observed (data not shown). We compared survival of UV-treated ES cells with MEFs, with the same genetic NER defect (Figure 1B). A total NER defect causes a comparable hypersensitivity to UV-induced DNA damage in both *Xpa*$^{-/-}$ ES cells and *Xpa*$^{-/-}$ MEFs (when corrected for effective dose in ES cells). While TC-NER-deficient *Csb*$^{-/-}$ MEFs show an almost similar UV survival as *Xpa*$^{-/-}$ MEFs, *Csb*$^{-/-}$ ES cells are just slightly more sensitivity to UV than wild type ES cells. In contrast, GG-NER-deficient *Xpc*$^{-/-}$ ES cells show a severe hypersensitivity

to UV, while *Xpc*$^{-/-}$ MEFs display an intermediate sensitivity. Taken together, we conclude that, as shown for human fibroblasts (7, 23, 24), UV sensitivity in MEFs is mainly associated with a TC-NER defect (hypersensitivity of *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs). In ES cells, UV sensitivity correlates to a large extent with GG-NER capacity (hypersensitivity of *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES cells)

5.3.3 Transcription-coupled repair is functional in ES cells

The severe sensitivity of *Xpc*$^{-/-}$ ES cells, as well as the almost complete lack of UV sensitivity of *Csb*$^{-/-}$ ES cells, compared to wild type ES cells, can be explained by a lack of TC-NER in ES cells, or by an overruling response due to damage in the global genome. It should be noted that these two options are not mutually exclusive. To test whether TC-NER is functional in ES cells, we measured RNA synthesis recovery after UV treatment, which is a

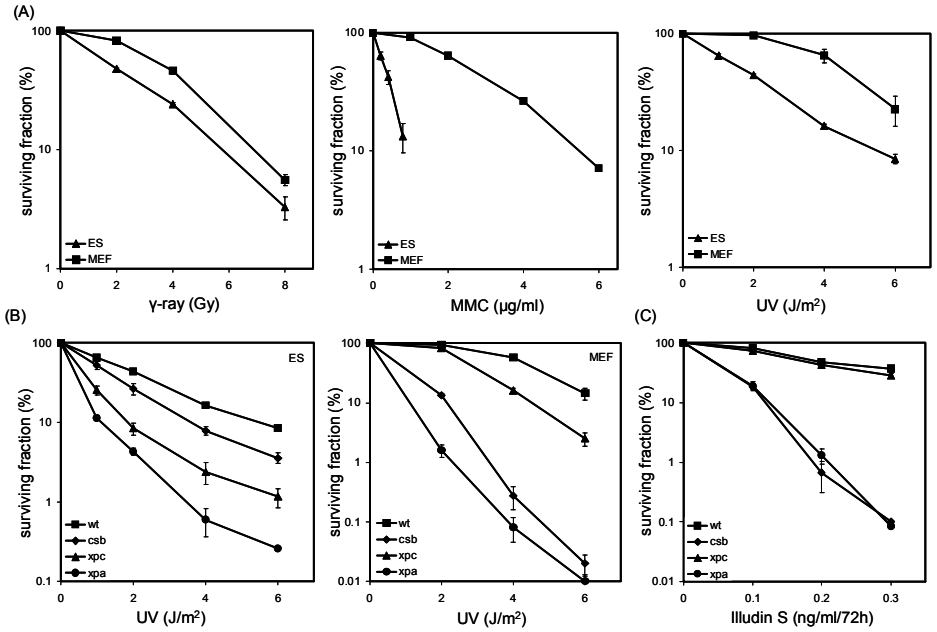

Figure 1

Survival of ES cells versus MEFs.

Panel A: Survival of wild type ES cells (triangles) and spontaneously transformed MEFs (squares) after exposure to γ-rays, mitomycin C or UV-light. Except for the UV survival of MEFs, which is determined by thymidine incorporation assay, survival is determined by colony assay. Panel B: Survival of wild type (squares), *Csb*$^{-/-}$ (diamonds), *Xpc*$^{-/-}$ (triangles) and *Xpa*$^{-/-}$ (circles) ES cells and transformed MEFs after exposure to increasing doses of UV-light (254 nm), as determined by colony assay and thymidine incorporation assay respectively.

Panel C: Survival of wild type (squares), *Csb*$^{-/-}$ (diamonds), *Xpc*$^{-/-}$ (triangles) and *Xpa*$^{-/-}$ (circles) ES cells after exposure to increasing doses of Illudin S for 72h, as determined by colony assay. For each cell-line, experiments were performed at least three times and for each genotype identical results were obtained with at least two independent cell-lines (data not shown). For all experiments, except UV survival of MEFs in panel B and Illudin S survival in panel C, the average of multiple experiments is shown. For the latter experiments representative examples are shown. Bars indicate the standard error of the mean.

wide used indicator for TC-NER capacity. Remarkably, we could not detect any block of RNA synthesis in UV treated wild type or *Csb*$^{-/-}$ ES cells at early time points (2 and 4h after up to 10 J/m^2 UV) (data not shown). In MEFs, a clear block in RNA synthesis has been reported at this dose and these timepoints. The lack of a rapid block in RNA synthesis in ES cells, together with the detachment of UV-treated ES cells as soon as 8 h after treatment from the surface, made it impossible to measure RNA synthesis recovery. To further test the relevance of TC-NER in ES cells, we treated wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells with Illudin S, which induces DNA lesions that appear to be recognized and repaired by TC-NER, only. As reported for fibroblasts (18), *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells are hypersensitive to Illudin S, while GG-NER-deficient *Xpc*$^{-/-}$ ES cells show the wild type sensitivity (Figure 1C), suggesting that TC-NER is functional in ES cells. This finding makes it unlikely that the UV-hypersensitivity of wild type ES cells and the relative small difference between wild type and *Csb*$^{-/-}$ ES cells is due to a general lack of TC-NER in ES cells.

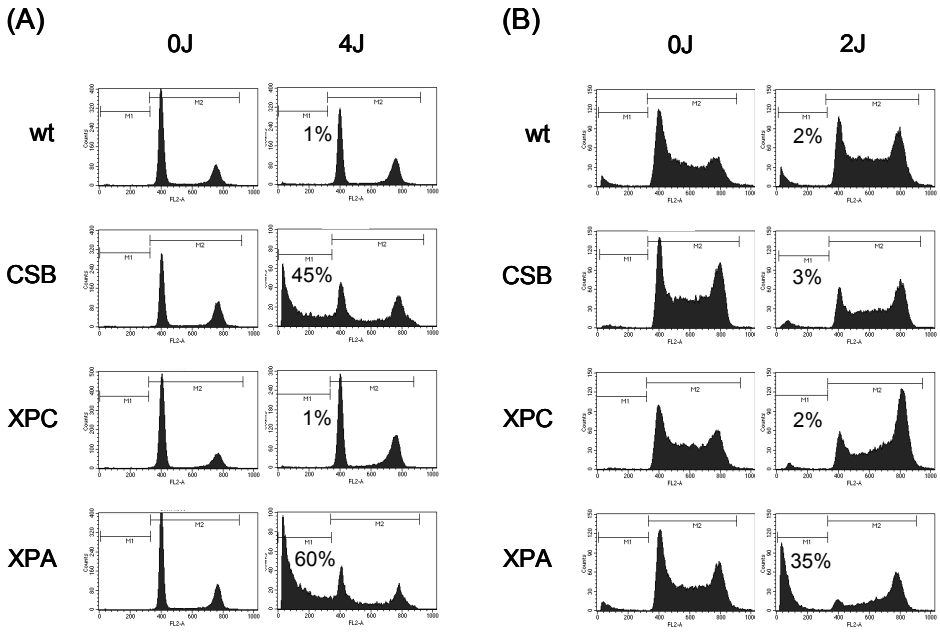

Figure 2
Apoptotic response of ES cells versus MEFs to exposure with UV light.
Apoptotic response of wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells and MEFs to exposure with UV-light (254nm) after 24 or 48 hours respectivicely. Apoptosis is shown by cell sorting of propidium iodide stained cells. Percentage of cells in apoptosis (subG1-fraction, marked in the figure with M1) is noted in the figure, after subtraction of background values (apoptosis in untreated sample). Cells marked with M2 are non-apoptotic cells, either in G1, S or G2 phase of the cell cycle. UV-dose is depicted above the columns; beside the rows genotypes are shown. Panel A: apoptotic response of MEFs. Panel B: apoptotic response of ES cells. For each cell-line, experiments were performed at least three times and for each genotype identical results were obtained with at least two independent cell-lines (data not shown).

5.3.4 Xpa$^{-/-}$, but not Xpc$^{-/-}$ or Csb$^{-/-}$ ES cells are hyperapoptotic

To test whether the observed UV sensitivity in ES cells is mainly caused by damage in the global genome, and to establish the mechanism underlying the observed hypersensitivity of (partly) NER-deficient ES cells, we examined the apoptotic reaction of these cells, using the subG1 assay. As reported for human fibroblasts (7), both *Xpa*$^{-/-}$ and (to a somewhat lesser extent) *Csb*$^{-/-}$ MEFs show a pronounced apoptotic response, judged from an increase in the subG1 population, measured 48h after exposure to 4J/m² UV (Figure 2A). The induction of apoptosis in *Csb*$^{-/-}$ and *Xpa*$^{-/-}$ MEFs correlates well with the observed hypersensitivity of these cells to UV-light.
Apoptosis induction in ES cells was determined after irradiation with 2 J/m² UV, which causes a comparable percentage of surviving wild type ES cells, compared to wild type MEFs irradiated with 4J/m² UV. While we show a strong increase in apoptosis in *Xpa*$^{-/-}$ ES cells 24h after UV-irradiation, only a marginal apoptosis-induction is observed in *Csb*$^{-/-}$ ES cells. Although *Xpc*$^{-/-}$ ES cell are almost as sensitive as *Xpa*$^{-/-}$ ES cells, we did not observe any induction of apoptosis in these cells (Figure 2B). Also, we do not observe any induction of apoptosis in *Xpc*$^{-/-}$ ES 48h after UV treatment (data not shown), suggesting that these cells lack UV induced apoptosis, rather than having a delayed apoptotic response. To exclude experimental artifacts, we confirmed these data by an AnnexinV assay (data not shown). In conclusion, we show that while *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ MEFs are hyperapoptotic after UV, only *Xpa*$^{-/-}$ ES cells attribute higher apoptotic levels after UV treatment. Surprisingly, *Xpc*$^{-/-}$ ES cells fail to show a *Xpa*-like apoptotic response, although the UV-sensitivity of these two lines is comparable.

5.3.5 S-phase delay in Xpa$^{-/-}$ and Xpc$^{-/-}$ ES cells upon UV exposure

Close examination of the cell-cycle profiles of UV-treated *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells suggests an accumulation of cells in late S/G2 phase 24h after UV treatment (Figure 2B). To further investigate this phenomenon, we analyzed the cell cycle profile of ES cells upon UV irradiation (Figure 3). The cell cycle kinetics of wild type and *Csb*$^{-/-}$ ES cells are similar, showing a synchronized release of cells from G1- into S-phase and a recovery of the normal cell cycle profile 24h after treatment. Since cells propagate into the S-phase, it seems that ES cells do not have a G1-block upon UV-irradiation. Moreover, these profiles suggest a transient S/G2 block, circumventing mitosis and thereby replenishing of the G1 population. *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES cells show a slower progression through the S-phase than the wild type ES cells, with a considerable percentage of cells that are blocked in late S/G2 phase 24h after treatment. Because only attached cells are harvested (and floating, and thus apoptotic cells are discarded), in these experiments the subG1-population in UV-treated *Xpa*$^{-/-}$ ES cells is not observed. In conclusion, we show a correlation between the lack of GG-NER and a slower S-phase progression after UV treatment in ES cells.

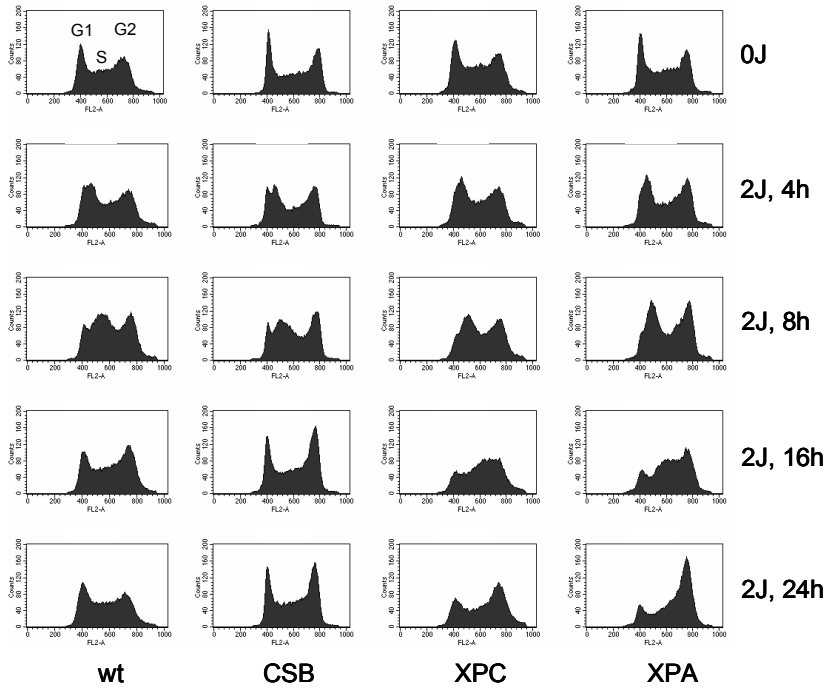

Figure 3
Cell cycle response of wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells to exposure with UV-light (254nm). Various timepoints after UV exposure, cells were stained by propidium iodide. Cells in G1, S and G2 phase are marked in the figure. UV-dose and time are depicted beside the rows and genotypes are depicted under the columns. For each cell-line, experiments were performed at least three times and for each genotype identical results were obtained with at least two independent cell-lines (data not shown).

5.3.6 Deficiency for TC-NER, but not for GG-NER, causes elevated UV-induced mutation rates in ES cells

To investigate whether the lack of repair in NER-deficient ES cells and the observed differences in sensitivity, apoptotic and cell cycle response of these cells influence mutagenesis, we performed a UV-induced mutation induction analysis on the HPRT gene. Surprisingly, in wild type, *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES cells UV-induced mutability is comparable; only *Csb*$^{-/-}$ ES cells appear hypermutable (Figure 4). This is due to the genetic defect on the UV-response, since background mutation rates were comparable for the different mutant ES cells. The HPRT gene in surviving ES clones was sequenced for identification of mutations (Table 1). *Xpa*$^{-/-}$ ES lines show mainly mutations arising from CC or TC sequences in the transcribed strand. In contrast, in *Xpc*$^{-/-}$ ES cells, in which TC-NER is normal, mutations predominantly arise from DNA damage on the non-transcribed strand. Finally, a strong bias to mutations arisen from lesion on the transcribed strand is observed in *Csb*$^{-/-}$ ES cells, in which TC-NER is abolished. In conclusion, only a *Csb* deficiency causes higher mutation rates in UV treated ES cells, compared to wild type ES cells.

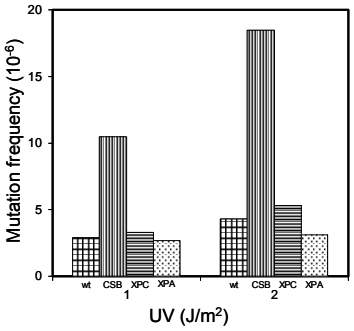

Figure 4
Mutation induction of UV light in wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells.
Mutation induction on the *Hprt*-gene in wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells after exposure to increasing doses of UV-light (254nm), as measured by 6-TG resistant clones. Mutation frequency is shown on the y-axis after subtraction of spontaneous mutation frequency in un-irradiated ES cells.

Table 1: Overview HPRT mutations

Genotype	Transitions	Trans-version	Other	pointmutations originate from NTS	TS
wt	5	1	6	2	4
CSB -/-	12	2	4	3	14
XPC -/-	5	4	4	5	1
XPA -/-	12		10	2	10

Table 1
Mutation induction of UV light in wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells.
Mutation induction on the *Hprt*-gene in wild type, *Csb*$^{-/-}$, *Xpc*$^{-/-}$ and *Xpa*$^{-/-}$ ES cells after exposure to increasing doses of UV-light (254nm), as measured by 6-TG resistant clones. Mutation frequency is shown in figure 4. The amount of measured transitions, transversions and other mutations are shown. Moreover, the amount of point mutations, originating from photoproducts in the transcribed or the non-transcribed strand of the HPRT gene, is noted.

5.4 Discussion

5.4.1 A hypersensitive response of ES cells to a wide variety of genotoxic treatments

In this study, we show that pluripotent undifferentiated ES cells respond differently to genotoxic stress when compared to more differentiated mouse embryonic fibroblasts. ES cells are more sensitive to UV (causing helix-distorting photoproducts), gamma rays (inducing double strand breaks and oxidative lesions) and mitomycin C (evoking mono-adducts, oxidative lesions, intra- and inter-strand cross-links), than MEFs. It is unlikely that the transformation process that MEFs underwent is the main cause of the relative resistance of these cells to a variety of genotoxic treatments, since UV sensitivity of primary MEFs is comparable to transformed MEFs (data not shown). Hypersensitivity could be caused by reduced repair capacity. However, in line with other reports we have shown that a deficiency for certain DNA repair factors in ES cells can cause hypersensitivity to various genotoxic agents, indicating that these repair pathways are active in ES cells (12, 43, 45). Moreover, using the repair replication assay it has been shown that NER kinetics at low doses (5 J/m^2) are comparable between ES cells and MEFs, although repair capacity seems to saturate already at 10 J/m^2 (45). In conclusion, these studies show that DNA repair is functional in ES cells, although the efficiency might be lower at more extreme doses.

5.4.2 Difference in contribution of TCR and GGR on cellular survival of MEFs and ES cells

UV sensitivity of MEFs and human fibroblasts correlates with the ability of these cells to perform TCR, as attributed by a severe UV-sensitivity in *Xpa*- and *Csb*-deficient fibroblasts (this study and (7, 23, 24). In contrast to MEFs, in ES cells, global genome repair-capacity is the major determinant of UV sensitivity, as shown by the marked UV sensitivity of $Xpa^{-/-}$ and $Xpc^{-/-}$ ES cells. This observation can be explained by either a lack of TC-NER in ES cells and/or an overruling response from damage that is normally repaired by GG-NER.
Non-functionality of TC-NER in ES cells does not seem to be likely, since we show that $Csb^{-/-}$ and $Xpa^{-/-}$ ES cells are hypersensitive for Illudin S, a DNA damaging agent that is specifically repaired by TC-NER. The extent of this hypersensitivity compares well with that observed in human fibroblasts (18). Therefore, TC-NER seems to be functional in ES cells, although the process might be overruled by damage responses originating from damage in the entire genome. In line with these observations, we previously showed that a TCR defect, associated with CSB deficiency, inflicts a more pronounced influence on sensitivity for oxidative lesions in MEFs than in ES cells (11).
The observed UV hypersensitivity of $Xpc^{-/-}$ and $Xpa^{-/-}$ ES cells suggests that a lack of GG-NER inflicts greater deleterious effects on ES cells than a lack of TC-NER, as discussed above. Since $Xpc^{-/-}$ ES cells are deficient for GG-NER, and $Xpa^{-/-}$ ES cells lack both TC-NER and GG-NER, it is tempting to speculate that the extra deficiency in TC-NER makes up the difference between $Xpa^{-/-}$ and $Xpc^{-/-}$ ES cells. Judging only from the slightly increased UV-sensitivity of $Xpa^{-/-}$ compared to $Xpc^{-/-}$ ES cells, this could be a valid explanation. However, the fact that we only observe a strong apoptotic response in $Xpa^{-/-}$ ES cells and not in $Xpc^{-/-}$ or $Csb^{-/-}$ ES cells indicates that the response of $Xpa^{-/-}$ and $Xpc^{-/-}$ ES cell lines differs fundamentally. Not only lack of GG-NER, but as well lack of either XPA- or XPC-protein determines the UV response of ES cells. This suggests that XPA and/or XPC proteins, beside their repair function, might have an additive role in apoptosis and/or cell cycle regulation.

5.4.3 XPC might be involved in apoptotic signaling in ES cells

In contrast to $Xpa^{-/-}$ ES cells, $Csb^{-/-}$ ES cells do not display a strong apoptotic response after UV treatment, arguing that the trigger for DNA damage-induced apoptosis in ES cells mainly arises from damage in the entire genome and not specifically from damage in the transcribed strand, as has been shown in fibroblasts (this study and (7)). The fact that $XPC^{-/-}$ ES cells are not triggered for apoptosis, but instead show a cell cycle block, suggests that in ES cells the XPC protein itself might be involved in sensing DNA damage and executing the apoptotic response. Contrarily, one could argue that XPA is needed to execute the cell cycle block and that a lack of this protein causes the switch-on of a safeguard mechanism by committing apoptosis. A strong argument against this second hypothesis is the fact that the low number of $Xpa^{-/-}$ ES cells that do not undergo apoptosis, show an identical cell cycle behavior as the $Xpc^{-/-}$ ES cell after UV treatment. Therefore, we hypothesize that the primary UV response is induction of apoptosis via a XPC mediated

pathway. The function of XPC in this pathway is unclear. A possibility could be that a partly assembled NER complex is a trigger for apoptosis. Since *Xpc*$^{-/-}$ ES cells miss the first step of damage recognition (42, 46), the complex is not formed and apoptosis is not induced. In this regard, analysis of ES cell lines lacking both XPA and XPC would be of great interest. Moreover, since it is disputed whether p53 plays any function in the protection of ES cells against genotoxic insults (1, 8) it is interesting to investigate whether this main apoptotic pathway in ES cells is p53 dependent.

5.4.4 Xpc$^{-/-}$ ES cells are hypersensitive to UV in an apoptotic independent fashion

Upon UV-irradiation, *Xpc*$^{-/-}$ ES cells as well as the fraction of *Xpa*$^{-/-}$ ES cells that did not undergo apoptosis, display a slower S-phase progression and probably arrest at late S/G2 phase. The fate of these arresting ES cells is unclear. They might die later (although induction of apoptosis could not be observed up to 48h after UV-exposure) or stay in a senescent state. Analysis of these remaining cells is complicated by possible overgrowth of surviving cells. Also in hamster cells, it has been shown that DNA damage can cause S-phase delay (34). It would be interesting to see whether p53 is needed for this response, since in fibroblasts it has been shown that both p53$^{-/-}$ and wild type mouse fibroblasts attribute a DNA replication arrest in response to UV (15). In conclusion, we suggest that in ES cells a sensitive apoptosis checkpoint as well as a cell cycle block, originating from un-repaired UV damage in the entire genome, protect against genotoxic assaults.

5.4.5 A double safeguard mechanism in ES cells protects against mutation accumulation

We failed to show a difference in the UV induced HPRT mutation rate between wild type, *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES cells. This observation is in strong contrast with the previously observed UV-hypermutability of *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ fibroblasts (22, 27, 28). This indicates that the strong apoptotic response in *Xpa*$^{-/-}$ ES cells is likely to counteract mutation accumulation by eliminating premutagenic cells and thereby compensates for the mutagenic consequences of a total NER defect. Moreover, the observed S-phase delay in combination with a potential cell cycle block, as observed in *Xpa*$^{-/-}$ and *Xpc*$^{-/-}$ ES cells, is so effective that a deficiency in apoptosis induction is efficiently compensated. The observed safeguard mechanism in ES cells might be absent in other cell types. This might partly explain why *Xpc*$^{-/-}$ animals show an elevated mutation rate (30, 48, 49).
However, CSB deficient ES cells are hypermutable, which might be due to the fact that although GG-NER is proficient, these cells keep a higher damage load in the transcribed strand, due to a deficiency in TC-NER. Escape from the cell cycle and apoptosis is probably relying on damage in the transcribed strand via a mechanism comparable to that observed in differentiated cell types (7, 23, 24). As known for these cell types, this mechanism is not able to completely protect cells from mutation induction (22). Moreover, ES cells lack a proficient G1 block (this study, (1, 36)), which is probably associated with a much simpler G1-S transition in ES cells. For example cyclinD/cdk4 and hypophosphorylated Rb are hardly detectable in ES cells (39, 40). Strikingly, a bias to mutations arisen from the

transcribed strand in *Csb*$^{-/-}$ and from the non-transcribed strand in *Xpc*$^{-/-}$ reflects the specific repair deficiencies in these cell lines. In *Xpa*$^{-/-}$ ES cells, mutations are mainly found in the transcribed strand, as was shown previously for human fibroblasts and Chinese hamster cells.
In conclusion, progression of pre-mutagenic ES cells is strongly counteracted by apoptosis and cell cycle arrest, both relying on sensing DNA damage in the entire genome.

5.4.6 ES cells: a model cell type for stem cells in the adult organism?

The observed balance between the different genome caretaking mechanisms in ES cells is probably the extreme example of how an undifferentiated, pluripotent cell reacts to genotoxic stress (as summarized in Figure 5). On the right side of this figure, the classical view is depicted, showing how a more differentiated, proliferating cell type like a fibroblast copes with DNA damage. First, it attempts to repair the DNA lesions. To provide enough time for DNA repair, the cell can temporally arrests the cell cycle. Ultimately, when the repair capacity is exhausted, the cell might undergo apoptosis or a permanent cell cycle arrest. The left side of the figure presents a possible mechanism used by ES cells. These cells mainly rely on apoptosis and cell cycle delay, induced by damage in the global genome, to counteract the mutagenic effects of DNA damage. It is likely that in most cell types, a combination of these extremes can be observed.
The optimal balance between the different genome caretaking processes is dependent on the specific requirements of that particular cell-type. For example, in postmitotic cells, which only have to maintain the genomic integrity of transcribed genes, global genome repair is not observed and only repair of transcribed areas is detected (32, 33). In contrast, reliance on only TC-NER in ES cells would be detrimental, since non-transcribed genes in an ES cell will accumulate damage and mutations, which might underlie severe malformation of the organism. Therefore, damage responses in ES cells are mainly focused on the damage in the global genome. Evidently, the strategy used by one cell type does not have to be beneficial for another cell type.
As a consequence of stringent genome caretaking processes ES cells show a lower mutation frequency, compared to MEFs (6). The drawback of this strategy is the high incidence of apoptosis. It is reasonable to assume that MEFs cannot use the same strategy, since it might cause an exhaustion of repopulation capacity and therefore MEFs should tolerate more mutations. Moreover a permanent G1 block, leading to senescence might be a valuable strategy for terminally differentiated cells, which do not have to divide per sé. The balance towards apoptosis and cell cycle block in ES cells, might as well explain the hypersensitivity of ES cell derived teratocarcinomas to chemotherapeutic cancer treatments (25, 26). Moreover, a similar strategy of high apoptotic levels is probably used by other stem-cell like cells with high proliferative rates, including cells from early developmental embryos (14, 16, 37) and stem cells in the intestine (35). It would be interesting to investigate whether indeed also in these cells apoptosis induction mainly relies on damage in the entire genome.

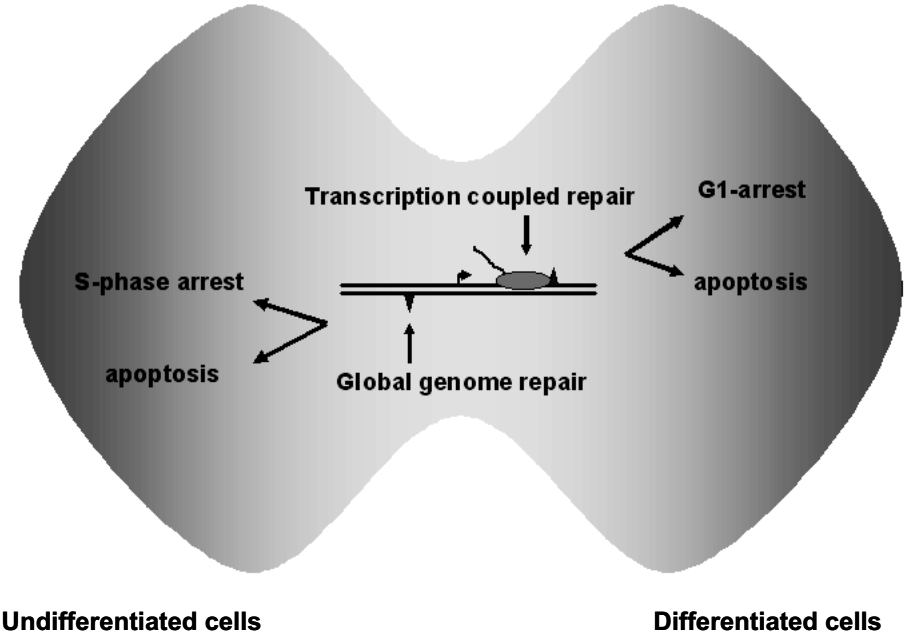

Figure 5
Hypothetic model for a cell-type specific response on NER lesions.
DNA damage can be repaired either by global genome repair or transcription-coupled repair. Persisting DNA damage in the transcribed strand blocks RNA polymerase II, which in turn can trigger apoptosis and/or a G1 block. This response is probably the major pathway on which differentiated proliferating cells rely to guard their genome. Contrarily, damage in non transcribed areas might as well fire apoptosis and/or arrest the cell cycle. This pathway is the main pathway in ES cells and might as well be in other stem cells. As shown in the model a balance between these different pathways to repair DNA damage or to quit the cell cycle will finally account for the specific response of a certain cell type.

5.4.7 Concluding remarks

In this study, we put forward alternative ways which an undifferentiated pluripotent ES cell uses to cope with genotoxic stress, safeguarding the entire organism for huge malformations and mutations. Knowledge on how this cell type, and presumable comparable cell types, handle DNA damage may shed light on processes as cancer and aging.

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Chapter 6

Concluding remarks and future directions

Concluding remarks and future directions

Since the integrity of the genome is continuously challenged by a wide variety of different genotoxic insults, cells are equipped with an extensive set of genome caretaking processes. These include (i) various DNA repair pathways to remove DNA lesions, (ii) cell cycle regulation mechanisms that provide the cell with the possibility to extent the time window for repair of DNA damage, and (iii) pathways (e.g. apoptosis, senescence and mitotic death) that remove cells with excessively damaged genomes. As mentioned in the introduction, a specific balance between these mechanisms might exist in different cell types, in order to allow a cell type specific response to DNA damage. Hereby, the particular needs of the cell and the organism can be met. For example, persistent DNA damage in proliferating cells (e.g. skin or intestinal epithelium) can lead to mutations and thereby cause cancer. To counteract this process, a cell is able to commit apoptosis and thereby prevent accumulation of pre-mutagenic cells. However, loss of cells due to a high level of apoptosis can result in the loss of tissue homeostasis. This is extremely evident for non-regenerative tissues such as the brain, where neuronal loss is not compensated by tissue renewal. But even in the case tissue regeneration can take place, like in the liver, excessive apoptosis in principle can exhaust the regenerative capacity as a result of stem cell depletion by senescence or apoptosis. This depletion of regenerative potential may underlie the aging process. In short, it seems that genome caretaking is a continuous balance between cancer and aging.

6.1 A cell type specific DNA damage response

The work presented in this thesis has uncovered a marked difference between different cell types in their response to genotoxic treatments. Pluripotent embryonic stem cells (ES cells) were shown to be more sensitive than more differentiated cell types like mouse embryonic fibroblasts (MEFs) for treatment with UV-light, ionizing radiation or mitomycin C (chapter 5). In contrast, keratinocytes are more resistant to UV-light and ionizing radiation than MEFs (chapter 3). Also, deficiencies for repair genes may have a different effect on cellular sensitivity in different cell types. We showed that whereas a deficiency for *Csb* causes γ-ray sensitivity in MEFs, keratinocytes and ES cells (chapter 2 and 3), a *Csa* deficiency causes γ-ray sensitivity in ES cells, but not in MEFs and keratinocytes (chapter 3). Moreover while *Xpa*$^{-/-}$ MEFs are not sensitive for ionizing radiation, *Xpa*$^{-/-}$ ES cells and mice are (chapter 2). Similarly, while *Csa*$^{-/-}$ MEFs are sensitive for mitomycin C, *Csa*$^{-/-}$ ES cells attribute a similar sensitivity as wt ES cells. Moreover, *Csa*- and *Csb*-deficient mice attribute a similar mitomycin C sensitivity, while in MEFs only a *Csa*-deficiency causes mitomycin C sensitivity (chapter 4). Finally, we showed that in ES cells regulation of apoptosis and cell cycle after UV-irradiation mainly depends on damage in the entire genome, whereas in MEFs, lesions that hinder transcription are a strong trigger for apoptosis and cell cycle regulation (chapter 5). This is reflected by the fact that the severest UV-sensitivity is found in GG-NER deficient ES lines and in TC-NER deficient MEFs. Moreover, although *Xpa*- and *Xpc*-deficient ES cell lines are both UV-sensitive, only *Xpa*$^{-/-}$ ES cells show a marked induction

in UV-induced apoptosis. Therefore we suggested in chapter 5 that probably XPC protein might play a role in apoptosis induction in ES cells.
In conclusion, we clearly showed a marked heterogeneity in stress response between different cell types. This is reflected both by the intrinsic sensitivity as well as by the effect of mutations in DNA repair genes on this sensitivity. Taken in account this heterogeneity it is difficult to extrapolate sensitivities from one cell type to another. Moreover, this can severely hinder extrapolation of data found in cell lines to the level of the whole organism. Studying only one cell type could therefore severely bias the conclusions. As a consequence, when studying the cause of certain diseases, the use of different cell types (ideally those cell types with relevance for the disease-etiology) is recommended.

6.2 Phenotypical differences between CSA and CSB?

Cockayne syndrome (CS), a rare inherited disorder characterized by UV sensitivity, severe neurodevelopmental and progeroid symptoms, is caused by mutations in the *CSA* or *CSB* gene. The loss of functional CSA or CSB proteins leads to a deficiency in the transcription-coupled repair pathway of NER. In contrast, mutations that cause a complete inactivation of the whole NER reaction (as in XPA) lead to the disease xeroderma pigmentosum. Although this disease is characterized by severe photosensitivity and increased carcinogenesis, XP does not share with CS the severe neurodevelopmental and progeroid symptoms, and can therefore be regarded as milder. These data suggest that the function of CS-proteins may not be restricted to TC-NER. Indeed, functions of CSB in transcription as well as in (transcription-coupled) repair of non-NER lesions have been put forward.
In line with these findings, we have provided evidence that *Csb*-deficient MEFs, keratinocytes, and ES cell are sensitive for oxidative DNA damage, which is not considered to be a classical NER-lesion (chapter 2 and 3). Moreover, $Csb^{-/-}$ mice are sensitive for oxidative DNA damage (chapter 2 and 3). Strikingly, completely NER-deficient $Xpa^{-/-}$ MEFs are not hypersensitive to γ-rays, whereas $Xpa^{-/-}$ ES cells and mice appear to be sensitive to oxidative DNA damage. This suggests that, at least in some cell types, repair of oxidative DNA damage requires classical TC-NER, while in other cell types a distinct non-NER related function of the CSB protein is needed.
Systematic differences in the symptoms between patients belonging to complementation group A or B have not been found. Similarly, *Csa*- and *Csb*-deficient mice attribute a comparable phenotype in all parameters studied thus far. Therefore, it has often been assumed that CSA and CSB function in the same pathway(s), and that mutations (or deletions) in the *CSA* or *CSB* genes cause the same phenotype. Interestingly, the work presented in this thesis for the first time uncovers a difference between CSA and CSB cells. We show that cells lacking *Csa*, in contrast to *Csb*-deficient MEFs and keratinocytes, are not sensitive for oxidative DNA damage. Similarly, $Csa^{-/-}$ mice appear not sensitive to the higher levels of 8-oxo-dG originating from a DEHP containing diet, whereas DEHP-evoked cytotoxic effects (detected as loss of body weight) are readily observed in $Csb^{-/-}$ mice (chapter 3). These findings seriously question the paradigm that CS is mainly caused by a defect in repair of oxidative DNA damage.

Differences in DNA damage sensitivity between CSA and CSB cells appear not restricted to oxidative DNA damage. We observed that *Csa*$^{-/-}$ MEFs attribute hypersensitivity to mitomycin C treatment, whereas the sensitivity of *Csb*$^{-/-}$ MEFs for this compound is within the wild type range (chapter 4). Therefore, we propose that the CSA and CSB proteins may function in different, not completely overlapping pathways. This hitherto unanticipated finding of mitomycin C sensitivity in *Csa*$^{-/-}$ MEFs, suggests a role of CSA in the cellular response to cross-links. Another NER-factor, ERCC1, has also been associated with cross-link repair, as indicated by the severe cross-link sensitivity in *Ercc1*-deficient cells. However, whereas *Csa*$^{-/-}$ mice posses a mild phenotype, *Ercc1* deficient mice are severely affected and die before weaning. Thus it appears that there is a different need for CSA and ERCC1 in cross-link repair. Therefore, it would be interesting to test the functionality of factors known to be involved in cross-link repair in a *Csa*-deficient background.

6.3 Future directions

The next paragraph lists a few issues that so far remained unsolved and that are interesting to be tackled.

The studies presented in this thesis have shown the existence of different balances between genome caretaking mechanisms in different cell types. These studies were all performed on in vitro cultured, and thus proliferating cells. Since proliferation could have a major impact on cellular sensitivity as well as on which genome caretaking processes are needed, it would be interesting to extend these studies to non-dividing cells, or to cells that only divide after specific triggers. Furthermore, the mechanisms that cause these differences between the various cell types are still fairly unknown. Closer examination of the cytotoxic and genotoxic effects of DNA damaging agents on the various cell types studied thus far, and extension of these assays to other (non-dividing) cell types or organotypic cultures, will further help to unravel these mechanisms. To our opinion, these studies should use a combination of different techniques. Classical genetics and cell biology should be combined with powerful new techniques such as transcriptomics and proteomics.
For example, to shed more light on the function of XPC in the UV response, a genetic approach can be taken. Here fore, ES lines defective for both *Xpa* and *Xpc* can be isolated. Classical experiments like survival, apoptosis and cell-cycle assays can already disclose part of the picture. However we strongly belief that for example expression profiling of the cellular response to stress at the RNA, as well as at the protein level can provide us with an more integrated picture. To our opinion such an integrated approach is not only recommended for this particular example, but can also easily be applied to more general questions like what causes the difference in stress responses in various cell types. To this end, we recently started to explore in our laboratory the transcriptional response of mouse dermal fibroblasts and embryonic stem cells to exposure to UV light in a dose and time dependent manner. These studies will probably be extrapolated to other cell types as well as expanded by proteomic studies.
Our findings that CSB is involved in the cellular response to oxidative DNA damage, and that in contrast CSA is involved in cellular resistance against mitomycin C, suggest an

additional role of these proteins in different pathways. So far, it is not exactly known how the CSA and CSB proteins function in the cellular response to oxidative DNA damage and interstrand cross-links, respectively. To unravel these questions, an integrated approach is needed. On the one hand, we need to establish how mutations in the *Csa* or *Csb* gene influence the cellular stress response to various genotoxic agents. To achieve this goal, the "–omics" approaches as well as classical cell biological and genetic tools can be used. On the other hand, detailed knowledge on the function of these proteins in the cell upon DNA damage should be gathered. To answer these questions, classical biochemistry as well as live cell imaging are powerful techniques. To this latter end, specific proteins can be tagged with fluorescent markers. A promising recent development in this field is the use of cells (and tissues) derived from knock-in mouse models that express fluorescent protein tagged repair proteins at physiological levels from the endogenous locus, rather than (over)expressing the tagged proteins by cDNA-transfection. In addition, the combined use of a fluorescent protein tag with an HA or his-tag will permit rapid isolation of the tagged protein (or protein complex) of interest from a variety of cells in a defined genetic background. Moreover, proteomic approaches could unravel complexes wherein these proteins are constituents and thereby provide knowledge on their possible roles. Integration of data obtained in these different experimental systems will eventually lead to a better understanding of the function(s) of these proteins and thereby also shed light on the underlying cause of the associated disease.

Last but not least, a major challenge for (molecular) biologists, geneticists and bioinformaticists will lie in the development of high-quality bioinformatics tools that integrate the wealth of heterogeneous data sets produced by the (semi) high throughput approaches as depicted above in order to provide a systemic picture of the biological processes that make up life.

Chapter 7

Samenvatting

Samenvatting

7.1 Inleiding

Het menselijke lichaam is opgebouwd uit vele honderden miljarden cellen, die afhankelijk van hun functie, kunnen worden onderverdeeld in ongeveer 200 verschillende celtypen (bijvoorbeeld huid-, spier-, bloed-, en zenuwcellen). Een groep van verschillende soorten cellen samen vormt een orgaan, dat een bepaalde functie in het lichaam vervult. De informatie die nodig is voor het functioneren van de cel en het bouwplan van de organen (en uiteindelijk het gehele organisme) is opgeslagen in de genen in het DNA, de drager van het erfelijke materiaal. Met de overdracht van één set DNA in de bevruchte eicel is alle erfelijke informatie aanwezig die nodig is om uit te groeien tot een compleet organisme. Elke cel bevat hetzelfde DNA, maar door het wel of niet actief zijn van verschillende genen kunnen cellen verschillende functies hebben. Met actief zijn van genen wordt bedoeld dat deze afgelezen (dat wil zeggen gekopieerd) worden (dit proces wordt transcriptie genoemd). Deze gekopieerde informatie, het RNA, wordt vervolgens door de cel vertaald in de functionele onderdelen (eiwitten) van de cel (dit proces heet translatie).
Het is van groot belang is dat het DNA intact blijft, echter de integriteit van het DNA wordt continu bedreigd. DNA schade ontstaat door exogene bronnen, zoals zonlicht, Röntgenstraling, chemotherapeutica en polycyclische koolwaterstoffen (onder andere aanwezig in sigarettenrook en uitlaatgassen), maar ook door endogene bronnen, zoals zuurstof radicalen die tijdens het normale cel metabolisme gevormd worden. Het kopiëren van beschadigd DNA tijdens de celdeling kan leiden tot permanente veranderingen in het DNA (mutaties). Mutaties kunnen ongeremde celgroei veroorzaken, waardoor een cel zich kan ontwikkelen tot een kwaadaardige tumor. Een ander nadelig effect van DNA schade is dat de schade ervoor kan zorgen dat de informatie in het DNA, zoals opgeslagen in de genen, niet goed afgelezen wordt en dat daardoor de cel zijn functie niet meer kan vervullen en sterft. Celsterfte als gevolg van DNA schade, en daarmee de teloorgang van de cellulaire opbouw van weefsels en organen, wordt gezien als een mogelijke oorzaak van het verouderingsproces.
Om het DNA intact te houden is de cel uitgerust met verschillende DNA herstel mechanismen. Elk mechanisme verwijdert een bepaalde klasse van DNA beschadigingen, al is er wel overlap tussen de verschillende herstel mechanismen (zie figuur 1 van de introductie). Om de cel voldoende tijd voor herstel te geven kan de celcyclus tijdelijk gestopt worden, waardoor de cel niet meer deelt en er dus ook geen mutaties kunnen ontstaan. Als echter de hoeveelheid DNA beschadigingen te groot is, kan de cel zelfmoord (apoptosis) plegen, waarmee voorkomen wordt dat er eventueel een tumor ontstaat.
Een van de herstel mechanismen, 'Nucleotide Excision Repair' (NER), herstelt schades veroorzaakt door zonlicht en door bijvoorbeeld polycyclisch koolwaterstoffen (onder andere aanwezig in sigarettenrook en uitlaatgassen). Voor het opsporen van DNA beschadigingen gebruikt NER twee subroutes van het NER systeem. In het ene geval zoekt het NER systeem het gehele genoom af naar beschadigingen, in het andere geval loopt de

transcriptiemachinerie vast op beschadigingen en draagt daarmee zorg voor de schade herkenning. Mutaties in genen betrokken bij NER veroorzaken een aantal erfelijke aandoeningen. Het bekendste voorbeeld is xeroderma pigmentosum (XP). Deze ziekte wordt gekenmerkt door een zon- en UV-licht gevoelige huid, met zeer veel moedervlekken op de blootgestelde delen van de huid en een verhoogde kans op zonlicht geïnduceerde huidkanker. Een andere ziekte die geassocieerd wordt met een defect in NER is Cockayne syndroom. Deze ziekte veroorzaakt net als XP zonlichtgevoeligheid, maar wordt vooral gekenmerkt door een scala van lichamelijke en geestelijke afwijkingen. De levensverwachting van een CS patiënt is gemiddeld slechts 12 jaar.

7.2 Dit proefschrift

7.2.1 Hoe wordt CS veroorzaakt?

XP kan veroorzaakt worden door mutaties in verschillende genen. Zowel defecten in het centrale mechanisme van NER als in de herkenning van beschadigingen in het gehele genoom kunnen leiden tot XP. CS wordt veroorzaakt door mutaties in het CSA of het CSB gen die leiden tot defecten in schadeherkenning via de vastlopende transcriptiemachinerie.
Het is vreemd dat het uitschakelen van één herkenningsmechanisme tot een ernstigere ziekte leidt dan het uitschakelen van de totale herstelcapaciteit. Daarom is er gesuggereerd dat CSA en CSB eiwitten ook betrokken zijn bij andere processen dan NER. Het is aangetoond dat cellen van een patiënt met een defect in het CSB gen gevoelig zijn voor ioniserende straling. Ioniserende straling veroorzaakt onder andere oxidatieve DNA beschadigingen, die ook door het normale metabolisme ontstaan. Deze schade wordt niet door NER maar door een ander herstel mechanisme gerepareerd. Het zou dus goed kunnen dat het CSB eiwit betrokken is bij schadeherstel van oxidatieve DNA beschadigingen en dat endogeen ontstane oxidatieve DNA beschadigingen bijdragen aan het ontstaan van de symptomen van CS.
In ons onderzoek hebben we vastgesteld dat verschillende soorten cellen, geïsoleerd uit muizen met een vergelijkbaar *Csb* defect als gevonden in een patiënt, gevoelig zijn voor oxidatieve DNA schade. Echter een *Csa* defect veroorzaakt geen gevoeligheid voor dit type schade. Deze vinding zet vraagtekens bij de veronderstelling dat CS veroorzaakt wordt door oxidatieve DNA schade.
Verder hebben we onderzocht of cellen met een defect in de *Csa* of *Csb* genen gevoelig zijn voor andere typen schade. Het bleek dat *Csa* deficiënte cellen gevoelig zijn voor het chemotherapeuticum mitomycin C. Op cel niveau leidt een defect voor *Csb* echter niet tot een verhoogde gevoeligheid voor mitomycin C, terwijl muizen met een defect in *Csa* of *Csb* beiden gevoelig zijn. Dus ook voor deze andere schade is er geen consistent beeld. Dit alles wijst erop dat CSA en CSB eiwitten blijkbaar in verschillende mechanismen kunnen functioneren.

7.2.2 Verschillen tussen cellen in de DNA schade respons

De vele verschillende celtypes in het lichaam kunnen ieder op hun eigen manier op DNA schade reageren. Een cel kan enerzijds proberen de schade te herstellen, waarbij de cel weliswaar in leven blijft maar wel de kans loopt op permanente veranderingen in het DNA, maar kan anderzijds ook besluiten over te gaan tot apoptosis, zodat potentiële kankercellen worden afgevoerd. In dit onderzoek hebben we gekeken naar de verschillen tussen cellen in hun repons op DNA schade. Hiertoe hebben we verschillende celtypes geïsoleerd. Naast de veelgebruikte fibroblasten (bindweefselcellen) hebben we ook gekeken naar keratinocyten (huidcellen) en embryonale stamcellen (ES cellen). ES cellen zijn de vroegste, nog totaal ongedifferentieerde cellen die het embryo vormen. Vanuit deze cellen ontstaan tijdens de groei en differentiatie van een embryo alle andere soorten cellen. Wij zagen dat ES cellen, vergeleken met de andere cel typen, extreem gevoelig waren voor verschillende soorten DNA schade. Vervolgens hebben we gekeken naar de functie van NER in deze cellen. Het bleek dat inactivatie van NER genen deze cellen nog gevoeliger maakt voor schadelijke gevolgen van UV licht. Ook in fibroblasten leidt het uitschakelen van NER tot een verhoogde UV gevoeligheid. Echter in fibroblasten veroorzaakt vooral het uitschakelen van de schadeherkenning door de vastlopende transcriptiemachinerie een verhoogde UV gevoeligheid, terwijl in ES cellen juist het uitschakelen van de algemene schadeherkenning er voor zorgt dat de cel erg gevoelig wordt voor UV. Het lijkt erop dat het voor een ES cel veel belangrijker is om het DNA intact te houden dan voor een fibroblast. Dit kan ermee te maken hebben dat een ES cel zijn DNA doorgeeft aan heel veel dochtercellen, terwijl fibroblasten het DNA alleen nog gebruiken om RNA af te schrijven en daarmee hun specifieke functie te vervullen. Het effect van deze verhoogde gevoeligheid is weliswaar meer celdood, maar de hersteldeficiënte ES cellen hebben een zelfde mutatiefrequentie als ES cellen zonder herstel defect.

List of abbreviations

ATM	ataxia telangiectasia mutated (gene)
BER	base excision repair
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
CSA/CSB	Cockayne syndrome A/B (protein)
DAR	differentiation associated repair
DDB	DNA damage binding protein
DEHP	di(2-ethylhexyl)phthalate
ss/dsDNA	single/double stranded DNA
DNA	deoxyribonucleic acid
DSB	double strand break
ERCC	human excision repair cross complementing (gene)
ES	embryonic stem (cell)
FA	Fanconi anemia
GG-NER	global genome NER
HNPCC	hereditary non-polyposis colorectal cancer
hHR23B	human homolog of *S.cerevisiae* repair protein RAD23B
HPLC	high performance liquid chromatography
HR	homologous recombination
kDa	kilodalton
MEF	mouse embryonic fibroblast
Mfd	mutation frequency decline
MMC	mitomycin C
MMR	mismatch repair
NER	nucleotide excision repair
NHEJ	non homologous end-joining
nt	nucleotide
8-oxodG	8-oxo-2'-deoxyguanosine
PCNA	proliferating cell nuclear factor
(6-4)PP	(6-4) pyrimidine-pyrimidone photoproduct
RFC	replication factor C
RNA	ribonucleic acid
ROS	reactive oxygen species
RPA	replication factor A
TC-NER	transcription-coupled NER
TCR	transcription-coupled repair
TFIIH	transcription factor IIH
TLS	translesion synthesis
TTD	trichothiodystrophy
UV	ultraviolet (light)
wt	wild type
XP	xeroderma pigmentosum
XPA-G,V	xeroderma pigmentsosum group A-G,V (protein)

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List of publications

de Waard H., de Wit J., Gorgels T.G.M.F., van den Aardweg G., Andressoo J.O., Vermeij M., van Steeg H., Hoeijmakers J.H.J., van der Horst G.T.J. Cell type specific hypersensitivity to oxidative damage in CSB and XPA mice, DNA repair 2 (2003) 13-25

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de Waard H., Sonneveld E., de Wit J., Esveldt-van Lange R., Hoeijmakers J.H.J, Vrieling H., van der Horst G.T.J. Repair or die; mutation prevention in embryonic stem cells, manuscript in preparation

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