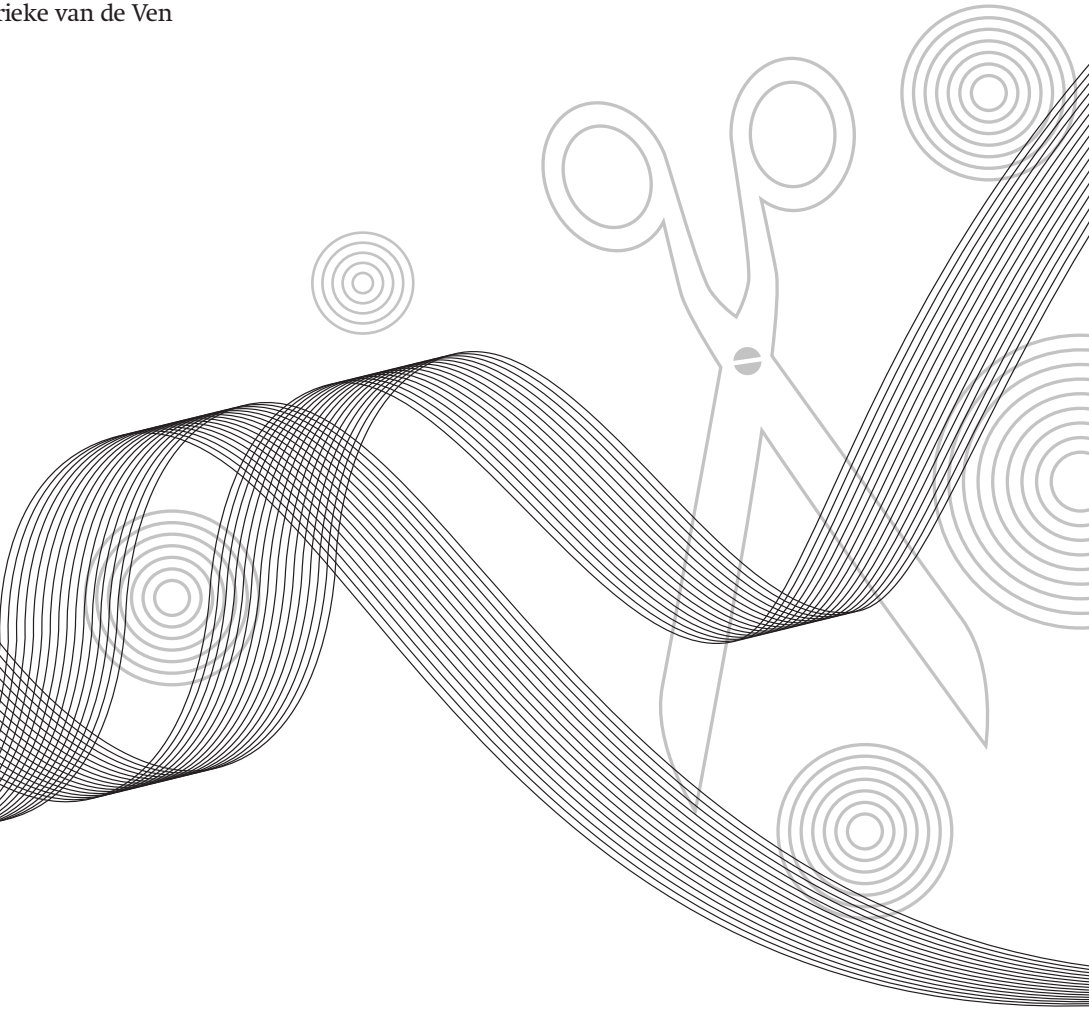
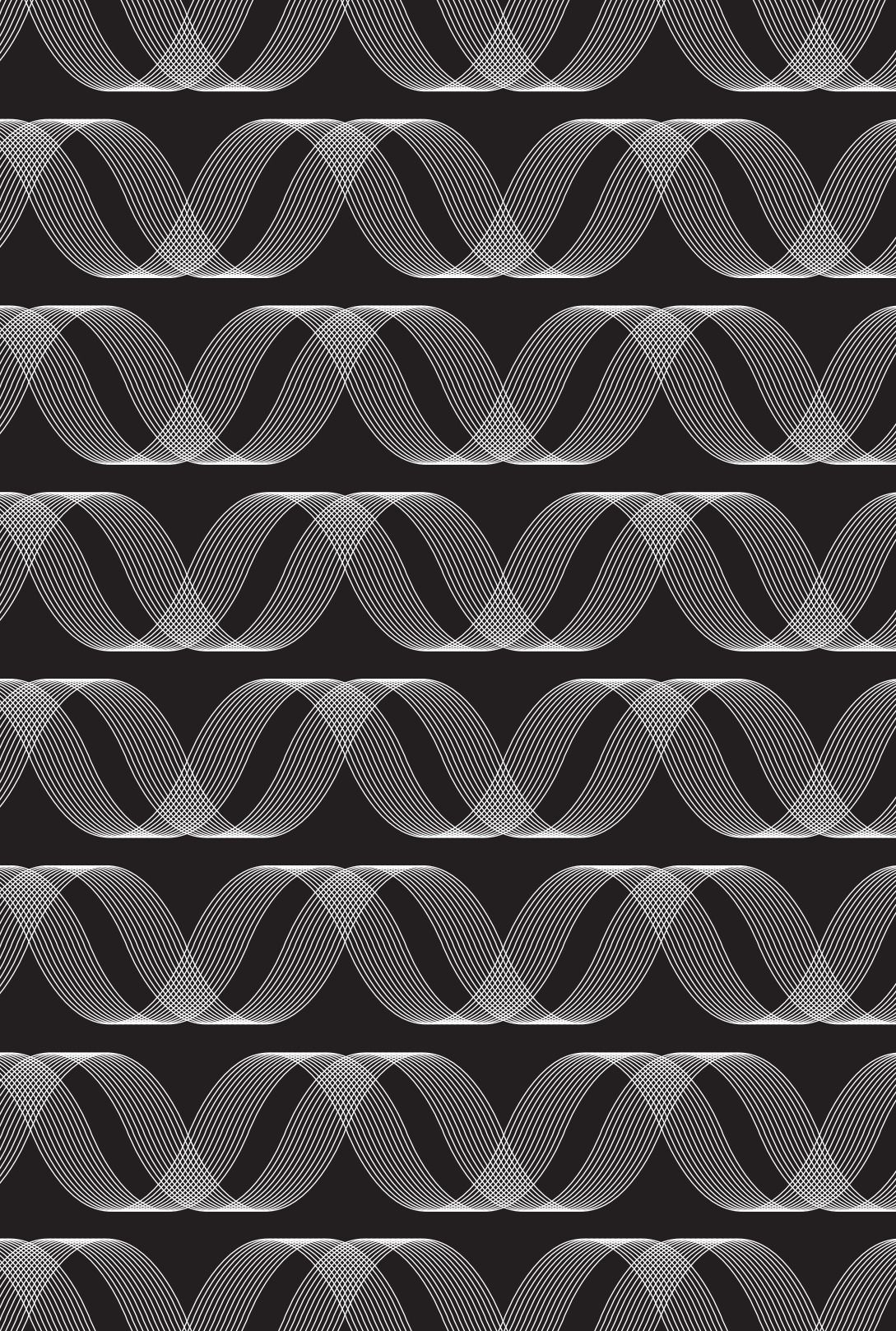
ucleotide Excision epair in Cancer, geing and tress Resistance

rieke van de Ven





Nucleotide Excision Repair in Cancer, Ageing and Stress Resistance

Nucleotide excisie herstel in kanker, veroudering en stress resistentie

ISBN 978 90 8570 285 6

Nucleotide Excision Repair in Cancer, Ageing and Stress Resistance

Nucleotide excisie herstel in kanker, veroudering en stress resistentie

PROEFSCHRIFT

~

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor
Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 20 mei 2009 om 09.45 uur

DOOR

~

Henrica Wilhelmina Maria van de Ven
(Marieke), geboren te Eindhoven

PROMOTIECOMMISSIE

Promoter:	Prof.dr. J.H.J. Hoeijmakers
Overige leden:	Prof.dr. J.A. Grootegoed Prof.dr. H. van Steeg Prof.dr. J.N.M. Ijzermans
Copromoter:	Dr. J.R. Mitchell

Dit proefschrift kwam tot stand binnen de vakgroep Genetica van de faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuidwest Nederland. Het onderzoek is financieel gesteund door het ZonMw programma RIDE. Bijdragen in de drukkosten zijn verkregen van de J.E. Jurriaanse Stichting te Rotterdam, HemoCue Diagnostics B.V. te Waalre en Greiner bio-one te Alphen a/d Rijn.

Contents **page**

1

Introduction in DNA repair.

~

Marieke van de Ven, Jaan-Olle Andressoo,
Valerie B. Holcomb, Paul Hasty, **Yousin Suh,**
Harry van Steeg, **George A. Garinis,**
Jan H.J. Hoeijmakers, James R. Mitchell

Introduction in DNA repair

The genetic information that takes care of the proper functioning of all cell types that makes up an organism is organized in DNA. This so-called blue print of life is continuously attacked by a variety of genotoxic agents and environmental stresses that can damage the DNA (Figure 1). For instance, ultraviolet radiation (UV) causes helix-distorting lesions, cis-syn-cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6,4)-pyrimidone products (6-4PPs) (Sancar, 1996). On the other hand, ionizing radiation (IR) can cause formation of single strand breaks (SSBs) and double strand breaks (DSBs) (van Gent et al., 2001). The organism's own metabolism generates reactive oxygen species (ROS) (including superoxide anions, hydrogen peroxide and hydroxyl radicals and their numerous subsequent reaction products) lipid peroxidation products, oestrogen metabolites, reactive carbonyl species, endogenous alkylating agents, spontaneous hydrolysis and deamination products (De Bont and van Larebeke, 2004). These result in DNA damages like oxidative DNA lesions, including 8-oxo-2'-deoxyguanosine (8-oxodG), thymine glycols, cyclopurines and SSBs and DSBs (Hoeijmakers, 2001). Finally, spontaneous modifications of nucleotides such as hydrolysis leading to abasic sites are common in cells. In total this adds up to 10^4-10^5 lesions per cell per day (Lindahl, 1993). The consequences of DNA damage can be severe and may lead to cellular malfunctioning caused by hampered transcription and replication, and may result in permanent cell cycle arrest (senescence) or cell death (apoptosis). If the cells don't get replaced, and the tissue or organismal homeostasis gets lost, it can result in (premature) ageing (Mitchell et al., 2003). However, when these DNA damages result in irreversible mutations or chromosomal aberrations, and therefore replication errors, these mutations can lead to carcinogenesis (Mitchell et al., 2003). A complex network of DNA repair pathways (first found in bacteria and yeast), each selective for a specific subset of DNA lesions has evolved to overcome early onset of cancer or ageing. However, the more complex the system, the more sensitive it is to errors and deficiencies.

Repair pathways

In mammals, multiple partially overlapping DNA repair mechanism exist, each with their own damage specificity (Essers et al., 2006). Base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double strand break repair are the four main DNA repair pathways. Each of these repair pathways uses different modes of damage recognition, which in most cases depends on the damage appearance on the DNA in terms of helix distortions, obstruction of DNA probing, or blockage of DNA transcription or replication (Hanawalt, 2002; Hoeijmakers, 2001; Jackson, 2002). Lesions that are recognised and repaired by BER or NER only affect one strand of the double helix. BER repairs non-bulky lesions produced by the alkylation, oxidation or deamination of bases, and single strand DNA breaks. A DNA glycosylase enzyme, followed by excision of the resulting sugar phosphate, removes the altered base in BER. The small gap left in the DNA helix is filled in by the sequential action of DNA polymerase and DNA ligase

(Parikh et al., 1997). NER can recognise a large range of structurally very different DNA damages. The recognition of DNA damage in NER is less specific than in BER and this process can thus repair a wider range of damage types. There are two modes of NER, global genome repair (GG-NER), which removes damage throughout the whole genome and transcription-coupled repair (TC-NER), which specifically repairs the transcribed strand of active genes from lesions that actually block transcription (de Laat et al., 1999; Hanawalt, 2002). A more extensive explanation into the mechanism or NER will follow in the next subsection. Double strand breaks (DSBs) are very problematic, because both strands are affected; therefore at least two major pathways have developed to cope with this problem. First, homologous recombination (HR), which occurs in S and G2 phase cells when an intact second copy of the sequence (sister chromatid) is available. A joint molecule between the homologous damaged and undamaged duplex is formed, followed by DNA synthesis, ligation and resolution of recombination intermediates (van Gent et al., 2001). Second, the less-accurate non-homologous end joining (NHEJ) is most relevant in the G1 phase of the cell cycle when a second copy of the sequence is not available. This pathway uses very limited or no sequence homology and is mediated by direct end-joining (Jackson, 2002). Finally, there is also a repair pathway that recognises and repairs the erroneous insertion, deletion and miss-incorporation of bases that can arise during DNA replication and recombination, as-well as repairing some forms of DNA damage. After recognition of the damage the MMR pathway in *E. coli* (bacteria) distinguishes between the correct (unmutated) and the 'wrong' strand (mutated strand), using the strand-specific methylation fingerprint. Excising the wrongly incorporated base and replacing it with the correct nucleotide to repair the damage (Fleck and Nielsen, 2004). Of all the repair systems, NER is the most all-round in terms of lesion recognition.

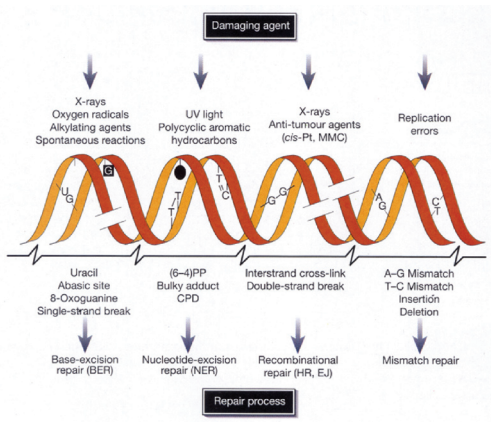


Figure 1.

The top row show DNA damaging agents, the middle row depict examples of DNA lesions induced by these agents, and the bottom row are the most relevant DNA repair mechanism responsible for the removal of the lesions. Abbreviations: cis-Pt and MMC, cisplatin and mitomycin C, respectively (both DNA-crosslinking agents); (6–4)PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light); BER and NER, base- and nucleotide-excision repair, respectively; HR, homologous recombination; EJ, end joining. This figure was adapted from Hoeijmakers (2001).

Nucleotide excision repair

Nucleotide excision repair (NER) removes a wide variety of DNA helix-distorting injuries of both exogenous and endogenous origin. It is a multistep pathway that is responsible for the removal of damage such as induced by UV irradiation. UV radiation contained in the solar light can be divided into three wavelengths: UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm). Shorter wavelengths (that are more highly absorbed by the ozone layer) are more effective in causing DNA damage than the higher wavelengths (Setlow, 1974). UVB and UVC can produce photolesions in the DNA like CPDs and 6-4PPs, which are recognised and repaired by NER. NER also removes bulky chemical adducts, intrastrand cross-links and several forms of oxidative lesions such as cyclopurines induced by reactive chemicals, ROS or cigarette smoke (Hoeijmakers, 2001; Smith and Pereira-Smith, 1996). There are two subpathways of NER, GG-NER and TC-NER. GG-NER has its focus of repair on the entire genome, whereas TC-NER only repairs lesions on actively transcribed parts of the DNA that arrest transcription. CPDs are mostly removed by TC-NER, and are therefore preferentially removed from transcriptionally active genes (Bohr et al., 1986), while (6-4)PP lesions, which distort the DNA more than CPDs, are more rapidly removed by GG-NER (Mullenders and Berneburg, 2001). In NER about 30 proteins are involved, most function in GG-NER as well as in TC-NER (Christmann et al., 2003). However they have different modes of damage recognition and therefore different proteins that recognize the damage. NER is a complex process in which at least the five following enzymatic steps can be distinguished: I) recognition of the DNA distortion; II) DNA unwinding around the damage; III) 'bubble' formation and multi protein complex stabilization; IV) incision on both sides of the damaged DNA; V) DNA repair synthesis and ligation of the newly synthesized strand (Figure 2).

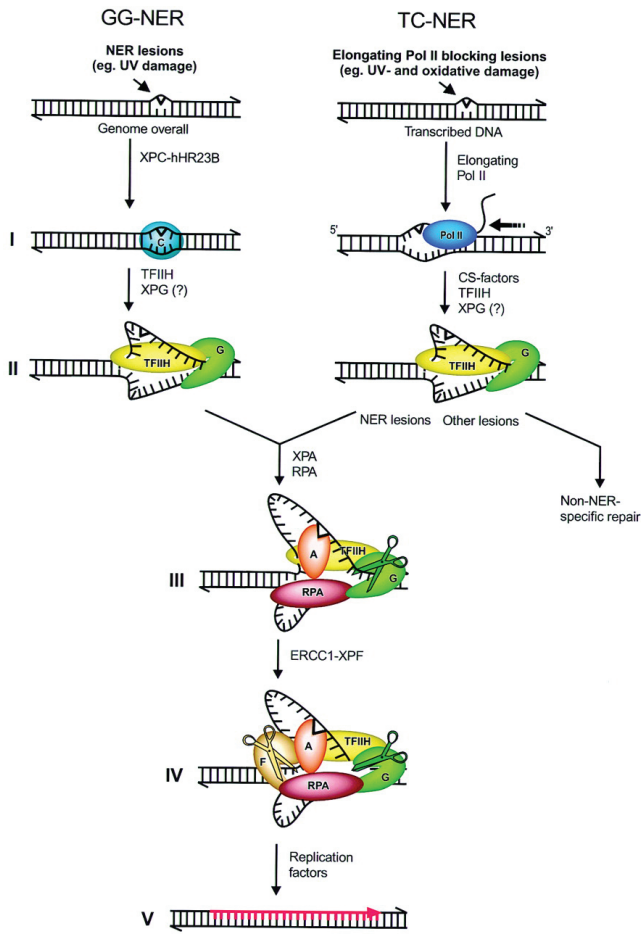


Figure 2.

NER and the proteins involved, divided in GG-NER and TC-NER. Steps I to V show the different proteins in action following UV damage. Step I: damage recognition (the blue C stands for XPC, and the brown E stands for XPE), step II: DNA unwinding (G (green), stands for XPG), step III: bubble formation (A (red), stands for XPA), step IV: incision (F (beige), stands for the ERCC1-XPF complex), step V: de novo synthesis. For further description see text. This figure is taken from de Laat et al. (1999).

Recognition

The first step in DNA repair is the recognition of the damage, this is also the step where GG-NER and TC-NER differ. GG-NER uses the XPC-hHR23B protein complex as the primary recognition factor (Hanawalt, 2002; Sugasawa et al., 1998; Volker et al., 2001). XPC-hHR23B has a high affinity to DNA damaged by UV. It recognises the damage and binds it, thereby locally distorting the DNA double helix and recruiting other factors of the NER pathway (de Laat et al., 1999; Gillet and Scharer, 2006; Trego and Turchi, 2006). Recently it has become clear that, for certain types of lesions, different proteins are responsible for the initial recognition. XPE (or DDB2) bound to DDB1 can recognise CPD lesions. The XPE-DDB1 complex recruits the XPC-hH23B complex to the lesion, where it gets exchanged by the XPC-hH23B complex and repair takes place (Kulaksiz et al., 2005). TC-NER lesion recognition occurs in a different manner compared to GG-NER recognition. In this case, the XPC-hHR23B protein complex is redundant since there already is 'bubble' formation at the location where RNA polymerase II (RNApolII) is stalled by a lesion (Hanawalt and Mellon, 1993; Thoma and Vasquez, 2003). Earlier it was believed that arrested transcription by RNApolII resulted in the recruitment of a large protein complex. This complex included the CSA and CSB proteins and some MMR proteins (Friedberg, 2001). However, more recent research suggests that CSA and CSB function in different protein complexes from each other. CSA is associated with RNApolII, and CSB is found in a different complex together with RNApolII. They both assist RNApolII, either blocking it, in the case of UV damage, or assisting the polymerase to progress in case of transcription blocks due to secondary DNA structure (Lehmann, 2003; van Gool et al., 1997). After recognition, CSB assembles the other NER proteins for the damage repair to take place.

DNA unwinding and bubble formation

In GG-NER the DNA lesion is recognised by XPC-hHR23B. Next, this complex attracts the rest of the NER proteins to further open up the DNA. XPC-hHR23B together with the multi-subunit transcription factor IIH (TFIIH) is responsible for the initial opening of the first 10 nucleotides. TFIIH consists of ten subunits, including XPB and XPD. XPB and XPD are ATP-dependent 3'-5' and 5'-3'directed helicases respectively, and play a key role in opening up the DNA around the damage (Evans et al., 1997), and transcription initiation of RNApolII at the promoter (Holstege et al., 1996). On the other hand, in TC-NER the stalled RNApolII has to be removed first before excision repair can proceed. CSA and CSB together with TFIIH and XPG can displace the stalled polymerase II, so the rest of the NER reaction can take place (de Laat et al., 1999). XPA and replication protein A (RPA) when bound together dislocate XPC-hHR23B from the damaged site since this protein complex is no longer needed (Fousteri et al., 2006). The full opening of about 25 nucleotides is also obtained with the help of XPA and RPA while XPG is there to stabilize the whole complex. This process is the same for GG-NER and TC-NER. The proteins have binding sites for the TFIIH complex, which is recruited in both types of NER.

Dual incision

After DNA is unwound, an oligonucleotide of 24 to 32 nucleotides containing the lesion is finally excised by ERCC1-XPF and XPG. ERCC1-XPF makes the incision at the 5' end of the lesion and XPG at the 3' end of the lesion. RPA interacts with both XPG and ERCC1 by ERCC1 at its 3'- oriented side and XPG at its 5'-oriented side. While bound to the undamaged strand, RPA coordinates strand specificity to ERCC1-XPF by strongly stimulating incisions in the damaged strand and therefore inhibiting cuts in the undamaged strand (de Laat et al., 1999). On the 3' incision site, RPA together with TFIIH positions XPG on the right strand (Evans et al., 1997; Iyer et al., 1996). Evans et al. (1997) suggested that XPB has an additional function in facilitating ERCC1-XPF in making the 5' incision. A patient missing only the C-terminus of XPB did not have a defect in open complex formation, but 5'-incisions were prevented (Evans et al., 1997). After the lesion has been removed, RPA stays associated with the excised DNA, initiating the assembly of resynthesis factors (Riedl et al., 2003).

Synthesis and ligation

The final step is synthesis of DNA for the excised patch, and to this end the intact strand is used as a template to fill in the gap. DNA polymerase δ, ε and the lately found κ, replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and RPA are all necessary for efficient repair synthesis (Lehmann et al., 2007; Shivji et al., 1995). The ligation of the newly synthesized DNA, that is probably done by DNA ligase I, is the final step of the NER reaction (Barnes et al., 1992).

Nucleotide excision repair deficiency syndromes

Any imaginable deficiency in one of the repair proteins can lead to serious disorders. Analysis of these disorders helped us to understand the mechanisms of DNA repair and highlighted their importance. For NER, there are three known deficiency syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). The three NER deficiency syndromes XP, CS and TTD are the result of mutations in the NER system. The genetics involved in the diseases contributed to unravel the NER pathway. Seven complementation groups have been identified in XP (XP-A to XP-G), two in CS (CS-A and CS-B), and three in TTD (XP-B, XP-D, and TTD-A) There is also a fourth syndrome, which is a combination of XP and CS, XPCS which will also be discussed in this section, and was identified in 4 complementation groups (XP-B, XP-D, XP-G and XP-F) (Broughton et al., 2001; Giglia-Mari et al., 2004; Hoeijmakers, 2001)

Xeroderma pigmentosum

XP is a group of rare autosomal-recessive inherited disorders characterized by a parchment skin (xeroderma) that is extremely sensitivity to ultraviolet (UV) light, abnormal skin pigmentation (pigmentosum), and high frequency of skin cancers, especially on sun-exposed areas. The mean age of onset of these symptoms is 2 years (Kraemer, 1997). Early clinical signs are erythema and blistering after exposure to sunlight followed by atrophy, scabbing and scarring of the skin, and degenerative alterations of the eyes (Weeda et al., 1998). Xeroderma pigmentosum (XP) was first described in 1874 by Hebra and Kaposi, and eight years later Kaposi coined the term xeroderma pigmentosum for the condition, referring to its characteristics (Norgauer et al., 2003). In 1968, Cleaver discovered that fibroblasts taken from the skin of XP patients carry out less UV-induced DNA synthesis than normal fibroblasts. He hypothesised that this was due to reduced capacity for repair of UV-DNA lesions (Cleaver, 1968). De Weerd-Kastelein et al. (1977), discovered that fusing cells from different XP patients could correct the repair defect. They discovered that there were different complementation groups for XP patients. More complementation studies showed the involvement of 7 genes in XP (XP-A to XP-G) (Bootsma et al., 1995). While XPC and XPE are specifically deficient in the GG-NER pathway (Venema et al., 1991), the other five complementation groups have a defect in both subpathways. The generally mild XP features (except for cancer risk) in XPC and XPE patients can therefore be explained by the fact that TC-NER is still functional. Moreover, many XP mutations do not cause complete inactivation of the protein, and therefore have residual repair capacity, which can explain the milder forms of XP. XP patients develop normally, but die of neoplasia at an average age of 30 years unless when exquisitely protected from sunlight (Kraemer et al., 1984). Neurological abnormalities are reported in about 18% of XP patients, likely caused by enhanced neuronal degeneration. Neurological symptoms only occur in XP patients belonging to complementation groups A and D (and C only when they become older), that have a severe loss of repair capacity (Rapin et al., 2000). The most severe early onset neurologic subtype of XP (mostly caused by mutations in XPA) is De Sanctis-Cacchione Syndrome (XP-DSC). In addition to the neurological symptoms described above, this disease involves dwarfing, hypogonadism and a 10-20 fold increase in frequency of several types of internal cancers (Kraemer et al., 1987).

The first mouse model successfully made was for XPA in 1995 (de Vries et al., 1995; Miyauchi-Hashimoto et al., 1996). Later, an XPC mouse model was made (Sands et al., 1995), these mice are fertile and mimic the XP patients' response to UV exposure, being highly susceptible to developing skin cancer at the UV exposed areas of their skin. Mouse models for other XP complementation groups quickly followed, like XPG and XPF; both mice showed a growth retardation and premature death (Harada et al., 1999; Tian et al., 2004). Attempts of making fully XPD-deficient mice have failed. This emphasizes the essential function of the XPD protein and is consistent with the notion that only subtle XPD mutations are found in XP, XPCS, and TTD patients (Andressoo et al., 2006a; de Boer et al., 1998).

Cockayne syndrome

Cockayne, in 1936, reported two siblings with dwarfism, microcephaly, mental retardation, deafness, a photosensitive dermatitis and a peculiar form of retinal pigmentation. Similar to XP, CS is an autosomal recessive disease, characterised by cutaneous photosensitivity. However, CS differs from XP by the occurrence of profound postnatal growth failure (cachexia), progressive neurological dysfunction and no UV-induced skin cancer predisposition (Rapin et al., 2000). However, skin fibroblasts from affected patients show sensitivity to sunlight. This low cancer predisposition is probably associated with functional GG-NER in CS cells and an increased apoptosis rate, which likely removes premutagenic cells (Ljungman and Lane, 2004). Also the mean age at death (12.5 years) (Nance and Berry, 1992), and the fact that the disease is noted very early in life could allow adequate protection. CS is a disease with clinical features that could range from mild to severe and are diverse. Therefore, patients are divided in differential clinical groups according to severity and onset of the disease: mild CS, classical CS (CS1), and severe CS (CSII) (Lowry, 1982), reviewed by (Rapin et al., 2000). In general, CS patients display skeletal abnormalities like kyphosis, bird-like face and early onset of osteoporosis, and also impaired sexual development, caries, cachexia, and mental retardation. Other symptoms found in CS patients are pigmentary retinopathy, thin hair and cataracts, and many of these symptoms classify CS as progeriod disease. The most common cause of death is pneumonia, resulting from an overall poor condition of patients and due to the severe neurological degeneration (Nance and Berry, 1992). Molecular research of CS patients showed that mutations in two genes, *CSA*, *CSB*, could cause the CS phenotype.

In 1997 CSB-deficient mice were generated, the mice display all off the CS repair characteristics: UV sensitivity, inactivation of TC-NER, unaffected GG-NER, and an inability to resume RNA synthesis after UV exposure. Growth failure and neurological dysfunction are present in a mild form. In contrast to the human syndrome, CSB-deficient mice show a slight increased susceptibility to skin cancer after UV exposure, but a normal lifespan (van der Horst et al., 1997). In 2002 CSA-deficient mice were generated, showing the same phenotype as the CSB-deficient mice (van der Horst et al., 2002). In 10% of reported CS cases, the disease is caused by mutations in *XPD*, *XPB* or *XPG* gene, which leads to the combined phenotype of XP and CS. In contrast to classical CS, these patients are more cancer-prone.

Trichothiodystrophy

The term trichothiodystrophy (TTD) was first introduced in 1980 by Price et al. for sulfur-deficient brittle hair. Patients with trichothiodystrophy have brittle hair and nails (because of reduced content of cysteine-rich matrix proteins), ichthyotic skin, physical and mental retardation, short stature and decreased fertility (Price et al., 1980). Other prominent features are an aged appearance, shortened lifespan, skeletal abnormalities (osteopenia, osteoporosis, kyphosis) (McCuaig et al., 1993). Brittle hair and nails are a

unique symptom in TTD patients, whereas other features are strikingly similar to CS, including the absense of cancer predisposition. Cell fusion experiments demonstrated that in all but 3 trichothiodystrophy (TTD) cell strains examined until 1993, the repair deficiency was in the same complementation group as is the defect in xeroderma pigmentosum group D (Stefanini et al., 1993). Later two other complementation groups were found for TTD, namely a mutation in the XPB gene found in siblings who were diagnosed with TTD at the age of three (Weeda et al., 1997b), and a third complementation group which is called TTD-A. The protein that is mutated in this latter complementation group is part of the TFIIH protein complex, like XPB and XPD (Giglia-Mari et al., 2004; Vermeulen et al., 2000).

To study the complex clinical symptoms and the paradoxical absence of skin cancer in NER-deficient TTD patients, a mouse model was generated with the same point mutation in the *XPD* gene as found in 5 patients. TTD mice reflect the pleiotropic features of the human disorder, including growth delay, reduced fertility shortened life span, cutaneous abnormalities, mild UV sensitivity of cultured fibroblasts and the remarkable brittle hair phenotype (de Boer et al., 1999). Recent studies with TTD mice have shown accelerated aging symptoms, likely due to accumulation of endogenously generated DNA damage and reduced transcription leading to cell death. Further the mice show signs of calorie restriction (CR) although their food intake is normal (Wijnhoven et al., 2005).

Genetically, XPB and XPD are not only involved in NER but also in transcription (as part of TFIIH), and mutations that causes TTD are all found in components of the TFIIH complex. Therefore, the assumption is made that the TTD phenotype is a combination of repair deficiency (UV-sensitivity, accelerated ageing) together with a transcription deficiency (brittle hair due to instable TFIIH) (Bootsma and Hoeijmakers, 1993). Interestingly, TTD is the third disease associated with XPB and XPD, clearly showing that specific mutations in these proteins can cause different diseases.

Xeroderma pigmentosum combined with Cockayne syndrome

Usually, XP and CS are clinically and genetically distinct. However, in rare cases, CS patients have been shown to have mutations in genes that were previously linked to the development of XP and show combined (XPCS) pathology (Lindenbaum et al., 2001). In total very few cases have been reported of XPCS patients. Rapin et al. reviewed XPCS in 2000, when there were only 9 cases known. The patients had mutations in XPB, XPD or XPG. Recently a XPCS mouse has been generated in our lab by copying a specific XPD point mutation of a patient. The XPCS mice had a shorter mean life span, a phenotype likely at least in part related to an earlier onset of spontaneous cancers, and this mouse displayed an extreme UV-induced cancer predisposition as in the human XPD patient. Thus, the patient-based XPD point mutation recapitulated in mice many elements of both the XP and CS components of the combined disorder (Andressoo et al., 2006b).

Compound heterozygosity

Compound heterozygotes are individuals carrying two different mutant alleles of the same gene in the absence of a dominant WT allele. Genetic interactions between the two differently mutated alleles could result in a wide variety of disease outcomes that are sometimes hard to dissect from the effects of differences in environment and/or genetic background. Because different mutations in *XPD* can give rise to different diseases, *XPD* is a good example of a gene where evidence of potential genetic interactions between two different recessive alleles can be found. For example, certain patients that are called compound heterozygous in their *XPD* gene have a mutation on one allele that in homozygous state gives an XPCS phenotype, and on the other allele is a certain presumed 'null allele'. This 'null allele' is named on the basis of their failure to support viability in a yeast complementation assay. The combination of this 'null allele' in which the XPCS allele gave milder disease symptoms, than what is seen for patients who are hemizygous for the XPCS mutation (only having one *XPD-XPCS* gene and no other) (Andressoo et al., 2006a; Broughton et al., 2001). With a lifespan that is ten times as long as the hemizyous XPCS patients, this presumed 'null allele' has a substantial impact on the disease outcome. Two patients with a combined TTD and XP phenotype are also thought to have phenotypic contributions from both alleles. These patients have one TTD allele, and the other XPD allele is interpreted as a 'null allele' although it partially rescues the typical hair phenotype seen in TTD patients (Andressoo et al., 2006a; Broughton et al., 2001).

To dissect the environmental and background effects from the effect of the two mutated alleles itself, Andressoo et al. (2006) crossed the TTD mouse model with XPCS and XP alleles generated in our lab that fail to support viability on their own in the homozygous state. These inviable alleles partially rescued the TTD-associated premature segmental ageing, cutaneous features, cellular DNA repair capacity, and UV survival when present in a compound heterozygote state together with the TTD allele (Andressoo et al., 2006a). Later crossings were made with a viable version of a XPCS allele and this allele even rescued the TTD phenotypes to a further extent (see also Chapter 6 of this thesis).

More NER discoveries

Many mice that are partially NER-deficient (TTD, ERCC1) or only deficient in TC-(NE)R like CSA and CSB mice, display ageing symptoms, while mice fully defective in NER (XPA) or only in GG-NER, like XPC mice, don't show this progeriod phenotype. An even more accelerated ageing phenotype originated when TTD mice where crossed with mice that have two XPA null alleles. These double mutant TTD|XPA mice were born normally but soon after birth displayed a severe accelerated ageing phenotype which resulted in growth failure, disproportionally big head and limbs, kyphosis, ataxia, spasticity, balance problems, disturbed gait, severe cachexia and death around 2 to 3 weeks of age

(de Boer et al., 2002). The same features were found when the double mutant XPCS|XPA, CSB|XPA, CSB|XPC or CSA|XPC were generated (Andressoo et al., 2006b; Murai et al., 2001; van der Pluijm et al., 2007). This is in contrast with XPC|XPA, CSB|CSA and XPC|TTD animals which are viable and appear to be quite normal. These findings suggest that a complete NER deficiency in combination with CSA, CSB or XPD is necessary to induce this severe premature ageing phenotype. ERCC1, XPF and XPG mutants also live only 3 weeks and have a similar, but not always identical phenotype (Harada et al., 1999; Tian et al., 2004; Weeda et al., 1997a). Compound heterozygous mice for XPD in an XPA background (TTD/XPCS|XPA, hereafter mentioned as CH|XPA) survive the stressful weaning period and show a partial rescue of the severe premature ageing phenotype as would be expected, due to phenotypic contributions of both alleles (see compound heterozygosity section above, and Chapter 3) (van de Ven et al., 2006).

Scope of this thesis

In Chapter 2 we extend this introduction with a review of our findings on longevity-associated and dietary-restricted (DR) characteristics, like lowering of the growth hormone (GH) and insulin-like growth factor (IGF-1) axis, in short–lived progeriod (NER) mouse models as a result of an adaptive stress/survival response. In Chapter 3 we show further phenotypic rescue of progeria in CH|XPA background and identify that also this genotype together with XPCS|XPA overlap with dietary restriction (DR), and the long-lived dwarfism phenotype in terms of repression of the GH and IGF-1 axis. In addition we looked at the acute oxidative stress resistance after DR by ischemia reperfusion injury and found that reduced signalling through insulin and IGF-1 caused by DR induces resistance to ischemia reperfusion injury (IRI) in mice (Chapter 4). In Chapter 5 we test if the adaptive stress response found in NER mice also protects against the acute oxidative stress by ischemia reperfusion injury. CSA and CSB mice were found to have an organ specific protection from acute oxidative stress. Later we looked at the different effects of XPD mutations on cancer and ageing and found out that in CH animals the accelerated aging and calorie restricted-like phenotypes are rescued to near completion. However, this is not the case for repair of UV lesions in various cell-based and in vivo assays. We therefore can say that defective repair of UV lesions is not the (only) relevant cause in the origin of the segmental progeria or calorie restricted-like phenotype (Chapter 6). Finally, we address a total different field where we test if the NER proteins are in fact required for DNA demethylation in a GADD45-dependent reaction and conclude that the TC-NER pathway this is necessary for recognizing the substrate. However, we find that different Xpd mutations behave differently in this activity (Chapter 7).

References

Andressoo, J.O., Jans, J., de Wit, J., Coin, F., Hoogstraten, D., van de Ven, M., Toussaint, W., Huijmans, J., Thio, H.B., van Leeuwen, W.J., et al. (2006a). Rescue of progeria in trichothiodystrophy by homozygous lethal Xpd alleles. PLoS Biol 4, e322.

Andressoo, J.O., Mitchell, J.R., de Wit, J., Hoogstraten, D., Volker, M., Toussaint, W., Speksnijder, E., Beems, R.B., van Steeg, H., Jans, J., et al. (2006b). An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. Cancer Cell 10, 121-132.

Barnes, D.E., Tomkinson, A.E., Lehmann, A.R., Webster, A.D., and Lindahl, T. (1992). Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents. Cell 69, 495-503.

Bohr, V.A., Okumoto, D.S., and Hanawalt, P.C. (1986). Survival of UV-irradiated mammalian cells correlates with efficient DNA repair in an essential gene. Proc Natl Acad Sci U S A 83, 3830-3833.

Bootsma, D., and Hoeijmakers, J.H. (1993). DNA repair. Engagement with transcription. Nature 363, 114-115.

Bootsma, D., Weeda, G., Vermeulen, W., van Vuuren, H., Troelstra, C., van der Spek, P., and Hoeijmakers, J. (1995). Nucleotide excision repair syndromes: molecular basis and clinical symptoms. Philos Trans R Soc Lond B Biol Sci 347, 75-81.

Broughton, B.C., Berneburg, M., Fawcett, H., Taylor, E.M., Arlett, C.F., Nardo, T., Stefanini, M., Menefee, E., Price, V.H., Queille, S., et al. (2001). Two individuals with features of both xeroderma pigmentosum and trichothiodystrophy highlight the complexity of the clinical outcomes of mutations in the XPD gene. Hum Mol Genet 10, 2539-2547.

Christmann, M., Tomicic, M.T., Roos, W.P., and Kaina, B. (2003). Mechanisms of human DNA repair: an update. Toxicology 193, 3-34.

Cleaver, J.E. (1968). Defective repair replication of DNA in xeroderma pigmentosum. Nature 218, 652-656.

de Boer, J., Andressoo, J.O., de Wit, J., Huijmans, J., Beems, R.B., van Steeg, H., Weeda, G., van der Horst, G.T., van Leeuwen, W., Themmen, A.P., et al. (2002). Premature aging in mice deficient in DNA repair and transcription. Science 296, 1276-1279.

de Boer, J., de Wit, J., van Steeg, H., Berg, R.J., Morreau, H., Visser, P., Lehmann, A.R., Duran, M., Hoeijmakers, J.H., and Weeda, G. (1998). A mouse model for the basal transcription/DNA repair syndrome trichothiodystrophy. Mol Cell 1, 981-990.

de Boer, J., van Steeg, H., Berg, R.J., Garssen, J., de Wit, J., van Oostrum, C.T., Beems, R.B., van der Horst, G.T., van Kreijl, C.F., de Gruijl, F.R., et al. (1999). Mouse model for the DNA repair/basal transcription disorder

References

trichothiodystrophy reveals cancer predisposition. Cancer Res 59, 3489-3494.

De Bont, R., and van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19, 169-185.

de Laat, W.L., Jaspers, N.G., and Hoeijmakers, J.H. (1999). Molecular mechanism of nucleotide excision repair. Genes Dev 13, 768-785.

de Vries, A., van Oostrom, C.T., Hofhuis, F.M., Dortant, P.M., Berg, R.J., de Gruijl, F.R., Wester, P.W., van Kreijl, C.F., Capel, P.J., van Steeg, H., et al. (1995). Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature 377, 169-173.

De Weerd-Kastelein, E.A., Keijzer, W., Rainaldi, G., and Bootsma, D. (1977). Induction of sister chromatid exchanges in xeroderma pigmentosum cells after exposure to ultraviolet light. Mutat Res 45, 253-261.

Essers, J., Vermeulen, W., and Houtsmuller, A.B. (2006). DNA damage repair: anytime, anywhere? Curr Opin Cell Biol 18, 240-246.

Evans, E., Moggs, J.G., Hwang, J.R., Egly, J.M., and Wood, R.D. (1997). Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J 16, 6559-6573.

Fleck, O., and Nielsen, O. (2004). DNA repair. J Cell Sci 117, 515-517.

Fousteri, M., Vermeulen, W., van Zeeland, A.A., and Mullenders, L.H. (2006). Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. Mol Cell 23, 471-482.

Friedberg, E.C. (2001). How nucleotide excision repair protects against cancer. Nat Rev Cancer 1, 22-33.

Giglia-Mari, G., Coin, F., Ranish, J.A., Hoogstraten, D., Theil, A., Wijgers, N., Jaspers, N.G., Raams, A., Argentini, M., van der Spek, P.J., et al. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat Genet 36, 714-719.

Gillet, L.C., and Scharer, O.D. (2006). Molecular mechanisms of mammalian global genome nucleotide excision repair. Chem Rev 106, 253-276.

Hanawalt, P., and Mellon, I.. Stranded in an active gene. Curr Biol 3, 67-69.

Hanawalt, P.C. (2002). Subpathways of nucleotide excision repair and their regulation. Oncogene 21, 8949-8956.

Harada, Y.N., Shiomi, N., Koike, M., Ikawa, M., Okabe, M., Hirota, S., Kitamura, Y., Kitagawa, M., Matsunaga, T., Nikaido, O., et al. (1999). Postnatal growth failure, short life span, and early onset of cellular senescence and subsequent immortalization in mice lacking the xeroderma pigmentosum group G gene. Mol Cell Biol 19, 2366-2372.

Hasty, P., Campisi, J., Hoeijmakers, J., van Steeg, H., and Vijg, J. (2003). Aging and genome maintenance: lessons from the mouse? Science 299, 1355-1359.

References

Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. Nature 411, 366-374.

Holstege, F.C., van der Vliet, P.C., and Timmers, H.T. (1996). Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. EMBO J 15, 1666-1677.

Iyer, N., Reagan, M.S., Wu, K.J., Canagarajah, B., and Friedberg, E.C. (1996). Interactions involving the human RNA polymerase II transcription/nucleotide excision repair complex TFIIH, the nucleotide excision repair protein XPG, and Cockayne syndrome group B (CSB) protein. Biochemistry 35, 2157-2167.

Jackson, S.P. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis 23, 687-696.

Kraemer, K.H. (1997). Sunlight and skin cancer: another link revealed. Proc Natl Acad Sci U S A 94, 11-14.

Kraemer, K.H., Lee, M.M., and Scotto, J. (1984). DNA repair protects against cutaneous and internal neoplasia: evidence from xeroderma pigmentosum. Carcinogenesis 5, 511-514.

Kraemer, K.H., Lee, M.M., and Scotto, J. (1987). Xeroderma pigmentosum. Cutaneous, ocular, and neurologic abnormalities in 830 published cases. Arch Dermatol 123, 241-250.

Kulaksiz, G., Reardon, J.T., and Sancar, A. (2005). Xeroderma pigmentosum complementation group E protein (XPE/DDB2): purification of various complexes of XPE and analyses of their damaged DNA binding and putative DNA repair properties. Mol Cell Biol 25, 9784-9792.

Lehmann, A.R. (2003). DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. Biochimie 85, 1101-1111.

Lehmann, A.R., Niimi, A., Ogi, T., Brown, S., Sabbioneda, S., Wing, J.F., Kannouche, P.L., and Green, C.M. (2007). Translesion synthesis: Y-family polymerases and the polymerase switch. DNA Repair (Amst) 6, 891-899.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature 362, 709-715.

Lindenbaum, Y., Dickson, D., Rosenbaum, P., Kraemer, K., Robbins, I., and Rapin, I. (2001). Xeroderma pigmentosum/cockayne syndrome complex: first neuropathological study and review of eight other cases. Eur J Paediatr Neurol 5, 225-242.

Ljungman, M., and Lane, D.P. (2004). Transcription - guarding the genome by sensing DNA damage. Nat Rev Cancer 4, 727-737.

Lowry, R.B. (1982). Early onset of Cockayne syndrome. Am J Med Genet 13, 209-210.

McCuaig, C., Marcoux, D., Rasmussen, J.E., Werner, M.M., and Gentner, N.E.

References

(1993). Trichothiodystrophy associated with photosensitivity, gonadal failure, and striking osteosclerosis. J Am Acad Dermatol 28, 820-826.

Mitchell, J.R., Hoeijmakers, J.H., and Niedernhofer, L.J. (2003). Divide and conquer: nucleotide excision repair battles cancer and ageing. Curr Opin Cell Biol 15, 232-240.

Miyauchi-Hashimoto, H., Tanaka, K., and Horio, T. (1996). Enhanced inflammation and immunosuppression by ultraviolet radiation in xeroderma pigmentosum group A (XPA) model mice. J Invest Dermatol 107, 343-348.

Mullenders, L.H., and Berneburg, M. (2001). Photoimmunology and nucleotide excision repair: impact of transcription coupled and global genome excision repair. J Photochem Photobiol B 65, 97-100.

Murai, M., Enokido, Y., Inamura, N., Yoshino, M., Nakatsu, Y., van der Horst, G.T., Hoeijmakers, J.H., Tanaka, K., and Hatanaka, H. (2001). Early postnatal ataxia and abnormal cerebellar development in mice lacking Xeroderma pigmentosum Group A and Cockayne syndrome Group B DNA repair genes. Proc Natl Acad Sci U S A 98, 13379-13384.

Nance, M.A., and Berry, S.A. (1992). Cockayne syndrome: review of 140 cases. Am J Med Genet 42, 68-84.

Norgauer, J., Idzko, M., Panther, E., Hellstern, O., and Herouy, Y. (2003). Xeroderma pigmentosum. Eur J Dermatol 13, 4-9.

Parikh, S.S., Mol, C.D., and Tainer, J.A. (1997). Base excision repair enzyme family portrait: integrating the structure and chemistry of an entire DNA repair pathway. Structure 5, 1543-1550.

Price, V.H., Odom, R.B., Ward, W.H., and Jones, F.T. (1980). Trichothiodystrophy: sulfur-deficient brittle hair as a marker for a neuroectodermal symptom complex. Arch Dermatol 116, 1375-1384.

Rapin, I., Lindenbaum, Y., Dickson, D.W., Kraemer, K.H., and Robbins, J.H. (2000). Cockayne syndrome and xeroderma pigmentosum. Neurology 55, 1442-1449.

Riedl, T., Hanaoka, F., and Egly, J.M. (2003). The comings and goings of nucleotide excision repair factors on damaged DNA. EMBO J 22, 5293-5303.

Sancar, A. (1996). DNA excision repair. Annu Rev Biochem 65, 43-81.

Sands, A.T., Abuin, A., Sanchez, A., Conti, C.J., and Bradley, A. (1995). High susceptibility to ultraviolet-induced carcinogenesis in mice lacking XPC. Nature 377, 162-165.

Setlow, R.B. (1974). The wavelengths in sunlight effective in producing skin cancer: a theoretical analysis. Proc Natl Acad Sci U S A 71, 3363-3366.

References

Shivji, M.K., Podust, V.N., Hubscher, U., and Wood, R.D. (1995). Nucleotide excision repair DNA synthesis by DNA polymerase epsilon in the presence of PCNA, RFC, and RPA. Biochemistry 34, 5011-5017.

Smith, J.R., and Pereira-Smith, O.M. (1996). Replicative senescence: implications for in vivo aging and tumor suppression. Science 273, 63-67.

Stefanini, M., Vermeulen, W., Weeda, G., Giliani, S., Nardo, T., Mezzina, M., Sarasin, A., Harper, J.I., Arlett, C.F., Hoeijmakers, J.H., et al. (1993). A new nucleotide-excision-repair gene associated with the disorder trichothiodystrophy. Am J Hum Genet 53, 817-821.

Sugasawa, K., Ng, J.M., Masutani, C., Iwai, S., van der Spek, P.J., Eker, A.P., Hanaoka, F., Bootsma, D., and Hoeijmakers, J.H. (1998). Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Mol Cell 2, 223-232.

Thoma, B.S., and Vasquez, K.M. (2003). Critical DNA damage recognition functions of XPC-hHR23B and XPA-RPA in nucleotide excision repair. Mol Carcinog 38, 1-13.

Tian, M., Shinkura, R., Shinkura, N., and Alt, F.W. (2004). Growth retardation, early death, and DNA repair defects in mice deficient for the nucleotide excision repair enzyme XPF. Mol Cell Biol 24, 1200-1205.

Trego, K.S., and Turchi, J.J. (2006). Pre-steady-state binding of damaged DNA by XPC-hHR23B reveals a kinetic mechanism for damage discrimination. Biochemistry 45, 1961-1969.

van de Ven, M., Andressoo, J.O., Holcomb, V.B., von Lindern, M., Jong, W.M., De Zeeuw, C.I., Suh, Y., Hasty, P., Hoeijmakers, J.H., van der Horst, G.T., et al. (2006). Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. PLoS Genet 2, e192.

van der Horst, G.T., Meira, L., Gorgels, T.G., de Wit, J., Velasco-Miguel, S., Richardson, J.A., Kamp, Y., Vreeswijk, M.P., Smit, B., Bootsma, D., et al. (2002). UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. DNA Repair (Amst) 1, 143-157.

van der Horst, G.T., van Steeg, H., Berg, R.J., van Gool, A.J., de Wit, J., Weeda, G., Morreau, H., Beems, R.B., van Kreijl, C.F., de Gruijl, F.R., et al. (1997). Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell 89, 425-435.

van der Pluijm, I., Garinis, G.A., Brandt, R.M., Gorgels, T.G., Wijnhoven, S.W., Diderich, K.E., de Wit, J., Mitchell, J.R., van Oostrom, C., Beems, R., et al. (2007). Impaired genome maintenance suppresses the growth hormone–insulin-like growth factor 1 axis in mice with Cockayne syndrome. PLoS Biol 5, e2.

References

van Gent, D.C., Hoeijmakers, J.H., and Kanaar, R. (2001). Chromosomal stability and the DNA double-stranded break connection. Nat Rev Genet 2, 196-206.

van Gool, A.J., Citterio, E., Rademakers, S., van Os, R., Vermeulen, W., Constantinou, A., Egly, J.M., Bootsma, D., and Hoeijmakers, J.H. (1997). The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex. EMBO J 16, 5955-5965.

Venema, J., van Hoffen, A., Karcagi, V., Natarajan, A.T., van Zeeland, A.A., and Mullenders, L.H. (1991). Xeroderma pigmentosum complementation group C cells remove pyrimidine dimers selectively from the transcribed strand of active genes. Mol Cell Biol 11, 4128-4134.

Vermeulen, W., Bergmann, E., Auriol, J., Rademakers, S., Frit, P., Appeldoorn, E., Hoeijmakers, J.H., and Egly, J.M. (2000). Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder. Nat Genet 26, 307-313.

Volker, M., Mone, M.J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., Hoeijmakers, J.H., van Driel, R., van Zeeland, A.A., and Mullenders, L.H. (2001). Sequential assembly of the nucleotide excision repair factors in vivo. Mol Cell 8, 213-224.

Weeda, G., de Boer, J., Donker, I., de Wit, J., Winkler, S.B., van der Horst, G.T., Vermeulen, W., Bootsma, D., and Hoeijmakers, J.H. (1998). Molecular basis of DNA repair mechanisms and syndromes. Recent Results Cancer Res 154, 147-155.

Weeda, G., Donker, I., de Wit, J., Morreau, H., Janssens, R., Vissers, C.J., Nigg, A., van Steeg, H., Bootsma, D., and Hoeijmakers, J.H. (1997a). Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. Curr Biol 7, 427-439.

Weeda, G., Eveno, E., Donker, I., Vermeulen, W., Chevallier-Lagente, O., Taieb, A., Stary, A., Hoeijmakers, J.H., Mezzina, M., and Sarasin, A. (1997b). A mutation in the XPB/ERCC3 DNA repair transcription gene, associated with trichothiodystrophy. Am J Hum Genet 60, 320-329.

Wijnhoven, S.W., Beems, R.B., Roodbergen, M., van den Berg, J., Lohman, P.H., Diderich, K., van der Horst, G.T., Vijg, J., Hoeijmakers, J.H., and van Steeg, H. (2005). Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. DNA Repair (Amst) 4, 1314-1324.

2

Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging

~

Marieke van de Ven, Jaan-Olle Andressoo,
Valerie B. Holcomb, Paul Hasty, **Yousin Suh,**
Harry van Steeg, **George A. Garinis,**
Jan H.J. Hoeijmakers, James R. Mitchell

Abstract

Semantic distinctions between "normal" aging, "pathological" aging (or age-related disease) and "premature" aging (otherwise known as segmental progeria) potentially confound important insights into the nature of each of the complex processes. Here we review a recent, unexpected discovery: the presence of longevity-associated characteristics typical of long-lived endocrine-mutant and dietary-restricted animals in short-lived progeroid mice. These data suggest that a subset of symptoms observed in premature aging, and possibly normal aging as well, may be indirect manifestations of a beneficial adaptive stress response to endogenous oxidative damage, rather than a detrimental result of the damage itself.

Definitions of aging: current focus on lifespan.

Most definitions of aging attempt to capture the irreversible, degenerative nature of the process by focusing on quite apparent symptoms like wrinkled skin and gray hair that affect different people in different ways as they age. A more general definition of aging avoids such individual variation and focuses on the one constant, the time-dependent increase in the probability of dying. The problem with this latter definition is that lifespan is only one component of aging and says nothing about a potentially more important aspect, the quality of life up until the point of death.

In the so-called "premature aging" disorders, or segmental progerias, lifespan is shortened and a number of characteristics, or "segments", of aging (in addition to a number of disease-specific pathologies) appear early or in exacerbated form (Martin and Oshima, 2000). The relation between progeria and "normal" aging is controversial, largely because there are so many ways to shorten lifespan that have nothing to do with the normal aging process (Miller, 2004). Also poorly defined is the connection between "normal" and "pathological" aging. Cancer or Alzheimer's both increase with age, but some people become very old without either and still die of "old age".

On the opposite side of premature or pathological aging, the connection between lifespan extension and "normal" aging is most often taken for granted. But perhaps it should not be. An increase in maximum lifespan can be achieved by slowing the rate of aging or merely by delaying its onset, with different implications for the underlying mechanism (Barger et al., 2003). Restricting the diet by reducing the total amount of food eaten (Weindruch and Walford, 1988) achieves both. Genetic models of lifespan extension, however, may simply delay the onset of ageing-related pathologies, some of which otherwise would limit lifespan (Barger et al., 2003).

What then is aging, and does broadening its definition beyond lifespan reveal anything useful about its nature? We gained an unexpected insight into this problem by performing detailed phenotypic analyses of segmental progeroid mice engineered with various defects in DNA damage repair.

Progeroid NER syndrome: longevity-associated traits in short-lived mice

Nucleotide excision repair (NER) is an evolutionarily conserved mechanism for the removal of bulky, helix-distorting lesions from DNA such as UV-induced damage. It functions by a "cut-and-patch" mechanism in which the damage is recognized, the DNA helix unwound, the damaged strand excised, and the remaining single-stranded region patched (Hoeijmakers, 2001). In humans, congenital defects in NER-associated components are uniformly associated with UV (sun) hypersensitivity. Specific defects in a subset of these components can also lead to the segmental progeroid disorders Cockayne syndrome and trichothiodystrophy (Bootsma et al., 2002). These recessive disorders display postnatal

onset of progressive neurodevelopmental pathology with overlapping progeroid features including reduced subcutaneous fat and small size (together known as cachectic dwarfism), sensorineural deafness, retinal degeneration, white matter hypomyelination and CNS calcification sometimes accompanied with premature appearance of neurofibrillary tangles (Itin et al., 2001; Nance and Berry, 1992; Rapin et al., 2000).

Mouse models of these diseases (Table 1 and references therein) display an overlapping set of progressive symptoms including cachectic dwarfism, reduced bone mineral density resembling osteoporosis, curvature of the spine (lordokyphosis) and failure to thrive. Cerebellar ataxia, a disease-specific pathology not associated with normal aging in mice, is sometimes accompanied by the loss of Purkinje neurons late in disease progression. Death usually occurs before weaning at about three weeks of age.

Recently we reported a surprising finding in *Xpd/Xpa* double homozygous mutant mice (van de Ven et al., 2006). In addition to the progeroid features listed above, these mice display characteristics usually associated with good health and extended lifespan, as in endocrine-deficient or dietary-restricted animals (Bartke and Brown-Borg, 2004). These characteristics, measured at two weeks of age when the pups are still nursing, include reduced weight, hypoglycemia, hypoinsulinemia, reduced serum insulin-like growth factor-1 (IGF-1) and reduced body temperature. In addition, a number of genes involved in the postnatal growth axis are downregulated in the livers of these animals, including growth hormone receptor. These features are observed in a variety of progeroid NER mice, including two different *Xpd/Xpa* (van de Ven et al., 2006) mutants, *Csb/Xpa* (van der Pluijm et al., 2006) and *Ercc1* (Niedernhofer et al., 2006) and are likely to be common to all of the progeroid NER mutants.

Adaptive response vs. constitutive defect

Constitutive defects in endocrine-mediated insulin signaling and dietary restriction can both extend longevity in a number of model organisms. In mice, both result in reduced size, hypoglycemia, hypoinsulinemia, reduced serum IGF-1 and reduced temperature (Bartke and Brown-Borg, 2004; Koubova and Guarente, 2003). One clear difference, however, is that these phenotypes are permanent in endocrine-deficient animals but reversible in dietary restricted animals. In short-lived progeroid NER mice, normal pituitaries (van der Pluijm et al., 2006) and normal growth hormone (Niedernhofer et al., 2006; van de Ven et al., 2006; van der Pluijm et al., 2006) are inconsistent with defects in hypothalamic or pituitary function. A more attractive hypothesis is that the alteration of energy metabolism via dampening of the growth hormone/IGF-1 axis in progeroid NER mice reflects an adaptive response in which reduction of mitochondrial ROS-derived oxidative DNA damage is the intended consequence. However, such a hypothesis is difficult to test in mice with an early onset, irreversible condition as in progeroid NER syndrome in which animals die within three weeks after birth.

Fortuitously, one particular combination of mutant alleles ($Xpd^{R722W/G602D}/Xpa^{-/-}$, Table 1) resulted in mice with each of the longevity-associated traits of dietary restriction in addition to all of the pathologies of progeroid NER syndrome, save one: instead of a three week lifespan, mutants survived past weaning with 80% penetrance. We hypothesize this to be due to complementation between the two different mutant *Xpd* alleles (Andressoo et al., 2006a). This allowed us to study these animals past early development and into adulthood. We noted additional phenotypes including normal to elevated food intake per gram body mass (like hypopituitary Ames dwarf mice (Bartke et al., 2001)) despite continuing dwarfism, time-dependent reduction in the mass of both white and brown adipose tissue deposits relative to total mass, progressive lordokyphosis, frequent loss of balance and a mean lifespan of approximately 5 months of age.

Blood glucose and serum IGF-1 levels of adult mutants gave us an unexpected clue about the nature of the effect on growth and metabolism observed at the earlier age of 2 weeks. By ten weeks of age, when control animals are past postnatal development and have reached sexual maturity, blood glucose and serum IGF-1 levels in the mutants are once again normal despite the remaining dwarfism (van de Ven et al., 2006).This is further evidence against any constitutive alteration of the growth hormone/IGF-1 axis or glucose homeostasis and strongly in favor of the interpretation that the downregulation of these components at two weeks of age reflects an adaptive response to stress.

SIRT6 KO mice: similar adaptive response to a common DNA repair defect?

SIRT6 deficiency results in a phenotype strongly overlapping progeroid NER syndrome. Like progeroid NER mice, SIRT6 KO mice are born normally but display early postnatal onset of growth retardation followed by lordokyphosis, cachexia and failure to thrive, with a maximum lifespan of 24 days (Mostoslavsky et al., 2006). Beginning at postnatal day 12, animals become increasingly hypoglycemic despite evidence of normal eating (milk in the stomach) and a normal ability to absorb glucose (R. Mostoslavsky, personal communication). Like in the NER progeroid mice, reduced serum IGF-1 (Mostoslavsky et al., 2006) despite normal growth hormone levels (D. Lombard, personal communication), suggestive of growth hormone insensitivity, is also present. Furthermore, ablation of the DNA damage response protein p53 does not affect lifespan in SIRT6 deficient mice (D. Lombard, R. Mostoslavsky, personal communication) or progeroid NER *Csb*/*Xpa* mice (H. van Steeg, unpublished observation).

Differences between SIRT6 deficiency and progeroid NER disorder also exist, but are minor compared to the overall similarities. These differences appear mainly in the end-of-life pathology. SIRT6 KO mice lose splenocytes, thymocytes and peripheral lymphocytes as the result of a systemic, non-cell autonomous defect, which may be attributable in part to the sensitivity of these cells to hypoglycemia and low IGF-1 (Alves et al., 2006; Mostoslavsky et al., 2006). The loss of Purkinje neurons typical of progeroid NER mice

may be due to a cell-type specific hypersensitivity to oxidative DNA damage combined with the systemic effects of reduced IGF-1, a neuronal survival factor. Decreased serum IGF-1 levels are associated with cerebellar ataxias of various etiologies in both humans and experimental rodent models (Busiguina et al., 2000; Torres-Aleman et al., 1996), and Purkinje neurons are hypersensitive to oxidative stress accompanying ischemic injury although relatively resistant to other types of stress such as hypoglycemia (Mohseni, 2001). Interestingly, different gene-specific pathologies also exist amongst progeroid NER disorders (Table 1). For example, liver- and kidney-specific pathologies exclusively in XPF- and ERCC1-deficient mice are probably due to the particular roles of these proteins outside of NER, such as interstrand crosslink repair or telomere maintenance; brittle hair is specific to the R722W-encoding mutation in *Xpd*, probably due to transcriptional deficiencies particular to this mutation (de Boer et al., 1999). Despite these differences, the overlapping phenotype of progeroid NER syndrome and SIRT6 deficiency is consistent with a common adaptive response to genotoxic stress during development.

Is there any evidence that this hypothetical shared adaptive response is triggered by a common genotoxic stress? SIRT6-deficient cells are hypersensitive to the effects of ROS generated by ionizing radiation or H_2O_2, and monoadducts by the alkylating agent MMS, but not to ultraviolet radiation, consistent with a defect in the base excision repair (BER) system (Mostoslavsky et al., 2006) or in other DNA damage response pathways. Although BER is functionally distinct from NER, there is recent genetic and biochemical evidence of a partial functional overlap between components previously thought to be specific to one system (NER components XPG and CSB) or the other (BER components OGG-1 and PARP-1)(Dianov et al., 2000; Licht et al., 2003; Osterod et al., 2002; Thorslund et al., 2005; Tuo et al., 2002). Although much further evidence is required, it is tempting to speculate that such a shared DNA repair defect can elicit a common adaptive response.

Adaptive stress response is not a general characteristic of genome instability

In addition to progeroid NER syndrome and SIRT6 deficiency, a number of genetically engineered mice have progeroid characteristics (reviewed in (Lombard et al., 2005)). At face value, these progeroid conditions appear to have much in common. The engineered mutations are mostly in genes involved in nucleic acid metabolism, for example, other types of DNA repair (Espejel et al., 2004), telomere maintenance (Lee et al., 1998), chromosome segregation (Baker et al., 2004), DNA methylation (Sun et al., 2004), mitochondrial DNA replication fidelity (Trifunovic et al., 2004), and the DNA damage response (Maier et al., 2004; Tyner et al., 2002). Also, the progeroid phenotypes have an overlapping set of characteristics including cachectic dwarfism, reduced fertility, hair loss and graying, curvature of the spine, cancer predisposition and shortened lifespan.

We asked whether other forms of genome instability in addition to excision repair defects could trigger a preservative organismal response through the postnatal growth/energy metabolism axis. We chose KU80-deficient mice, with a defect in repairing DNA

double-strand breaks via the non-homologous endjoining pathway. These animals are cachectic dwarfs throughout their lives and display many characteristics of premature senescence on both the cellular and organismal levels (Vogel et al., 1999). In two-week-old animals, however, we found no difference in blood glucose, serum IGF-1, or gene expression from the postnatal growth axis in the liver as there is in progeroid NER syndrome (van de Ven et al., 2006). Together these data suggest that whatever the apparent similarities amongst genomically unstable progeroid mice, on both the molecular and organismal levels different mechanisms, or possibly similar mechanisms with very different kinetics, are at work. In support of this conclusion, a different form of genome instability related to short telomeres produces a related subset of progeroid symptoms including hair loss and graying, osteoporosis and fingernail atrophy in a variety of otherwise unrelated progerias (Hofer et al., 2005).

Table 1.

Overlapping characteristics of excision repair deficiencies in man and mouse

Human progeroid syndrome	Mouse mutant	DNA repair defect	Lifespan	Genotype specific pathology
XFE (Niedernhofer et al., in press)	*Ercc1*$^{-/-}$ (McWhir et al., 1993; Weeda et al., 1997)	NER/TCR; ICLR; telomere maintenance (Zhu et al., 2003)	~3 weeks	Liver/kidney polyploidy; reduced hematopoietic reserves (Prasher et al., 2005)
	Xpf$^{-/-}$ (Tian et al., 2004)	NER/TCR; ICLR; telomere maintenance (Zhu et al., 2003)	~3 weeks	Liver/kidney polyploidy
XPCS	*Xpg*$^{-/-}$ (Harada et al., 1999; Sun et al., 2001)	NER/TCR	~2–3 weeks	Undeveloped small intestines
	Xpa$^{-/-}$|*Csb*$^{-/-}$ (Murai et al., 2001; van der Pluijm et al., 2007)	NER/TCR	~3 weeks	n.d.
	Xpa$^{-/-}$|*Xpd*$^{G602D/G602D}$ (Andressoo et al., 2006b)	NER/TCR	~3 weeks	n.d.
XPTTD	*Xpa*$^{-/-}$|*Xpd*$^{R722W/R722W}$ (de Boer et al., 2002)	NER/TCR	~3 weeks; survivors 4, 12 months	Cutaneous abnormalities, brittle hair
XPCS (mild)	*Xpa*$^{-/-}$|*Xpd*$^{R722W/G602D}$ (Andressoo et al., 2006b; van de Ven et al., 2006)	NER/TCR	~22 weeks	n.d.
?	*SIRT6*$^{-/-}$ (Mostoslavsky et al., 2006)	BER	~3 weeks	Osteopenia, lymphopenia

n.d.: none determined; NER: nucleotide excision repair; TCR: transcription coupled repair; ICRL: interstrand crosslink repair; BER: base excision repair.

Evolution of the preservative stress response

An adaptive stress response involving downregulation of growth and alteration of energy metabolism in favor of conservation may have evolved to cope with periods of reduced food availability or life-threatening disease. It may be thus best defined as a preservative stress response rather than a longevity stress response, because its primary purpose is to help animals through a period of stress that could occur during any stage of life (and thus could be selected for) rather than to extend lifespan past the reproductive years. A developmental stage-specific version of this response is also conserved in the worm *C. elegans*. During early larval development, food inadequacy triggers an adaptive response known as dauer formation in which metabolism is altered via the insulin-signaling pathway in an attempt to survive until environmental conditions are once again favorable (Kenyon et al., 1993).

The phenotypes of progeroid NER syndrome and SIRT6 deficiency suggest another way to trigger this response: a particular type of oxidative genotoxic stress during early development. The rapid postnatal onset suggests that birth stress, which involves an

increase in ROS levels (Randerath et al., 1997a; Randerath et al., 1997b) may trigger it and that rapid postnatal growth may further exacerbate it. Combined with the inability to repair certain endogenous lesions as in SIRT6 or NER deficiency, oxidative genotoxic stress may trigger an adaptive response intended to reduce generation of ROS through mitochondrial respiration and thus prevent further damage.

This stress response may also not be limited to aging or aging-related pathology, but may also occur in response to acute stress. In support of this, we have data implicating downregulation of IGF-1 and growth hormone receptor on the mRNA level in response to the acute oxidative stress of renal ischemia reperfusion injury (J.R. Mitchell, see Chapter 4). Furthermore, chronic exposure of mice to a peroxisome proliferator (resulting in elevated oxidative DNA damage (de Waard et al., 2004)) induces a similar response in the gene expression profile of livers of wild-type mice (van der Pluijm et al., 2006). A reduction of the growth hormone/IGF-1 may thus be a more general marker of both chronic and acute oxidative stress. However, as is clear from the shortened lifespan of progeroid NER and SIRT6 deficient mice, reduced IGF-1 on its own cannot ensure a beneficial outcome; the nature and duration of the stress must also be taken into consideration (Figure 1).

Terminal senescent weight loss: an adaptive stress response to normal aging?

If the reaction to certain types of genome instability resembles a stress response, does the oxidative DNA damage that accumulates with age also trigger such a beneficial response? There is some evidence that normal aging evokes a similar response to stress. In the absence of age-related disease such as cancer or diabetes, people at advanced age often enter a period of weight loss culminating in death. This syndrome is known as geriatric failure to thrive (Sarkisian and Lachs, 1996). The cause of the weight loss is currently unknown. Rats that live to advanced age also display a related phenomenon known as senescent terminal weight loss. Although originally believed to be caused by decreased food intake, new data indicate this not to be the case (Black et al., 2003). The growth hormone/IGF-1 axis is already greatly reduced by this age, and thermoregulation and blood glucose may also be altered in this terminal senescent period (Black et al., 2003; van de Ven et al., 2006). In light of the data reviewed here, perhaps its not surprising then that rats (Black et al., 2003) (and probably mice (Miller et al., 2005)) that experience this senescent terminal weight loss “syndrome” actually live significantly longer than those that do not. In other words, we propose that terminal senescent weight loss and geriatric failure to thrive, despite their foreboding names, are probably components of a beneficial, adaptive response triggered very late in life in response to the accumulated oxidative damage to macromolecules including DNA.

Conclusions

Everything changes with age, and few of them for the better. Most things, from our muscles to our short-term memory, deteriorate over time. Here we emphasize the potential importance of another component of the aging process: the body's own response to deterioration. This adaptive response probably evolved to combat other stresses such as starvation early in life, but may be activated in cases of premature, pathological and natural aging. A more nuanced and useful definition of aging should thus include at least four basic components: underlying mechanisms including molecular oxidation (Harman, 1988), genetic background (from progeroid to centenarian) defining susceptibility to such damage, time-dependent primary effects of stochastic oxidative macromolecular damage, and secondary adaptive organismal attempts to counterbalance these effects. In the future, a better understanding of these components and their interactions will tell us more, not just about how we age, but about how we function at all stages of our lives.

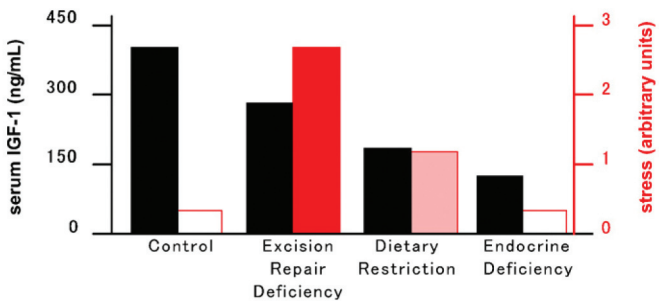


Figure 1.

Correlations between serum IGF-1, genotoxic stress and lifespan. In dietary restricted and endocrine-deficient mice, reduced serum IGF-1 (black bars; y axis on the left) correlates with increased longevity. In progeroid NER and SIRT6 knockout (excision repair deficient) mice, this correlation doesn't hold. To explain this apparent paradox, we add the presumed cause of the reduced IGF-1 in excision repair deficient and dietary restricted animal: stress (red bars; y axis on the right). Different shadings indicate the different nature of the stressors (unrepaired endogenous DNA damage, dark red; reduced energy intake, light red; baseline stress in control and endocrine-deficient animals, empty). Constitutive unrepaired genotoxic stress may overrule the efficacy of reduced IGF-1 signalling in excision repair-deficient progeroid mice, while in dietary restriction or endocrine deficiency the stress is either of a different nature, or absent relative to controls as in constitutive endocrine deficiency.

Acknowledgements

The authors would like to thank Joris Pothof, David Lombard and Raul Mostoslavsky for informative discussions and critical reading of the manuscript. This work was supported in part by the following grants: National Institutes of Health (1PO1 AG17242-02); Association of International Cancer Research Grant Award (05-280); and Netherlands Organization for Health Research and Development, Research Institute for Diseases in the Elderly (60-60400-98-004).

References

Alves, N.L., Derks, I.A., Berk, E., Spijker, R., van Lier, R.A. and Eldering, E. (2006) The Noxa/Mcl-1 axis regulates susceptibility to apoptosis under glucose limitation in dividing T cells. Immunity 24, 703-16.

Andressoo, J.O., Jans, J., de Wit, J., Coin, F., Hoogstraten, D., van de Ven, H.W.M., Toussaint, W., Huijmans, J., Thio, B., van Leeuwen, W.J., de Boer, J., Egly, J.-M., Hoeijmakers, H.J.H., van der Horst, G.T.J. and Mitchell, J.R. (2006a) Rescue of progeria in trichothiodystrophy by homozygous lethal Xpd alleles. PLoS Biology 4.

Andressoo, J.O., Mitchell, J.R., de Wit, J., Hoogstraten, D., Volker, M., Toussaint, W., Speksnijder, E., Beems, R.B., van Steeg, H., Jans, J., de Zeeuw, C.I., Jaspers, N.G., Raams, A., Lehmann, A.R., Vermeulen, W., Hoeijmakers, J.H. and van der Horst, G.T. (2006b) An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. Cancer Cell 10, 121-32.

Baker, D.J., Jeganathan, K.B., Cameron, J.D., Thompson, M., Juneja, S., Kopecka, A., Kumar, R., Jenkins, R.B., de Groen, P.C., Roche, P. and van Deursen, J.M. (2004) BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. Nat Genet 36, 744-9.

Barger, J.L., Walford, R.L. and Weindruch, R. (2003) The retardation of aging by caloric restriction: its significance in the transgenic era. Exp Gerontol 38, 1343-51.

Bartke, A. and Brown-Borg, H. (2004) Life extension in the dwarf mouse. Curr Top Dev Biol 63, 189-225.

Bartke, A., Brown-Borg, H., Mattison, J., Kinney, B., Hauck, S. and Wright, C. (2001) Prolonged longevity of hypopituitary dwarf mice. Exp Gerontol 36, 21-8.

Black, B.J., Jr., McMahan, C.A., Masoro, E.J., Ikeno, Y. and Katz, M.S. (2003) Senescent terminal weight loss in the male F344 rat. Am J Physiol Regul Integr Comp Physiol 284, R336-42.

Bootsma, D., Kraemer, K.H., Cleaver, J.E. and Hoeijmakers, J.H. (2002) Nucleotide Excision Repair Syndromes:Xeroderma Pigmentosum, Cockayne Syndrome, and Trichothiodystrophy. In Vogelstein, B. and Kinzler, K.W. (eds.), The Genetic Basis of Human Cancer, McGraw-Hill Medical Publishing Division, New York, pp. 211-237.

Busiguina, S., Fernandez, A.M., Barrios, V., Clark, R., Tolbert, D.L., Berciano, J. and Torres-Aleman, I. (2000) Neurodegeneration is associated to changes in serum insulin-like growth factors. Neurobiol Dis 7, 657-65.

de Boer, J., Andressoo, J.O., de Wit, J., Huijmans, J., Beems, R.B., van Steeg, H., Weeda, G., van der Horst, G.T., van Leeuwen, W., Themmen, A.P., Meradji, M. and Hoeijmakers, J.H. (2002) Premature aging in mice deficient in DNA repair and transcription. Science 296, 1276-9.

References

de Boer, J., van Steeg, H., Berg, R.J.W., Garssen, J., de Wit, J., van Oostrom, C.T.M., Beems, R.B., van der Horst, G.T.J., van Kreijl, C.F., de Gruijl, F.R., Bootsma, D., Hoeijmakers, J.H.J. and Weeda, G. (1999) Mouse model for the DNA repair/basal transcription disorder trichothiodystrophy reveals cancer predisposition. Cancer Res. 59, 3489-3494.

de Waard, H., de Wit, J., Andressoo, J.O., van Oostrom, C.T., Riis, B., Weimann, A., Poulsen, H.E., van Steeg, H., Hoeijmakers, J.H. and van der Horst, G.T. (2004) Different effects of CSA and CSB deficiency on sensitivity to oxidative DNA damage. Mol Cell Biol 24, 7941-8.

Dianov, G.L., Thybo, T., Dianova, II, Lipinski, L.J. and Bohr, V.A. (2000) Single nucleotide patch base excision repair is the major pathway for removal of thymine glycol from DNA in human cell extracts. J Biol Chem 275, 11809-13.

Espejel, S., Martin, M., Klatt, P., Martin-Caballero, J., Flores, J.M. and Blasco, M.A. (2004) Shorter telomeres, accelerated ageing and increased lymphoma in DNA-PKcs-deficient mice. EMBO Rep 5, 503-9.

Harada, Y.N., Shiomi, N., Koike, M., Ikawa, M., Okabe, M., Hirota, S., Kitamura, Y., Kitagawa, M., Matsunaga, T., Nikaido, O. and Shiomi, T. (1999) Postnatal growth failure, short life span, and early onset of cellular senescence and subsequent immortalization in mice lacking the xeroderma pigmentosum group G gene. Mol Cell Biol 19, 2366-72.

Harman, D. (1988) Free radicals in aging. Mol Cell Biochem 84, 155-61.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. Nature 411, 366-74.

Hofer, A.C., Tran, R.T., Aziz, O.Z., Wright, W., Novelli, G., Shay, J. and Lewis, M. (2005) Shared phenotypes among segmental progeroid syndromes suggest underlying pathways of aging. J Gerontol A Biol Sci Med Sci 60, 10-20.

Itin, P.H., Sarasin, A. and Pittelkow, M.R. (2001) Trichothiodystrophy: update on the sulfur-deficient brittle hair syndromes. J Am Acad Dermatol 44, 891-920; quiz 921-4.

Kenyon, C., Chang, J., Gensch, E., Rudner, A. and Tabtiang, R. (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366, 461-4.

Koubova, J. and Guarente, L. (2003) How does calorie restriction work? Genes Dev 17, 313-21.

Lee, H.W., Blasco, M.A., Gottlieb, G.J., Horner, J.W., 2nd, Greider, C.W. and DePinho, R.A. (1998) Essential role of mouse telomerase in highly proliferative organs. Nature 392, 569-74.

Licht, C.L., Stevnsner, T. and Bohr, V.A. (2003) Cockayne syndrome group B cellular and biochemical functions. Am J Hum Genet 73, 1217-39.

References

Lombard, D.B., Chua, K.F., Mostoslavsky, R., Franco, S., Gostissa, M. and Alt, F.W. (2005) DNA repair, genome stability, and aging. Cell 120, 497-512.

Maier, B., Gluba, W., Bernier, B., Turner, T., Mohammad, K., Guise, T., Sutherland, A., Thorner, M. and Scrable, H. (2004) Modulation of mammalian life span by the short isoform of p53. Genes Dev 18, 306-19.

Martin, G.M. and Oshima, J. (2000) Lessons from human progeroid syndromes. Nature 408, 263-6.

McWhir, J., Seldridge, J., Harrison, D.J., Squires, S. and Melton, D.W. (1993) Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. Nat. Gen. 5, 217-223.

Miller, R.A. (2004) 'Accelerated aging': a primrose path to insight? Aging Cell 3, 47-51.

Miller, R.A., Buehner, G., Chang, Y., Harper, J.M., Sigler, R. and Smith-Wheelock, M. (2005) Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell 4, 119-25.

Mohseni, S. (2001) Hypoglycemic neuropathy. Acta Neuropathol (Berl) 102, 413-21.

Mostoslavsky, R., Chua, K.F., Lombard, D.B., Pang, W.W., Fischer, M.R., Gellon, L., Liu, P., Mostoslavsky, G., Franco, S., Murphy, M.M., Mills, K.D., Patel, P., Hsu, J.T., Hong, A.L., Ford, E., Cheng, H.L., Kennedy, C., Nunez, N., Bronson, R., Frendewey, D., Auerbach, W., Valenzuela, D., Karow, M., Hottiger, M.O., Hursting, S., Barrett, J.C., Guarente, L., Mulligan, R., Demple, B., Yancopoulos, G.D. and Alt, F.W. (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124, 315-29.

Murai, M., Enokido, Y., Inamura, N., Yoshino, M., Nakatsu, Y., van der Horst, G.T., Hoeijmakers, J.H., Tanaka, K. and Hatanaka, H. (2001) Early postnatal ataxia and abnormal cerebellar development in mice lacking Xeroderma pigmentosum Group A and Cockayne syndrome Group B DNA repair genes. Proc Natl Acad Sci U S A 98, 13379-84.

Nance, M.A. and Berry, S.A. (1992) Cockayne syndrome: Review of 140 cases. Am. J. Med. Genet. 42, 68-84.

Niedernhofer, L.J., Garinis, G.A., Raams, A., Lalai, A.S., Robinson, A.R., Appeldoorn, E., Odijk, H., Oostendorp, R., Ahmad, A., Leeuwen, W.v., Theil, A.F., Vermeulen, W., Horst, G.T.J.v.d., Meinecke, P., Kleijer, W.J., Vijg, J., Jaspers, N.G.J. and Hoeijmakers, J.H.J. (2006) A novel progeroid syndrome reveals genotoxic stress suppresses the somatotroph axis. Nature in press.

References

Osterod, M., Larsen, E., Le Page, F., Hengstler, J.G., Van Der Horst, G.T., Boiteux, S., Klungland, A. and Epe, B. (2002) A global DNA repair mechanism involving the Cockayne syndrome B (CSB) gene product can prevent the in vivo accumulation of endogenous oxidative DNA base damage. Oncogene 21, 8232-9.

Prasher, J.M., Lalai, A.S., Heijmans-Antonissen, C., Ploemacher, R.E., Hoeijmakers, J.H., Touw, I.P. and Niedernhofer, L.J. (2005) Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1-/- mice. Embo J 24, 861-71.

Randerath, E., Zhou, G.D. and Randerath, K. (1997a) Organ-specific oxidative DNA damage associated with normal birth in rats. Carcinogenesis 18, 859-66.

Randerath, K., Zhou, G.D., Monk, S.A. and Randerath, E. (1997b) Enhanced levels in neonatal rat liver of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-hydroxydeoxyguanosine), a major mutagenic oxidative DNA lesion. Carcinogenesis 18, 1419-21.

Rapin, I., Lindenbaum, Y., Dickson, D.W., Kraemer, K.H. and Robbins, J.H. (2000) Cockayne syndrome and xeroderma pigmentosum. Neurology 55, 1442-9.

Sarkisian, C.A. and Lachs, M.S. (1996) "Failure to thrive" in older adults. Ann Intern Med 124, 1072-8.

Sun, L.Q., Lee, D.W., Zhang, Q., Xiao, W., Raabe, E.H., Meeker, A., Miao, D., Huso, D.L. and Arceci, R.J. (2004) Growth retardation and premature aging phenotypes in mice with disruption of the SNF2-like gene, PASG. Genes Dev 18, 1035-46.

Sun, X.Z., Harada, Y.N., Takahashi, S., Shiomi, N. and Shiomi, T. (2001) Purkinje cell degeneration in mice lacking the xeroderma pigmentosum group G gene. J Neurosci Res 64, 348-54.

Thorslund, T., von Kobbe, C., Harrigan, J.A., Indig, F.E., Christiansen, M., Stevnsner, T. and Bohr, V.A. (2005) Cooperation of the Cockayne syndrome group B protein and poly(ADP-ribose) polymerase 1 in the response to oxidative stress. Mol Cell Biol 25, 7625-36.

Tian, M., Shinkura, R., Shinkura, N. and Alt, F.W. (2004) Growth retardation, early death, and DNA repair defects in mice deficient for the nucleotide excision repair enzyme XPF. Mol Cell Biol 24, 1200-5.

Torres-Aleman, I., Barrios, V., Lledo, A. and Berciano, J. (1996) The insulin-like growth factor I system in cerebellar degeneration. Ann Neurol 39, 335-42.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly, Y.M., Gidlof, S., Oldfors, A., Wibom, R., Tornell, J., Jacobs, H.T. and Larsson, N.G. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429, 417-23.

References

Tuo, J., Chen, C., Zeng, X., Christiansen, M. and Bohr, V.A. (2002) Functional crosstalk between hOgg1 and the helicase domain of Cockayne syndrome group B protein. DNA Repair (Amst) 1, 913-27.

Tyner, S.D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C., Hee Park, S., Thompson, T., Karsenty, G., Bradley, A. and Donehower, L.A. (2002) p53 mutant mice that display early ageing-associated phenotypes. Nature 415, 45-53.

van de Ven, M., Andressoo, J.O., Holcomb, V.B., von Lindern, M., Jong, W., de Zeeuw, C.I., Hasty, P., Suh, Y., Hoeijmakers, J.H.J., van der Horst, G.T.J. and Mitchell, J.R. (2006) Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. PLoS Genetics in press.

van der Pluijm, I., Garinis, G.A., Brandt, R., Gorgels, T., Diderich, K.E.M., Wijnhoven, S., de Wit, J., Mitchell, J.R., van Steeg, H., van Oostrom, C., Beems, R., Niedernhofer, L.J., Velasco, S., Friedberg, E., Tanaka, K., Hoeijmakers, J.H.J. and van der Horst, G.T.J. (2006) Premature aging mice link impaired genome maintenance with GH/IGF1suppression. PLoS Biology in press.

Vogel, H., Lim, D.S., Karsenty, G., Finegold, M. and Hasty, P. (1999) Deletion of Ku86 causes early onset of senescence in mice. Proc Natl Acad Sci U S A 96, 10770-5.

Weeda, G., Donker, I., de Wit, J., Morreau, H., Janssens, R., Vissers, C.J., Nigg, A., van Steeg, H., Bootsma, D. and Hoeijmakers, J.H.J. (1997) Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. Curr Biol 7, 427-39.

Weindruch, R. and Walford, R.L. (1988) The Retardation of Aging and Disease by Dietary Restriction. Charles C Thomas Pub Ltd, pp. 436.

Zhu, X.D., Niedernhofer, L., Kuster, B., Mann, M., Hoeijmakers, J.H. and de Lange, T. (2003) ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. Mol Cell 12, 1489-98.

3

Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice

~

Marieke van de Ven , Jaan-Olle Andressoo,
Valerie B. Holcomb, Marieke von Lindern,
Willeke Jong, Chris I. De Zeeuw, Yousin Suh,
Paul Hasty, Jan H. J. Hoeijmakers,
Gijsbertus T. J. van der Horst, James R. Mitchell

Abstract

How congenital defects causing genome instability can result in the pleiotropic symptoms reminiscent of aging but in a segmental and accelerated fashion remains largely unknown. Most segmental progerias are associated with accelerated fibroblast senescence suggesting that cellular senescence is a likely contributing mechanism. Contrary to expectations, neither accelerated senescence nor acute oxidative stress hypersensitivity was detected in primary fibroblast or erythroblast cultures from multiple progeroid mouse models for defects in the nucleotide excision DNA repair (NER) pathway, which share premature aging features including postnatal growth retardation, cerebellar ataxia and death before weaning. Instead, we report a prominent phenotypic overlap with long-lived dwarfism and calorie restriction during postnatal development (two weeks of age), including reduced size, reduced body temperature, hypoglycemia and perturbation of the growth hormone/insulin-like growth factor-1 (IGF-1) neuroendocrine axis. These symptoms were also present at two weeks of age in a novel progeroid NER mouse model ($XPD^{G602D/R722W}$/$XPA^{-/-}$) that survived weaning with high penetrance. However, despite persistent cachectic dwarfism, blood glucose and serum IGF-1 levels returned to normal by ten weeks, with hypoglycemia reappearing near their premature death at five months of age. These data strongly suggest changes in energy metabolism as part of an adaptive response during the stressful period of postnatal growth. Interestingly, a similar perturbation of the postnatal growth axis was not detected in another progeroid mouse model, the double strand DNA break repair deficient *Ku80*$^{-/-}$ mouse. Specific (but not all) types of genome instability may thus engage a conserved response to stress that evolved to cope with environmental pressures such as food shortage.

Introduction

Congenital defects in genome stability-promoting mechanisms such as DNA repair often lead to elevated cancer predisposition and/or the accelerated appearance of some but not all characteristics ("segments") observed in natural aging (Martin, 2005). Among these so-called segmental progerias, different defects lead to the acceleration or exaggeration of different segments of aging. Typical examples include Werner syndrome, in which diabetes, osteoporosis and increased cancer incidence are observed; and Cockayne syndrome, characterized by neurological dysfunction and cachectic dwarfism. While the causative genetic defects of many progerias are known, the molecular mechanisms underlying pleiotropic disease symptoms remain elusive and their relevance to normal aging controversial (Miller, 2004). However, because cultured fibroblasts from almost all reported mouse models of segmental progeria (as well as many of the corresponding human progerias) exhibit limited proliferative capacity (Lombard et al., 2005), cell-based mechanisms including premature senescence and enhanced apoptosis have emerged as strong candidates underlying pleiotropic disease symptoms (Hasty et al., 2003; Mitchell et al., 2003).

The evolutionarily conserved nucleotide excision repair (NER) pathway removes helix-distorting DNA damage (such as UV lesions) in a complex 'cut and patch" mechanism. Inborn NER defects can lead to the skin cancer syndrome xeroderma pigmentosum (XP) or the segmental progerias Cockayne syndrome (CS) and trichothiodystrophy (TTD) (Bootsma et al., 2002). An increase in the mutation rate caused by defects in one of at least 7 different NER proteins (XPA-G) explains the cancer predisposition in XP (Cleaver, 2000); each of these proteins participates in lesion removal anywhere in the genome (global genome NER). In contrast, how alterations in any of 5 NER-associated proteins (XPB, XPD, XPG, CSA, CSB) can cause progeria remains largely obscure. Current genetic evidence points to a link with defective transcription-coupled repair (TCR) (Andressoo and Hoeijmakers, 2005; Andressoo et al., 2006b; de Boer et al., 1998; Spivak and Hanawalt, 2005), a subpathway of NER that specifically removes lesions on the transcribed strand of active genes that interfere with transcription thus promoting cellular survival from DNA damage (Hanawalt, 2002; Svejstrup, 2003).

The *XPD* gene, encoding a helicase subunit of the transcription/NER-associated TFIIH complex, is unique amongst NER genes in that different point mutations are associated with cancer (XP), progeria (TTD) or a combination (XP combined with Cockayne syndrome [XPCS] ot trichothiodystrophy [XPTTD]) (Andressoo and Hoeijmakers, 2005; Kraemer, 2004; Lehmann, 2001). Although the hallmark feature of TTD, brittle hair, is not observed in CS or XPCS, the neurodevelopmental features of the disease are similar (Itin et al., 2001; Nance and Berry, 1992). In most cases these features include (i) normal *in utero* development followed by postnatal growth failure in the first year (including reduced postnatal brain growth (Rapin et al., 2000)), (ii) progressive gait abnormalities caused by a combination of cerebellar ataxia, joint contractures, spasticity and frequent kyphosis, (iii) sensorineural hearing loss, (iv) progressive loss of subcutaneous fat

(cachexia) yielding an "aged" appearance and (v) the conspicuous lack of (skin) cancer predisposition despite sun sensitivity due to a cellular UV repair defect (Itin et al., 2001; Nance and Berry, 1992).

We mimicked point mutations found in human TTD (Xpd^{TTD}) (de Boer et al., 1998) and XPCS (Xpd^{XPCS}) patients (Andressoo et al., 2006b) in the mouse and reported phenotypic overlap between homozygous mutant animals and the corresponding human syndromes. Crossing mutant *Xpd* animals allowed us to address the effects of compound heterozygosity, or the presence of two different mutant alleles of the same gene, on disease symptoms. Compound heterozygosity between Xpd^{TTD} and Xpd^{XPCS} alleles surprisingly ameliorated developmental delay and age-related premature cachexia and kyphosis associated with each homozygous mutant mouse model as well as the cutaneous abnormalities specific to the TTD model (Andressoo et al., 2006a).

Exacerbation of the repair defect by *Xpa* ablation, on the other hand, results in a much more severe phenotype including dramatic postnatal growth retardation, progressive cerebellar ataxia, lack of subcutaneous fat and death around weaning in several of these mouse models (e.g. XPCS/XPA, TTD/XPA and CSB/XPA) (Andressoo et al., 2006b; de Boer et al., 2002; Murai et al., 2001). **We refer to the above segmental progeroid phenotypes** as "progeroid NER syndrome". **Here, we tested the ability of compound heterozygosity** at the *Xpd* locus in an XPA-deficient background ($Xpd^{TTD/XPCS}/Xpa^{-/-}$) to ameliorate the severe symptoms of progeroid NER syndrome associated with the corresponding double homozygous ($Xpd^{TTD/TTD}/Xpa^{-/-}$ and $Xpd^{XPCS/XPCS}/Xpa^{-/-}$) animals.

Table 1

Genotype	DNA Repair Defect	Postnatal Growth Defect	Progressive Neurological Dysfunction	Lifespan	Additional Pathology
$Xpg^{-/-}$ [24,28]	NER/TCR	Yes	Cerebellar ataxia	~2–3 wk	Undeveloped small intestines
XpgD811stop (Δ360) [56]	NER/TCR	Yes	Slightly less severe than $Xpg^{-/-}$	22/24 within 30 d	Undeveloped small intestines
$XpgΔex15|Xpa^{-/-}$ [27]	NER/TCR	Yes	n.d.	23/25 within 30 d	n.d.
$Xpa^{-/-}|Xpd^{TTD/TTD}$ [21]	NER/TCR	Yes	Ataxia	10/12 within 22 d; survivors 4, 12 mo	Cutaneous abnormalities, brittle hair
$Xpa^{-/-}|Xpd^{XPCS/XPCS}$ [9]	NER/TCR	Yes	Cerebellar ataxia	~3 wk	n.d.
$Xpa^{-/-}|Csb^{-/-}$ [20]	NER/TCR	Yes	Cerebellar ataxia	~3 wk	n.d.
$Xpc^{-/-}|Csa^{-/-}$, $Xpc^{-/-}|Csb^{-/-}$ [57]	NER/TCR	Yes	Yes	~3 wk	n.d.
$Ercc1^{-/-}$ [25,30]	NER/TCR; ICLR; telomere maintenance[58]	Yes	Ataxia	~3 wk	Liver polyploidy; reduced hematopoetic reserves [54]
$Xpf^{-/-}$ [29]	NER/TCR; ICLR; telomere maintenance[58]	Yes	n.d.	~3 wk	Liver polyploidy

ICLR, interstrand cross-link repair; n.d., not determined; NER, nucleotide excision repair; TCR, transcription-coupled repair.

Results

XPD compound heterozygotes in an XPA-deficient background

A comparison amongst the subset of single and combined NER defects resulting in a severe segmental progeroid phenotype is presented in Table 1. Intercrossing of $Xpd^{+/TTD}/Xpa^{-/-}$ and $Xpd^{+/XPCS}/Xpa^{-/-}$ animals resulted in double homozygous $Xpd^{TTD/TTD}/Xpa^{-/-}$ and $Xpd^{XPCS/XPCS}/Xpa^{-/-}$ mutant mice (subsequently referred to as TTD/XPA and XPCS/XPA animals, respectively) as well as compound heterozygous $Xpd^{XPCS/TTD}/Xpa^{-/-}$ (CH/XPA) mice. Expected at a Mendelian frequency of 25%, XPCS/XPA and TTD/XPA mice were instead observed at 15% and 5%, respectively, upon genotyping at postnatal day 9-10. CH/XPA animals were present at 20%, closer to the expected ratio of 25%. $Xpd^{wt/XPCS\ or\ TTD}/Xpa^{-/-}$ (subsequently referred to as XPA) were used as controls in subsequent experiments since XPA deficiency on its own has no or relatively minor effects on growth, development or fecundity (de Vries et al., 1995; Nakane et al., 1995).

Although indistinguishable from control littermates after birth indicative of normal *in utero* development, CH/XPA mice were visibly smaller than XPA littermates by two weeks of age (Fig. 1A and data not shown), similar to XPCS/XPA and TTD/XPA mice (Andressoo et al., 2006b; de Boer et al., 2002). **CH/XPA mice also displayed abnormal gait and tremors** (data not shown) consistent with cerebellar ataxia as previously observed in several other progeroid NER-deficient mouse models (Table 1). However, unlike XPCS/XPA and TTD/XPA, CH/XPA mice survived the stressful weaning period with high penetrance (~80%), reaching an average body weight about half that of littermate controls (Fig. 1A).

Despite their small size, we consistently noted unusual daytime feeding **behavior in** CH/XPA mice, prompting an investigation into their average daily food intake. At 6 weeks of age, mutant mice consumed 23% more food per gram of body weight than littermate controls (p=0.001; Fig. 1B). In a repeat experiment with 7 week-old mice from a different litter, CH/XPA animals ate 12% more than littermate controls (p=0.09). In both cases, food consumption per gram of body weight was similar between CH/XPA mice and younger (3-5 week old) control mice having similar weights but growing faster (Fig 1B and data not shown). We conclude that food consumption corrected for body weight in CH/XPA mice was equal to or greater than littermate controls, and thus does not explain their smaller size. Furthermore at ten weeks of age, CH/XPA weighed on average 71% of littermate XPA controls and had a cachectic appearance. Corrected for body weight, white adipose tissue deposits were on average 48% of controls (perigonadal 41%, mesenteric 52%, adrenal 37%, subcutaneous 57%; overall p=0.01). Interestingly, however, interscapular brown adipose tissue deposits corrected for body weight were similar between CH/XPA and littermate controls at ten weeks of age. Together these data suggest that CH/XPA mice differ from control mice in either the extraction or use of energy from their food.

Between four and six months of age, we observed a rapid and general decline in the overall condition of CH/XPA mice. Prior to sacrifice due to poor health status, mice were

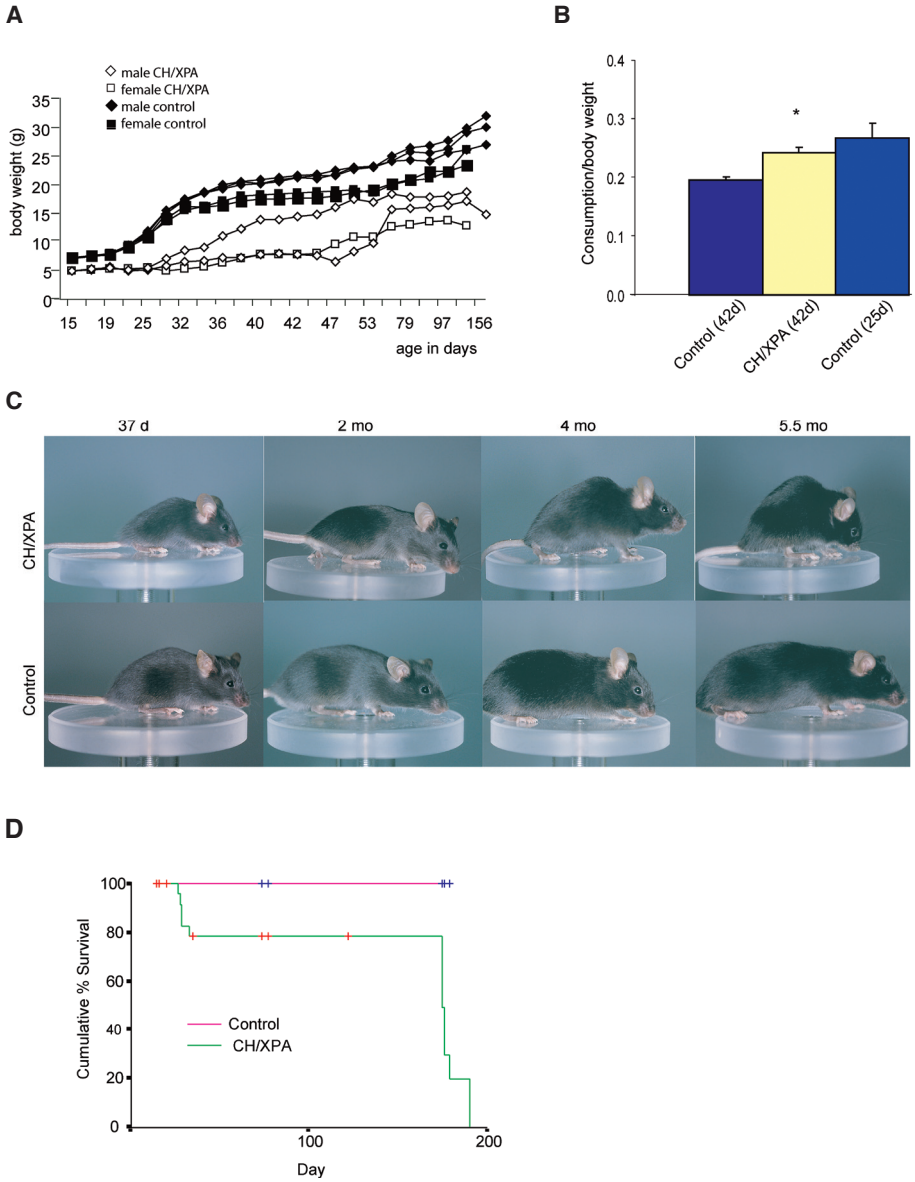


Figure 1. Compound heterozygosity at the Xpd locus partially rescues severe progeroid NER syndrome.

A. Growth curves of individual CH/XPA and control XPA animals from a representative litter.
B. Food intake of CH/XPA animals vs. XPA littermate controls (42 days old) and 25 day-old controls expressed per gram body weight. Asterisks indicate statistical significance ($p<0.001$) vs. 42-day-old controls; error bars indicate SEM.
C. Photographs of male littermate CH/XPA and control XPA mice over 5.5 months.
D. Kaplan-Meier survival curve of CH/XPA and littermate control XPA mice.

on average 56% of littermate weight and increasingly cachectic, with white adipose tissue corrected for body weight on average 43% of controls (perigonadal 43%, mesenteric 32%, adrenal 20%, subcutaneous 63%; overall p=0.02) and also reduced interscapular brown adipose tissue (62% of control, p=0.01). This decline was accompanied by other progeroid features including disturbed gait, frequent loss of balance and kyphosis (Fig. 1C). Kaplan Meier survival plots revealed a significant difference between CH/XPA mice and XPA littermate controls (log rank p<0.01) (Fig. 1D). Mortality was observed primarily at two points, the first around weaning (approximately one month of age) and the second around five months of age. Mean lifespan was approximately five months, which is proportional to the percent lifespan of the related human progeria, Cockayne syndrome (Nance and Berry, 1992). Thus amongst the reported progeroid NER mouse models summarized in Table 1, this mutant is unique in its ability to reach adulthood with high penetrance despite ataxia and cachectic dwarfism.

Accelerated fibroblast senescence undetected in multiple progeroid NER syndromes

Among NER-deficient mouse models for which cellular growth parameters have been reported, an inverse correlation appears to exist between cellular proliferative capacity *in vitro* and progeroid features *in vivo*. For example, primary mouse embryonic fibroblasts (MEFs) from *Xpg*$^{-/-}$ (Harada et al., 1999) and *Ercc1*$^{-/-}$ (Weeda et al., 1997) embryos undergo premature senescence when cultured under atmospheric (~20%) oxygen tension. XPA MEFs, on the other hand, have normal proliferative capacity (Parrinello et al., 2003) and the corresponding animals, despite their NER deficiency, lack profound symptoms of progeroid NER syndrome (de Vries et al., 1995).

We tested this correlation in XPD/XPA MEFs by measuring proliferation in log-phase cultures at atmospheric (20%) oxygen tension as well as low (3%) oxygen tension in which cellular senescence is rescued in wildtype MEFs (Parrinello et al., 2003). Despite the severe progeroid symptoms in XPCS/XPA and TTD/XPA mice in vivo, we found no difference in the long-term proliferative capacity of XPCS/XPA or TTD/XPA MEFs in vitro relative to control or single homozygous mutant littermate controls (Fig. 2A, B). We also tested MEFs from another severely affected progeroid NER model, CSB/XPA (Table 1), and found no difference between control, single or double homozygous mutant MEFs (Fig. 2C). As a positive control, *Ercc1*$^{-/-}$ MEFs (n=4) displayed reduced proliferative capacity vs. heterozygous or wt controls (n=4) at 20% oxygen tension (data not shown) as previously reported (Weeda et al., 1997).

In order to see if this unexpected lack of premature cellular senescence extended to cells prepared from adult animals or to another cell type, we tested the proliferative capacity of dermal fibroblasts prepared from the tails of 10 week old CH/XPA and erythroblasts prepared from embryonic day 13.5 fetal livers. Relative to XPA control littermates, no proliferative defects were observed in either cell type (Fig 2 D, E). We further tested erythroblasts from double mutant CSB/XPA as well as single mutant CSB and XPA fetal livers and found no genotype-specific proliferative differences (Fig 2F).

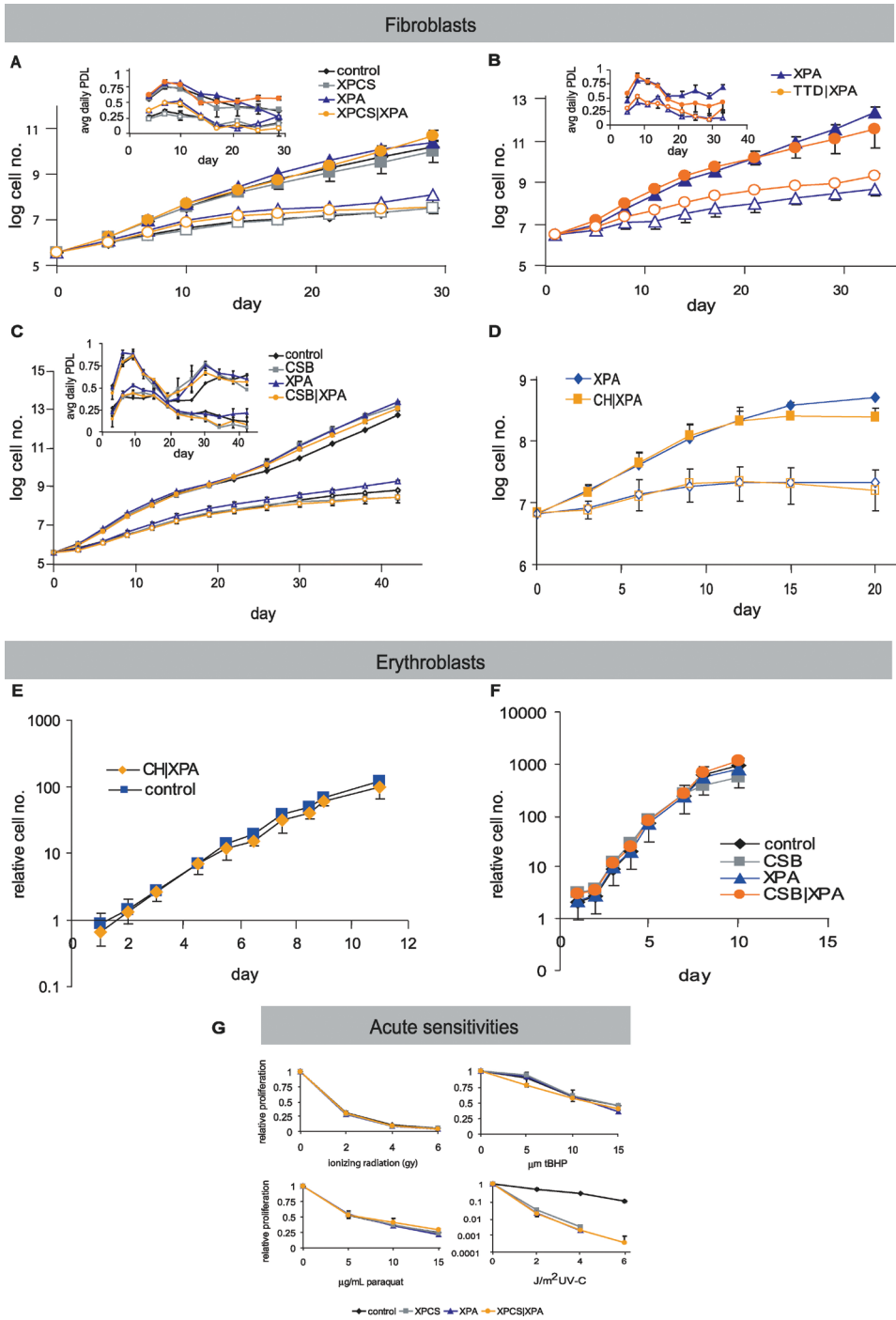

Fibroblasts
A
control
XPCS
XPA
XPCS|XPA
log cell no.
avg daily PDL
day
B
XPA
TTD|XPA
log cell no.
avg daily PDL
day
C
control
CSB
XPA
CSB|XPA
log cell no.
avg daily PDL
day
D
XPA
CH|XPA
log cell no.
day
Erythroblasts
E
CH|XPA
control
relative cell no.
day
F
control
CSB
XPA
CSB|XPA
relative cell no.
day
G
Acute sensitivities
relative proliferation
ionizing radiation (gy)
μm tBHP
μg/mL paraquat
J/m2 UV-C
control
XPCS
XPA
XPCS|XPA

Figure 2. Normal proliferation of progeroid NER primary cells upon chronic and acute oxidative stress in vitro.

A-C. Serial passaging of MEFs from individual litters at 3% (filled symbols) vs. 20% (open symbols) oxygen tension. Lines are compiled from the indicated number of fibroblast cultures derived from single embryos and plotted as the log of the cumulative cell number over time; error bars indicate SEM. Insets in A-C are the same data plotted to indicate the rate of growth; an average daily population doubling (avg daily PDL) of 1 indicates that the cells divide on average once a day. A. Black diamonds, control (n=3); blue triangles, XPA (n=1); grey squares, XPCS (n=2); orange circles, XPCS/XPA (n=2). **B. Blue triangles, XPA (n=4); orange circles, TTD/XPA (n=2). C. Black diamonds, control (n=1);** blue triangles, XPA (n=2); grey squares, CSB (n=2); orange circles, CSB|XPA (n=3).
D. Serial passaging of MDFs prepared from tails of 10 week old animals at 3% (filled symbols) vs. 20% (open symbols) oxygen tension. Lines are compiled from the indicated number of fibroblast cultures derived from single animals and plotted as the log of the cumulative cell number over time; error bars indicate SEM.
E, F. Serial passaging of erythroblasts prepared from E12.5 embryos from a representative litter. E. **Blue squares,** XPA (n=5); orange diamonds, CH/XPA (n=4). F. Black diamonds, control (n=2); blue triangles, XPA (n=3); grey squares, CSB (n=1); orange circles, CSB|XPA (n=2).
G. Proliferative capacity upon acute treatment with paraquat, ionizing radiation, tert-butylhydroperoxide and UV-C as indicated is expressed as a percentage of growth of the untreated samples. **Black diamonds, control (n=3); blue** triangles, XPA (n=1); grey squares, XPCS (n=2); orange circles, XPCS/XPA (n=2); error bars indicate SEM.

Finally, we tested hypersensitivity of primary embryonic fibroblasts to acute oxidative stress induced by **ionizing radiation, paraquat and tert-butyl hydroperoxide. Similar to** *Xpg*$^{-/-}$ MEFs (Harada et al., 1999; Shiomi et al., 2005), we found no significant differences between any of the genotypes (Fig. 2H and data not shown). As a control, all single and double homozygous mutants tested were hypersensitive to UV-C, consistent with their NER deficiencies (Fig. 2H and data not shown). In conclusion, it is unlikely that premature senescence or cell death observed in proliferating primary cell cultures under conditions of chronic or acute oxidative stress are related to the symptoms common amongst the various progeroid syndromes listed in Table 1.

Purkinje cell death is a late event in disease progression

We next looked for differences in cell death or proliferation *in vivo* using one of the severe progeroid NER syndromes, XPCS/XPA, in comparison to single mutant (XPCS and XPA) and heterozygous (control) littermates at postnatal days 5, 12 and 15 coincident with growth retardation and ataxia but prior to signs of weight loss or morbidity. We focused on this double homozygous mutant due to the 100% penetrance of mortality around weaning (Table 1). We first examined cerebellar Purkinje neurons, one of the few cell types consistently affected in severe progeroid NER syndromes as reported in XPG, CSB/XPA and XPCS/XPA (Table 1). Despite disturbed gait and balance problems consistent with cerebellar ataxia, CH/XPA mice showed no detectable degeneration or loss of Purkinje neurons or abnormalities in cerebellar morphology upon sacrifice at the age of 5.5 months (Fig. 3A). Similarly, Purkinje neurons were morphologically intact in all mutant and control XPD/XPA animals at postnatal day 15 (Fig. 3B). These results were unexpected based on the almost complete loss of Purkinje cells by postnatal day 20 in XPCS/XPA mice (Andressoo et al., 2006b) as well as XPG mice (Sun et al., 2001).

Except for cellular loss in the cerebellum very late in disease progression, gross pathological analysis of double mutant XPCS/XPA mice did not reveal overt defects in any other major organ system investigated, similar to reports in TTD/XPA and CSB/XPA mice (de Boer et al., 2002; Murai et al., 2001). **Furthermore, we failed to observe polyploidization** of liver nuclei (Fig. 3B) as in *Ercc1*$^{-/-}$ and *Xpf*$^{-/-}$ mice (McWhir et al., 1993; Tian et al., 2004; Weeda et al., 1997). **Cellular proliferation, as measured by BrdU incorporation, Ki67 and** PCNA immunohistochemistry in 5 and 12 day old mice did not reveal evident genotype-specific differences in the number of replicating cells in the intestine, liver, kidney, heart or lung (data not shown). Nor did we find evidence of increased cell death/apoptosis in hematoxylin and eosin- or TUNEL-stained sections of various organs or following sensitive ligation-mediated PCR-based analysis of DNA from organs including liver, cerebellum, heart and eye (Fig. 3C and data not shown). Taken together, these *in vitro* and *in vivo* data support neither a vicious cycle of cell death and proliferative exhaustion nor a general cell-autonomous proliferative defect *per se* as causative of the cachectic dwarfism and failure to thrive in the developmental period that precedes severe pathology and death before weaning in most progeroid NER mice.

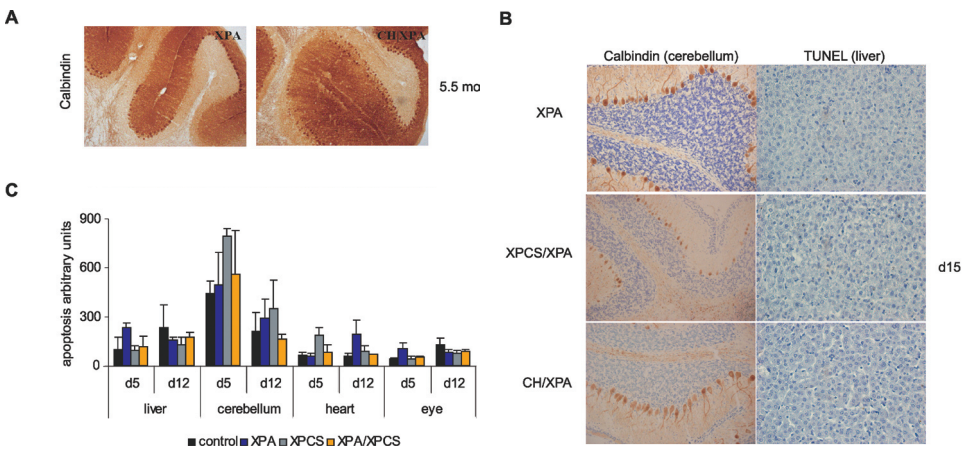


Figure 3. Proliferation and cell death do not correlate with onset or severity of progeroid NER syndrome.

A. Anti-calbindin staining (brown) of Purkinje neurons of 5.5-month-old CH/XPA and control XPA mice (magnification 100X).

B. TUNEL staining (brown) with hematoxylin co-stain (blue) of liver sections and anti-calbindin immunostaining (brown) with hematoxylin co-stain (blue) of cerebellar sections of representative XPCS/XPA, CH/XPA and control XPA mice at 15 days of age (magnification 400X).

C. Ligation-mediated PCR-based analysis of apoptosis in liver, cerebellum, heart and eye of postnatal d5 and d12 control (n=2-3), XPA (n=3), XPCS (n=3) and XPA/XPCS (n=3-4) mice. The relative amount of laddered PCR products representing apoptosis is presented in arbitrary units on the y axis; error bars indicate SEM.

Symptoms of progeroid NER syndrome resemble long-lived dwarfism and calorie restriction

We next looked for parameters that were partially rescued by *Xpd* compound heterozygosity in CH/XPA mice compared to XPCS/XPA mice in order to elucidate those parameters that are important in disease etiology. We focused initially on metabolic parameters including body weight, temperature and blood glucose in anesthetized mice. As shown in Fig. 4A, significant differences between mutants and controls were observed in body weight, temperature and blood glucose in anesthetized mice upon sacrifice at postnatal day 15. Interestingly, however, these differences did not reach statistical significance between XPCS/XPA and CH/XPA animals despite the large difference in lifespan.

Growth retardation, hypothermia, hypoglycemia (Fig. 4A) and reduced body mass despite normal to increased food intake per gram body weight (Fig. 1B) are phenotypes shared by long-lived mice with defects in growth hormone signalling, including growth hormone deficient Ames and Snell dwarfs and growth hormone resistant growth hormone receptor knock-out (GHR-KO) mice (Bartke and Brown-Borg, 2004; Longo and Finch, 2003). Growth hormone mediates its effects largely through the increased secretion of insulin-like growth factor 1 (IGF-1) biosynthesis in target tissues including the liver. To determine the status of the growth hormone/IGF-1 axis in progeroid NER syndrome, we measured total IGF-1 in serum of postnatal day 15 animals (Fig. 4B). Compared to normally sized XPA animals, serum IGF-1 was reduced in both XPCS/XPA and CH/XPA pups, although only to a level of statistical significance in XPCS/XPA animals ($p<0.05$). IGF-1 mRNA from liver, the major source of circulating IGF-1, was significantly reduced in both (Fig. 4C, $p<0.05$). As a control, relative abundance of IGF-1 and a number of other mRNAs in single mutant XPA, XPCS and TTD livers were not significantly different from heterozygous controls (data not shown).

We next measured growth hormone in the serum (Fig. 4B). Despite considerable inter-animal variation, likely due to the pulsatile nature of growth hormone secretion from the pituitary (MacLeod et al., 1991), levels were in the range of control XPA animals independent of genotype. Thus, dwarfism in NER progeria is not likely a primary pituitary defect as in Ames and Snell dwarf mice, in which the anterior pituitary fails to develop properly (Cheng et al., 1983). Consistent with this, no histological or functional differences were detected in the pituitary gland of another progeroid NER mutant, CSB/XPA (van der Pluijm et al. 2007). Growth hormone insensitivity, as in GHR-KO mice, can also cause dwarfism (Hauck et al., 2001; Zhou et al., 1997) even in the presence of excess growth hormone. Indeed, liver GHR mRNA abundance was significantly reduced in both XPCS/XPA and CH/XPA pups. Despite a range of expression levels, maintenance of the strong correlation between GHR and IGF-1 mRNA in individual CH/XPA ($r=0.84$, $n=12$) and XPCS/XPA ($r=0.76$, $n=8$) animals relative to control XPA animals ($r=0.96$, $n=12$) suggests that coordinated transcriptional regulation is intact in progeroid NER syndrome (Fig. 4C, inset) and that GHR mRNA levels are a relevant indicator of GHR protein function.

Calorie restriction and growth hormone deficiency/insensitivity both result in hypoglycemia and hypoinsulinemia. Increased sensitivity to the effects of insulin is thought to underlie the low blood sugar and may also be a key to extended longevity (Bartke and Brown-Borg, 2004). We measured serum insulin from day 15 animals and found, similar to dwarves and calorie-restricted animals, no evidence of hyperinsulinemia as a cause of hypoglycemia (Fig. 4B). IGF-1, IGF-1 binding protein 3 (IGF-BP3, also known as growth hormone-dependent binding protein) and acid-labile subunit (ALS) form a ternary complex in the serum and are all reduced in growth hormone insensitivity disorders in proportion to the severity of growth hormone resistance (Savage et al., 2005). In XPCS/XPA and CH/XPA animals, liver IGF-BP3 and ALS mRNA levels were reduced in addition to IGF-1, although the reduction in ALS expression in CH/XPA did not meet the criteria for statistical significance (Fig. 4C and data not shown). Other key genes involved in postnatal growth via the somatotroph axis, including deiodinase 1, which activates thyroid hormone precursor, and the prolactin hormone receptor, were significantly reduced as well (Fig. 4C). Within this signature group of five somatotroph axis genes, mRNA expression levels were significantly different not only between NER progeroid animals and controls, but also between XPCS/XPA and CH/XPA animals; in each case, downregulation was more severe in XPCS/XPA. Thus, on the level of gene expression, downregulation of the somatotropic IGF-1-GH axis correlated with the severity of NER progeria.

IGF-1, ALS and prolactin receptor are three of a surprisingly limited number of liver transcripts reported to be altered in the long-lived GHR-KO dwarf (only 10 genes at a significance of $p<0.001$) while in Snell dwarf mice and calorie-restricted mice hundreds of liver transcripts are differentially expressed using the same experimental platform and statistical criteria (Miller et al., 2002). From this list of 10 transcripts, we additionally tested IGF-BP2, flavin-containing monoxygenase 3 (FMOX3) and apolipoprotein A-IV (APOA4) expression. Circulating concentrations of IGF-BP2 are inversely related to growth hormone status and were significantly upregulated in progeroid NER mice, further underlining the down-regulation of the somatotropic axis. FMOX3, which is involved in the oxidative breakdown of xenobiotic compounds and downregulated in GHR-KO mice, was also down-regulated in progeroid NER mice. Finally, APOA4, a protein involved in serum lipid transport that is ~10 fold reduced in GHR-KO mice but 4-5 fold increased upon 24-48 hours of starvation (Bauer et al., 2004), was 3-6 fold increased in NER-deficient mice. In conclusion, many but not all characteristics of both CH/XPA and XPCS/XPA mice resembled either calorie restriction or genetic dwarfism.

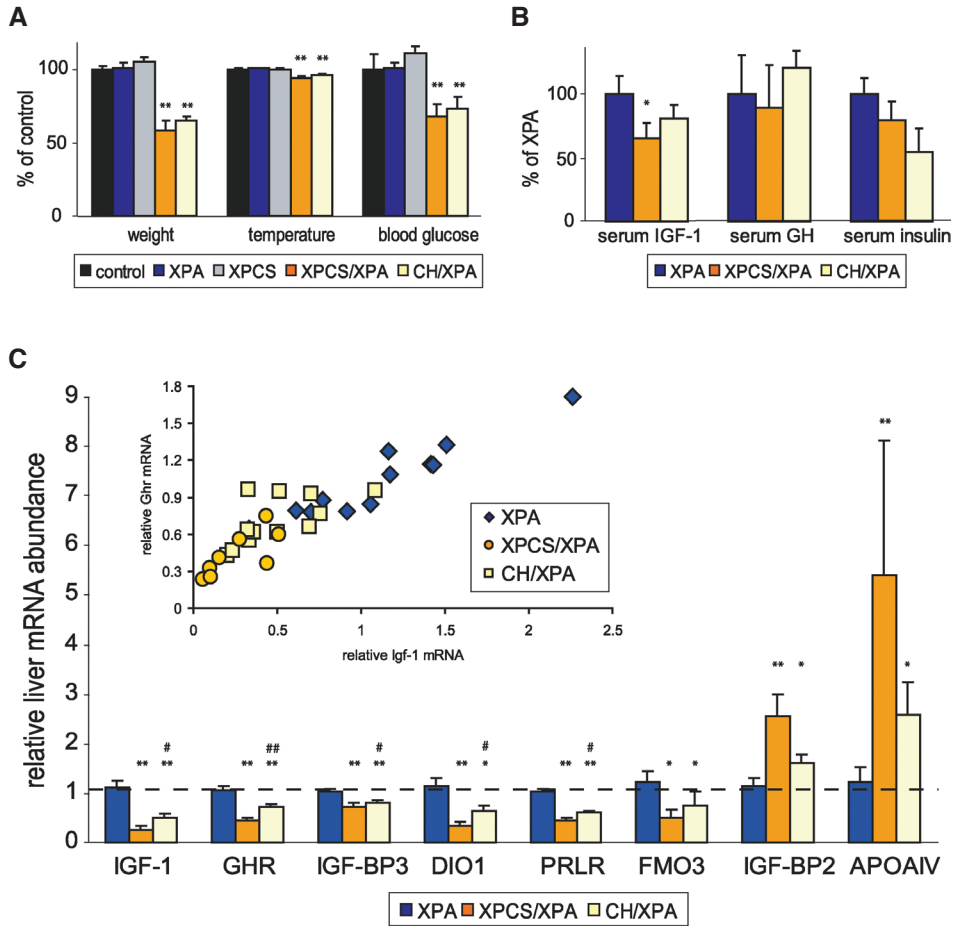


Figure 4. Perturbation of growth hormone/IGF-1 axis correlates with severity of NER progeria.

A. Body weight, temperature and whole blood glucose of anesthetized wildtype control (n=4, black); single mutant XPA (n=23, blue) and XPCS (n=6, grey); and double mutant XPCS/XPA (n=6, orange) and CH/XPA (n=13, beige) animals relative to wildtype controls. Asterisks indicate statistical significance vs. wt controls (** p<.01); error bars indicate SEM.

B. Serum IGF-1, growth hormone and insulin as determined by ELISA in XPCS/XPA (n=5) and CH/XPA (n=7) relative to XPA (n=6). Asterisks indicate statistical significance vs. XPA controls (* p<.05); error bars indicate SEM.

C. Abundance of liver mRNAs encoding insulin-like growth factor 1 (IGF-1), growth hormone receptor (GHR), IGF-1 binding protein 3 (IGF-BP3), deiodinase 1 (DIO1), prolactin receptor (PRLR), flavin-containing monoxygenase 3 (FMO3), IGF-1 binding protein 2 (IGF-BP2), and apolipoprotein A-IV (APOAIV) in XPCS/XPA (n=8) and CH/XPA (n=12) relative to XPA (n=12) as determined by quantitative RT-PCR. Asterisks indicate statistical significance vs. XPA controls (* p<.05, ** p<.01); number signs indicate statistical significance between XPCS/XPA and CH/XPA (# p<.05, ## p<.01); error bars indicate SEM. Inset: correlation between relative IGF-1 and GHR mRNA expression values in individual animals; correlation coefficients per group: XPA, 0.96 (blue diamonds); XPCS/XPA, 0.76 (orange circles); CH/XPA, 0.84 (yellow squares); overall, 0.92.

An adaptive response to stress in progeroid NER syndrome

In genetic dwarfism, differences in serum IGF-1 and glucose metabolism are caused by congenital, irreversible defects in components of the postnatal growth axis itself. In calorie-restricted animals, these differences are reversible and represent an adaptive response to the stress of nutritional deprivation. We followed blood glucose and serum IGF-1 levels in CH/XPA mice over their lifespan to see if the phenotypic overlap with dwarf and calorie-restricted animals was more a structural defect or an adaptive response. At ten weeks of age, blood glucose and serum IGF-1 levels were normal in CH/XPA animals relative to wt and XPA controls (Fig. 5). This strongly suggests that the reduction of these components during early postnatal development (Fig. 4) represents an adaptive response rather than a structural defect. At the end of their lives, IGF-1 levels remained within the normal range while blood glucose levels once again fell significantly below control wt and XPA levels. Low serum glucose levels were also observed in the long-term survivors of a cohort of wt mice at the end of their lives (approximately two years; Fig. 5).

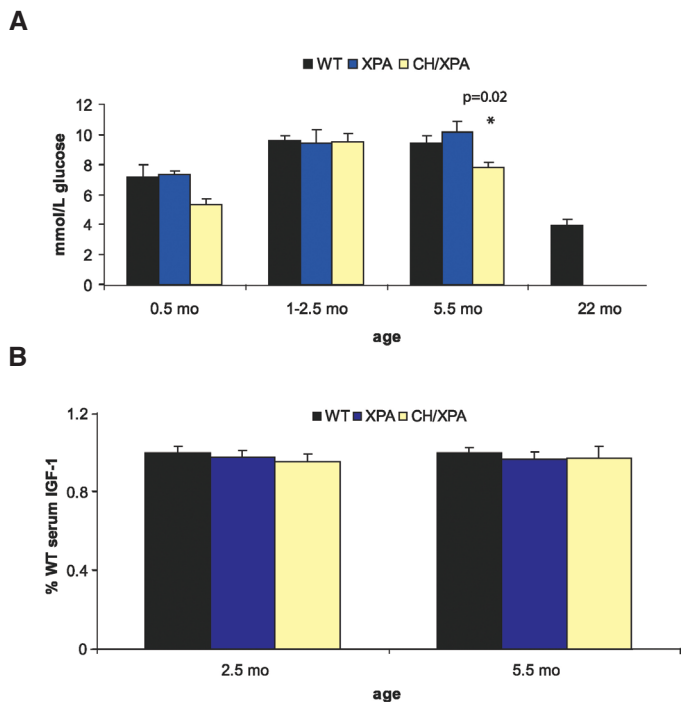


Figure 5. Kinetics of blood glucose and serum IGF-1 levels throughout the life of CH/XPA mice.

Longitudinal measurements of (A.) blood glucose and (B.) serum IGF-1 in CH/XPA vs. XPA and wt control mice. Error bars indicate SEM; 5-15 animals were used per genotype per timepoint. P value indicates the significance of the difference relative to WT at that timepoint.

Is somatotroph axis dampening common to progeroid genome instability disorders?

Cachectic dwarfism is common in mouse models of genome instability with segmental progeria, and has been attributed in many cases to cell-autonomous proliferative defects (premature senescence) as observed in cultured MEFs (Lombard et al., 2005). We next asked whether the dampening of the somatotroph axis observed here in progeroid NER disorder is common to cachectic dwarfism caused by a different primary DNA repair defect. *Ku80*$^{-/-}$ mice, which are defective in the repair of double-strand DNA breaks via the non-homologous endjoining pathway, are smaller than control littermates from E17.5 on (Nussenzweig et al., 1996) and display multiple symptoms of premature senescence including kyphosis, atrophic skin, hepatocellular degeneration and reduced lifespan due to cancer and sepsis (Vogel et al., 1999). We looked at blood glucose and serum IGF-1 levels in 2-week-old animals and found no differences between *Ku80*$^{-/-}$ and wildtype or heterozygous littermate control mice (Fig 6A). Next, we analysed liver mRNA expression of signature somatotroph axis genes *Ku80*$^{-/-}$ vs. littermate controls using QRT-PCR, the method found above to be most sensitive at identifying subtle differences between progeroid NER mice. Despite 50-60% reduced weight relative to littermate controls, *Ku80*$^{-/-}$ mice did not displayed no evidence of somatotropic axis dampening as observed in progeroid NER mice of the same age (Fig 6B). Nor was involvement of long-lived dwarf GHR-KO signature genes such as *ApoA4* detected. We also found no evidence of somatotroph dampening in *Ku80*$^{-/-}$ mice at 10 weeks of age when the activity of this axis should be near its peak. As a control for the ability to detect somatotropic axis dampening in 10 wk old mice, we compared them to older animals (~1 year of age) from the same stable and observed the expected age-related reduction in transcript abundance from somatotroph axis genes including IGF-1 and DIO1. In conclusion, unlike in NER progeria, we found no evidence of reduced GH/IGF-1 signalling contributing to cachectic dwarfism in *Ku80*$^{-/-}$ mice.

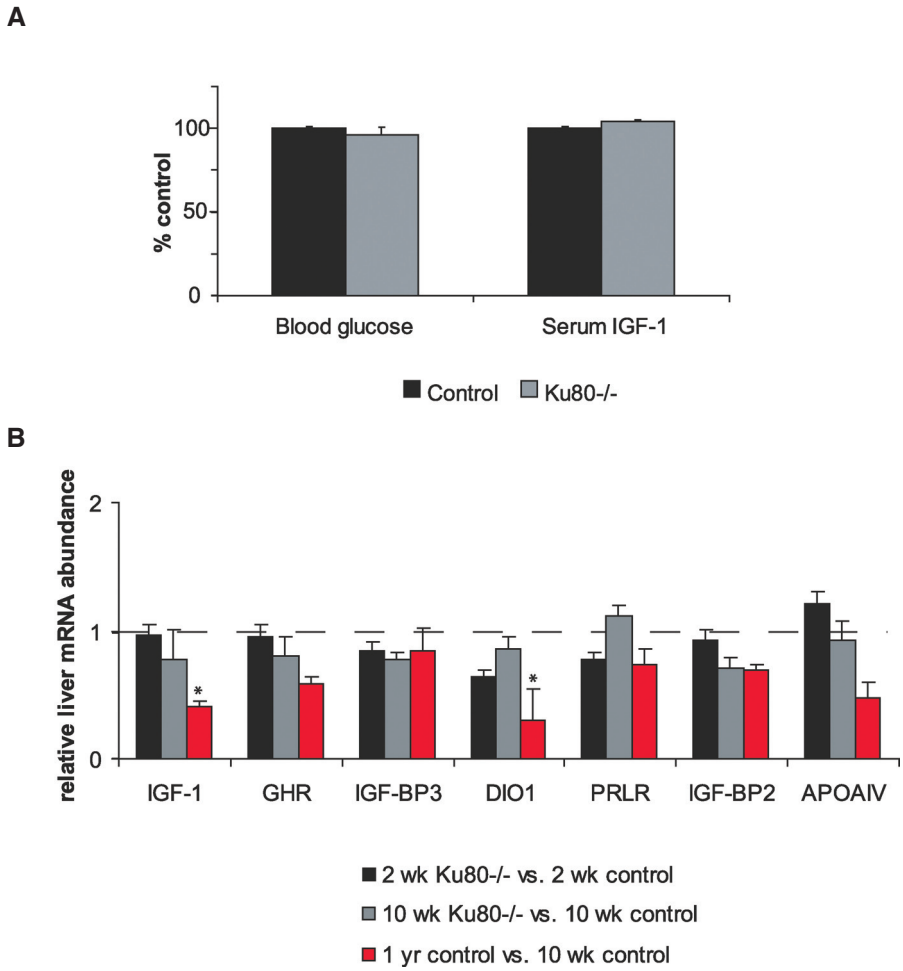


Figure 6. Analysis of blood glucose, serum IGF-1 and liver gene expression profiling in NHEJ-deficient Ku80$^{-/-}$ mice.

A. Glucose and IGF-1 levels in the blood serum of 2-week-old ku80$^{-/-}$ vs. wt or heterozygous littermate controls. Error bars indicate SEM; six animals were used per group.

B. Abundance of liver mRNAs encoding insulin-like growth factor 1 (IGF-1), growth hormone receptor (GHR), IGF-1 binding protein 3 (IGF-BP3), deiodinase 1 (DIO1), prolactin receptor (PRLR), flavin-containing monoxygenase 3 (FMO_3), IGF-1 binding protein 2 (IGF-BP_2), and apolipoprotein A-IV (APOAIV) in 2-weekold ku80$^{-/-}$ (n=6) vs. 2-weekold wt or heterozygous littermate controls (n=6); and both 10-week-old ku80$^{-/-}$ (n=4) and ~1 year-old vs. 10-week-old controls (n=5) as determined by quantitative RT-PCR. Error bars indicate SEM between experiments.

Discussion

We describe a new mouse model for progeroid NER syndrome created by combining two different mutant *Xpd* alleles in an *Xpa*$^{-/-}$ background. A major benefit of this model over existing models (Table 1) is its ability to survive weaning with high penetrance and a lifespan proportional to relevant progeroid NER disorders including CS. Furthermore, it is simpler than XPG, XPF and ERCC1 models which additionally have profound cell autonomous proliferative defects. Using this model we found evidence for an adaptive response to genome instability during postnatal development involving dampening of the somatotropic GH/IGF-1 axis and perturbation of glucose homeostasis. This response appears so far specific to excision repair disorders, as it was not observed in NHEJ deficient mice despite cachectic dwarfism and several progeroid features.

Uncoupling of dwarfism and progeria from premature fibroblast senescence in progeroid NER syndrome

There is a strong correlation between genomic instability disorders with symptoms of progeria and premature senescence of the corresponding cultured fibroblasts, for example in Werner syndrome, Hutchinson-Gilford progeria and ataxia telangiectasia in man (Kipling and Faragher, 1997) and BUB1, PASG, DNA-PK, ERCC1 and KU80 mutants in mouse (Lombard et al., 2005). As a result, cell-autonomous proliferative defects are generally regarded as strong candidates underlying progeroid phenotypes, including cachectic dwarfism (Lombard et al., 2005; Nussenzweig et al., 1996). Mechanistically, this may be due not only to reduced proliferative capacity *per se* but also altered characteristics of senescent cells such as secretion of inflammatory cytokines and extracellular matrix-degrading enzymes (Campisi, 2005). Here, in related NER progerias (CH/XPA, XPCS/XPA, TTD/XPA and CSB/XPA), we demonstrated that cell-autonomous proliferative defects as observed in two different primary cell types, fibroblasts and erythroblasts, are not an absolute prerequisite for postnatal growth retardation or progeria. This makes these NER-associated progerias exceptional amongst mouse progeroid syndromes caused by genome instability. Consistent with these findings in mice, CS and TTD are peculiar amongst human progerias not only due to their lack of cancer predisposition but also the failure of cells to undergo premature senescence in culture (Kipling and Faragher, 1997). Taken together, these data suggest that defects particularly in transcription-coupled NER are not growth limiting in proliferating cells. This may be due to their ability to repair (or tolerate) DNA damage by a variety of replication-dependent, NER-independent mechanisms. On the contrary, non-dividing post-mitotic cells such as neurons may be at greater risk to transcription-coupled NER deficiencies precisely because they do not have the opportunity to "clean" their genes or dilute their damage by DNA replication, while at the same time they appear to downregulate their ability to repair lesions via the global genome NER pathway (Nouspikel and Hanawalt, 2002).

Instead of any cell autonomous proliferative defect, our data reveal dampening of the postnatal GH-IGF-1 axis, making this a much more likely explanation underlying reduced postnatal growth and small size. The inability to detect gross differences in cell death or

proliferation in vivo except in specific cell types (i.e. cerebellar granular neurons (Murai et al., 2001)) or at the end stage of disease (i.e. cerebellar Purkinje loss (Andressoo et al., 2006b)) does not rule out the contribution of small but significant differences in the number of proliferating or dying cells due to a lack of growth and survival signals. Such differences are likely to be found in specific cell compartments such as slowly cycling (label-retaining) cells of the germinal layer or chondrocytes in the proliferative zone of the growth plate responsible for longitudinal bone growth that are stimulated by growth hormone and IGF-1, respectively (Ohlsson et al., 1992).

Perturbation of somatotropic GH/IGF-1 axis and glucose homeostasis: structural defect or adaptive response?

We observed a number of similarities with long-lived dwarf and calorie-restricted mice, including small size, hypoglycemia and reduced serum IGF-1 indicative of dampening of the postnatal GH/IGF-1 growth axis. In a number of model organisms including worms and mice, suppression of the GH/IGF-1 axis is associated with increased stress resistance and extended longevity (Bartke and Brown-Borg, 2004). Reduced serum IGF-1 can be caused by genetic defects in structural components of the somatotropic axis itself (Ames, Snell, GHR-KO mice) or by an adaptive response to environmental cues such as starvation or calorie restriction. For example, Ames and Snell dwarf mice suffer congential hypopituitarism and lack a number of pituitary hormones including GH from birth. Upon dietary restriction, the only intervention known to extend lifespan in mammals, reduced blood glucose and IGF-1 appear to be key components of an adaptive response to stress (Koubova and Guarente, 2003; Weindruch and Walford, 1988) and are entirely reversible. The apparent logic behind this reversible response is to shift the metabolism away from energy consumption directed at growth or reproduction toward energy conservation and somatic maintenance with a concomitant increase in the ability of cells to cope with stress, with an overall aim of enhancing survival until favourable conditions return.

In CH/XPA mice, blood glucose and serum IGF-1 were reduced relative to controls at two weeks of age but returned to normal by 10 weeks of age. These data strongly argue against any structural defect in the components of the somatotropic axis or glucose homeostasis itself. This also makes it quite different from what has been proposed for progeroid SIRT6-/- mice. Based on the animals' low blood glucose and serum IGF-1, failure to thrive and death with high penetrance before weaning (Mostoslavsky et al., 2006), the authors proposed an important role for SIRT6 in maintaining organismal homeostasis in addition to a role in DNA repair via the base excision repair pathway. In CH/XPA mice, we believe that the reduced blood sugar and serum IGF-1 during early postnatal development are components of an adaptive response to some form of stress experienced during this period before weaning. Although the nature of this stress is unknown, it appears to be initiated soon after birth and may involve the dramatic transition from prenatal to postnatal environment, including a different oxygen concentration, a switch in energy acquisition from the mother's blood via her bloodstream to the mother's milk via the pup's own digestive system, combined with a period of rapid growth.

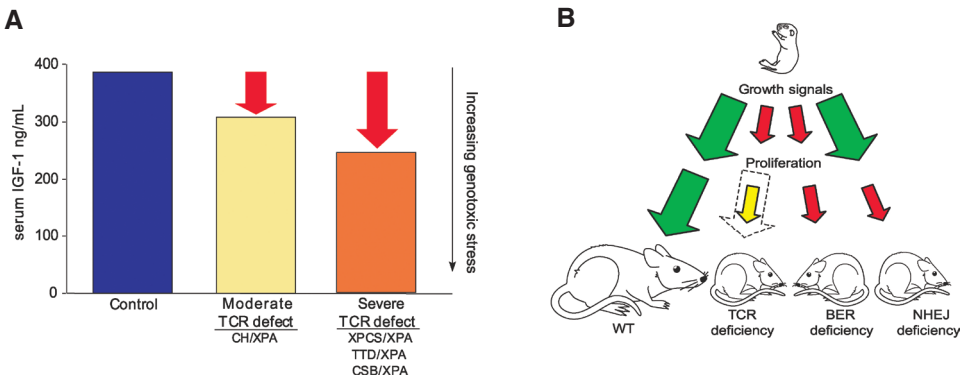


Figure 7. Model for GH/IGF-1 axis and cell proliferation involvement in segmental progeroid genome instability disorders.

A. Inverse correlation between IGF-1 and lifespan in progeroid NER syndrome. Decreased IGF-1 signalling usually correlates positively with increased longevity; in XPD/XPA mutants lower serum IGF-1 (y axis on the left) instead correlates inversely with lifespan. To explain this apparent paradox, we interpret serum IGF-1 levels as indicative of the magnitude of the perceived genotoxic stress (red arrows, y axis on the right). This genotoxic stress may overrule the efficacy of reduced IGF-1 in XPCS/XPA and TTD/XPA animals, while in CH/XPA animals compound heterozygosity partially complements the defect, relieving genotoxic stress and reducing the corresponding stress response.

B. Model for the differential contribution of cell autonomous proliferative defects and systemic growth axis perturbation to progeria in genome instability disorders. Growth signals from the IGF-1/GH somatotropic axis are dampened in progeroid NER syndrome (small red arrow) leading to postnatal growth deficiency; cells removed from this environment and provided adequate growth signals proliferate normally (dotted arrow represents wildtype proliferative capacity). In other segmental progerias, such as SIRT6 deficiency with a reported defect in base excision repair (BER), both appear to be affected. Alternately, cellular proliferation can be affected without apparent involvement of the somatotropic axis as in NHEJ-deficient *Ku80*$^{-/-}$ mice.

Interestingly, although serum IGF-1 levels remained in the normal range at least some time before the death of the CH/XPA animals, blood glucose levels dropped significantly below age-matched controls, indicating that low IGF1 levels do not always precede low glucose. We also observed reduced glucose levels in 15 of the oldest survivors of a longevity cohort of wt mice. Recently it was reported that senescent terminal weight loss in rats correlated with extended longevity (Black et al., 2003). It is therefore tempting to speculate that reduced size and decreased blood glucose are components of an adaptive response intended to extend longevity in both normal and some forms of pathological aging rather than the unintended consequences of random degenerative processes.

Differences between progeroid NER mice and long-lived dwarf mice, on the other hand, may be better explained by defects in the transcription function of the TFIIH complex to which XPD belongs. For example, altered fat metabolism in CH/XPA mice may be related to reported defects in the activation of peroxisome proliferator-activated receptors implicated in lipid metabolism (Compe et al., 2005).

Paradox of IGF-1 reduction: extended vs. shortened lifespan

Although reduced serum IGF-1 on its own does not result in dwarfism or extended longevity in mice (liver IGF-1 KO; (Liu et al., 2000)), it correlates with both in most reported cases. Comparative liver transcriptome analyses of long-lived dwarfism vs. calorie restriction also point to IGF-1 as one of the few consistent predictors of extended longevity (Miller et al., 2002). If reduced IGF-1 mRNA correlates with increased longevity, why were liver IGF-1 mRNA levels observed here lowest in XPCS/XPA animals with the shortest lifespan and intermediate in CH/XPA mice with intermediate longevity? Our data demonstrate that IGF-1 dampening is reversible (at least in the CH/XPA mice) and thus most likely an adaptive response to genotoxic stress. When considering the benefits of reduced IGF-1, one must also in this case consider the magnitude of the stress that triggers this response. At some level of damage, this stress may overrule the efficacy of the response even at its maximal beneficial level. According to this interpretation, the magnitude of the dampening of the postnatal growth axis does not correlate directly to lifespan extension, but to the magnitude of the perceived stress, be it starvation or genome instability, and is independent of whether that stress response is successful in extending longevity (Fig. 7A). Thus in XPCS/XPA and TTD/XPA mice, this results in death before weaning with high penetrance despite the maximal stress response; compound heterozygosity at the *Xpd* locus, which partially complements this defect, results in a less robust stress response due to less or slower damage accumulation resulting in longer lifespan. Whether or not this response is adaptive or maladaptive and what actually triggers it (i.e. CNS dysfunction or malnutrition) remain important questions for future research.

Uncoupling of postnatal growth axis dampening from dwarfism in progeroid genome instability disorders

In contrast to certain progeroid NER disorders, cultured *Ku80*$^{-/-}$ fibroblasts display cell autonomous proliferative defects (Parrinello et al., 2003). Furthermore, *Ku80*$^{-/-}$ dwarfism is not exclusively postnatal (animals are already smaller at birth (Nussenzweig et al., 1996)), and we found no evidence of perturbation of the GH-IGF-1 postnatal growth axis at two weeks, as in progeroid NER disorder, or at 10 weeks near the peak of somatotroph axis activity. This suggests a model in which cachectic dwarfism and progeria can be caused by at least two fundamentally distinct mechanisms: perturbation of postnatal growth signaling **independent of cell autonomous proliferative defects, as in NER progeria, or** cell autonomous proliferative defects without perturbation of the somatotroph axis, as in KU80 deficiency. Because these two scenarios are not necessarily mutually exclusive (for example, *Ercc1*$^{-/-}$ and *Xpg*$^{-/-}$ mice display symptoms of NER progeria (Table 1) but also have cell autonomous proliferative defects; SIRT6-/- mice with a reported BER deficiency also display cell autonomous proliferative defects in addition to reduced serum IGF-1), dwarfism can likely result from combinations of both. In Fig. 7B, we summarize how defects in growth factor environment and cell-autonomous proliferative capacity may independently or together produce the symptoms of segmental progeria due to NER dysfunction or other defects in genome maintenance.

Conclusions

Taken together, these data reveal perturbation of the growth hormone/IGF-1 axis as a novel mechanism underlying the common symptoms of progeroid NER syndromes. Downregulation of IGF-1 may thus be part of a general stress response activated not only upon conditions such as food scarcity but also in response to the accumulation of certain types of DNA damage via congenital repair defects and possibly natural aging. In support of this interpretation, end-of-life pathology in TTD mice is consistent with both segmental accelerated aging (e.g. increased lipofuscin accumulation in the liver) as well as calorie restriction (e.g. reduced incidence of pituitary adenomas) (Wijnhoven et al., 2005); TTD liver gene expression also resembled that of long-lived dwarf mice (Suh et al. submitted). Similarly, transcriptome analysis of CSB/XPA livers revealed significant downregulation of the somatotroph axis as well as upregulation of antioxidant defense (van der Pluijm et al. submitted). Finally, reduced serum IGF-1 and glucose levels were observed in segmental progeroid SIRT6 knockout mice, with a tentative link to a defect in DNA repair via the base excision repair pathway (Mostoslavsky et al., 2006). Extension of these findings from mouse models to the related human disorders remains to be determined. Downregulation of neuroendocrine IGF-1-GH signaling has not been a consistent finding in CS or TTD patients, although the relative paucity of both patients and data prevent firm conclusions from being drawn (Itin et al., 2001; Nance and Berry, 1992; Rapin et al., 2000). However, as evidenced by the reduced cancer incidence in hypopituitary dwarf mice (Bartke and Brown-Borg, 2004), it is a plausible mechanism contributing to the lack of cancer observed in these individuals (Itin et al., 2001; Nance and Berry, 1992; Rapin et al., 2000).

Materials and methods

Mice

Xpa$^{-/-}$, *Xpd*^{TTD} (Xpd^{R722W}) and *Xpd*^{XPCS} (Xpd^{G602D}) mice were generated as described previously (Andressoo et al.; de Boer et al., 1998; de Vries et al., 1995). Mice were in a mixed 129Ola|C57BL/6|FVB background. All experiments involving mice were evaluated and approved by the national committee for genetic identification of organisms and the animal ethical committee and were conducted according to national and international guidelines.

Cells

Embryonic fibroblasts from d13.5 embryos were dispersed by mechanical disruption with an Ultra-Turrax T25 Basic and cultured in a 50:50 mix of DMEM:Ham's F10 supplemented with 10% fetal bovine serum and antibiotics (pen/strep) in a mixed gas incubator with 5% CO_2 and 3% O_2 (passage 0), unless otherwise noted. For long term proliferation assays, cells were counted using a Coulter Multisizer Z2, seeded at 375,000 cells per 10 cm round bottom TC petridish (Greiner Bio-One) and cultured at both 3% and atmospheric oxygen tensions. For subsequent passages, cells were trypsinized before they reached ~80% confluence. For acute stress sensitivity assays, early passage cells (P1-5) were plated in duplo in 6 well plates (Greiner Bio-One) at a density of 20,000 cells per well and treated with the appropriate agent the following day. Cells were counted 4-5 days after treatment using the Multisizer Z2 and plotted as a percentage of the total number of cells from the corresponding untreated cell line. Dermal fibroblasts were prepared from the isolated tail skin of euthanized animals. Following mincing with a razor, tail skin was incubated overnight in a mixed gas incubator with 5% CO_2 and 3% O_2 in DMEM supplemented with 20% fetal bovine serum and antibiotics and 40mg/mL Type II Collagenase (Invitrogen Corporation). The next day, cells were resuspended, passaged through a 40 micron filter, washed, and replated in media without collagenase. Long-term proliferation assays were performed as above except that cells were plated at a slightly higher density (500,000 cells per 10 cm TC petridish). Erythroblast cultures were derived from fetal livers removed from d13.5 embryos. Progenitors were cultured as previously described (Prasher et al., 2005) under normoxic conditions.

Apoptosis

Apoptosis was measured by ligation-mediated PCR using the Apo-Alert LM-PCR Ladder Assay Kit in combination with Advantage cDNA Polymerase mix (both from Clonetech, Palo Alto, CA) according to the manufacturer's instructions. TUNEL assays were performed using the ApoTag Plus Peroxidase In Situ Apoptosis Detection Kit (S7101, Chemicon International) according to manufacturer's instructions.

Immunohistochemistry

For histological examination, mouse samples fixed in formalin were embedded in paraffin, sectioned, rehydrated and stained with hematoxylin and eosin or monoclonal antibodies against PCNA, Ki67 or calbindin and visualized based on HRP conjugated

secondary antibodies. TUNEL staining was performed using ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions.

Physiologic parameters

Food intake was measured by weighing remaining dry food given to individually housed animals (3 per genotype) and the corresponding animals themselves each day over the period of four days; the experiment was repeated twice with different litters at different times. Body temperature was measured in animals immediately following anaesthetization with Isofluran by inserting a surgical thermometer into a small hole cut in the abdominal cavity or inserted into the rectum. Blood was collected following decapitation. Glucose was measured in whole blood using the Freestyle Mini blood glucose meter (Abbott Laboratories, Amersfoort, Nederland) with a range of detection between 1.1 and 27.8 mmol/L. Serum was prepared by low speed centrifugation following clotting. Serum IGF-1, growth hormone and insulin were measured by ELISA using Mouse/Rat IGF-I (DSL-10-2900) with a range of detection between 50 and 3500 ng/mL, Mouse/Rat GH (DSL-10-72100) with a range of detection between 1 and 100 ng/mL, both from Diagnostic Systems Laboratories (DSL Deutschland), and Ultrasensitive Mouse Insulin ELISA (10-1150-01) with a range of detection between 0.188 and 3.75 ug/L from Mercodia (Uppsala, Sweden), according to manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from the liver using TRIzol reagent (Invitrogen) and oligodT or hexamer-primed cDNA synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using an Opticon2 DNA Engine (MJ Research) or a MyIQ (BioRad) with SYBR Green incorporation. Relative expression was calculated using the equation $1.8^{(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$ (Pfaffl, 2001). Each sample was tested in duplo at least two times.

Acknowledgements

We are grateful to Ruud Koppenol for photography; Eliza Haaskijk, Wendy Toussaint, Renata Brandt and Roel Janssens for expert technical assistance; Bjorn Schumacher, George Garinis and Harm de Waard for critical reading of the manuscript; Henk Visser and Leo van de Brande for informative discussions; Linda Verhagen for assistance with fat isolation; and Mattijs Arts for original artwork.

References

1. Martin GM (2005) Genetic modulation of senescent phenotypes in Homo sapiens. Cell 120: 523-532.
2. Miller RA (2004) 'Accelerated aging': a primrose path to insight? Aging Cell 3: 47-51.
3. Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, et al. (2005) DNA repair, genome stability, and aging. Cell 120: 497-512.
4. Mitchell JR, Hoeijmakers JH, Niedernhofer LJ (2003) Divide and conquer: nucleotide excision repair battles cancer and ageing. Curr Opin Cell Biol 15: 232-240.
5. Hasty P, Campisi J, Hoeijmakers J, van Steeg H, Vijg J (2003) Aging and genome maintenance: lessons from the mouse? Science 299: 1355-1359.
6. Bootsma D, Kraemer KH, Cleaver JE, Hoeijmakers JH (2002) Nucleotide Excision Repair Syndromes:Xeroderma Pigmentosum, Cockayne Syndrome, and Trichothiodystrophy. In: Vogelstein B, Kinzler KW, editors. The Genetic Basis of Human Cancer. New York: McGraw-Hill Medical Publishing Division. pp. 211-237.
7. Cleaver JE (2000) Common pathways for ultraviolet skin carcinogenesis in the repair and replication defective groups of xeroderma pigmentosum. J Dermatol Sci 23: 1-11.
8. Andressoo JO, Hoeijmakers JH (2005) Transcription-coupled repair and premature ageing. Mutat Res 577: 179-194.
9. Andressoo JO, Mitchell JR, de Wit J, Hoogstraten D, Volker M, et al. (2006) An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. Cancer Cell 10: 121-132.
10. de Boer J, de Wit J, van Steeg H, Berg RJW, Morreau M, et al. (1998) A mouse model for the basal transcription/DNA repair syndrome trichothiodystrophy. Mol Cell 1: 981-990.
11. Spivak G, Hanawalt PC (2005) Host cell reactivation of plasmids containing oxidative DNA lesions is defective in Cockayne syndrome but normal in UV-sensitive syndrome fibroblasts. DNA Repair (Amst).
12. Hanawalt PC (2002) Subpathways of nucleotide excision repair and their regulation. Oncogene 21: 8949-8956.
13. Svejstrup JQ (2003) Rescue of arrested RNA polymerase II complexes. J Cell Sci 116: 447-451.
14. Kraemer KH (2004) From proteomics to disease. Nat Genet 36: 677-678.
15. Lehmann AR (2001) The xeroderma pigmentosum group D (XPD) gene: one gene, two functions, three diseases. Genes Dev 15: 15-23.
16. Nance MA, Berry SA (1992) Cockayne syndrome: Review of 140 cases. Am J Med Genet 42: 68-84.

References

17. Itin PH, Sarasin A, Pittelkow MR (2001) Trichothiodystrophy: update on the sulfur-deficient brittle hair syndromes. J Am Acad Dermatol 44: 891-920; quiz 921-894.
18. Rapin I, Lindenbaum Y, Dickson DW, Kraemer KH, Robbins JH (2000) Cockayne syndrome and xeroderma pigmentosum. Neurology 55: 1442-1449.
19. Andressoo JO, Jans J, de Wit J, Coin F, Hoogstraten D, et al. (2006) Rescue of progeria in trichothiodystrophy by homozygous lethal Xpd alleles. PLoS Biology 4.
20. Murai M, Enokido Y, Inamura N, Yoshino M, Nakatsu Y, et al. (2001) Early postnatal ataxia and abnormal cerebellar development in mice lacking Xeroderma pigmentosum Group A and Cockayne syndrome Group B DNA repair genes. Proc Natl Acad Sci U S A 98: 13379-13384.
21. de Boer J, Andressoo JO, de Wit J, Huijmans J, Beems RB, et al. (2002) Premature aging in mice deficient in DNA repair and transcription. Science 296: 1276-1279.
22. Nakane H, Takeuchi S, Yuba S, Saijo M, Nakatsu Y, et al. (1995) High incedence of ultraviolet-B- or chemical-carcinogen-induced skin tomours in mice lacking the xeroderma pigmentosum group A gene. Nature 377: 165-168.
23. de Vries A, van Oostrom CTM, Hofhuis FMA, Dortant PM, Berg RJW, et al. (1995) Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature 377: 169-173.
24. Harada YN, Shiomi N, Koike M, Ikawa M, Okabe M, et al. (1999) Postnatal growth failure, short life span, and early onset of cellular senescence and subsequent immortalization in mice lacking the xeroderma pigmentosum group G gene. Mol Cell Biol 19: 2366-2372.
25. Weeda G, Donker I, de Wit J, Morreau H, Janssens R, et al. (1997) Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. Curr Biol 7: 427-439.
26. Parrinello S, Samper E, Krtolica A, Goldstein J, Melov S, et al. (2003) Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nat Cell Biol 5: 741-747.
27. Shiomi N, Mori M, Kito S, Harada YN, Tanaka K, et al. (2005) Severe growth retardation and short life span of double-mutant mice lacking Xpa and exon 15 of Xpg. DNA Repair (Amst) 4: 351-357.
28. Sun XZ, Harada YN, Takahashi S, Shiomi N, Shiomi T (2001) Purkinje cell degeneration in mice lacking the xeroderma pigmentosum group G gene. J Neurosci Res 64: 348-354.

References

29. Tian M, Shinkura R, Shinkura N, Alt FW (2004) Growth retardation, early death, and DNA repair defects in mice deficient for the nucleotide excision repair enzyme XPF. Mol Cell Biol 24: 1200-1205.
30. McWhir J, Seldridge J, Harrison DJ, Squires S, Melton DW (1993) Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. Nat Gen 5: 217-223.
31. Bartke A, Brown-Borg H (2004) Life extension in the dwarf mouse. Curr Top Dev Biol 63: 189-225.
32. Longo VD, Finch CE (2003) Evolutionary medicine: from dwarf model systems to healthy centenarians? Science 299: 1342-1346.
33. MacLeod JN, Pampori NA, Shapiro BH (1991) Sex differences in the ultradian pattern of plasma growth hormone concentrations in mice. J Endocrinol 131: 395-399.
34. Cheng TC, Beamer WG, Phillips JA, 3rd, Bartke A, Mallonee RL, et al. (1983) Etiology of growth hormone deficiency in little, Ames, and Snell dwarf mice. Endocrinology 113: 1669-1678.
35. Hauck SJ, Hunter WS, Danilovich N, Kopchick JJ, Bartke A (2001) Reduced levels of thyroid hormones, insulin, and glucose, and lower body core temperature in the growth hormone receptor/binding protein knockout mouse. Exp Biol Med (Maywood) 226: 552-558.
36. Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, et al. (1997) A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). Proc Natl Acad Sci U S A 94: 13215-13220.
37. Savage MO, Blair JC, Jorge AJ, Street ME, Ranke MB, et al. (2005) IGFs and IGFBPs in GH insensitivity. Endocr Dev 9: 100-106.
38. Miller RA, Chang Y, Galecki AT, Al-Regaiey K, Kopchick JJ, et al. (2002) Gene expression patterns in calorically restricted mice: partial overlap with long-lived mutant mice. Mol Endocrinol 16: 2657-2666.
39. Bauer M, Hamm AC, Bonaus M, Jacob A, Jaekel J, et al. (2004) Starvation response in mouse liver shows strong correlation with life-span-prolonging processes. Physiol Genomics 17: 230-244.
40. Nussenzweig A, Chen C, da Costa Soares V, Sanchez M, Sokol K, et al. (1996) Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. Nature 382: 551-555.
41. Vogel H, Lim DS, Karsenty G, Finegold M, Hasty P (1999) Deletion of Ku86 causes early onset of senescence in mice. Proc Natl Acad Sci U S A 96: 10770-10775.
42. Kipling D, Faragher RG (1997) Progeroid syndromes: probing the molecular basis of aging? Mol Pathol 50: 234-241.

References

43. Campisi J (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 120: 513-522.
44. Nouspikel T, Hanawalt PC (2002) DNA repair in terminally differentiated cells. DNA Repair (Amst) 1: 59-75.
45. Ohlsson C, Nilsson A, Isaksson OG, Lindahl A (1992) Effect of growth hormone and insulin-like growth factor-I on DNA synthesis and matrix production in rat epiphyseal chondrocytes in monolayer culture. J Endocrinol 133: 291-300.
46. Koubova J, Guarente L (2003) How does calorie restriction work? Genes Dev 17: 313-321.
47. Weindruch R, Walford RL (1988) The Retardation of Aging and Disease by Dietary Restriction: Charles C Thomas Pub Ltd. 436 p.
48. Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, et al. (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124: 315-329.
49. Black BJ, Jr., McMahan CA, Masoro EJ, Ikeno Y, Katz MS (2003) Senescent terminal weight loss in the male F344 rat. Am J Physiol Regul Integr Comp Physiol 284: R336-342.
50. Compe E, Drane P, Laurent C, Diderich K, Braun C, et al. (2005) Dysregulation of the peroxisome proliferator-activated receptor target genes by XPD mutations. Mol Cell Biol 25: 6065-6076.
51. Liu JL, Yakar S, LeRoith D (2000) Mice deficient in liver production of insulin-like growth factor I display sexual dimorphism in growth hormone-stimulated postnatal growth. Endocrinology 141: 4436-4441.
52. Wijnhoven SW, Beems RB, Roodbergen M, van den Berg J, Lohman PH, et al. (2005) Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. DNA Repair (Amst).
53. Andressoo JO, Mitchell JR, de Wit J, Hoogstraten D, Volker M, et al. An Xpd mouse model for the combined Xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and accelerated aging. Cancer Cell In press.
54. Prasher JM, Lalai AS, Heijmans-Antonissen C, Ploemacher RE, Hoeijmakers JH, et al. (2005) Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1-/- mice. Embo J 24: 861-871.
55. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.
56. Shiomi N, Kito S, Oyama M, Matsunaga T, Harada YN, et al. (2004) Identification of the XPG region that causes the onset of Cockayne syndrome by using Xpg mutant mice generated by the cDNA-mediated knock-in method. Mol Cell Biol 24: 3712-3719.

References

57. Friedberg EC, Meira LB (2004) Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage (Version 6). DNA Repair (Amst) 3: 1617-1638.

58. Zhu XD, Niedernhofer L, Kuster B, Mann M, Hoeijmakers JH, et al. (2003) ERCC1/XPF removes the 3' overhang from uncapped telomeres and represses formation of telomeric DNA-containing double minute chromosomes. Mol Cell 12: 1489-1498.

4

Fasting offers rapid and robust protection against ischemia reperfusion injury in mice

~

James R. Mitchell, Marielle Verweij,
Marieke van de Ven, Natascha Goemaere,
Sandra van den Engel, Timothy Chu, Flavio Forrer,
Cristina Müller, Marion de Jong, Jan N. M. IJzermans,
Jan H. J. Hoeijmakers, Ron W. F. de Bruin

Abstract

Dietary restriction, or reduced food intake without malnutrition, is synonymous with extended longevity and increased stress resistance in many model organisms. While the kinetics of onset and loss of benefits are rapid in lower organisms, they are unknown in mammals. Using a mouse model of surgical ischemia reperfusion injury to the kidney and liver, we found profound benefits of short-term dietary restriction, including four weeks of thirty-percent reduced food intake as well as three days of water-only fasting, against organ dysfunction and death. Significant protection occurred within one day, increased for up to three days of water-only fasting and lasted for several days beyond the fasting period. The benefits of fasting were not adversely affected by glucose-water ingestion during the fasting period in either kidney or liver, suggesting that the benefits of preoperative fasting are not necessarily incompatible with the current practice of preoperative carbohydrate loading. The mechanisms of protection overlap those associated with long-term dietary restriction, including reduced insulin/IGF-1 signaling, improved insulin sensitivity, improved antioxidant defense and reduced inflammation. Our data suggest that benefits of dietary restriction occur rapidly in mammals, with implications for protection against acute organ stress in the clinic.

Introduction

Dietary restriction (DR) is defined as reduced food intake without malnutrition and is associated with the benefits of extended longevity, prolonged healthspan and improved multiple stress resistance in model organisms including yeast, worms, flies and rodents (Bishop and Guarente, 2007; Brown-Borg, 2006; Masoro, 2003; Sinclair, 2005; Weindruch et al., 1986). In non-human primates and people, effects on longevity and acute stress protection are not yet known but the benefits on general health are clear, including improved cardiovascular fitness, body-mass index and insulin sensitivity (Fontana and Klein, 2007; Weiss et al., 2006).

In experimental rodents, DR is achieved by either restricting total food intake (often called calorie restriction, or CR) or by altering temporal patterns of food availability, generally referred to as intermittent fasting (IF), and including such diets as alternating days of ad libitum feeding and fasting (also known as every-other-day fasting). Because animals on CR diets are hungry and consume their food allotment quickly when fed, CR is usually accompanied by extended periods of fasting between meals, with the period of fasting depending on how often food is provided (typically once daily to thrice weekly) (Masoro, 2004; Masoro et al., 1995; Weindruch et al., 1986). Similarly, IF is often associated with reduced total food intake and reduced body weight typical of CR in some rodent strains, but still produces tangible health benefits in rodents that maintain or even gain weight (Varady and Hellerstein, 2007). Thus although conceptually distinct, the relative contributions of periods of fasting versus reduction of total calorie intake to the benefits of DR, and whether they act by different mechanisms, remain unknown (Mattson, 2005).

An understanding of the underlying mechanistic basis of DR in experimental organisms is far from complete, particularly in mammals. However, reduced signaling through insulin and insulin-like growth factor (IGF) receptors appears to be one evolutionarily conserved mechanism regulating the beneficial effects of DR. This may occur in part through a transcriptional program that is normally repressed by insulin/IGF signaling, including upregulation of stress response genes, altered energy metabolism and reduced inflammation. In mammals, heterozygous deletion of the IGF-1R or adipose tissue-specific homozygous deletion of the insulin receptor results in extended longevity and/or increased stress resistance (Bluher et al., 2003; Holzenberger et al., 2003). DR is similarly characterized by reduced serum levels of IGF-1 and insulin, and presumably reduced signaling through these pathways, but also by improved sensitivity to their action. Whether this improved sensitivity is simply a marker of DR or contributes to its beneficial effects remains unresolved.

In the fruit fly, the maximal benefits of DR on longevity, measured as a function of daily mortality rate, are achieved within 1-3 days of switching from a normal to a restricted diet (Mair et al., 2003). Similarly, the benefits are lost rapidly upon switching from a restricted to a normal diet. Because thousands of animals are required for accurate assessment of

daily mortality rates, such experiments using longevity as an endpoint in mammals are not considered practical. We thus chose to assess the speed of onset and loss of another benefit of DR, namely increased stress resistance. As an acute stress, we chose ischemia reperfusion injury (IRI), which is initiated by a lack of blood flow (ischemia) resulting in a state of tissue oxygen and nutrient deprivation characterized chiefly by ATP depletion, loss of ion gradients across membranes and buildup of toxic byproducts. Restoration of blood flow (reperfusion) causes further damage at first by inappropriate activation of cellular oxidases and subsequently by inflammatory mediators in response to tissue damage (Friedewald and Rabb, 2004). IRI can occur both naturally (stroke, myocardial infarction, hypovolemic shock) or unavoidably during surgery (cardiovascular, transplantation). Previously it has been shown that long-term DR regimes, including both CR and IF regimes for between 3 months and 1 year, are effective at reducing ischemic injury to the heart and brain in rodent models (Ahmet et al., 2005; Chandrasekar et al., 2001; Yu and Mattson, 1999). Here we tested the ability of short-term nutritional interventions (defined here as less than 1 month), including periods of reduced daily food intake and water-only fasting, to protect against renal ischemia reperfusion in mice.

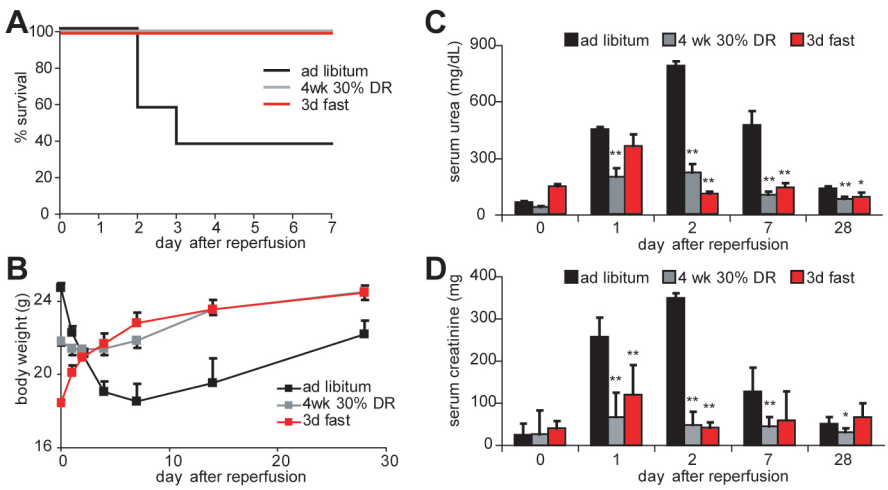


Figure 1. DR protects against the damaging effects of renal IRI.

A. Survival curves of wildtype male mice fed ad libitum, restricted to 70% of ad libitum food consumption for 4 weeks, or fasted for 3 days prior to induction of 37 minutes of bilateral renal IRI (n=10 per group). No further mortality was observed beyond day 4 after surgery. Both dietary treatments led to a significant survival advantage by Kaplan Meier analysis (log rank test, $p<0.01$).

B. Body weight of mice over a 28-day time course following renal IRI.

C, D. Kidney function as measured by serum urea (C) and creatinine (D) concentrations on the indicated days following surgery. Asterisks indicate the significance of the difference as compared to the ad libitum group at the same time point (* $p<0.05$; ** $p<0.01$).

Results

Short-term dietary restriction and fasting protect against ischemia-reperfusion injury

Adult male mice were fed 70% of their normal food intake once a day (30% dietary restriction, DR) for four weeks prior to challenge with renal IRI. Both renal pedicles were clamped for 37 minutes (bilateral occlusion), followed by clamp removal to reinitiate blood flow to the kidneys. Under these conditions, 60% of control mice fed ad libitum prior to surgery died or were sacrificed due to morbidity indicative of irreversible kidney failure by the fourth day following surgery.

In striking contrast, survival in the DR group was 100% (Fig 1A). Two weeks of 30% DR resulted in the same strong protection against mortality (100% survival 1 week after reperfusion). Intermittent fasting regimens with reported health benefits in rodents vary from every-other-day feeding (Goodrick et al., 1982) to four days fasting once every two weeks (Sogawa and Kubo, 2000). We chose to test the potential benefits of three days of fasting. Despite significant weight loss, mice fasted for 3 days prior to surgery had 100% survival rates following 37 minutes of bilateral renal IRI, similar to 4 or 2 weeks of 30% DR (Fig 1A). Both fasted and restricted mice also gained weight faster after IRI and with similar kinetics, reaching their preoperative/pretreatment weight of approximately 24 grams within two weeks; survivors fed ad libitum prior to IRI failed to reach their starting weight for up to one month following IRI (Fig 1B). Mortality following renal IRI usually occurs 2-4 days after the injury due to kidney dysfunction and the associated buildup of toxic waste products in the blood. We analyzed kidney function indirectly by measuring serum metabolites normally cleared by the kidneys. In the ad libitum group, serum urea concentrations continued to rise for at least 2 days following the injury and remained significantly elevated above preoperative levels for up to one month in surviving animals (Fig 1C). In both fasted and 30% restricted animals, blood urea values increased only slightly following the injury and returned to preoperative levels within seven days. A similar profile was seen for serum creatinine (Fig1D), a byproduct of muscle metabolism removed by the kidneys and largely unaffected by feeding state.

Protection against ischemia is rapidly gained and lost

In lower organisms such as the fruit fly *D. melanogaster*, the effects of dietary restriction on daily mortality rate are gained and partially lost in the time frame of 1-3 days (Mair et al., 2003). We next tested how rapidly the protective effects of fasting appeared in mice. To do this, as well as to extend our results from the bilateral renal ischemia model, we used a unilateral occlusion model in which the left kidney was clamped for 37 minutes. After clamp release, the right (undamaged) kidney was removed so that kidney function and animal survival depended on the single damaged kidney. Under these conditions, 90% of wildtype male ad libitum fed mice died or were sacrificed due to morbidity indicative of irreversible kidney failure by the fourth day following IRI. However, mice fasted for three days, two days or even overnight had significantly elevated survival rates (100%,

100% and 90%, respectively; Fig 2A). Despite reaching maximal survival by two days of fasting, protection against kidney dysfunction increased in a dose-dependent manner for up to three days of fasting (Fig 2B). Interestingly, despite robust increase in survival by overnight fasting, significant effects on kidney function were observed only on the third day following the injury. On this critical day, the ad libitum group continued to worsen while the overnight fasted group showed signs of recovery.

The protective effects of fasting and their rapid onset were further confirmed with a more direct measure of kidney function. The uptake of technetium-99m-radiolabeled dimercaptosuccinic acid (^{99m}Tc-DMSA) by the kidneys is directly related to their function (namely tubular reabsorption) and can be determined by measuring the radioactivity that collects in kidneys with a gamma counter. 24 hours after IRI we observed significantly less accumulation of ^{99m}Tc-DMSA in all groups relative to the mock-treated animals; however, mice fasted 48 and 72 hours prior to IRI accumulated significantly more ^{99m}Tc-DMSA and thus retained better renal function than ad libitum fed animals (Fig S1).

In order to determine the half-life of fasting-based protection from renal ischemia reperfusion, we tested the effects of refeeding after fasting on survival and organ function following renal IRI. To do this, we fasted animals for 3 days and then allowed ad libitum access to food for between two hours and seven days prior to induction of bilateral renal IRI. Animals fed ad libitum or fasted for 3 days without refeeding prior to surgery served as negative and positive controls, respectively. Significant benefits on animal survival remained for at least two days after refeeding (Fig 2C). Despite these lingering survival benefits, significant protection from kidney dysfunction was lost by as little as two hours of refeeding prior to surgery (Fig 2D). These results suggest that the benefits of fasting on kidney function are separable from those on animal survival. A similar separation of benefits was observed previously in the protection afforded by overnight fasting, namely improved survival but no significant effect on kidney function on the first two days following reperfusion (Fig 2A, B, Fig S1). Taken together, these data suggest that the total protection afforded by fasting against renal IRI is both gained and lost within a period of just a few days. This is consistent with findings in lower organisms of the rapid onset of benefits of DR against mortality and acute stress (Chippindale et al., 1993; Mair et al., 2003).

Nutrition-based protection is a preconditioning effect

Restricted mice were typically observed eating soon after awaking from anesthesia and, likely as a result, maintained or gained weight in the days following the surgery (Fig 1B). Mice fed ad libitum prior to the surgery were never observed exhibiting this postoperative eating behavior and lost weight in the days following the surgery (Fig 1B). We thus asked if the survival and functional differences were due to diet-induced changes present at the time of renal IRI (preconditioning) or due to differences in eating behavior occurring after the injury. To do this, we prevented access to food for one day following the surgery in ad libitum fed animals vs. those fasted for two days prior to bilateral renal IRI. Although

both groups continued to lose weight following the surgery (Fig 3A), animals fasted for two days prior to IRI had a significant survival advantage (Fig 3B) and significantly better renal function on the second day following the injury (Fig 3C). We conclude that the impact of diet on the ability to withstand renal IRI is largely due to a preconditioning effect. The best known example of this is ischemic preconditioning, the phenomenon by which a brief period of ischemia followed by reperfusion protect against a longer bout of subsequent ischemia reperfusion injury (Murray et al., 1986). We thus refer to effects of short-term DR and fasting observed here as "nutritional preconditioning".

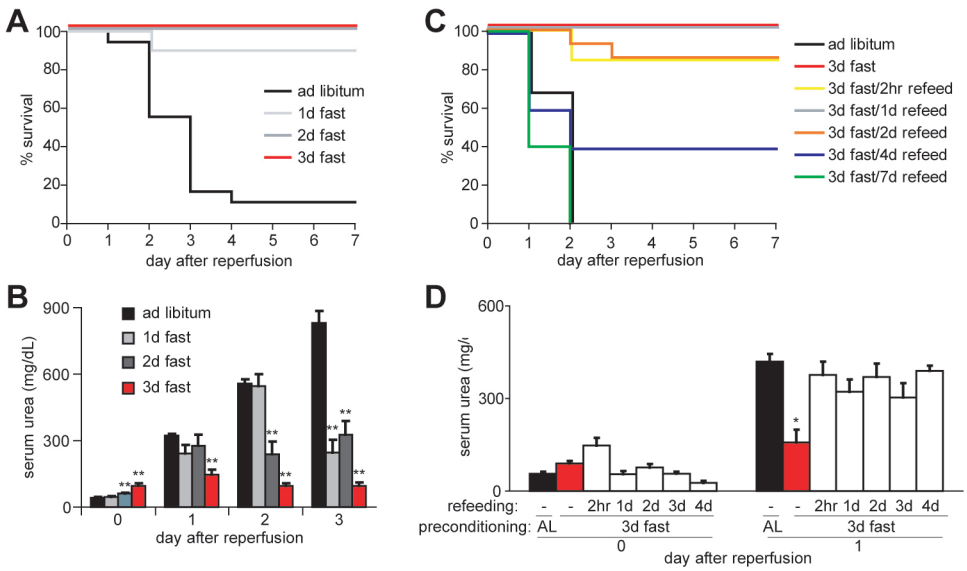


Figure 2. Rapid onset and loss of protective effects of fasting.

A, B. Rapid onset: A. Survival curves of wildtype male mice fed ad libitum or fasted for 1-3 days prior to induction of 37 minutes of unilateral renal IRI with contralateral nephrectomy (n=10-18 per group). B. Kidney function as measured by serum urea following 37 minutes of unilateral renal IRI in the indicated groups (serum from 4-10 individual animals was sampled per data point). Asterisks indicate the significance of the difference as compared to the ad libitum group at the same time point (** $p<0.01$).

C, D. Rapid partial loss: C. Survival curves of the indicated groups. Survival of animals refed for 2hr, 1 and 2 days was significantly different than ad lib fed animals ($p<0.002$); survival of animals refed for 4 and 7 days was not significantly different than ad lib fed animals. D. Kidney function as measured by serum urea prior to and one day following IRI. Data from three independent experiments with 4-12 animals per group are averaged. Asterisk indicates significant difference between 3 days of fasting without refeeding and ad libitum (AL) fed animals as well as each of the refed groups ($p<0.05$). There were no significant differences between the ad libitum group and any of the refed groups on day 1 following renal IRI

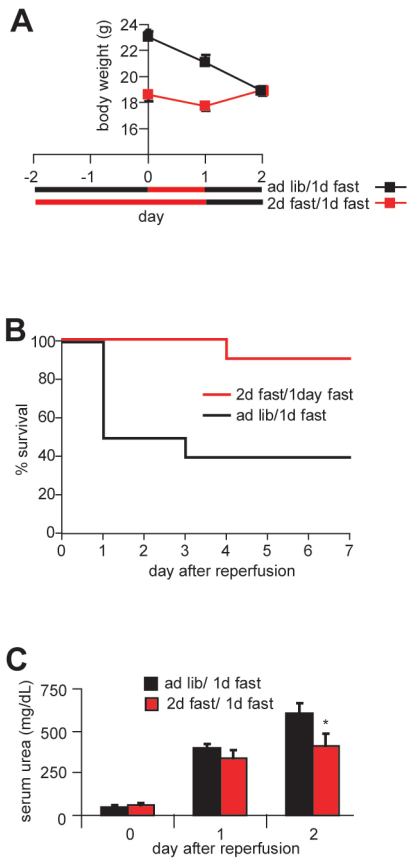


Figure 3. Benefits of fasting do not require refeeding in the first 24 hours after surgery.

A. Black bars indicate periods of free access to chow; red bars indicate periods without access to chow. Body weights of the two groups around the time of surgery (day 0) are indicated.
B. Survival following renal IRI; fasted animals retained their survival advantage ($p < 0.02$) despite lack of refeeding for one day following renal IRI.
C. Kidney function as measured by serum urea before and after IRI. Asterisk indicates a significant difference between the fasted and ad libitum fed groups ($p<0.05$) on the second day following surgery.

Preoperative carbohydrate loading does not interfere with nutritional preconditioning

Benefits of preoperative fasting against IRI as shown above are in apparent conflict with reported benefits of a very different nutritional intervention: preoperative carbohydrate loading. The use of liquid carbohydrate beverages up to two hours prior to surgery has been reported to improve postoperative outcomes including insulin resistance and subjective well-being (Ljungqvist and Soreide, 2003). We thus tested the effects of glucose water ingestion on nutritional preconditioning against renal IRI in mice. Four groups of mice were given access to either water, 54% glucose water, chow and water or chow and 54% glucose water. Mice with ad libitum access to 54% glucose water during the three day fasting period lost less weight than the water-only fasted group, but had the same advantages in terms of survival and kidney function (Fig S2) versus the chow fed groups either with or without glucose water. In conclusion, although preoperative glucose consumption had no clear benefits on organ function or survival in this model, neither did it interfere with the benefits of nutritional preconditioning. Importantly, with respect to optimization of preoperative nutrition, these data suggest that the potential benefits of fasting and nutritional enhancement with liquid carbohydrates are not mutually exclusive.

Nutritional preconditioning is not organ specific

To find out if the protection afforded by brief fasting against IRI was specific to the kidney or more broadly applicable, we used a model of liver IRI in which the portal triad is clamped for 75 minutes, resulting in ischemic damage to approximately 75% of the liver. Because no mortality is associated with this model, we monitored ischemic liver damage by measuring the release of the liver-specific enzyme ALAT from dead or damaged cells into the blood for up to 24 hours (Fig 4A) and hemorrhagic necrosis in histological sections prepared 24 hours after reperfusion (Fig 4B). As in the renal ischemia model, 1-3 days of water-only fasting significantly protected against ischemic damage to the liver. Furthermore, significant protection against necrosis was observed in animals with access to glucose water during the 3 day preoperative fasting period (Fig 4B). Thus, nutritional preconditioning against IRI by fasting is not specific to the kidney.

Overlap between mechanisms of DR-induced longevity and protection against ischemia-reperfusion injury

Although the molecular mechanisms underlying extended lifespan/healthspan by long-term DR remain largely unknown in mammals, a number of potential candidates have been identified, including reduced insulin/IGF-1 signaling, increased insulin sensitivity, increased antioxidant protection and reduced inflammation. Proposed mechanisms of protection against ischemic insult by long-term DR are overlapping and include attenuation of oxidative stress, upregulation of stress proteins and reduced inflammation resulting in less cell death (Ahmet et al., 2005; Chandrasekar et al., 2001; Yu and Mattson, 1999). We asked if mechanisms of nutritional preconditioning by short-term DR and fasting against renal IRI overlap these as well.

We followed markers of insulin/IGF signaling, antioxidant protection and inflammation on the transcriptional level using quantitative real-time PCR on kidney cDNA from 3 day fasted vs. ad libitum fed animals at baseline and over a 2-day time course following renal IRI (Fig 5A). Transcription of Igf-1 is dependent on binding of growth hormone to its cognate receptor, Ghr. At baseline, steady-state levels of kidney Ghr and Igf-1 mRNAs were significantly reduced in fasted animals. In the hours following reperfusion, Ghr and Igf-1 mRNA levels fell below baseline in both groups, but stabilized (Ghr) or rose above baseline (Igf-1) only in the fasted group. This is consistent with an increase in local Igf-1 production relative to baseline in fasted but not ad libitum fed mice following injury.

Reduced signaling through insulin and insulin-like peptides including Igf-1 is thought to represent an evolutionarily conserved switch away from growth and reproduction and toward conservation and cytoprotection. In mammals, this includes increased expression of genes such as the inducible form of hemeoxygenase (HO-1) and components of the glutathione system, including glutathione reductase (Gsr). HO-1 and Gsr are both involved in protection from oxidative stress, and both were significantly elevated in fasted kidneys relative to ad libitum controls at baseline. Following injury, HO-1 was strongly induced in both groups but to significantly lower levels and with a more rapid return to baseline in the fasted group. Gsr mRNA levels fell in the fasted group and increased in the fed group over the 48-hr time course following reperfusion.

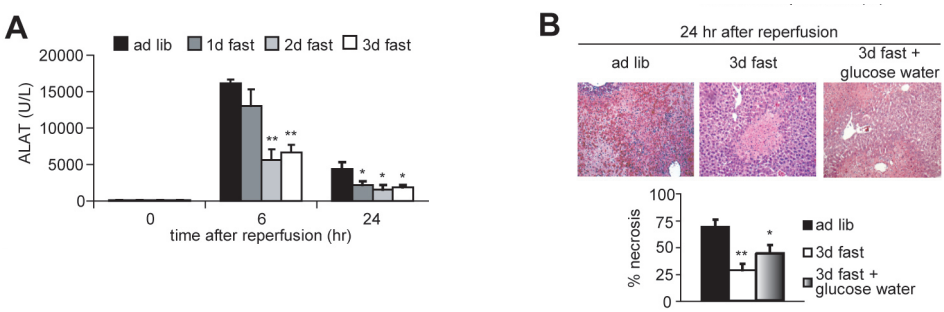


Figure 4. Protection from IRI by fasting is not specific to the kidney.

A. Reduced injury markers upon liver IRI in fasted mice. Mice (5-8 animals per group) were fasted for the indicated times prior to induction of 75 minutes of warm ischemia to the liver. Serum concentration of the liver-specific enzyme alanine aminotransferase (ALAT) indicative of liver damage was measured at the indicated times following reperfusion. Asterisks indicate the significance of the difference as compared to the ad libitum group at the same time point using a Mann-Whitney U-test (* $p<0.05$; ** $p<0.01$).

B. Representative hematoxylin/eosin-stained liver sections from mice 24 hours after reperfusion. Note the large areas of hemorrhagic necrosis (in red) in the mouse fed ad libitum prior to IRI and its relative absence in the mouse fasted for 3 days prior and those with access to glucose water during the fasting period. Magnification 100X. Below: Quantification of hemorrhagic necrosis. Asterisks indicate the significance of the difference as compared to the ad libitum group (* $p<0.05$; ** $p<0.01$). There was no significant difference in % hemorrhagic necrosis in the fasted group between those with access to normal water (n=11) vs. glucose water (n=6; p=0.14).

Inflammation is a significant mediator of cell death and organ dysfunction following IRI in multiple organs (Friedewald and Rabb, 2004). Reduced markers of inflammation generally correlate with improved outcome. Following renal IRI, markers of inflammation including the proinflammatory cytokine IL-6 and the neutrophil-recruiting endothelial adhesion molecule P-selectin were significantly activated in both groups with similar kinetics, but to a significantly lower degree in the fasted group.

Changes in glucose metabolism, including improved ability to clear glucose from the blood upon challenge with either glucose or insulin, are further hallmarks of extended longevity by long-term DR. To test these parameters in our model, we performed glucose tolerance and insulin sensitivity tests by injecting a bolus of glucose or insulin, respectively, and then monitoring blood glucose levels in mice fed ad libitum, fasted overnight or for 3 days, or 30% restricted for 4 weeks (Figs 5B, C). The area under each curve is inversely proportional to that group's ability to clear glucose or respond to insulin following glucose or insulin challenge, respectively. Fasting and short-term DR resulted in improved glucose disposal and improved insulin sensitivity relative to ad libitum fed mice, correlating with improved outcome following renal IR among our experimental groups. We therefore reasoned that if improved insulin sensitivity is a consistent marker of, or possibly causally related to, improved outcome following IR injury, then this should also not be adversely affected by ingestion of glucose water during a 3-day fast. Glucose tolerance and insulin sensitivity tests were thus performed on ad libitum fed or 3-day fasted animals with access to either normal water or glucose water. Consistent with the lack of an effect of glucose water ingestion on the course of IR injury, glucose water ingestion changed baseline glucose levels but did not significantly alter the relative insulin sensitivities of the fed vs. fasted groups (Fig S3).

Increased antioxidant capacity and reduced inflammation are predictive of reduced damage and death at the cellular level. To confirm this, we followed time-dependent leakage of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the blood and histological evidence of acute tubular necrosis as injury markers. Although LDH levels were significantly elevated above baseline at 2 and 6 hours following reperfusion in all groups, at 24 hours they remained significantly elevated only in the ad libitum group (Fig 5D). Comparisons between treatments at individual time points revealed significantly lower LDH in the DR vs. ad libitum groups at 2 hrs, and significantly elevated LDH at baseline in the fasted vs. ad libitum group. Acute tubular necrosis was scored on a scale of 1-5 in a blinded fashion with histological sections from kidneys before and one and two days after IRI. Both short-term DR and 3 day fasting resulted in significantly less acute tubular necrosis on days 1 and 2 following IRI (Fig 5E). To look at replacement of lost cells, we measured the proliferative markers PCNA and Ki67 on the second day following reperfusion, coincident with the onset of organ regeneration. Surprisingly, we observed as much proliferation in the ad libitum group as in the DR and fasted groups (Fig 5F). However, considering the significantly higher damage load in the ad libitum group, the ratio of living to dead cells was higher in the DR and fasted groups. This is consistent with better preservation of organ function, faster return to function and improved survival in nutritionally preconditioned animals.

Taken together, these data suggest a model of protection against acute IR injury by fasting and short-term DR involving reduced insulin/IGF signaling, concomitant upregulation of cellular stress resistance mechanisms and downregulation of inflammatory mediators (Fig 5G). As a consequence, oxidative injury, cell death and inflammation are all reduced subsequent to IR injury. Our data further suggest a relative increase in insulin/IGF signaling above baseline following IR injury in the fasted group. This unexpected finding is consistent with the beneficial role of these factors in survival signaling and regeneration, for example following myocardial infarction and cerebral ischemia (Guan et al., 2003; Suleiman et al., 2007).

Discussion

Fasting has been reputed to have healthful benefits at least since the time of Hippocrates but is largely absent from Western medicine today. Instead, fasting is often associated with malnutrition, which was first reported in 1936 to be a risk factor for postoperative recovery and survival following abdominal surgery (Studley, 1936) and has since been associated with negative outcomes including poor wound healing, increased risk of infection and multiple organ failure (Chung, 2002). One of the few modern clinical applications of fasting is to reduce the risk of pulmonary aspiration of regurgitated stomach contents prior to operations involving anesthesia. While the origins in the 1960s of the standard "nil by mouth after midnight" preoperative fast are somewhat obscure (Maltby, 2006), today even this relatively short fast is perceived as overcautious and possibly detrimental to patient subjective well-being and postoperative recovery (Diks et al., 2005). Current more liberal guidelines allow consumption of solids up to six hours prior to surgery and recommend consumption of liquids, including carbohydrate-rich beverages, up to two hours prior to surgery (Soreide et al., 2005). In rodents, preoperative fasting for this purpose is considered unnecessary since they lack the ability to vomit. Our data suggest that slightly longer periods of DR and/or fasting prior to surgery may be beneficial for an entirely different purpose – protection against certain types of acute organ stress. Importantly, these benefits do not appear to be mutually exclusive with existing preoperative nutritional strategies including carbohydrate supplementation. This may be due in part to the different endpoints addressed by these two different nutritional interventions. Whereas fasting may activate endogenous stress resistance, carbohydrate beverage consumption may ameliorate measures of subjective well-being such as thirst, hunger, irritability and headache associated with fasting (Crenshaw and Winslow, 2002). The fact that consumption of calories in the form of glucose water does not interfere with the benefits of fasting on protection from IRI suggests that the lack of carbohydrates (or calories) in the diet on its own is not the trigger underlying the benefits of fasting.

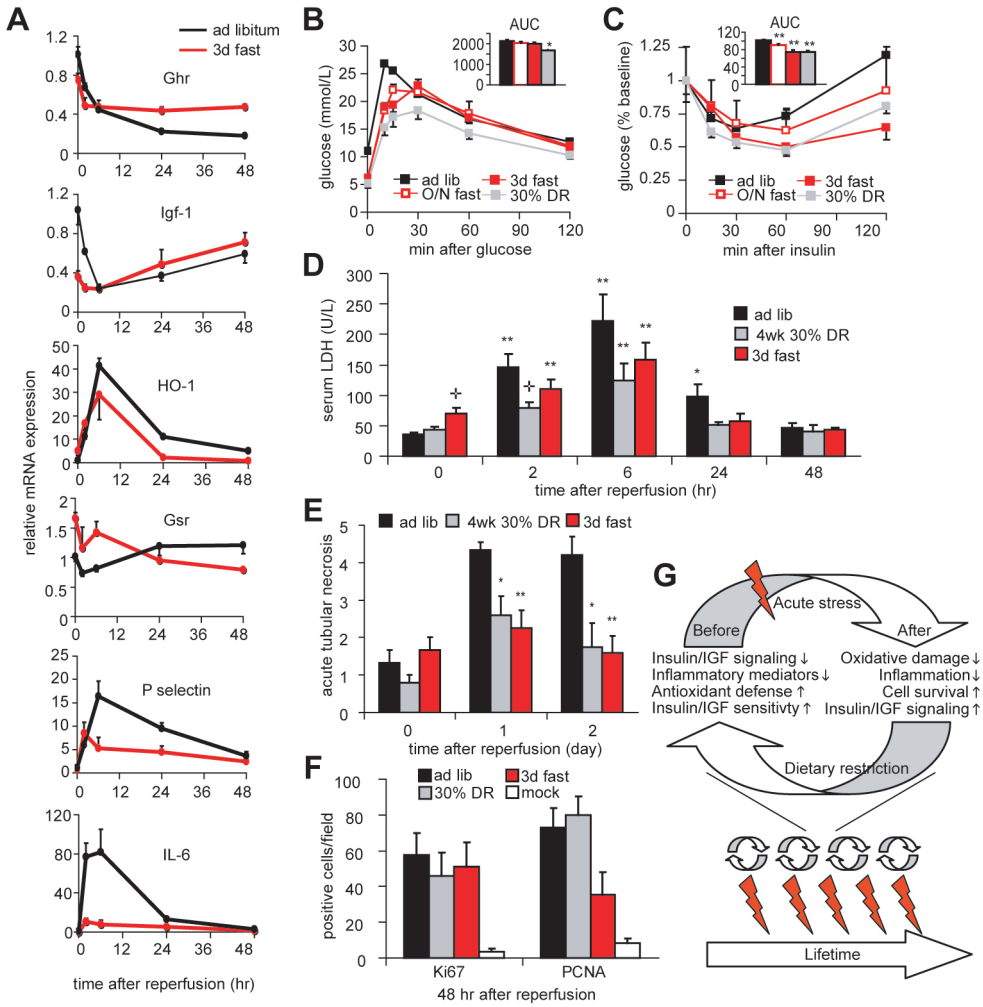


Figure 5. Mechanisms of protection against renal IRI by nutritional preconditioning.

A. Fasting results in differential expression of markers of antioxidant protection, inflammation and somatotroph axis at baseline and in response to renal IR. Changes in steady state mRNA levels of the indicated genes over a two day time course following renal ischemia reperfusion by quantitative real-time PCR. All data points are expressed relative to the ad libitum group at t=0 prior to renal IR. Each data point represents the mean expression value from 5 animals. HO-1, hemeoxygenase-1; Gsr, glutathione reductase; IL-6, interleukin-6; Ghr, growth hormone receptor; Igf-1, insulin-like growth factor-1.

B, C. Improved glucose tolerance and insulin sensitivity in preconditioned mice. Whole blood glucose levels at the indicated timepoints following intraperitoneal injection of glucose (B) or insulin (C) into animals following the indicated preconditioning regimens. Insets: areas under the curves (AUC). Statistically significant differences relative to the ad libitum group are indicated by asterisks (** $p<0.01$; * $p<0.05$).

Figure 5. Mechanisms of protection against renal IRI by nutritional preconditioning.

D. Blood serum LDH of the indicated groups over a time course following reperfusion. Asterisks indicate significant differences relative to time 0 of the same treatment; crosses represent significant differences relative to the ad libitum group at the indicated time point.
E. Quantification of acute tubular necrosis on a 5 point scale before and after renal IRI based on blind scoring of hemotoxylin/eosin stained kidney sections as described previously (Leemans et al., 2005). Asterisks represent significant differences relative to the ad libitum group at the indicated time point (* $p<0.05$; ** $p<0.01$).
F. Percentage of cells expressing Ki67 or PCNA proliferative markers in a microscopic field of the indicated group on the second day following IRI. All groups were significantly elevated vs. the mock control.
G. Model for nutritional preconditioning. Dietary restriction produces a preconditioned (before) state of reduced insulin signaling resulting in improved cytoprotective gene expression, reduced inflammation and improved insulin/IGF-1 sensitivity. After an acute stress such as ischemia reperfusion injury, ROS cause less tissue damage and less subsequent inflammation. Improved insulin/IGF-1 sensitivity from the preconditioned state allows better survival signaling and enhanced cell division/regeneration after the injury. Extension of this model to a lifetime of acute stressors incorporates the benefits of both reduced and increased insulin/IGF signaling, but separated by time relative to an acute stress.

Previous reports of benefits of donor fasting in a rat model of liver transplantation following warm and cold organ preservation (Nishihara et al., 1997; Sumimoto et al., 1993) remain controversial with little if any followup reported on their potential translation to the clinic. There are likely several reasons for this. First is the aforementioned association between fasting and malnutrition, a known risk factor for postoperative recovery. Second, the reported benefits of fasting prior to organ transplantation are at apparent odds with reports of increased injury to organs from fasted donors using isolated perfusion models (Arnault et al., 2002; Domenicali et al., 2001). Third, the reported benefits of preoperative carbohydrate loading have been proposed to work by avoiding a catabolic state, such as that induced by fasting, altogether (Ljungqvist and Soreide, 2003). While the benefits of long-term DR (including both CR and IF feeding regimes and lasting between 3 months and one year) against ischemia reperfusion injury to the brain and heart in rodent models (Ahmet et al., 2005; Anson et al., 2003; Chandrasekar et al., 2001; Yu and Mattson, 1999) are not controversial, their practical translational potential is considered extremely limited for the purposes of preoperative nutrition. This is due both to the extended periods of time thought to be required for benefit as well as the difficulty in voluntary compliance with reduced calorie diets over extended periods of time. Furthermore, long-term DR is not necessarily beneficial in all cases, as it exacerbates gut IRI (Ueno et al., 2005) and has been interpreted as detrimental in models of chemically-induced peritonitis due to reduced host neutrophil exudation (Ikeda et al., 2001). It remains to be seen if the benefits of short-term DR or fasting against renal or hepatic IRI observed in rodents will translate to humans. However, the fact that long-term DR extends healthspan in both non-human primates (Colman et al., 2008) as well as in humans (Fontana and Klein, 2007) suggests that it might. The observation that the kinetics of onset and loss of DR benefits are similarly rapid in rodents (Fig 2) as they are in fruit flies (Mair et al., 2003) further suggests that this may be an evolutionarily conserved characteristic of DR applicable in humans as well.

Fasting and short-term DR are known to induce many of the transcriptional changes observed upon long-term DR including activation of genes involved in cellular stress resistance and repression of those involved in the inflammatory response (Bauer et al., 2004). Reduced insulin/IGF-1 signaling is one evolutionarily conserved mechanism through which DR may mediate such changes (Tatar et al., 2003). In *C. elegans*, reduced signaling through the insulin/insulin-like growth factor receptor Daf-2 results in extended longevity and increased stress resistance, both of which depend on relocalization of the Daf16 transcription factor to the nucleus and upregulation of target genes including those involved in antioxidant defense. However, depending on the method of DR employed, extended longevity in worms is dependent either on Daf16 (Greer et al., 2007) or Pha4 (Panowski et al., 2007).Whether the mammalian Daf 16 or Pha4 homologs FoxO or FoxA, respectively, are required for any of the benefits of DR in mammals remains to be determined.

The so-called IGF-aging paradox points out apparent inconsistencies between the longevity effects of reduced insulin/IGF signaling on the one hand and the pro-growth, pro-survival effects of these factors on the other (Rincon et al., 2004; Tang, 2006). Our data suggest that the benefits of reduced and increased insulin/IGF signaling can be separated by time relative to an acute injury, and are thus not mutually exclusive. The enigmatic role of improved insulin/IGF sensitivity in DR may be to facilitate improved survival signaling following an acute stress, as summarized in Fig 5G. An extension of this model from acute stress protection to extended longevity would involve repetition of this cycle over a lifetime of acute stress events. Accordingly, DR could extend lifespan by increasing the likelihood of surviving an acute stress event such as an ischemic insult on any given day. This may occur independently of its ability to reduce the oxidative molecular damage that accumulates with age as a consequence of normal metabolism.

The ability of dietary restriction to extend longevity was first reported in rats in 1935 (McCay et al., 1935). Nearly eight decades later, basic questions about the relative importance of energy restriction vs. nutrient restriction (Mair et al., 2005) or the role of fasting between meals (Mattson, 2005) still remain. Nonetheless, a consensus has begun to emerge linking DR to hormesis, the phenomenon first described in toxicology in which low doses of toxic substances elicit beneficial effects. In the case of DR, nutrient or energy restriction is the low-level stressor eliciting an evolutionarily conserved adaptive responses resulting in stress resistance and extended longevity (Sinclair, 2005). One characteristic of hormetic substances is that they induce resistance to multiple forms of stress. Consistent with this notion, two days of fasting in mice was recently shown to protect against a stress very different than ischemic insult: acute toxicity associated with chemotherapeutic agents (Raffaghello et al., 2008). Fasting may thus represent a non-invasive, cost-free method of protecting against multiple types of acute stress including surgical ischemia reperfusion injury unavoidably encountered in elective surgeries including living-donor organ transplantation, cardiac surgery, vascular surgery and liver resection.

Materials and Methods

Mice

Male C57BL/6J mice in the age/weight range of 22-25 grams/10-14 weeks were purchased from Charles River Laboratories, Maastricht, the Netherlands. Animals were kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 h light/12 h dark) with 3-4 animals per cage and allowed free access to water and food (Hope Farms, Woerden, The Netherlands) except where noted. All experiments were performed with the approval of the appropriate local ethical board.

Dietary regimens

The amount of food eaten ad libitum was approximately 3.5 gram/day as determined by weighing the remaining food on a daily basis for one week. DR was applied for 2-4 weeks by feeding mice 70% of this amount on a daily basis. Fasting was applied by transferring mice to a fresh cage without food for 1-3 days.

Renal ischemia model

Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially and then 2-2.5% with oxygen for maintenance). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. Following a midline abdominal incision, the left renal pedicle was localized and the renal artery and vein were dissected. An atraumatic microvascular clamp was used to occlude the left kidney for 37 minutes. For bilateral occlusion, the procedure was repeated immediately on the right kidney. After inspection for signs of ischemia (purple color), the wound was covered with phosphate-buffered saline (PBS)-soaked cotton and the animal placed under an aluminum foil blanket to maintain body temperature. After release of the clamp, restoration of blood-flow was inspected by return of the kidney to normal color. In the case of unilateral occlusion, a contralateral nephrectomy was performed immediately following clamp release. The abdominal wound was closed in two layers using 5/0 sutures. Animals were given 0.5 mL PBS subcutaneously for maintenance of fluid balance and kept warm under a heat lamp. All animals were observed to have regained consciousness before moving their cages from the operating room to the stable.

Kidney function analysis

Unlabeled DMSA kits were purchased from GE Healthcare (Roosendaal, the Netherlands) and radiolabeled with ^{99m}Tc according to manufacturer's instructions. Mice were injected in a lateral tail vein with 30 MBq ^{99m}Tc-DMSA. Four hours post injection the mice were sacrificed, the kidney was removed, and the absorbed radioactivity was measured in a gamma counter (Perkin Elmer, Groningen, the Netherlands) and expressed as percentage of injected dose per kidney (%ID/kidney).

Liver ischemia model

Liver IRI was performed by visualizing the liver hilus and clamping the portal triad with an atraumatic microvascular clamp for 75 minutes. In this model, 70% of the liver tissue

becomes ischemic, and blood outflow from the small intestine is preserved through the right anterior and caudate liver lobes. Mortality associated with this amount of ischemic damage to the liver was not observed. **Liver samples were fixed in formalin for 24 hr** prior to embedding in paraffin. 4 µm sections were cut and stained with hematoxylin and eosin. Liver necrosis was scored blindly on a scale from 0-4, with 4 representing 100% of the area covered by hemorrhagic necrosis.

Serum measurements

Blood samples were collected by retro-orbital puncture. Serum urea and creatinine levels were measured using QuantiChrom assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICT-500, Gentaur, Brussels, Belgium). Serum ALAT levels were determined using an ELAN analyzer (Eppendorf Merck, Hamburg, Germany) with Ecoline S+ reagents (Diagnostic Systems GmbH, Holzheim, Germany) according to manufacturer's instructions. Serum LDH levels were determined using Ecoline S+ reagents according to manufacturer's instructions in a 96-well format on a Varioskan microplate reader (Thermo Scientific).

Histology

Organs were harvested, fixed for 24 hr in formalin and embedded in paraffin. 3 µm sections were stained with hematoxylin and eosin. Tubular injury was assessed in a blind fashion on a five point scale as described previously.

Glucose tolerance and insulin sensitivity tests

Following baseline blood glucose determination from tail blood of conscious, restrained mice, animals were injected with a bolus of glucose (1.5 mg/g body weight) or insulin (Novorapid; 0.75 U/kg body weight) into the intraperitoneal cavity. Blood glucose determinations were performed at the indicated times after injection using a HemoCue glucose 201 RT blood glucose analyzer (HemoCue, Ängelholm, Sweden) according to the manufacturer's instructions.

Quantitative real time PCR

Total RNA was extracted from frozen kidney tissue using TRIzol reagent (Invitrogen) and oligodT or hexamer-primed cDNA synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a MyIQ (BioRad) with SYBR Green incorporation. Relative expression was calculated using the equation $1.8^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$. Each sample was tested in duplo at least two times.

Statistics

Data are expressed as the mean ± SEM. Statistical analyses of data on urea, creatinine, histomorphology, immunohistochemistry and immunoblot was preformed using a Student's T test unless otherwise indicated. Survival was analyzed by Kaplan-Meier (SPSSv11). Area under the curve was calculated using GraphPad Prism 4.0, and the significance calculated using a Student's T test.

Acknowledgements

Thanks to Joris Pothof, Karl Brand, Bas Zwaan, Rudi Westendorp, Henk Roest and Steven Russell for discussions and critical reading of the manuscript, and to Paula van Heijningen for technical assistance. This work was supported by grants from the Netherlands Organization for Scientific Research (Healthy Ageing stimulation grant 05040202 from the Netherlands Genomics Initiative), the Netherlands Organization for Health Research (Research Institute of Diseases in the Elderly 60-60400-98-004), the United States National Institutes of Health (1PO1 AG17242-02), the European Commission RISC-RAD contract number FI6R-CT-2003-508842, the Association of International Cancer Research (05-0280) and the Ellison Medical Foundation. M.V. was supported by a grant from the Dutch Kidney Foundation (C07-2206).

References

Ahmet, I., Wan, R., Mattson, M.P., Lakatta, E.G., and Talan, M. (2005). Cardioprotection by intermittent fasting in rats. Circulation 112, 3115-3121.

Anson, R.M., Guo, Z., de Cabo, R., Iyun, T., Rios, M., Hagepanos, A., Ingram, D.K., Lane, M.A., and Mattson, M.P. (2003). Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proc Natl Acad Sci U S A 100, 6216-6220.

Arnault, I., Bao, Y.-M., Dimicoli, J.-L., Lemoine, A., Sebagh, M., and Adam, R. (2002). Combined effects of fasting and alanine on liver function recovery after cold ischemia. Transpl Int 15, 89-95.

Bauer, M., Hamm, A.C., Bonaus, M., Jacob, A., Jaekel, J., Schorle, H., Pankratz, M.J., and Katzenberger, J.D. (2004). Starvation response in mouse liver shows strong correlation with life-span-prolonging processes. Physiol Genomics 17, 230-244.

Bishop, N.A., and Guarente, L. (2007). Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nat Rev Genet 8, 835-844.

Bluher, M., Kahn, B.B., and Kahn, C.R. (2003). Extended longevity in mice lacking the insulin receptor in adipose tissue. Science 299, 572-574.

Brown-Borg, H.M. (2006). Longevity in mice: is stress resistance a common factor? AGE 28, 145-162.

Chandrasekar, B., Nelson, J.F., Colston, J.T., and Freeman, G.L. (2001). Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 280, H2094-2102.

Chippindale, A.K., Leroi, A.M., Kim, S.B., and Rose, M.R. (1993). Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. Journal of Evolutionary Biology 6, 171-193.

Chung, A. (2002). Perioperative nutrition support. Nutrition 18, 207-208.

Colman, R.J., Beasley, T.M., Allison, D.B., and Weindruch, R. (2008). Attenuation of sarcopenia by dietary restriction in rhesus monkeys. J Gerontol A Biol Sci Med Sci 63, 556-559.

Crenshaw, J.T., and Winslow, E.H. (2002). Preoperative fasting: old habits die hard. Am J Nurs 102, 36-44; quiz 45.

Diks, J., van Hoorn, D.E., Nijveldt, R.J., Boelens, P.G., Hofman, Z., Bouritius, H., van Norren, K., and van Leeuwen, P.A. (2005). Preoperative fasting: an outdated concept? JPEN J Parenter Enteral Nutr 29, 298-304.

References

Domenicali, M., Caraceni, P., Vendemiale, G., Grattagliano, I., Nardo, B., Dall'Agata, M., Santoni, B., Trevisani, F., Cavallari, A., Altomare, E., et al. (2001). Food deprivation exacerbates mitochondrial oxidative stress in rat liver exposed to ischemia-reperfusion injury. J Nutr 131, 105-110.

Fontana, L., and Klein, S. (2007). Aging, adiposity, and calorie restriction. Jama 297, 986-994.

Friedewald, J.J., and Rabb, H. (2004). Inflammatory cells in ischemic acute renal failure. Kidney Int 66, 486-491.

Goodrick, C.L., Ingram, D.K., Reynolds, M.A., Freeman, J.R., and Cider, N.L. (1982). Effects of intermittent feeding upon growth and life span in rats. Gerontology 28, 233-241.

Greer, E.L., Dowlatshahi, D., Banko, M.R., Villen, J., Hoang, K., Blanchard, D., Gygi, S.P., and Brunet, A. (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol 17, 1646-1656.

Guan, J., Bennet, L., Gluckman, P.D., and Gunn, A.J. (2003). Insulin-like growth factor-1 and post-ischemic brain injury. Prog Neurobiol 70, 443-462.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P.C., Cervera, P., and Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 421, 182-187.

Ikeda, S., Saito, H., Fukatsu, K., Inoue, T., Han, I., Furukawa, S., Matsuda, T., and Hidemura, A. (2001). Dietary restriction impairs neutrophil exudation by reducing CD11b/CD18 expression and chemokine production. Arch Surg 136, 297-304.

Leemans, J.C., Stokman, G., Claessen, N., Rouschop, K.M., Teske, G.J., Kirschning, C.J., Akira, S., van der Poll, T., Weening, J.J., and Florquin, S. (2005). Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 115, 2894-2903.

Ljungqvist, O., and Soreide, E. (2003). Preoperative fasting. Br J Surg 90, 400-406.

Mair, W., Goymer, P., Pletcher, S.D., and Partridge, L. (2003). Demography of dietary restriction and death in Drosophila. Science 301, 1731-1733.

Mair, W., Piper, M.D., and Partridge, L. (2005). Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS Biol 3, e223.

Maltby, J.R. (2006). Fasting from midnight–the history behind the dogma. Best Pract Res Clin Anaesthesiol 20, 363-378.

References

Masoro, E.J. (2003). Subfield history: caloric restriction, slowing aging, and extending life. Sci Aging Knowledge Environ 2003, RE2.

Masoro, E.J. (2004). Caloric intake versus temporal pattern of food intake. Aging Clin Exp Res 16, 423-424.

Masoro, E.J., Shimokawa, I., Higami, Y., McMahan, C.A., and Yu, B.P. (1995). Temporal pattern of food intake not a factor in the retardation of aging processes by dietary restriction. J Gerontol A Biol Sci Med Sci 50A, B48-53.

Mattson, M.P. (2005). The need for controlled studies of the effects of meal frequency on health. Lancet 365, 1978-1980.

McCay, C.M., Crowel, M.F., and Maynard, L.A. (1935). The effect of retarded growth upon the length of the life span and upon the ultimate body size. J Nutr 10, 63-79.

Nishihara, V., Sumimoto, R., Fukuda, Y., Southard, J.H., Asahara, T., and Dohi, K. (1997). Inhibition of warm ischemic injury to rat liver, pancreas, and heart grafts by controlling the nutritional status of both donor and recipient. Surg Today 27, 645-650.

Panowski, S.H., Wolff, S., Aguilaniu, H., Durieux, J., and Dillin, A. (2007). PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. Nature 447, 550-555.

Raffaghello, L., Lee, C., Safdie, F.M., Wei, M., Madia, F., Bianchi, G., and Longo, V.D. (2008). Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. Proc Natl Acad Sci U S A 105, 8215-8220.

Rincon, M., Muzumdar, R., Atzmon, G., and Barzilai, N. (2004). The paradox of the insulin/IGF-1 signaling pathway in longevity. Mech Ageing Dev 125, 397-403.

Sinclair, D.A. (2005). Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev 126, 987-1002.

Sogawa, H., and Kubo, C. (2000). Influence of short-term repeated fasting on the longevity of female (NZB x NZW)F1 mice. Mech Ageing Dev 115, 61-71.

Soreide, E., Eriksson, L.I., Hirlekar, G., Eriksson, H., Henneberg, S.W., Sandin, R., and Raeder, J. (2005). Pre-operative fasting guidelines: an update. Acta Anaesthesiol Scand 49, 1041-1047.

Studley, H.O. (1936). Percentage of weight loss: a basic indicator of surgical risk in patients with chronic peptic ulcer. JAMA 106, 458-460.

Suleiman, M.S., Singh, R.J., and Stewart, C.E. (2007). Apoptosis and the cardiac action of insulin-like growth factor I. Pharmacol Ther 114, 278-294.

References

Sumimoto, R., Southard, J.H., and Belzer, F.O. (1993). Livers from fasted rats acquire resistance to warm and cold ischemia injury. Transplantation 55, 728-732.

Tang, B.L. (2006). SIRT1, neuronal cell survival and the insulin/IGF-1 aging paradox. Neurobiol Aging 27, 501-505.

Tatar, M., Bartke, A., and Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. Science 299, 1346-1351.

Ueno, C., Fukatsu, K., Maeshima, Y., Moriya, T., Shinto, E., Hara, E., Nagayoshi, H., Hiraide, H., and Mochizuki, H. (2005). Dietary restriction compromises resistance to gut ischemia-reperfusion, despite reduction in circulating leukocyte activation. JPEN J Parenter Enteral Nutr 29, 345-351; discussion 351-342.

Varady, K.A., and Hellerstein, M.K. (2007). Alternate-day fasting and chronic disease prevention: a review of human and animal trials. Am J Clin Nutr 86, 7-13.

Weindruch, R., Walford, R.L., Fligiel, S., and Guthrie, D. (1986). The retardation of ageing in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. Journal of Nutrition 116, 641–654.

Weiss, E.P., Racette, S.B., Villareal, D.T., Fontana, L., Steger-May, K., Schechtman, K.B., Klein, S., and Holloszy, J.O. (2006). Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr 84, 1033-1042.

Yu, Z.F., and Mattson, M.P. (1999). Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. J Neurosci Res 57, 830-839.

Supplementary figures

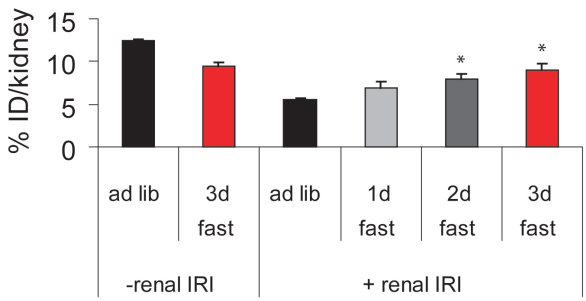


Fig. S1.

Mice fasted for 0-3 days (4-10 animals per group) were analyzed for kidney function either in the absence of renal ischemia (-renal IRI) or one day following 37 minutes renal ischemia (+renal IRI) by measuring radioactivity in the kidney with a gamma-counter 4 h after injection of ^{99m}Tc-DMSA, expressed as a percentage of the injected dose per one kidney (% ID/kidney). Note the reduced percentage of ^{99m}Tc-DMSA in the kidneys of the ad libitum group 24 hours after renal IRI (12.4% to 5.6%), indicative of renal dysfunction. Asterisks indicate the significance of the difference as compared to the ad libitum group one day after renal IRI ($p<0.05$).

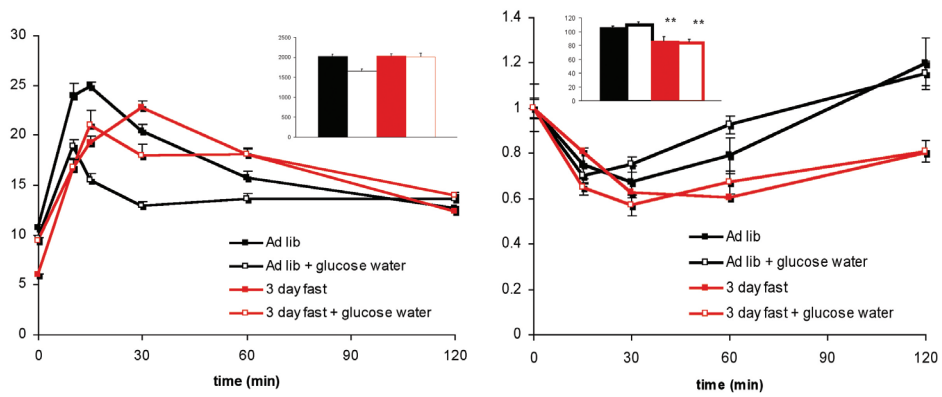


Fig. S3. Neutral effects of glucose-water ingestion on insulin sensitivity.

Whole blood glucose levels at the indicated timepoints following intraperitoneal injection of glucose (upper) and insulin (lower) into animals following the indicated preconditioning regimes. Insets: Area under the curve. Statistically significant differences between glucose water and normal water controls within ad libitum fed or fasted groups are indicated by asterisks (** $p<0.01$).

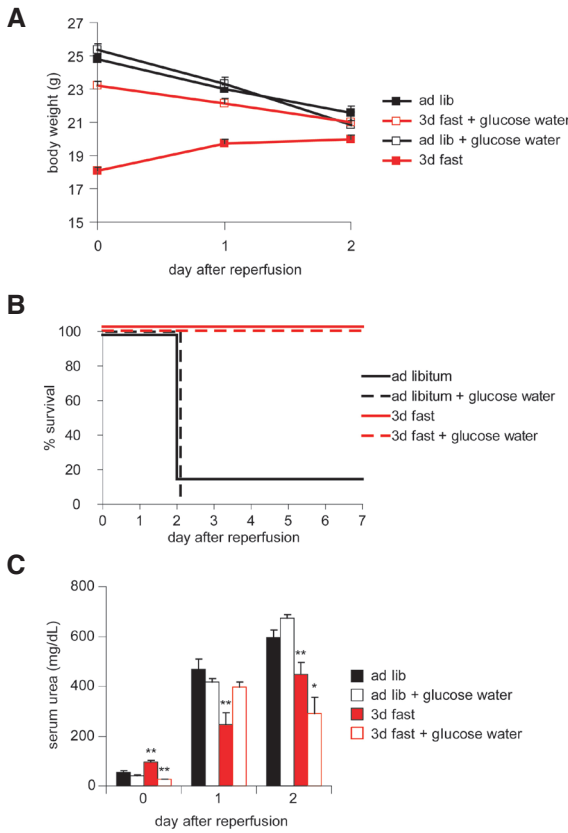


Fig. S2. Neutral effects of glucose-water ingestion on nutritional preconditioning.

A. Preoperative, 1d and 2d postoperative weights as indicated of mice allowed ad libitum access to chow and water, chow and 54% glucose water, water only or 54% glucose water only for 3 days prior to 37 min bilateral renal ischemia (n=8 per group). In the ad libitum groups, animals with access to 54% glucose water drank equal volumes of liquid (3.5 +/- 0.4 mL glucose water vs. 3.5 +/- 0.3 mL water per mouse per day) and consumed significantly less chow (2.0 +/- 0.2 vs. 4.0 +/-0.2 g/mouse/day; p=0.00002) without any significant effect on body weight over the three day period. In the fasted groups, animals with access to glucose water drank significantly more than those with access only to normal water (5.2 +/- 0.2 mL glucose water vs. 0.4 +/- 0.1 mL water per mouse per day; p=0.00004) and lost significantly less weight during the 3 day fast as a result. Following surgery, all animals had ad libitum access to chow and water. Both ad libitum groups (with and without prior access to glucose water) continued to lose weight at similar rates. Among fasted mice, postoperative weights increased in the water-only group as observed previously (Fig 1B) but decreased significantly in the sugar water-only group, albeit proportionately less than in either of the ad libitum groups.

B. Kaplan-Meier survival data indicate significant differences between fed and fasted groups, but no significant effects of glucose water ingestion on survival in either group.

C. Serum urea values of the corresponding groups as indicated. Asterisks represent significant differences relative to the ad libitum group at the indicated time point (* $p<0.05$; ** $p<0.01$). Consistent with the survival data, glucose water ingestion had no consistent effects on renal function in either the ad libitum fed or fasted groups in the two days following renal IRI.

5

Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice

~

Denis Susa, James R. Mitchell, Marielle Verweij,
Marieke van de Ven, Henk Roest, Sandra van den Engel,
Ingeborg Bajema, Kirsten Mangundap, Jan N. M. IJzermans,
Jan H. J. Hoeijmakers and Ron W. F. de Bruin

Abstract

Cockayne syndrome and other segmental progerias with inborn defects in DNA repair mechanisms are thought to be due in part to hypersensitivity to endogenous oxidative DNA damage. The accelerated aging-like symptoms of this disorder include dysmyelination within the central nervous system, progressive sensineuronal hearing loss and retinal degeneration. We tested the effects of congenital nucleotide excision DNA repair deficiency on acute oxidative stress sensitivity *in vivo*. Surprisingly, we found mouse models of Cockayne syndrome less susceptible than wildtype animals to surgically-induced renal ischemia reperfusion injury, a multifactorial injury mediated in part by oxidative damage. Renal failure-related mortality was significantly reduced in *Csb*$^{-/-}$ mice, kidney function was improved and proliferation was significantly higher in the regenerative phase following ischemic injury. Protection from ischemic damage correlated with improved baseline glucose tolerance and insulin sensitivity and a reduced inflammatory response following injury. Protection was further associated with genetic ablation of a different Cockayne syndrome-associated gene, *Csa*. Our data provide the first functional *in vivo* evidence that congenital DNA repair deficiency can induce protection from acute stress in at least one organ. This suggests that while specific types of unrepaired endogenous DNA damage may lead to detrimental effects in certain tissues, they may at the same time elicit beneficial adaptive changes in others and thus contribute to the tissue specificity of disease symptoms.

Introduction

The case for the involvement of oxidative damage to macromolecules in the etiology of aging, aging-related disorders and so-called "premature aging" disorders (here referred to as segmental progerias) is compelling. The free radical theory as originally proposed by Harman (Harman 1956) and extended in recent years (Beckman and Ames 1998) posits that reactive oxygen species (ROS), byproducts of endogenous cellular metabolism, inflict damage on lipids, protein and DNA. Despite defense systems to prevent or repair such damage, oxidized macromolecules accumulate over time in vivo and are thought to underlie both normal and pathological aging. When combined with congenital defects in certain of these defense systems, for example genome stability mechanisms, endogenous oxidative damage may also contribute to the symptoms of segmental progerias, which display some but not all of the characteristics of normal aging in accelerated or exacerbated forms (Martin and Oshima 2000).

Nucleotide excision DNA repair (NER) is one such defense system that removes a range of damages, including helix distorting lesions induced either by exogenous ultraviolet radiation (e.g. pyrimidine dimers) or endogenous oxidative radicals (e.g. cyclopurines) (Brooks et al. 2000). Two basic modes of lesion recognition have been defined. In the first, lesions occurring anywhere in the genome are recognized by damage-binding proteins such as the XPC-HR23A/B-CEN1 complex. This triggers the assembly of the multiprotein NER machinery which functions via a cut and patch mechanism to remove the damage and fill the remaining single-strand gap (Hoeijmakers 2001). In the second mode of recognition, transcription-coupled (TC)-NER, lesions that block an elongating RNA polymerase trigger assembly of the NER machinery in a process which depends on the chromatin remodeling protein CSB (Citterio et al. 2000) and a ubiquitin ligase complex containing the TC-NER specific protein CSA (Groisman et al. 2003; Fousteri et al. 2006).

Defects in CSA or CSB can give rise to Cockayne syndrome, a severe neurodevelopmental disease marked by photosensitivity (but curiously without skin cancer predisposition), dysmyelination within the central nervous system, progressive sensineuronal hearing loss, retinal degeneration and cachectic dwarfism resulting in an aged appearance (Nance and Berry 1992). Mouse models lacking Csa or Csb function recapitulate some progeroid characteristics of the human disease, including cachexia and progressive loss of photoreceptor cells, but with a normal lifespan and an overall milder phenotype than in humans (van der Horst et al. 1997; van der Horst et al. 2002; Dolle et al. 2006). In support of the role of oxidative stress hypersensitivity in Cockayne syndrome etiology, an increase in photoreceptor cell loss following whole-body ionizing radiation was observed in both *Csa*$^{-/-}$ and *Csb*$^{-/-}$ mice (Gorgels et al. 2006). Thus, at least particular CS cell types appear hypersensitive to the effects of oxidative DNA damage, a property that may underlie tissue-specific disease phenotypes.

Contrary to the notion of hypersensitivity to oxidative stress in Cockayne syndrome is the observation that mouse models of Cockayne syndrome and related NER-deficient

segmental progerias (NER progerias) display characteristics of hypopituitary dwarf mutants or dietary restricted wildtype mice (Wijnhoven et al. 2005; Niedernhofer et al. 2006; van de Ven et al. 2006; van der Pluijm et al. 2006; Schumacher et al. 2008). In dwarf and dietary restricted mice, these phenotypes, including hypoglycemia, reduced body weight and temperature, hypoglycemia, hypoinsulinemia and reduced serum IGF-1, are correlated not only with extended longevity but also resistance to acute oxidative stress (Bartke and Brown-Borg 2004). In progeroid NER mice, these phenotypes have been interpreted as an adaptive response to unrepaired endogenous DNA damage engaged to protect from further oxidative stress injury (van de Ven et al. 2007). One important prediction of this interpretation is that mice should be resistant rather than hypersensitive to acute oxidative stress.

Ischemia reperfusion injury is a complex insult initiated by loss of blood flow to an organ. During the ischemic period, the lack of molecular oxygen as an electron acceptor in oxidative phosphorylation prevents ATP generation and compromises processes with high energy demand, such as maintenance of ion gradients across intracellular membranes. Reinitiation of oxygenated blood flow, or reperfusion, results in inappropriate activation of cellular oxidases and ROS generation, which can affect not only the reperfused organ but distant sites in the body as well (Kelly 2003). Following reperfusion, a maladaptive inflammatory response mediated in part by the release of ROS from neutrophils infiltrating the tissue causes further injury (Friedewald and Rabb 2004). In the kidney, ischemia reperfusion injury is associated with cell death primarily in the stripe between the cortex and medulla consisting mainly of tubular epithelial cells via necrosis or apoptosis, depending on the severity of the insult (Padanilam 2003). Recovery and a return to proper kidney function depends on regeneration of the tubular epithelial cells and remodeling of the renal tubules, a process which takes place in the days and weeks following the injury.

We used surgically-induced renal ischemia reperfusion injury to test whether mouse models of Cockayne syndrome are susceptible to acute oxidative stress, as predicted by photoreceptor sensitivity to ionizing radiation, or protected from it, as predicted by physiological similarities between other short-lived progeroid NER mice and long-lived, stress resistant mice. Here we report resistance to renal ischemia reperfusion injury in mouse models of Cockayne syndrome.

Results

Warm ischemia was induced in the left kidney of male wildtype and *Csb*$^{-/-}$ (subsequently referred to as WT and CSB, respectively) mice for 37 minutes by clamping the renal artery/vein with a nontraumatic surgical clamp. The 37 minute time point was chosen based on the results of preliminary experiments in which ischemia times were titrated between 30 and 45 minutes. Following clamp release, the undamaged right kidney was removed so that animal survival depended on the function of the damaged kidney; mice entirely lacking kidney function build up toxic compounds in their blood and die within 2-4 days. Survival of CSB mice was significantly higher than WT (Fig. 1A). On post-operative day (POD) 7, survival was 56% in CSB mice but only 30% in the WT group (log rank score p<0.0033; n=24 CSB, 40 WT).

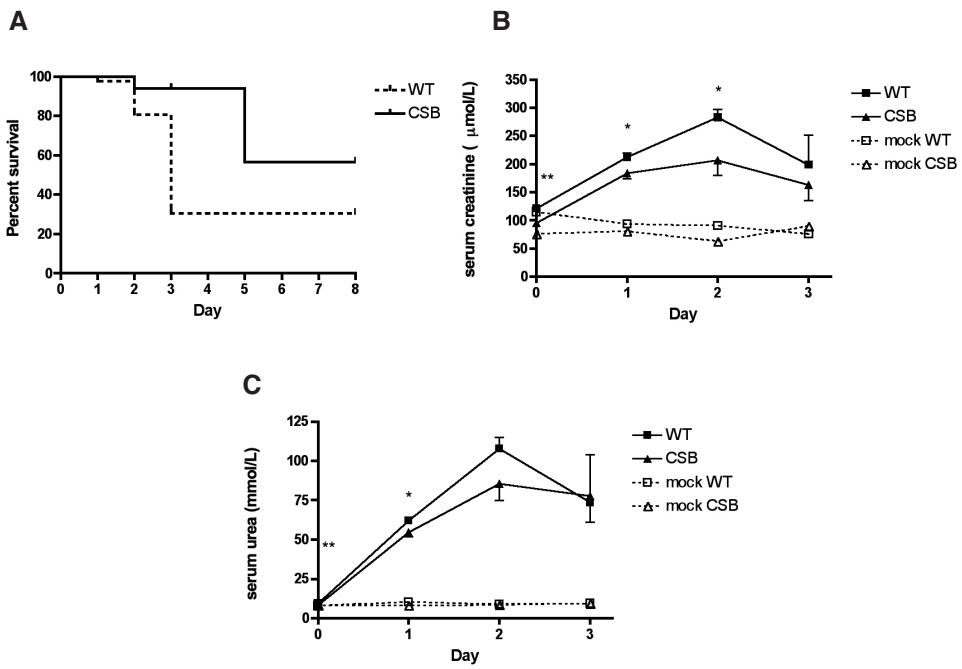


Figure 1. CSB mice are better protected against warm renal ischemia reperfusion injury than WT.

A. Kaplan-Meier survival curves of CSB (n=24) and WT (n=40) animals; a log rank score of p<0.0033 indicates a highly significant difference between the genotypes.
B, C. Kidney function following IRI as determined by serum levels of urea (B) and creatinine (C). Statistically significant differences in magnitude observed at individual timepoints between genotypes following injury are indicated by asterisks (** p<0.01, * p<0.05). Groups receiving a mock treatment that did not involve ischemia are indicated.

Kidney function was determined by measuring the concentrations of urea and creatinine in the blood serum. High levels of these waste products indicate an inability of the kidney to remove them from the blood and thus correlate inversely with kidney function. Interestingly, pre-operative urea and creatinine values were slightly but significantly lower in CSB mice than in WT mice (urea 8.5 ± 1.3 mmol/L (CSB) vs 9.7 ± 1.2 mmol/L (WT), p=0.0007; creatinine 95 ± 17 umol/L (CSB) vs. 122 ± 34 umol/L (WT), p=0.002). Following ischemia-reperfusion injury, these markers of kidney function rose with similar kinetics, suggesting maximal dysfunction on POD 2 and a return to function beginning on POD 3. However, average values were significantly lower in CSB animals on POD 1 for urea and on PODs 1 and 2 for creatinine (Fig 1B, C), consistent with better survival in the CSB group.

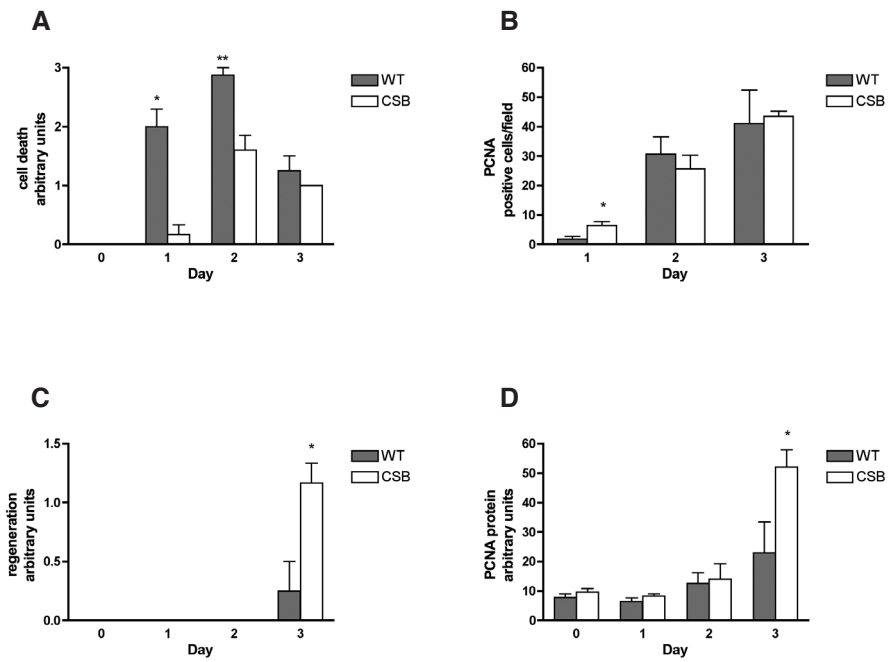


Figure 2. Cell loss and regeneration following 37 min warm renal ischemia reperfusion injury.

A. Cell death as determined by H&E-stained paraffin sections on the indicated day following IR. Day 0 kidneys were contralateral kidneys harvested immediately after clamp release.
B-D. Regeneration as determined by mitotic index in H&E stained sections (B); relative number of cells expressing PCNA as determined by IHC (C); and total PCNA protein level as determined by immunoblotting on the indicated days following ischemia reperfusion injury (D). Statistically significant differences between CSB and WT on a given day are indicated by asterisks (** p<0.01, * p<0.05).

We next analyzed cell death at various timepoints after the injury to look for differences that could explain the observed survival and functional benefits in CSB animals vs. WT controls. We used histology to score for acute tubular necrosis, the major form of cell death due to this type of injury (Fig. 2A). In both groups, acute tubular necrosis peaked on POD2; in the CSB group, average total cell death was lower at all time points, reaching statistical significance on PODs 1 and 2. The high mortality due to kidney dysfunction in the wildtype group prevented meaningful comparisons beyond POD 3.

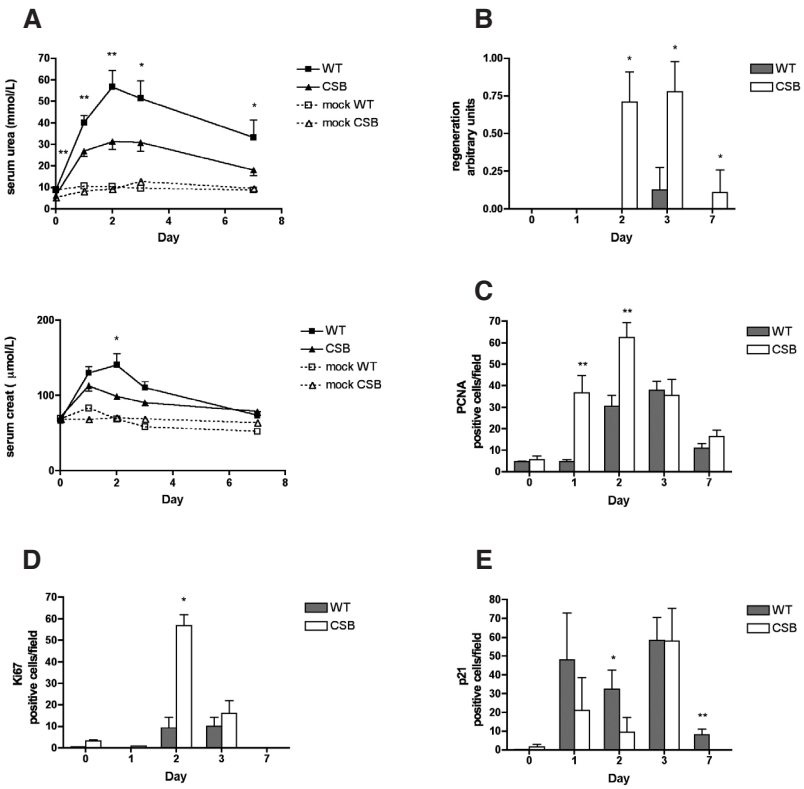


Figure 3. Kidney function, cell death and regeneration following 25 min warm renal ischemia reperfusion injury in CSB and WT animals.

A. Kidney function of the indicated genotypes following IRI or mock treatment as determined by concentration of serum urea (top) or creatinine (bottom). Statistically significant differences between CSB and WT on a given day are indicated by asterisks (** $p<0.01$, * $p<0.05$).
B-E. Regeneration as indicated by mitotic figures in H&E stained sections (B) and immunohistochemistry against PCNA (C), Ki67 (D), and p21 (E) as indicated. Statistically significant differences between CSB and WT on a given day are indicated by asterisks (** $p<0.01$, * $p<0.05$).

Following cell death and clearance of cellular debris by shedding into the lumen of the tubules, kidneys damaged by ischemia reperfusion injury may undergo a regenerative phase in which cellular proliferation can be observed. Since the survival benefit of the CSB mice was likely only partially explained by the lower cell death on PODs 1 and 2, we next asked whether regeneration of the kidney was enhanced. We used three different assays to gauge this parameter: histology, or the presence of mitotic figures in H&E stained sections; immunohistochemistry against the proliferating cell nuclear antigen (PCNA); and PCNA immunoblotting of total kidney homogenates to assess total levels of this protein. In both groups, mitotic figures were first seen on POD 3, with significantly higher scores in CSB mice (Fig. 2B; $p = 0.025$). We next looked at PCNA on the single-cell level by immunohistochemistry. The number of PCNA positive cells was significantly increased in the CSB group on POD 1 (Fig. 2C; $p = 0.035$), possibly indicative of its additional role in DNA repair. We further analyzed PCNA levels in total kidney homogenates by immunoblot. Although pre-operative PCNA levels were similar between groups, significantly higher levels of PCNA were observed on POD 3 in the CSB group (Fig. 2D; $p = 0.042$). Taken together, these results are consistent with enhanced proliferation leading to better regeneration and survival in CSB vs. WT animals.

Previously it was reported that wildtype C57BL/6 mice subjected to 30-50 minutes of warm ischemia to one kidney followed by contralateral nephrectomy had a one-week survival of approximately 80%, with no statistically significant differences between ischemia times (Megyesi et al. 2002). We consistently found mortality of approximately 70% due to kidney failure following 30-37 minutes warm ischemia (Fig 1A and data not shown), making it difficult to study the course of organ recovery beyond POD3. To circumvent this problem, we repeated our experiments in WT and CSB mice using 25 minutes of warm renal ischemia. With this amount of ischemic damage, survival was 100% in both WT and CSB groups (data not shown). However, 25 minutes of ischemia resulted in significantly less kidney dysfunction in mice lacking the CSB protein relative to WT controls at all timepoints following ischemic damage as measured by serum urea (Fig. 3A, top). A similar trend was seen for creatinine (Fig. 3A, bottom).

Analysis of total cell death by histology revealed a significant elevation above preoperative values in CSB and WT (although less than after 37 min warm ischemia) on all PODs examined, but no significant differences between genotypes (data not shown). Despite similar low amounts of cell death between groups, we observed large differences in proliferation following 25 min warm renal ischemia. As with 37 min ischemia, proliferation was elevated in CSB vs. WT mice. Regeneration scored by mitotic figures in histological sections was first seen in wildtype mice on POD 3, while regeneration was already significantly elevated relative to baseline in CSB mice on PODs 2 and 3 (Fig. 3B; $p=0.035$ and $p=0.021$, respectively). The number of PCNA positive cells on PODs 1 and 2 was also significantly increased in the CSB group (Fig. 3C; $p = 0.005$ and $p = 0.002$, respectively). Because PCNA is involved in DNA repair as well as proliferation, we stained sections for an additional proliferative marker, Ki-67, which is expressed during all active phases of the cell cycle (G1, S, G2 and mitosis) but is absent from resting cells (Go)

(Scholzen and Gerdes 2000). Ki-67 positive cells were also increased in CSB mice, with a maximum on POD 2 (Fig. 3D; p = 0.016).

The cyclin-dependant kinase inhibitor p21, which binds stoichiometrically to PCNA and inhibits its action in DNA replication, is a key regulator of proliferation following renal ischemic injury. Mice lacking p21 display hyperproliferation and greatly reduced survival (Megyesi et al. 2002). We tested p21 protein levels by immunohistochemistry (Fig. 3E). Consistent with increased proliferation in CSB relative to wildtype on PODs 1 and 2, the number of p21 positive cells was significantly reduced in CSB mice (POD 2, p = 0.016). Interestingly, p21 staining was equal in wildtype and CSB on POD3, coincident with a decline in the first wave of proliferation following injury.

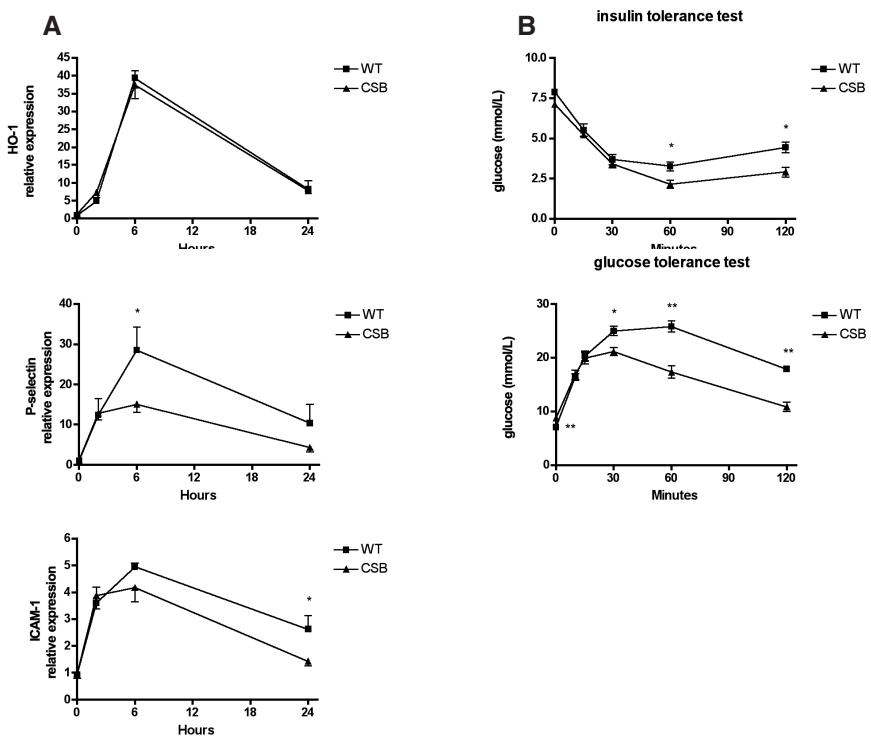


Figure 4. Mechanisms of reduced susceptibility to renal ischemia reperfusion injury in CSB vs WT mice.

A. Time course of hemeoxygenase-1 (HO-1), P-selectin and ICAM-1 mRNA expression as indicated in the kidney following 37 min renal ischemia reperfusion injury using quantitative real time PCR. Statistically significant differences between genotypes at a given time point day are indicated by an asterisk (* p<0.05).
B. Improved glucose clearance and insulin sensitivity in CSB vs. WT mice as indicated. Whole blood glucose levels at the indicated timepoints following intraperitoneal injection of insulin (E; n=11 WT, 7 CSB) or glucose (D, n= 9 WT, 5 CSB) into overnight fasted animals. Statistically significant differences between genotypes at a given time point day are indicated by asterisks (** p<0.01, * p<0.05).

We next turned to potential mechanisms of protection against renal ischemia reperfusion injury specific to CSB animals. Addition of antioxidants is a proven way to ameliorate the effects of this injury (Devarajan 2005). Furthermore, antioxidant capacity is increased in multiple organs in both genetic dwarfism and dietary restriction, a property thought to contribute to stress resistance and increased longevity in these mice (Bartke and Brown-Borg 2004). We thus looked for evidence of increased antioxidant capacity on the level of gene expression. We used quantitative RT-PCR over a time course before and after renal ischemia reperfusion injury to analyze antioxidant defense capabilities as represented by the steady state levels of candidate mRNAs including superoxide dismutase 1, glutathione reductase and hemoxygenase 1 (Fig 4A and data not shown). At baseline, steady state amounts of these mRNAs were not significantly different between WT and CSB groups. Similarly, over a 24 hour period following 25 or 37 minutes warm renal ischemia, some of these genes (e.g. hemoxygenase-1) were significantly upregulated above baseline but none were consistently, significantly differentially regulated between CSB and WT groups (Fig. 4A and data not shown).

Another proven way to reduce the effects of ischemia reperfusion injury is to prevent the inflammatory response that follows tissue injury, for example by neutralization of cellular adhesion molecules that serve to recruit neutrophils to the site of tissue injury by genetic or antibody-based methods (Takada et al. 1997). We examined the expression of P-selectin and ICAM-1, endothelial adhesion molecules that are upregulated upon tissue injury and whose expression correlates negatively with survival and functional outcomes. We observed a significant increase in both markers following renal IRI above baseline levels; this increase, however, was less in the CSB animals (Fig. 4A). An area-under-the-curve analysis revealed significant differences in expression of both P-selectin ($p=0.02$) and ICAM-1 ($p=0.05$) following renal ischemia reperfusion injury, consistent with less damage subsequent to the reperfusion and a better outcome.

Finally, enhanced sensitivity to the effects of insulin on glucose metabolism is a property shared by dwarf and dietary restricted mice that had been proposed to underlie longevity benefits observed in these models (Bartke and Brown-Borg 2004). We chose to analyze glucose metabolism by performing both glucose tolerance and insulin sensitivity tests in overnight fasted CSB and WT mice. We found a significant increase in the ability of CSB animals to clear glucose from the circulation in response to a bolus injection of insulin or glucose, respectively (Fig. 4B). It should be noted that despite the slight but significant elevation of fasting glucose levels observed at baseline in CSB animals subject to the glucose tolerance test, no significant difference was observed in the insulin tolerance test between CSB and WT ($p=0.34$) nor in the combined data set ($p=0.41$). This lack of a significant difference is consistent with previous reports of fed glucose levels of single mutant CS and XPCS mice at postnatal day 15 prior to weaning, while in more severe XPA-deficient double mutant animals hypoglycemia was observed (van de Ven et al. 2006; van der Pluijm et al. 2006)

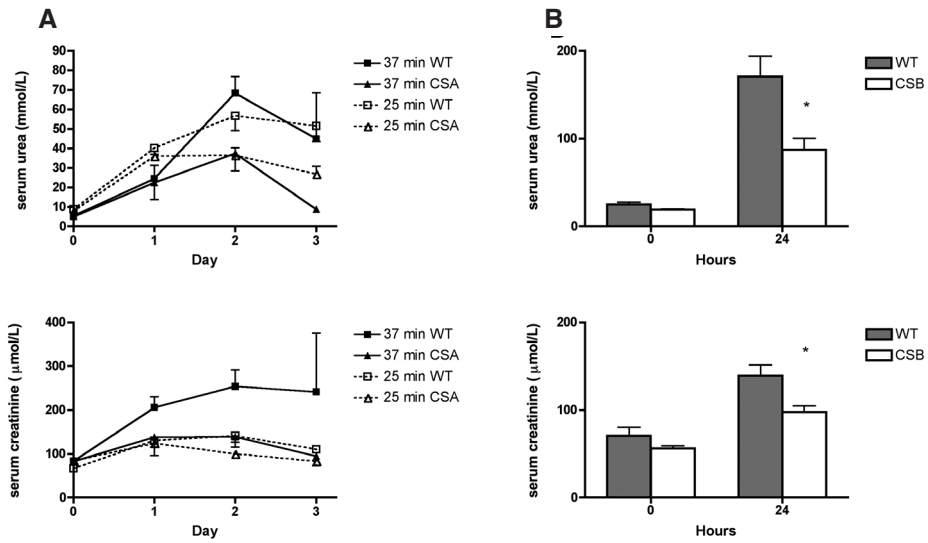


Figure 5. Protection from renal ischemia reperfusion correlates with genetic defects in TC-NER and extends to CSB females.

A. Functional response of TC-NER deficient CSA vs. WT animals to 25 and 37 min warm IRI according to serum urea (top) and creatinine (bottom) values on the indicated days after IRI. Statistically significant differences between genotypes at a given time point day are indicated by asterisks (** $p<0.01$, * $p<0.05$).
B. Functional response of WT vs. CSB females to 37 min warm renal IRI as measured by serum urea (top) and creatinine (bottom) values prior to and 24 hours after injury.

Cockayne syndrome can also be caused by defects in the *CSA* gene. In mice, *Csa*$^{-/-}$ (subsequently referred to as CSA) mice are nearly indistinguishable from CSB mice (van der Horst et al. 2002). We tested the resistance of CSA mice to renal ischemia reperfusion injury and found evidence of protection against both 25 and 37 min warm ischemia in CSA mice vs. WT controls as at the level of kidney function (Fig. 5A).

No sex bias has been previously reported for Cockayne syndrome (Nance and Berry 1992)or for mouse models of this disease. To see whether the protective effects we observed are specific to males, we tested CSB females for resistance to 37 minutes of warm renal ischemia. Female mice are in general less susceptible to this injury than males (Kher et al. 2005), and as expected we observed less evidence of kidney dysfunction and inflammation following 37 min warm ischemia in females than in males. However, CSB females displayed evidence of increased resistance to injury, including significantly lower urea and creatinine in the serum 24 hours after reperfusion (Fig. 5B).

Discussion

Although the genetic lesions causative of Cockayne syndrome are known, the molecular defect(s) leading to the observed symptoms remains unclear. The prominent inability to repair UV-induced DNA lesions in Cockayne cells from both man and mouse models has led to the hypothesis that hypersensitivity to DNA damage, presumably oxidative in nature, is primarily responsible for disease symptoms. Consistent with this, CSB mice are hypersensitive to the effects of whole-body ionizing irradiation, a potent oxidative stress (de Waard et al. 2003), and both CSA and CSB mice demonstrate an increase in photoreceptor cell loss following ionizing radiation (Gorgels et al. 2006). However, primary CSB cells (van de Ven et al. 2006) as well as immortalized CSA cells (de Waard et al. 2004) are not hypersensitive to oxidative stress.

A number of recent reports have described overlap between short-lived segmental progeroid NER models and long-lived hypopituitary dwarfs or dietary restricted wildtype mice on the level of physiology and gene expression (Wijnhoven et al. 2005; Niedernhofer et al. 2006; van de Ven et al. 2006; van der Pluijm et al. 2006; van de Ven et al. 2007; Schumacher et al. 2008). These phenotypes have been interpreted as an adaptive response to genotoxic stress overlapping with the starvation response. A previously untested prediction of this interpretation is increased resistance rather than hypersensitivity to acute oxidative stress in these models.

Consistent with this prediction, we showed here that mouse models of Cockayne syndrome deficient in either Csa or Csb are less susceptible to renal ischemia reperfusion injury than wildtype mice. Susceptibility was measured in terms of animal survival, kidney function and histological evidence of damage and organ regeneration. In all cases, CSB mice did significantly better than WT mice. On the molecular level, we observed less upregulation of the cell-cycle inhibitor p21 after injury in CSB mice, consistent with less damage and/or better proliferative potential. Upregulation of inflammatory markers ICAM-1 and P selectin were also significantly reduced following injury in CSB mice, consistent with either reduced damage and/or a dampened ability to mount an inflammatory response. Finally, improved glucose tolerance and insulin sensitivity at baseline were observed in overnight fasted CSB animals relative to WT controls. Taken together with previous studies demonstrating hypersensitivity to ionizing radiation in certain tissues, these data suggest that hypersensitivity and resistance to oxidative stress can coexist in the same animal, depending on the tissue or cell type involved.

Although the mechanism of protection against acute stress in the long-lived dwarf or dietary restricted models is not known, a number of plausible candidate mechanisms have been identified. These include increased antioxidant capacity, reduced inflammation and improved glucose homeostasis due to heightened insulin sensitivity (Bartke and Brown-Borg 2004). The induction of genes including hemeoxygenase-1 upon ischemic injury demonstrated that the capacity of CSB animals to mount a transcriptional response is normal despite a defect in the repair of RNA polymerase-stalling bulky DNA lesions.

However, we did not observe any differences in antioxidant capacity on the level of gene expression between WT and CSB mice. We did find a difference in the upregulation of two markers of inflammation, P selectin and ICAM-1. Reduced inflammation is associated with improved outcome following renal ischemia (Friedewald and Rabb 2004), suggesting that these differences may in part underlie the stress resistance observed in the CSB model.

Glucose tolerance and insulin sensitivity tests were both consistent with improved response of CSB animals to the effects of insulin. Improved insulin sensitivity is associated with extended longevity in various models including genetic dwarfism and dietary restriction and is usually associated with reduced IGF-1 and improved glucose metabolism. How improved insulin sensitivity might underlie protection from acute stress observed here, or extended longevity observed in other models, remains unknown. As insulin and insulin-like growth factor signaling are pro-survival, and growth factor delivery can improve outcome after ischemic injury to other organs including the brain (Tang 2006), we speculate that increased sensitivity to these growth factors may result in both better cell survival as well as improved proliferation following ischemia reperfusion injury.

Which defective biochemical activity (or activities) of CSB is causative of disease phenotypes, possibly including protection from renal ischemic insult? We speculate that a defect in TC-NER is the likely culprit based on the following genetic argument. CSB and other proteins implicated in Cockayne syndrome are multifunctional and have roles in various cellular processes, defects in any of which could in principle give rise to disease symptoms. Nonetheless the symptoms of Cockayne syndrome are remarkably similar regardless of the underlying genetic lesion, particularly in mice including CSA, CSB, XPCS or TTD models in an XPA deficient background, as well as in XPG, XPF and ERCC1 single mutant animals (van de Ven et al. 2007). We speculate that this common phenotype likely stems from a defect in a shared process in which each of these proteins participates directly. To date, the only common pathway in which each of these multifunctional proteins has a direct role is TC-NER. It should be noted, however, that the presumed unrepaired lesion or repair intermediate remains to be identified.

We propose a model in which a congenital DNA repair deficiency results in activation of systemic protective mechanisms including a reduced inflammatory response and improved insulin sensitivity. These mechanisms would then lend resistance to certain forms of acute stress in some tissues (renal ischemia reperfusion injury to the kidney, as shown here) but not others (ionizing radiation in the photoreceptor layer of the eye (Gorgels et al. 2006)) . We can envision at least two distinct mechanisms by which this organ/tissue specificity could occur. Based on the neuronal phenotypes in Cockayne patients and mouse models, TC-NER deficiency could in principle result in neuronal deficiencies causing a state of real or perceived dietary restriction, for example through defective function of neurons involved in nutrient sensing, or neuronal control of the gut leading to malabsorption. Extended periods of dietary restriction in wildtype mice have been shown to protect against ischemic damage to organs including the

heart (Chandrasekar et al. 2001) and the brain (Yu and Mattson 1999). Alternately, unrepaired TC-NER substrates in the genome may elicit an adaptive stress response similar to dietary restriction but in the absence of reduced food intake. Consistent with this latter interpretation is the adaptive response involving transient alterations in glucose homeostasis and serum IGF-1 levels observed in a related TC-NER deficient mouse model during the potentially stressful period of postnatal development (van de Ven et al. 2006).

Hormesis is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has net beneficial effects on the cellular and/or organismal level, including protection against subsequent, higher dose exposures as well as to different types of stress (Murray et al. 1986; Mattson 2008). Dietary restriction has been proposed to act as a mild stressor that extends longevity through hormetic mechanisms (Turturro et al. 2000; Sinclair 2005). Interestingly, ischemic preconditioning, a procedure used to protect against ischemic insult that entails brief period(s) of ischemia prior to a longer ischemia time, is also thought to function via hormesis (Arumugam et al. 2006). Our data suggest that specific types of unrepaired endogenous DNA lesions may also be hormetic in nature. The interplay between tissue-specific sensitivities to endogenous DNA damage and the resulting adaptive responses may underlie the complex phenotypes observed in segmental disorders such as Cockayne syndrome. Having identified such unexpected benefits associated with DNA repair deficiency, further elucidation of underlying mechanisms may allow exploitation of the benefits without suffering the severe complications associated with congenital DNA repair insufficiencies.

Materials and Methods

Animals

Animals were allowed free access to food and water throughout the experiments. All experiments were performed with the approval of the appropriate ethical board. **Male** *Csb*$^{-/-}$ (van der Horst et al. 1997) and *Csa*$^{-/-}$ (van der Horst et al. 2002) mice in a C57BL/6J background were bred at the animal facility of the Erasmus Medical Centre; C57BL/6J mice were purchased from Harlan, Horst, the Netherlands.

Ischemia model

Mice between 12 and 16 weeks of age were anaesthetized by isoflurane inhalation. Following a midline abdominal incision, an atraumatic microvascular clamp was used to occlude the left kidney for 25-37 minutes. After release of the clamp a contralateral nefrectomy was performed.

Kidney functional measurements

Blood samples were collected by retro-orbital puncture. Blood serum urea and creatinine levels were measured using QuantiChrom assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICT-500, Gentaur, Brussels, Belgium) according to the manufacturer's instructions, or were determined using an ELAN analyzer (Eppendorf Merck, Hamburg, Germany) with Ecoline S+ reagents (Diagnostic Systems GmbH, Holzheim, Germany) according to manufacturer's instructions.

Histology

Kidneys were harvested, bisected longitudinally, fixed for 24 hr in formalin and embedded in paraffin. 3 µm sections were stained with hematoxylin and eosin, modified Jones staining and periodic acid Schiff (PAS). Immunohistochemistry was performed on deparaffinzed sections following antigen retrieval in boiling 10 mM sodium citrate.

mRNA expression analysis

Total RNA was extracted from frozen kidney using TRIzol reagent (Invitrogen) and oligodT or hexamer-primed cDNA synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using an Opticon2 DNA Engine (MJ Research) or a MyIQ (BioRad) with SYBR Green incorporation. Each sample was tested in duplo at least two times.

Glucose tolerance and insulin sensitivity tests

Mice were fasted overnight prior to testing. Following baseline blood glucose determination from tail blood of conscious, restrained mice, animals were injected with a bolus of glucose (1.5 mg glucose/gram body weight) or insulin (0.75 U/kg body weight) into the intraperitoneal cavity. Blood glucose determinations were performed as above at the indicated times following injection using a HemoCue glucose 201 RT blood glucose analyzer (HemoCue, Ängelholm, Sweden).

Materials and Methods

Statistics

Data are expressed as the mean ± SEM. Statistical analyses of data on urea, creatinine, histomorphology, immunohistochemistry and immunoblot was preformed using a Student's T test. Survival was analyzed by Kaplan-Meier (SPSSv11).

Acknowledgements

This research was supported in part by the Netherlands Organization for Health Research and Development, Research Institute for Diseases of the Elderly (60-60400-98-004), the National Institutes of Health (1PO1 AG17242–02), the Association for International Cancer Research (05–280) and the European Commission RISC-RAD (FI6R-CT-2003-508842). JRM was a fellow of the Damon Runyon Cancer Research Fund (DRG 1677). MV was supported by a grant from the Dutch Kidney Foundation (C04.2105).

References

Arumugam, T.V., M. Gleichmann, S.C. Tang, and M.P. Mattson. 2006. Hormesis/preconditioning mechanisms, the nervous system and aging. Ageing Res Rev 5: 165-78.

Bartke, A. and H. Brown-Borg. 2004. Life extension in the dwarf mouse. Curr Top Dev Biol 63: 189-225.

Beckman, K.B. and B.N. Ames. 1998. The free radical theory of aging matures. Physiol Rev 78: 547-81.

Brooks, P.J., D.S. Wise, D.A. Berry, J.V. Kosmoski, M.J. Smerdon, R.L. Somers, H. Mackie, A.Y. Spoonde, E.J. Ackerman, K. Coleman, R.E. Tarone, and J.H. Robbins. 2000. The oxidative DNA lesion 8,5'-(S)-cyclo-2'-deoxyadenosine is repaired by the nucleotide excision repair pathway and blocks gene expression in mammalian cells. J Biol Chem 275: 22355-62.

Chandrasekar, B., J.F. Nelson, J.T. Colston, and G.L. Freeman. 2001. Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 280: H2094-102.

Citterio, E., V. Van Den Boom, G. Schnitzler, R. Kanaar, E. Bonte, R.E. Kingston, J.H. Hoeijmakers, and W. Vermeulen. 2000. ATP-dependent chromatin remodeling by the Cockayne syndrome B DNA repair-transcription-coupling factor. Mol Cell Biol 20: 7643-53.

de Waard, H., J. de Wit, J.O. Andressoo, C.T. van Oostrom, B. Riis, A. Weimann, H.E. Poulsen, H. van Steeg, J.H. Hoeijmakers, and G.T. van der Horst. 2004. Different effects of CSA and CSB deficiency on sensitivity to oxidative DNA damage. Mol Cell Biol 24: 7941-8.

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

Devarajan, P. 2005. Cellular and molecular derangements in acute tubular necrosis. Curr Opin Pediatr 17: 193-9.

Dolle, M.E., R.A. Busuttil, A.M. Garcia, S. Wijnhoven, E. van Drunen, L.J. Niedernhofer, G. van der Horst, J.H. Hoeijmakers, H. van Steeg, and J. Vijg. 2006. Increased genomic instability is not a prerequisite for shortened lifespan in DNA repair deficient mice. Mutat Res 596: 22-35.

Fousteri, M., W. Vermeulen, A.A. van Zeeland, and L.H. Mullenders. 2006. Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. Mol Cell 23: 471-82.

Friedewald, J.J. and H. Rabb. 2004. Inflammatory cells in ischemic acute renal failure. Kidney Int 66: 486-91.

References

Gorgels, T.G., I. van der Pluijm, R.M. Brandt, G.A. Garinis, H. van Steeg, G. van den Aardweg, G.H. Jansen, J.M. Ruijter, A.A. Bergen, D. van Norren, J.H. Hoeijmakers, and G.T. van der Horst. **2006. Retinal** degeneration and ionizing radiation hypersensitivity in a mouse model for Cockayne syndrome. Mol Cell Biol.

Groisman, R., J. Polanowska, I. Kuraoka, J. Sawada, M. Saijo, R. Drapkin, A.F. Kisselev, K. Tanaka, and Y. Nakatani. 2003. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell 113: 357-67.

Harman, D. 1956. Aging: a theory based on free radical and radiation chemistry. J Gerontol 11: 298-300.

Hoeijmakers, J.H. 2001. Genome maintenance mechanisms for preventing cancer. Nature 411: 366-74.

Kelly, K.J. 2003. Distant effects of experimental renal ischemia/reperfusion injury. J Am Soc Nephrol 14: 1549-58.

Kher, A., K.K. Meldrum, M. Wang, B.M. Tsai, J.M. Pitcher, and D.R. Meldrum. 2005. Cellular and molecular mechanisms of sex differences in renal ischemia-reperfusion injury. Cardiovasc Res 67: 594-603.

Martin, G.M. and J. Oshima. 2000. Lessons from human progeroid syndromes. Nature 408: 263-6.

Mattson, M.P. 2008. Hormesis defined. Ageing Res Rev 7: 1-7.

Megyesi, J., L. Andrade, J.M. Vieira, Jr., R.L. Safirstein, and P.M. Price. 2002. Coordination of the cell cycle is an important determinant of the syndrome of acute renal failure. Am J Physiol Renal Physiol 283: F810-6.

Murray, C.E., R.B. Jennings, and K.A. Reimer. 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74: 1124-1136.

Nance, M.A. and S.A. Berry. 1992. Cockayne syndrome: Review of 140 cases. Am. J. Med. Genet. 42: 68-84.

Niedernhofer, L.J., G.A. Garinis, A. Raams, A.S. Lalai, A.R. Robinson, E. Appeldoorn, H. Odijk, R. Oostendorp, A. Ahmad, W. van Leeuwen, A.F. Theil, W. Vermeulen, G.T. van der Horst, P. Meinecke, W.J. Kleijer, J. Vijg, N.G. Jaspers, and J.H. Hoeijmakers. 2006. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 444: 1038-43.

Padanilam, B.J. 2003. Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. Am J Physiol Renal Physiol 284: F608-27.

Scholzen, T. and J. Gerdes. 2000. The Ki-67 protein: from the known and the unknown. J Cell Physiol 182: 311-22.

References

Schumacher, B., I. van der Pluijm, M.J. Moorhouse, T. Kosteas, A.R. Robinson, Y. Suh, T.M. Breit, H. van Steeg, L.J. Niedernhofer, W. van Ijcken, A. Bartke, S.R. Spindler, J.H. Hoeijmakers, G.T. van der Horst, and G.A. Garinis. 2008. Delayed and accelerated aging share common longevity assurance mechanisms. PLoS Genet 4: e1000161.

Sinclair, D.A. 2005. Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev 126: 987-1002.

Takada, M., K.C. Nadeau, G.D. Shaw, K.A. Marquette, and N.L. Tilney. 1997. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. J Clin Invest 99: 2682-90.

Tang, B.L. 2006. SIRT1, neuronal cell survival and the insulin/IGF-1 aging paradox. Neurobiol Aging 27: 501-5.

Turturro, A., B.S. Hass, and R.W. Hart. 2000. Does caloric restriction induce hormesis? Hum Exp Toxicol 19: 320-9.

van de Ven, M., J.O. Andressoo, V.B. Holcomb, P. Hasty, Y. Suh, H. van Steeg, G.A. Garinis, J.H. Hoeijmakers, and J.R. Mitchell. 2007. Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging. Mech Ageing Dev 128: 58-63.

van de Ven, M., J.O. Andressoo, V.B. Holcomb, M. von Lindern, W.M. Jong, C.I. Zeeuw, Y. Suh, P. Hasty, J.H. Hoeijmakers, G.T. van der Horst, and J.R. Mitchell. 2006. Adaptive Stress Response in Segmental Progeria Resembles Long-Lived Dwarfism and Calorie Restriction in Mice. PLoS Genet 2: e192.

van der Horst, G.T., L. Meira, T.G. Gorgels, J. de Wit, S. Velasco-Miguel, J.A. Richardson, Y. Kamp, M.P. Vreeswijk, B. Smit, D. Bootsma, J.H. Hoeijmakers, and E.C. Friedberg. 2002. UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. DNA Repair (Amst) 1: 143-57.

van der Horst, G.T.J., H. van Steeg, R.J.W. Berg, A. van Gool, J. de Wit, G. Weeda, H. Morreau, R.B. Beems, C.F. van Kreijl, F.R. de Gruijl, D. Bootsma, and J.H.J. Hoeijmakers. 1997. Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell 89: 425-35.

van der Pluijm, I., G.A. Garinis, R.M. Brandt, T.G. Gorgels, S.W. Wijnhoven, K.E. Diderich, J. de Wit, J.R. Mitchell, C. van Oostrom, R. Beems, L.J. Niedernhofer, S. Velasco, E.C. Friedberg, K. Tanaka, H. van Steeg, J.H. Hoeijmakers, and G.T. van der Horst. 2006. Impaired genome maintenance suppresses the growth hormone--insulin-like growth factor 1 axis in mice with Cockayne syndrome. PLoS Biol 5: e2.

References

Wijnhoven, S.W., R.B. Beems, M. Roodbergen, J. van den Berg, P.H. Lohman, K. Diderich, G.T. van der Horst, J. Vijg, J.H. Hoeijmakers, and H. van Steeg. 2005. Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. DNA Repair (Amst).

Yu, Z.F. and M.P. Mattson. 1999. Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. J Neurosci Res 57: 830-9.

6

Different effects of XPD mutations on cancer and ageing in mouse models

~

Marieke van de Ven, Jaan-Olle Andressoo,
Gijsbertus T.J. van der Horst,
Jan H.J. Hoeijmakers, James R. Mitchell.

Abstract

XPD is a helicase subunit of the transcription factor IIH, a ten-protein complex involved in a wide range of cellular activities including transcription initiation by RNA polymerase II and I, activated transcription via nuclear hormone receptor activation, and various DNA repair systems. Some mutations in the *XPD* gene can lead to xeroderma pigmentosum (XP), a disease characterized by an extreme predisposition to sun-induced skin cancer. Different mutations in *XPD* can lead to trichothiodystrophy (TTD), a severe neurodevelopmental disorder with multiple symptoms of accelerated aging and characteristic brittle hair and nails, but with a distinct lack of cancer predisposition. Also XPCS mutations are known, this is XP combined with the progeroid disorder Cockayne syndrome.

We have reported that mouse models of two *XPD*-related human disorders, XPCS and TTD, share a subset of metabolic phenotypes, like lower body weight and reduced metabolism, resembling calorie-restricted wildtype animals. Furthermore, the phenotypes are largely rescued in compound heterozygous animals (CH) derived from crosses between these two different mutant XPD animals. Here we tested the effects of compound heterozygosity on the repair of distorting DNA lesions, such as those induced by UV, using a variety of cell-based and *in vivo* assays including UV carcinogenicity and cellular survival. We found no correlation between the ability to repair UV lesions and the rescue of metabolic phenotypes in the CH state, suggesting that inability to repair bulky DNA lesions is not a driving force behind the segmental progeria/calorie restricted-like phenotype observed in these disease models.

Introduction

DNA contains the genetic information of an organism and should be passed on in an error free manner from generation to generation. To preserve genome integrity, a network of genome maintenance mechanisms has evolved, including repair processes. Defects in genome maintenance can lead to different pathologies ranging from cancer to metabolic syndrome to premature ageing (van de Ven et al., 2007). One of these repair processes is nucleotide excision repair (NER), which eliminates a wide diversity of helix distorting DNA lesions, including cyclobutane pyrimide dimers, (6-4) photoproducts (main damage induced by UV light), intrastrand cross-links, and bulky chemical adducts. In addition, NER has been implicated in the repair of lesion, including several forms of oxidative lesions such as cyclo-purines (Brooks, 2007). NER has two sub pathways with partly distinct substrate specificity: global genome NER (GG-NER) surveys the entire genome for distorting lesions, and transcription-coupled nucleotide excision repair (TC-NER) focuses only on distorting damage in the transcribed strand of active genes that blocks elongating RNA polymerase (Batty and Wood, 2000; de Laat et al., 1999; Hanawalt and Spivak, 2008; Tornaletti and Hanawalt, 1999).

XPD is a helicase component of basal transcription/DNA repair factor IIH (TFIIH). TFIIH consists of ten subunits, which together form a multifunctional complex that is essential for multiple processes, of which basal transcription initiation and DNA damage repair via the NER pathway are two (Giglia-Mari et al., 2004; Schaeffer et al., 1993). Mutations in *XPD* affect TFIIH function and are associated with different UV-sensitive, disorders like xeroderma pigmentosum (XP), XP combined with Cockayne syndrome (CS) (XPCS), and trichothiodystrophy (TTD) (Broughton et al., 2001; Hoeijmakers, 2001; Lehmann, 2001). XP is associated with a dramatic incidence of sun-induced skin cancer. The frequency of internal tumors is modestly elevated, and in addition accelerated neurodegeneration is often noted (Lehmann, 2001). CS, on the other hand, is a TC-NER specific disorder, and is caused by mutations in the *CSA* or *CSB* genes. The disorder is characterized by progressive post-natal growth failure and primary demyelization, resulting in severe neurodysfunction, but without a clear cancer predisposition (Nakura et al., 2000). XPCS is displaying both the cancer predisposition of XP, and the neurodevelopmental complications of CS (Lehmann, 2001). Finally, TTD is a condition sharing many symptoms with CS, but with the additional hallmarks of brittle hair, nails and scaly skin (Itin and Pittelkow, 1990).

Previously, TTD and XPCS mouse models, mimicking known human point mutations (R722W and G602D respectively) in the mouse *Xpd* locus, have been made. These mice exhibit striking phenotypic resemblance with the human syndromes (Andressoo et al., 2006b; de Boer et al., 1999). XPCS mice, like XPCS patients, show a mild accelerated ageing phenotype, including cachexia and have a high UV-induced cancer susceptibility. TTD patients also show this accelerated ageing phenotype but, despite their partial NER deficiency, do not seem to be cancer prone. TTD mice develop UV-induced skin cancers when subjected to a high dose of UV, and are therefore considered more susceptible to UV carcinogenesis than wildtype (WT) mice (de Boer et al., 1999). This modest skin

cancer susceptibility is only apparent after extensive UV exposure. Spontaneous cancer frequency in TTD mice, particularly lymphoma's and pituitary adenomas are reduced (Wijnhoven et al., 2005). TTD mice, like patients, also show segmental accelerated ageing and have a calorie restricted-like phenotype with liver gene expression profiles that resemble those of long-lived dwarf mice (Park et al., 2008; van der Pluijm et al., 2007; Wijnhoven et al., 2005). Unfortunately, the initial studies on UV-sensitivity and skin cancer susceptibility have been performed in mice and cells in varying genetic backgrounds, therefore making it difficult to accurately compare these experimental outcomes.

Many NER patients, including approximately half of those in the XPD complementation group, are compound heterozygotes carrying a different mutation in each of the two *Xpd* alleles. In the absence of a WT allele, genetic interactions between recessive alleles (referred to here as "biallelic" effects) can result in a variety of phenotypic outcomes (Andressoo et al., 2006a; Oh et al., 2006). Because genetic heterogeneity and environmental exposure are impossible to control for in patients (Lehmann et al., 1988; Stefanini et al., 1992), we demonstrated this the occurrence of biallelic effects in NER-related disorders using a variety of *Xpd* mutant mouse models. Intercrosess of mice heterozygous for viable TTD mutations and homozygous lethal XPCS or XP mutations result in compound heterozygous (CH) mice with a TTD mutation in one allele and a XPCS or XP mutation in the other. For some phenotypes associated with these alleles in the homozygous state (brittle hair in TTD homozygotes; embryonic lethality in XPCS and XP homozygotes due to reduced expression of the engineered allele) the compound heterozygous mice are more like wildtype than either of the corresponding homozygous animals, consistent with interallelic complementation. In other words the combined mutated alleles can do what neither one in homozygous state can do on its own. Other phenotypes (UV sensitivity, developmental delay) show dominance of one mutant allele (TTD) over the other (XPCS, XP) (Andressoo et al., 2006a).

Previously, we showed that a NER deficiency in mice is associated with a number of overlapping features of segmental progeria and dietary restriction. These phenotypes include changes in energy metabolism, reduced blood glucose and serum insulin-like growth factor (IGF-1), and reduced growth hormone receptor (GHR) signaling (Niedernhofer et al., 2006; Schumacher et al., 2008; van de Ven et al., 2006; van der Pluijm et al., 2007). Furthermore, in the severely affected XPD|XPA (this is a *Xpd* mutant with a simultaneous inactivation of the *Xpa* gene (de Vries et al., 1995; Niedernhofer et al., 2006), which is central for GG-NER and TC-NER of bulky distorting DNA lesions), many of these phenotypes were partially rescued in the context of *Xpd* compound heterozygosity (van de Ven et al., 2006). XPD|XPA double mutant mice have growth retardation and are hypoglycemic around the time of weaning.

Here, we report the use of homozygous viable *Xpd* alleles of TTD and XPCS in an attempt to understand which activity of *Xpd* is complemented in compound heterozygous mutants. To do this, we first looked for evidence of complementation of calorie restricted/progeroid

phenotypes, as previously reported in related studies (Andressoo et al., 2006a; van de Ven et al., 2006). Next we measured a variety of endpoints of DNA repair capacity both *in vitro* and *in vivo*. Finally, we asked if the ability of compound heterozygosity to rescue calorie restricted/progeroid phenotypes correlated with its ability to complement measures of DNA repair. We used TTD and XPCS mice, which both show segmental progeria/calorie restriction-like symptoms as well as differential UV sensitivity in the homozgygous state (Andressoo et al., 2006b; van de Ven et al., 2006; Wijnhoven et al., 2005) in an isogenic C57Bl/6J background to isolate the effects of the NER mutations from effects of genetic background.

Results

Compound heterozygosity of TTD|XPCS partially rescues calorie restricted-like phenotype.

We investigated the effects of compound heterozygosity between TTD and XPCS alleles at the *Xpd* locus on metabolic phenotypes, including body weight and glucose metabolism in adult male animals using littermate controls in an isogenic C57BL6 background. First we measured the body weight of the different adult (10 week old) mutant animals. TTD and XPCS mice had a significantly lower weight than WT animals ($p = 0.0164$ and 0.0355 respectively) (Figure 1A). CH animals were not significantly different from either WT or XPA mice, indicating that reduced body weight in homozygous *Xpd* mutants is rescued by compound heterozygosity. No differences were found in insulin levels or levels of IGF-1 in the serum of these different groups of animals. We next performed glucose tolerance and insulin sensitivity tests as a measure of glucose metabolism. It is known that dietary restriction improves glucose tolerance and insulin sensitivity and these measurements are therefore a good test for our segmental progeria/calorie restricted-like phenotypes. Furthermore, mouse models of Cockayne syndrome have improved glucose tolerance and insulin sensitivity (Susa et al., 2009). These tests have never been performed in the XPD/XPA animals, because these very short lived double mutants are to weak and cannot be fasted overnight before weaning. To this end, mice of each genotype (WT, XPA, XPCS, TTD, CH) were injected with a bolus of either glucose or insulin and measurements of blood glucose were taken over a time course following injection (Figure 1B,C). The area under each curve (AUC) is a measurement of the overall response or sensitivity, with smaller areas representing improved glucose tolerance or insulin sensitivity, respectively (Figure 1B,C). TTD and XPCS mice showed improved glucose tolerance, as measured by individual time points in the glucose curve (XPCS $p < 0.05$ at $t = 120$min.; TTD $p < 0.01$ at $t = 60, 120$ min.) and overall according to the AUC (Figure 1B). TTD and XPCS mice also displayed improved insulin sensitivity according to the AUC (Figure 1C). CH mice were not significantly different than WT or XPA in either test. We conclude that the calorie restricted-like phenotype is rescued in CH animals. Taken together, differential body weight and glucose metabolism are phenotypes present in both homozygous single mutant animals that are complemented by compound heterozygosity.

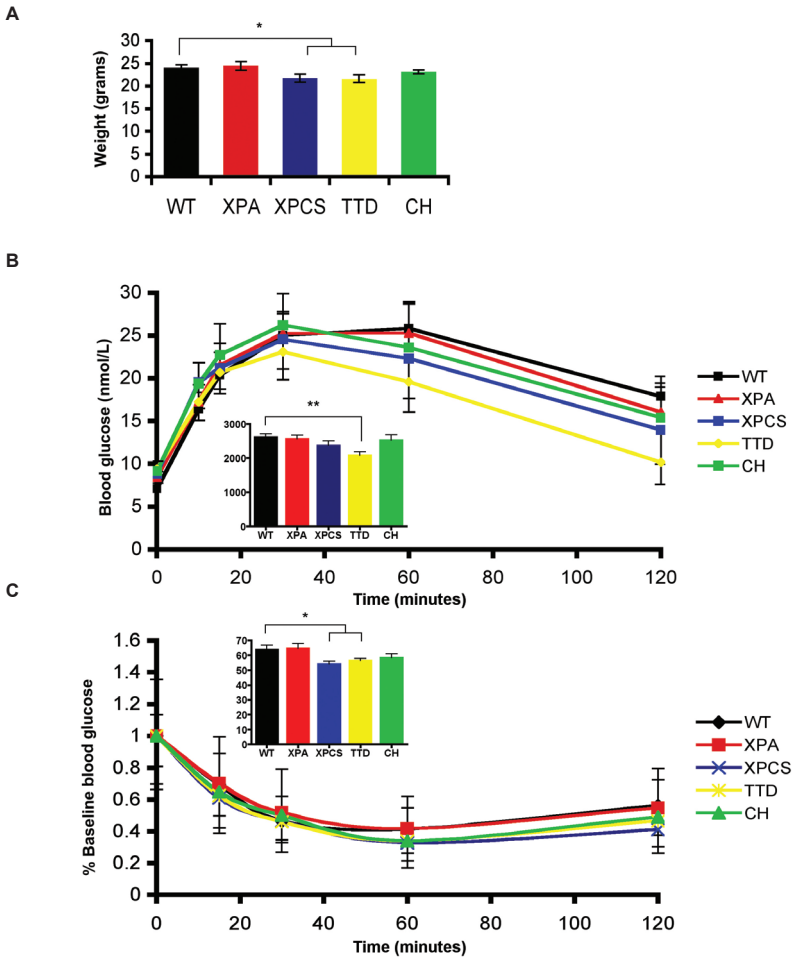


Figure 1. Partial reversal of the calorie restricted-like phenotype by interallelic complementation.

A. Bodyweights of ten week old XPCS and male TTD mice (n=9). Error bars indicate SD. Note the significant difference between WT and TTD and XPCS homozygous but not compound heterozygous mutants. Error bars indicate SEM (* $p < 0.05$).

B. Glucose tolerance test (male mice n=9): whole blood glucose levels at the indicated time points following intraperitoneal injection of glucose (1.5 mg per gram body weight). At 60 and 120 minute time points blood glucose is significantly lower in TTD vs. WT ($p < 0.01$) and at the 120 minute time point lower in XPCS vs. WT ($p < 0.05$). Insert: the area under each curve is represented in arbitrary units. Asterisk indicates the significance of the difference vs. WT (** $p < 0.01$). Error bars indicate SD.

C. Insulin sensitivity test (male mice n=9): whole blood glucose levels at the indicated time points following intraperitoneal injection of insulin (0.75 U/kg body weight). Insert: the area under each curve is represented in arbitrary units. Asterisk indicates the significance of the difference vs. WT (* $p < 0.05$). Error bars indicate SD.

Dominance of TTD allele over XPCS allele in measures of UV-sensitivity.

We next looked on both the organismal and cellular sensitivity to UV irradiation as a measure of NER capacity. UV-induced inflammation is expressed as the minimal UV-B dose required to induce erythema, or edema (minimal erythemal dose, MED). As shown in Figure 2A, TTD mice have a slightly reduced MED compared to WT (1200 J/m^2 versus 1500 J/m^2). The MED of XPCS animals was more comparable to the one of XPA animals (100 J/m^2 versus 150 J/m^2 respectively). However, the MED of CH mice was 1000 J/m^2, between XPCS and TTD animals, but much closer to the value observed for TTD mice. Thus, the TTD allele is dominant over the XPCS allele with respect to MED.

Another measure of acute UV sensitivity is hyperplasia, or the thickening of the epidermal layer that occurs as an adaptive response to acute UV exposure. To measure this, mice were depilated on the back and exposed to UV-B light with a dose of 200 J/m^2/day for 4 days. Histological analyses of skin sections were made 1 week after the start of UV-B treatment (3 days after the final treatment) (Figure 2B). XPCS and XPA skin revealed pronounced epidermal hyperplasia, consisting of an increased number of cell layers (acanthosis) as shown before (Andressoo et al., 2006b; de Vries et al., 1995) and with no significant difference observed between them. Neither erythema nor hyperplasia was observed in the UV-exposed skin of WT or TTD animals (de Boer et al., 1999). Only minor hyperplasia was found in sections of UV-exposed CH skin. Unirradiated skin of all animals served as a negative control. In conclusion, the XPCS mutation induces severe photosensitivity in mice, and the TTD allele is dominant in CH animals, with the difference in CH vs. wildtype likely due to dosage effects.

Next we determined skin cancer susceptibility, fifteen XPCS and XPA animals and five CH animals were exposed daily to an incremental low UV-B dose up to a maximum of 100 J/m2/day, until a total amount of 11 kJ/m^2 was reached. For the remaining ten CH mice and fifteen WT and TTD mice a higher daily and cumulative dose was selected (650 J/m2/day and 103 kJ/m^2 respectively), because of the higher MED and reduced skin cancer susceptibility (de Boer et al., 1999). In Figure 3A the percentage of tumor free mice (that is, the time when the first tumor was noted) is depicted. In all cases soon after the treatment stopped the first tumors appeared. There was no significant difference in latency time of developing tumors between CH and TTD mice (Log rank 0.2579) or between XPA and XPCS mice (Log rank = 0.9969). Significant differences were detected between WT and TTD or CH mice (Log rank = 0.0013 and 0.0060 respectively) and between CH and XPCS or XPA animals (Log rank = 0.0099 and 0.0054 respectively). In Figure 3B, the time until at least one tumor reached a diameter of 4 mm (upon which the animal was sacrificed) is depicted. Outcomes were approximately uniform (Log rank WT/TTD = 0.0031, WT/CH = 0.0055, CH/XPA = 0.0063, CH/XPCS = 0.0093, XPA/XPCS = 0.7836), except for TTD and CH animals which were significantly different (Log rank = 0.0299). This could be because of the observed slow tumor growth and therefore increased tumor yield in TTD animals. This higher tumor yield was also noted by de Boer et al. (1999). An example of smaller tumors in TTD mice vs. XPCS, XPA, CH and WT mice is shown in Figure 3C. All tumors

were histopathologically identified as squamous cell carcinomas (SCCs) (data not shown) consistent with previous studies in which only SCCs were found in UV-irradiated TTD and XPA mice (de Boer et al., 1999; de Vries et al., 1995) and only few squamous cell papillomas (SSPs) were found in XPCS mice (Andressoo et al., 2006b). During the experiment, corneal opacity and bulging of the eyes were also observed in XPA and XPCS mice but not in TTD, CH or WT mice (data not shown). In conclusion, in all UV sensitivity experiments the TTD allele is dominant over the XPCS allele, on the organismal as well as cellular level.

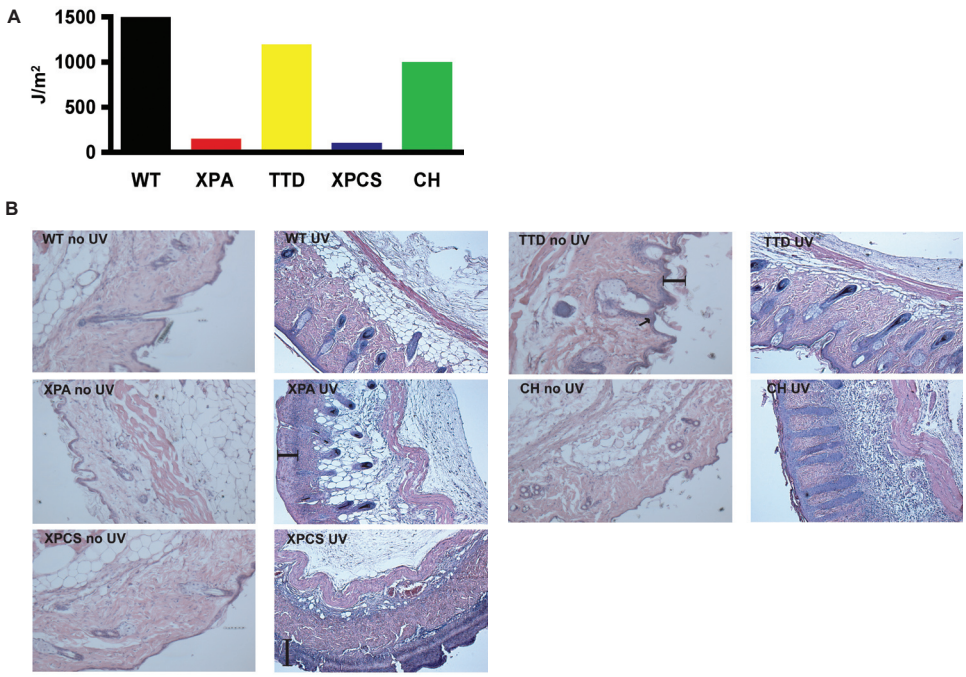


Figure 2. Acute effects of UV-B on the skin in mice with different *Xpd* mutations *in vivo*.

A. The Minimal Erythemal Dose (MED), a quantification of UV-induced inflammation or sunburn as determined by an increase in skin thickness following acute UV-B. Ear skin was exposed to different doses of UV-B. Ear thickness was measured before and 24h after UV-B exposure using an engineer's micrometer. The lowest dose that was able to induce a significant increase in thickness was designated the MED for that genotype (n = 9 per genotype 3 per dose).

B. Depilated backs of isogenic C57BL6 mice of the indicated genotypes were exposed to 200 J/m² UV-B daily for four consecutive days and sacrificed one week after the start of the treatment. Note the pronounced epidermal hyperplasia (purple, indicated by horizontal and vertical bar), consisting of increased number of viable cell layers (acanthosis and hyperkeratosis), in the XPA and XPCS skin after UV treatment. The prominent cornified layer (indicated by horizontal bar) and thicker granular layer (indicated by an arrow) in TTD skin (scaling skin) is also clearly visible (n = 4 per genotype).

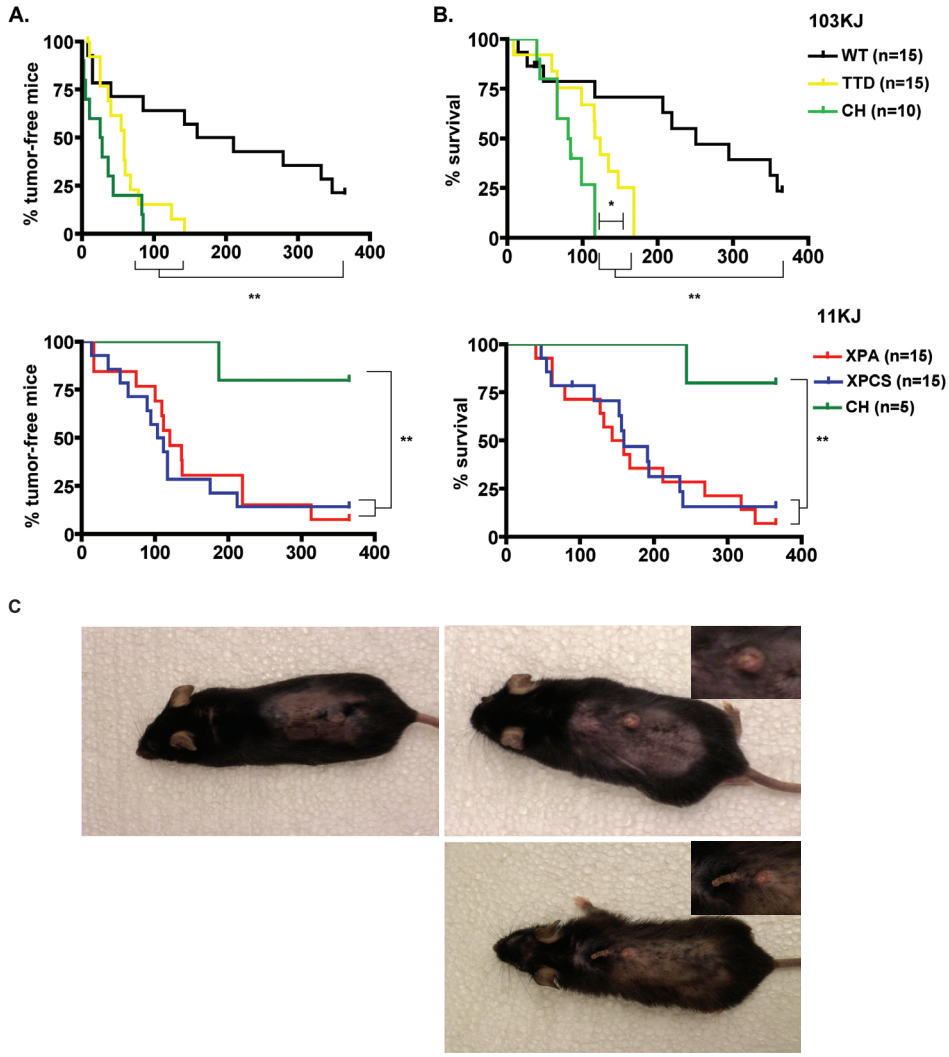


Figure 3. Differential UV sensitivities of mice with different *Xpd* mutations *in vivo*.

A,B. UV-induced carcinogenesis was studied in isogenic C57BL6/J mice of the indicated genotype by chronically exposing the depilated backs of mice with an incremental-dose to 650 J/m²/day (WT, TTD, and CH animals; 103KJ total (n = 15 per genotype)), or 100 J/m²/day (XPA, XPCS and CH animals; 11KJ total (n = 15 per genotype)). The percent of mice without a visible tumor from onset of UV exposure is plotted in **A**. In **B**, percent survival represents the time from the onset of UV exposure until at least one tumor was > 4 mm diameter and the mouse was sacrificed. Note the significant difference between TTD and CH animals in percent survival (* $p < 0{,}05$; ** $p < 0.01$).
C. UV-induced skin cancers in WT (top left), XPCS (top right) and TTD (bottom right) mice. Note the lack of tumors in the first mouse; the second showing a tumor after UV-B in a XPCS mouse that is typical of that seen in all the genotypes except TTD; and third, small, slower growing tumors typical of TTD mice.

We next turned to cell-based endpoints of radiation exposure in primary dermal fibroblasts prepared from each genotype in order to further focus in on the DNA repair characteristics of these different genotypes. Using a proliferation-based assay, we found that primary MDFs from XPCS and XPA mice were hypersensitive to UV irradiation, and that TTD and CH MDFs were far less sensitive, consistent with the *in vivo* data (Figure 4A). Using the same assay, no difference in sensitivity to ionizing radiation was observed in any of the mutant cells relative to the WT MDFs (Figure 4B). This is consistent with the lack of oxidative stress sensitivity in any of these NER mutants (van de Ven et al., 2006), although it also induces single stranded and double stranded breaks of different kinds.

We next focused more specifically on NER characteristics of primary MDFs derived from the tail skin of adult mice of each genotype. The UV-induced unscheduled DNA synthesis (UDS) assay reflects GG-NER of bulky lesions anywhere in the genome. Specifically, this assay measures the incorporation of radiolabelled nucleotides that constitute the "patch" part of the NER reaction following removal of the lesion 2 hours after UV irradiation. XPA cells, which lack GG-NER altogether, were used as negative controls. As found previously in dermal fibroblasts from patients as well as in mouse embryonic fibroblasts (MEFs), XPCS MDFs retained ~40% of WT UDS activity (Andressoo et al., 2006b; Theron et al., 2005). This is a result of "phantom UDS", which is dependent on the NER factor XPA but does not reflect efficient repair of UV lesions (Andressoo et al., 2006b; van Hoffen et al., 1999). TTD and CH had a UDS around 60% of WT and therefore significantly differed in the amount of UDS as compared to XPCS ($p = 0.0004$ and 0.00002, respectively) but were not significantly different from each other ($p = 0.2518$). In conclusion, the TTD allele is dominant over the XPCS allele with respect to GG-NER capacity in MDFs.

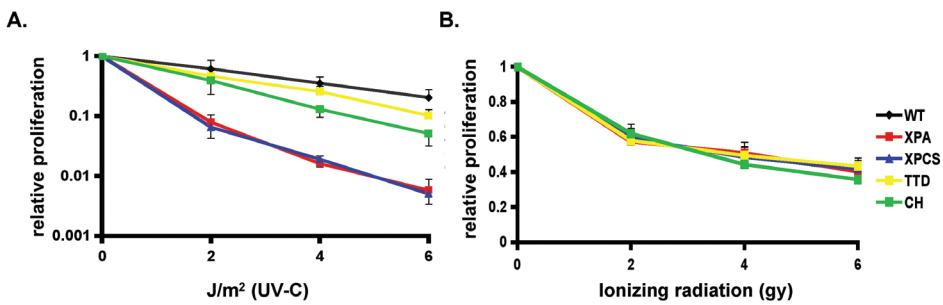


Figure 4. Differential UV sensitivities of cells with different *Xpd* mutations *in vitro*.

A. Early passage C57BL6 mouse dermal fibroblasts (MDFs) from the indicated genotypes were treated with the indicated dose of UV-C (**A.**) or ionizing radiation (**B.**). The number of cells remaining after three days was plotted as a percentage of the total number of cells from the corresponding untreated cell line. Note that the outcome of the UV cell survival curve (but not ionizing radiation) follows the same pattern as the skin cancer survival curve, with NER-deficient XPA and XPCS both highly hypersensitive, and CH and TTD much closer to WT. Error bars indicate SEM.

To confirm these UDS data with a different assay, we followed the phosphorylated histone H2AX (γH2AX) 3hr after UV treatment of confluent, non-dividing MDFs. γH2AX staining reflects the incision activity of NER, which is low in XPA deficient cells (O'Driscoll et al., 2003; Theron et al., 2005) and in TTD cells (Andressoo et al., 2006b). In MDFs, γH2AX staining was consistent with previous results reported on MEFs (Andressoo et al., 2006b) as well as the relative percent of UDS activities (Figure 5A, B). As with the UV-UDS assay, XPCS cells displayed γH2AX staining unrelated to their repair capacity, with an increased number of incisions despite reduced UDS. This is also consistent with what has been previously reported in XPCS cells from both mice and humans (Andressoo et al., 2006b).

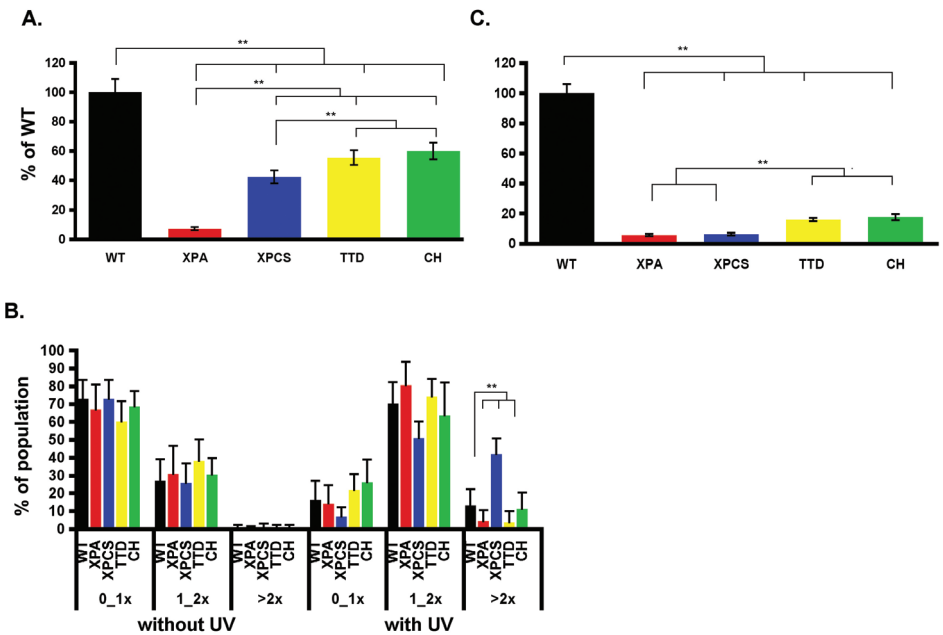


Figure 5. UV-induced DNA damage repair characteristics.

A. UV-induced DNA repair synthesis capacity. Cells were irradiated with 16 J/m² UV-C followed by incubation with [methyl-³H]thymidine for 2 hours. Incorporation of radioactivity was counted in individual cells (n = two cell lines per genotype; 25 nuclei per line). Error bars indicate SEM. Asterisks indicate significant differences between indicated lines (** $p < 0.01$).

B. Quantification of γH2AX immunofluorescence indicative of DNA breaks in MDF nuclei with and without UV-C irradiation.1x relative intensity represents the average amount of γH2AX immunofluorescence in the corresponding unirradiated cell line. Error bars indicate SD. Asterisks indicate significant differences between indicated lines (** $p < 0.01$).

C. RNA synthesis recovery in MDFs, measured 16 hours after exposure to 10 J/m² UV-C followed by a 1hr pulse labeling with [³H]uridine. UDS and RRS are each expressed as a percentage of WT. Error bars represent SEM. Asterisks indicate significant differences between indicated lines (** $p < 0.01$).

Recovery of RNA synthesis (RRS) is a specific measure of TC-NER in transcribed regions of the genome. It is measured by incorporation of radiolabelled uridine 16 hours after UV irradiation. Similar to XPA cells, which lack TC-NER capacity entirely, XPCS cells had no detectable RRS (Figure 5C). However, a small but significant residual TC-NER activity (~15%) was detectable in CH and TTD cells.

Dominance of TTD allele over XPCS allele in UV-induced stress response.

Steady state levels of insulin-like growth factor 1 receptor (IGF-1R) mRNA and protein are reduced after UV irradiation in WT MDFs. This reduction is prolonged in cells from NER progeria models largely correlated with the severity of the progeroid phenotype *in vivo* (Garinis et al., 2009). We thus analyzed this parameter to explore if level of IGF-1R mRNA correlated with progeroid characteristics in our mutants. To this end we measured the amount of IGF-1R mRNA in the primary MDFs of the different cell lines in a time course following UV irradiation by quantitative RT-PCR. Figure 6 shows the relative amount of IGF-1R mRNA of each cell line at the different time points after the different doses of UV. IGF-1R expression in WT cells was reduced within 6 hours after UV exposure in a dose dependent manner, and returned to baseline levels 12 hours after exposure. The IGF-1R downregulation in the mutant MDFs was exacerbated, and the reduction lasted longer in each of the NER mutant lines. These effects were most pronounced in XPCS and XPA cells, with an intermediate phenotype in CH and TTD cells. This measure of the UV-induced stress response correlates in these mutants with most of the former UV-based experiments (in which XPA=XPCS < CH=TTD<WT). From these data we conclude that the severity of progeriod symptoms in XPCS, TTD and CH mice does not correlate with the UV-induced down regulation of IGF-1R.

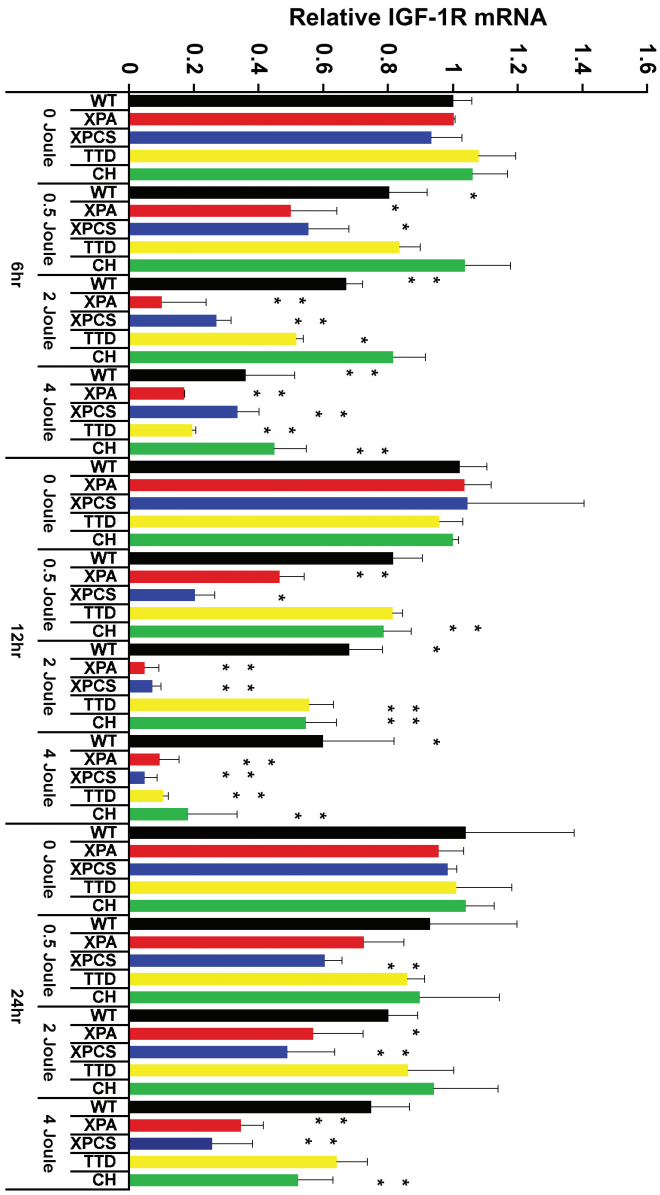


Figure 6. Relative IGF-1R abundance in MDFs after UV treatment.

Steady-state levels of IGF-1R in different MDF cell lines 6, 12 or 24hr after indicated amount of UV as determined by quantitative real-time PCR. Within each time point the value of each cell line is set relative to its own 0 joule value. Each value off each cell line is set relative to the 0 Joule outcome of that cell line at each time point. Error bars indicate SD. Asterisks indicate statistical significance versus 0 Joule controls (* $p < 0.05$; ** $p < 0.01$).

Discussion

Here, using mouse models containing different non-lethal mutations in the *Xpd* gene, we show for the first time that, compound heterzygosity complemented the progeriod and calorie restricted-like phenotypes found in TTD and XPCS mice. However, this complementation was not found for the repair of UV lesions in various cell-based and *in vivo* assays. We therefore conclude that defects in transcription-coupled nucleotide excision DNA repair activity *in vitro* and *in vivo* are unlikely to be causative of progeroid/ calorie restricted-like phenotypes.

Complementation of the calorie restricted-like phenotype in compound heterozygous mice.

The aim of this study was to investigate the previous found interallelic complementation of calorie-restricted like phenotypes by analysis of body weight and glucose metabolism of (WT, XPA, TTD, XPCS and CH) mice. Previous studies reported calorie restricted-like phenotypes that are observed in these accelerated aging models (Niedernhofer et al., 2006; van de Ven et al., 2006; van der Pluijm et al., 2007), likely as an adaptive survival response to endogenous genotoxic stress. First, we confirmed that a number of calorie restricted-like phenotypes, like reduced body weight and insulin sensitivity, were complemented in these mice. Previously, when looking at compound heterozygous mice with a lethal XPCS or XP allele also a rescue of developmental delay and progeriod features such as premature cachexia and loss of bone mineral density was found (Andressoo et al., 2006a). Later, a partial rescue of progeriod features was found in compound heterozygous animals with a viable XPCS allele in a XPA deficient background (van de Ven et al., 2006). Here we show that in the compound heterozygous progeny (CH), derived from crosses between XPCS an TTD animals (and thus carrying two different viable mutant *Xpd* alleles in the same animal without the XPA background), the accelerated aging and calorie restricted-like phenotypes were rescued to near completion, as shown in Figure 1A and B. While the mechanism of these rescues is currently unknown, we believe it to involve complementation between the two mutant proteins, so that together they can complete a required XPD activity relevant to aging whereas neither can on its own (Andressoo et al., 2006a).

Dominance of the TTD allele over the XPCS allele in UV sensitivity of the mouse skin.

We next tested the mechanistic hypothesis that defective NER of bulky lesions, and in particular TC-NER, is causative of these phenotypes by measuring parameters of DNA repair in CH vs. control mice. These studies were based on the logic that the XPD activity underlying the calorie-restricted phenotype should be rescued or partially rescued in the compound heterozygous state in which these phenotypes are complemented *in vivo*. To this end we performed an in-depth analysis of the effects of compound heterozygosity on the repair of UV-lesions in various cell-based and *in vivo* assays. Contrary to our hypothesis, not a single measure of TC-NER (or GG-NER) of UV induced lesions *in vivo* nor *in vitro* was complemented in CH animals. Instead, the TTD allele dominated in all of these readouts.

This means that defects in transcription-coupled DNA repair activity IN VITRO and *in vivo* are unlikely to be causative of progeroid/calorie restricted-like phenotypes.

Repair deficient TTD patients seem to be free of cancer, despite their NER defect, and occasionally mild photosensitivity (Botta et al., 1998; Itin and Pittelkow, 1990). This contrasts XPCS patients, which are highly cancer prone while having mutations in the same gene (Broughton et al., 1995; Lafforet and Dupuy, 1978; Lindenbaum et al., 2001). A similar situation is observed for XPCS mice, which are cancer prone (Andressoo et al., 2006b). In contrast with TTD mice which are found to be only mildly cancer prone when exposed to a high dose of UV-B (de Boer et al., 1999), however they have a lower spontaneous tumor incidence than WT mice (Wijnhoven et al., 2005). Explanations for the low UV sensitivity could be the thick cornified skin of TTD patients and mice, which will protect them for the UV irradiation, however there were also no eye lesions registered. Keratinocytes of the mouse TTD skin have a defect in later stage of terminal differentiation, and therefore tumor cell transformation could be different (de Boer et al., 1998). Another important explanation for the absence of cancer predisposition could be that TTD patients die young because of the severe ness of the disease; which does not allow time to develop skin tumors (de Boer et al., 1999). XPCS mice seem to be equally cancer prone as completely NER deficient XPA mice, despite the residual UDS activity. However this residual UDS does not mean that lesions are actually removed (Andressoo et al., 2006b; Theron et al., 2005). This is in contrast with previous data, where XPCS mice were found to be more cancer prone than XPA mice (Andressoo et al., 2006b). This could be due to differences in background of the mice compared in that experiment. Our data fit those of de Boer et al. (1999), he also found a higher tumor yield in TTD mice compared to WT mice. As with the acute UV sensitivity, CH mice show a tumor incidence comparable to TTD animals, only the tumor growth, and therefore tumor yield are different.

Dominance of the TTD allele over the XPCS allele in UV sensitivities of cells.

Looking at UV sensitivity of mutant MDFs, a same picture is drawn as for the comparison of UV sensitivity of the different mutant mice, with TTD cells mildly sensitive to UV compared to WT, and the CH MDFs a bit more sensitive, but comparable to TTD, while XPA and XPCS are evenly high sensitive to UV.

Dominance of the TTD allele over the XPCS allele in UV-induced repair characteristics.

As found earlier, unlike in TTD or other NER deficient cells UDS (a GG-NER readout) failed to correlate with lesion removal in XPCS cells. This is a property only found in XPCS cells, mutated in *XPD*. While XPCS cells fail to remove UV lesions, they still show an uncoupled cut and path mechanisms resulting in high γH2AX foci and 'phantom' UDS (Andressoo et al., 2006b; Theron et al., 2005). Meaning that the level of UDS is high despite the lack of removal of UV lesions. The UDS level of CH and TTD relative to WT cells may still suggest lesion removal but at a reduced rate. This also counts in a lower degree for the RRS experiment (a readout for TC-NER), however no residual activity is found in XPA and XPCS cells.

Dominance of the TTD allele in UV-induced stress response.

Down regulation of the IGF-1 axis, including the IGF-1 receptor on the level of mRNA, has been proposed as a potential general adaptive stress response mechanism that could be activated by a number of stressors including food scarcity or accumulation of DNA damage (Niedernhofer et al., 2006; Schumacher et al., 2008; van de Ven et al., 2007; van de Ven et al., 2006; van der Pluijm et al., 2007). Previously, the duration of reduction of IGF-1R steady state mRNA levels upon UV has been proposed to correlate with the severity of progeria *in vivo*, such that cells from double mutant CSB-XPA and ERCC1 animals with the most severe phenotypes had reduced IGF-1R for the longest period of time after UV (Garinis et al., 2009). Similarly, we found the greatest magnitude and duration of IGF-1R mRNA reduction after UV treatment to be associated with the most UV sensitive genotypes, XPCS and XPA. However, this correlation between *in vivo* progeroid/calorie-restricted like phenotypes and cellular IGF-1R down regulation after UV was lost in TTD and CH MDFs. Despite the rescue of *in vivo* progeriod/calorie-restricted like phenotypes in CH vs. TTD mice, the dampening of the IGF-1R mRNA level was similar in both and less pronounced and shorter than in XPA or XPCS cells. In other words, in these genotypes the mRNA level of IGF-1R nicely correlated with the UV sensitivity of the cells, but not with the progeroid phenotype. If our hypothesis that the XPD activity complemented in the compound heterozygous state is causally related to the progeroid/calorie restricted-like symptoms is correct, then defects in transcription-coupled DNA repair activity as measured in skin fibroblasts *in vitro* and skin *in vivo* are unlikely to be causative of progeroid/calorie restricted-like phenotypes.

Conclusion

Taken together, the severity of progeria and the presence of calorie restricted-like symptoms are tightly linked in the different NER mutant mouse models (XPCS=TTD >> CH > XPA=WT). The calorie restricted-like phenotypes observed in both XPCS and TTD mutant animals (reduced weight, improved insulin sensitivity) were partially reversed in XPCS/TTD or so-called CH mice, likely by interallelic complementation. The UV sensitivity tests performed on mice and cells *in vivo* (sunburn, cancer) and *in vitro* (UDS, RRS and γH2AX) did not show a rescue or complementation. Instead, the TTD allele was shown dominant over the XPCS allele with respect to these parameters. Therefore, defects in transcription-coupled DNA repair activity of bulky lesions *in vitro* and *in vivo* are unlikely to be causative of progeroid/calorie restricted-like phenotypes. However, interstrand cross-links are also found to be repaired by TC-NER (Ahn et al., 2004). We therefore hypothesize that it could well be defects in interstrand cross-link repair that is causative of progeriod/calorie restricted-like phenotypes. However, to test this hypothesis, more studies need to be conducted.

Materials and methods

Mice

C57Bl/6J mice (10-16 weeks of age with the start of the experiment) were kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12h light/12h dark) with 3-4 animals per cage before and after the experiment, and allowed free access to water and food (Hope Farms, Woerden, The Netherlands) except where noted differently. XPA, XPCS, TTD mice are used that have been described earlier (Andressoo et al., 2006b; de Boer et al., 1998; de Vries et al., 1995). CH mice are derived from crosses between mice that are heterozygous for XPCS and TTD. All animal experiments were performed with the approval of the appropriate ethical board.

Glucose tolerance and insulin sensitivity tests

Mice were fasted overnight prior to testing. Following baseline glucose determination from tail blood of restrained mice, animals were injected with a bolus of glucose 18 mmol/kg body weight (D (+)-glucose monohydrate, Fluka Biochemica, Sigma-Aldrich Chemie GmbH, Steinheim), or insulin 0.75 U/kg body weight (Insuline aspart rDNA, NovoRapid, Novo Nordisk A/S, Novo Allé, DK-2880 Bagsveard, Denmark) into the peritoneal cavity. Blood glucose determinations were performed as above at the indicated times following injection using a HemoCue glucose 201 RT blood glucose analyzer (HemoCue, Ängelholm, Sweden) according to the manufacturer's instructions.

MED

Ear skin was exposed to a broadband UVB radiation from a filtered (Schott-WG305 filter) Hanovia Kromayer Lamp Model 10S (Slought, UK), because ear skin does not contain fur. Ear thickness was measured prior to and 24 hrs after UVB exposure using an engineer's micrometer (Mitutoyo model 193-10, Veenendaal, The Netherlands). The lowest dose that was able to induce a significant swelling response (i.e edema reaction) was denoted to be the MED for that strain of mice.

UV-induced carcinogenesis

The depilated backs of 15 animals from each genotype were exposed to an incremental dose starting with 40 J/m^2 a day for XPCS and XPA (plus 5 CH animals) gradually increasing to 100 J/m^2. For CH, WT and TTD animals we started with 100 J/m^2 a day till 650 J/m^2 (250-400 nm). Timer-controlled American Philips F40 sunlamps were positioned 33 cm above the cage and yielded a dose rate of 13.3 J/m^2/min. Mice were checked for tumor appearance once a week. Skin tumors were routinely processed for histopathological examination.

Cells

Dermal fibroblasts were prepared from isolated tail skin of euthanized animals. Following mincing with a razor, tail skin was incubated overnight in a mixed gas incubator with 5% CO_2 and 3% O_2 in DMEM supplemented with 20% fetal bovine serum and antibiotics and 40 mg/ml Type II collagenase (Invitrogen, http://www.invitrogen.com) (van de Ven et al.,

2006). The next day, cells were resuspended, passaged through a 40 µm filter, washed, and replated in medium without collagenase.

Cell survival

Early passage MDFs were plated in duplo in six well plates a a density of 20,000 cells per well and treated with the appropriate dose of UV or IR the following day. Cells were counted 3-4 days after treatment using the Coulter Multisizer Z2 (Beckman Coulter, http://www.beckmancoulter.com), and plotted as a percentage of the total number of cells from the corresponding untreated cell line.

UDS

UV-induced UDS assays were performed on primary MDFs as described previously (de Waard et al., 2003; Vermeulen et al., 1994). In brief, coverslip-grown cells were exposed to 16 J/m^2 of 254 nm UV light and labeled with [^{3}H]thymidine. Repair capacity was quantified by grain counting after radiography and expressed as percentage of WT. Experiment repeated twice; each time at least 30 nuclei were counted.

RRS

UV-induced RRS assays were performed on primary MDFs as described previously (Ma et al., 1997). In short, coverslip-grown cells were exposed to 10 J/m^2 of 254 nm UV light, allowed recover for 16hr, labeled with [^{3}H]uridine, and processed for autoradiography. The relative rate of RNA synthesis was expressed as a percentage of WT. Experiment repeated twice; each time at least 30 nuclei were counted.

γH2AX

Cells were grown to confluency and checked if the primary MDFs stopped dividing by Ki67 staining. Cells were treated with 10 joules of UV and after 3 hrs washed twice with cold PBS and subsequently fixed and lysed by addition of PBS containing 2% formaldehyde for 15 min. and 2x 10 min. PBS with 0.1% Triton X-100 (Fluka). Cells were washed twice with cold PBS+. Then cells were incubated with the appropriate primary antibodies (diluted 1:1000 in PBS+) for 2 hr at room temperature, and washed three times and two times 10 min. with PBS with 0.1% Triton X-100. Incubation with secondary antibodies (diluted 1:1000 in PBS+) was performed at room temperature for 1 hr, followed by three wash steps and 2x 10 min. PBS with 0.1% Triton X-100. Cells were mounted in mounting medium containing DAPI-Vectashield (50/50). After staining at least 50 cells for each genotype were randomly chosen. Of these, total nuclear intensity of γH2AX signal was measured using Adobe Photoshop.

Quantitative real time PCR

Total RNA was extracted from the primary MDFs cells from a 6 well plate using TRIzol reagent (Invitrogen) and hexamerprimed cDNA synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a Bio-Rad MyIQ Real time PCR machine with Bio-Rad IQ5 program (Bio-Rad, http://www.bio-rad.com) with SYBR Green incorporation (primers: Tubg2 F:

CAGACCAACCACTGCTACAT, R: AGGGAATGAAGTTGGCCAGT, mHprt F: CGAAGTGT TGG ATACAGGCC, R: GGCAACATCAACAGGACTCC, IGF-1R F:ACGACAACACAACGTGCGT, R: AACGAAGCCATCCGAGTCA). Relative expression was calculated using the equation (Pfaffl, 2001) ratio = $(E_{target})^{\Delta CPtarget\ (control\text{-}sample)} / (E_{ref}))^{\Delta CPref\ (control\text{-}sample)}$ where E is calculated by $E = 10^{(-1/slope)}$. Each sample was tested in duplo at least two times.

References

Ahn, B., Kang, D., Kim, H., and Wei, Q. (2004). Repair of mitomycin C cross-linked DNA in mammalian cells measured by a host cell reactivation assay. Mol Cells 18, 249-255. Andressoo, J.O., Jans, J., de Wit, J., Coin, F., Hoogstraten, D., van de Ven, M., Toussaint, W., Huijmans, J., Thio, H.B., van Leeuwen, W.J., et al. (2006a). Rescue of progeria in trichothiodystrophy by homozygous lethal Xpd alleles. PLoS Biol 4, e322.

Andressoo, J.O., Mitchell, J.R., de Wit, J., Hoogstraten, D., Volker, M., Toussaint, W., Speksnijder, E., Beems, R.B., van Steeg, H., Jans, J., et al. (2006b). An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. Cancer Cell 10, 121-132.

Batty, D.P., and Wood, R.D. (2000). Damage recognition in nucleotide excision repair of DNA. Gene 241, 193-204.

Botta, E., Nardo, T., Broughton, B.C., Marinoni, S., Lehmann, A.R., and Stefanini, M. (1998). Analysis of mutations in the XPD gene in Italian patients with trichothiodystrophy: site of mutation correlates with repair deficiency, but gene dosage appears to determine clinical severity. Am J Hum Genet 63, 1036-1048.

Brooks, P.J. (2007). The case for 8,5'-cyclopurine-2'-deoxynucleosides as endogenous DNA lesions that cause neurodegeneration in xeroderma pigmentosum. Neuroscience 145, 1407-1417.

Broughton, B.C., Berneburg, M., Fawcett, H., Taylor, E.M., Arlett, C.F., Nardo, T., Stefanini, M., Menefee, E., Price, V.H., Queille, S., et al. (2001). Two individuals with features of both xeroderma pigmentosum and trichothiodystrophy highlight the complexity of the clinical outcomes of mutations in the XPD gene. Hum Mol Genet 10, 2539-2547.

Broughton, B.C., Thompson, A.F., Harcourt, S.A., Vermeulen, W., Hoeijmakers, J.H., Botta, E., Stefanini, M., King, M.D., Weber, C.A., Cole, J., et al. (1995). Molecular and cellular analysis of the DNA repair defect in a patient in xeroderma pigmentosum complementation group D who has the clinical features of xeroderma pigmentosum and Cockayne syndrome. Am J Hum Genet 56, 167-174.

de Boer, J., de Wit, J., van Steeg, H., Berg, R.J., Morreau, H., Visser, P., Lehmann, A.R., Duran, M., Hoeijmakers, J.H., and Weeda, G. (1998). A mouse model for the basal transcription/DNA repair syndrome trichothiodystrophy. Mol Cell 1, 981-990.

de Boer, J., van Steeg, H., Berg, R.J., Garssen, J., de Wit, J., van Oostrum, C.T., Beems, R.B., van der Horst, G.T., van Kreijl, C.F., de Gruijl, F.R.,

References

et al. (1999). Mouse model for the DNA repair/basal transcription disorder trichothiodystrophy reveals cancer predisposition. Cancer Res 59, 3489-3494.

de Laat, W.L., Jaspers, N.G., and Hoeijmakers, J.H. (1999). Molecular mechanism of nucleotide excision repair. Genes Dev 13, 768-785.

de Vries, A., van Oostrom, C.T., Hofhuis, F.M., Dortant, P.M., Berg, R.J., de Gruijl, F.R., Wester, P.W., van Kreijl, C.F., Capel, P.J., van Steeg, H., et al. (1995). Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature 377, 169-173.

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

Garinis, G.A., Uittenboogaard, L.M., Stachelscheid, H., Fousteri, M., van Ijcken, W., Breit, T.M., van Steeg, H., Mullenders, L.H.F., van der Horst, G.T.J., Brüning, J.C., et al.,. (2009) Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. Nat Cell Bio (article in press)

Giglia-Mari, G., Coin, F., Ranish, J.A., Hoogstraten, D., Theil, A., Wijgers, N., Jaspers, N.G., Raams, A., Argentini, M., van der Spek, P.J., et al. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat Genet 36, 714-719.

Hanawalt, P.C., and Spivak, G. (2008). Transcription-coupled DNA repair: two decades of progress and surprises. Nat Rev Mol Cell Biol 9, 958-970.

Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. Nature 411, 366-374.

Itin, P.H., and Pittelkow, M.R. (1990). Trichothiodystrophy: review of sulfur-deficient brittle hair syndromes and association with the ectodermal dysplasias. J Am Acad Dermatol 22, 705-717.

Lafforet, D., and Dupuy, J.M. (1978). [Photosensitization and DNA repair. Possible nosologic relationship between Xeroderma pigmentosum and Cockayne's syndrome]. Arch Fr Pediatr 35, 65-74.

Lehmann, A.R. (2001). The xeroderma pigmentosum group D (XPD) gene: one gene, two functions, three diseases. Genes Dev 15, 15-23.

Lehmann, A.R., Arlett, C.F., Broughton, B.C., Harcourt, S.A., Steingrimsdottir, H., Stefanini, M., Malcolm, A., Taylor, R., Natarajan, A.T., Green, S., et al. (1988). Trichothiodystrophy, a human DNA repair disorder with heterogeneity in the cellular response to ultraviolet light. Cancer Res 48, 6090-6096.

References

Lindenbaum, Y., Dickson, D., Rosenbaum, P., Kraemer, K., Robbins, I., and Rapin, I. (2001). Xeroderma pigmentosum/cockayne syndrome complex: first neuropathological study and review of eight other cases. Eur J Paediatr Neurol 5, 225-242.

Ma, G.T., Roth, M.E., Groskopf, J.C., Tsai, F.Y., Orkin, S.H., Grosveld, F., Engel, J.D., and Linzer, D.I. (1997). GATA-2 and GATA-3 regulate trophoblast-specific gene expression in vivo. Development 124, 907-914.

Nakura, J., Ye, L., Morishima, A., Kohara, K., and Miki, T. (2000). Helicases and aging. Cell Mol Life Sci 57, 716-730.

Niedernhofer, L.J., Garinis, G.A., Raams, A., Lalai, A.S., Robinson, A.R., Appeldoorn, E., Odijk, H., Oostendorp, R., Ahmad, A., van Leeuwen, W., et al. (2006). A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 444, 1038-1043.

O'Driscoll, M., Ruiz-Perez, V.L., Woods, C.G., Jeggo, P.A., and Goodship, J.A. (2003). A splicing mutation affecting expression of ataxia-telangiectasia and Rad3-related protein (ATR) results in Seckel syndrome. Nat Genet 33, 497-501.

Oh, K.S., Khan, S.G., Jaspers, N.G., Raams, A., Ueda, T., Lehmann, A., Friedmann, P.S., Emmert, S., Gratchev, A., Lachlan, K., et al. (2006). Phenotypic heterogeneity in the XPB DNA helicase gene (ERCC3): xeroderma pigmentosum without and with Cockayne syndrome. Hum Mutat 27, 1092-1103.

Park, J.Y., Cho, M.O., Leonard, S., Calder, B., Mian, I.S., Kim, W.H., Wijnhoven, S., van Steeg, H., Mitchell, J., van der Horst, G.T., et al. (2008). Homeostatic imbalance between apoptosis and cell renewal in the liver of premature aging Xpd mice. PLoS ONE 3, e2346.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H., Chambon, P., and Egly, J.M. (1993). DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. Science 260, 58-63.

Schumacher, B., van der Pluijm, I., Moorhouse, M.J., Kosteas, T., Robinson, A.R., Suh, Y., Breit, T.M., van Steeg, H., Niedernhofer, L.J., van Ijcken, W., et al. (2008). Delayed and accelerated aging share common longevity assurance mechanisms. PLoS Genet 4, e1000161.

Stefanini, M., Giliani, S., Nardo, T., Marinoni, S., Nazzaro, V., Rizzo, R., and Trevisan, G. (1992). DNA repair investigations in nine Italian patients affected by trichothiodystrophy. Mutat Res 273, 119-125.

References

Theron, T., Fousteri, M.I., Volker, M., Harries, L.W., Botta, E., Stefanini, M., Fujimoto, M., Andressoo, J.O., Mitchell, J., Jaspers, N.G., et al. (2005). Transcription-associated breaks in xeroderma pigmentosum group D cells from patients with combined features of xeroderma pigmentosum and Cockayne syndrome. Mol Cell Biol 25, 8368-8378.

Tornaletti, S., and Hanawalt, P.C. (1999). Effect of DNA lesions on transcription elongation. Biochimie 81, 139-146.

van de Ven, M., Andressoo, J.O., Holcomb, V.B., Hasty, P., Suh, Y., van Steeg, H., Garinis, G.A., Hoeijmakers, J.H., and Mitchell, J.R. (2007). Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging. Mech Ageing Dev 128, 58-63.

van de Ven, M., Andressoo, J.O., Holcomb, V.B., von Lindern, M., Jong, W.M., De Zeeuw, C.I., Suh, Y., Hasty, P., Hoeijmakers, J.H., van der Horst, G.T., et al. (2006). Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. PLoS Genet 2, e192.

van der Pluijm, I., Garinis, G.A., Brandt, R.M., Gorgels, T.G., Wijnhoven, S.W., Diderich, K.E., de Wit, J., Mitchell, J.R., van Oostrom, C., Beems, R., et al. (2007). Impaired genome maintenance suppresses the growth hormone–insulin-like growth factor 1 axis in mice with Cockayne syndrome. PLoS Biol 5, e2.

van Hoffen, A., Kalle, W.H., de Jong-Versteeg, A., Lehmann, A.R., van Zeeland, A.A., and Mullenders, L.H. (1999). Cells from XP-D and XP-D-CS patients exhibit equally inefficient repair of UV-induced damage in transcribed genes but different capacity to recover UV-inhibited transcription. Nucleic Acids Res 27, 2898-2904.

Vermeulen, W., Scott, R.J., Rodgers, S., Muller, H.J., Cole, J., Arlett, C.F., Kleijer, W.J., Bootsma, D., Hoeijmakers, J.H., and Weeda, G. (1994). Clinical heterogeneity within xeroderma pigmentosum associated with mutations in the DNA repair and transcription gene ERCC3. Am J Hum Genet 54, 191-200.

Wijnhoven, S.W., Beems, R.B., Roodbergen, M., van den Berg, J., Lohman, P.H., Diderich, K., van der Horst, G.T., Vijg, J., Hoeijmakers, J.H., and van Steeg, H. (2005). Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. DNA Repair (Amst) 4, 1314-1324

7

GADD45a demethylation and its function in DNA repair

~

Marieke van de Ven, Wendy Toussaint,
Nils Wijgers, Wim Vermeulen,
Jan H.J. Hoeijmakers, James R. Mitchell

Abstract

DNA methylation is a covalent postreplicative modification of genomic DNA that induces and maintains a transcriptionally silent state of gene expression. It has been implicated in development, differentiation and cancer. GADD45a (Growth arrest and DNA damage inducible gene) has recently been shown to be a key regulator of active DNA demethylation (Barreto et al, 2007). The GADD45a gene is stress inducible and functions in numerous biological processes, including the cell cycle, senescence, apoptosis and NER (nucleotide excision DNA repair) (Hollander and Fornace, 2002), but its mechanistic role in these processes remains unclear. Barreto et al. (2007) found that GADD45a is targeted to specific sites of demethylation and recruits the DNA repair machinery through core NER components XPG and XPB, thus linking the processes of UV-induced DNA demethylation and DNA repair through excision of methylated cytosines. We explored the genetics of GADD45a-dependent demethylation using cell lines from a variety of NER-deficient mice. We found that all core NER components tested were required for GADD45a demethylation activity. Furthermore, GADD45-dependent demethylation was absent in CSA- and CSB-deficient cells but not XPC-deficient cells, suggesting initiation by the transcription-coupled rather than the global genome branch of the NER pathway. We also found that previously identified 'phantom UDS' observed upon UV in XPCS cell lines is partially dependent on GADD45, and thus likely reflects NER activity at sites of DNA methylation rather than removal of DNA damage.

Introduction

Mammalian cells are continuously exposed to damage by genotoxic agents against which they have evolved a variety of defense mechanisms in order to maintain their genomic integrity. In dividing cells, key components of this defense mechanism are cell cycle arrest checkpoints, which give cells time to repair DNA, and programs of activated cell death in cases in which damage is irreparable (Hartwell and Weinert, 1989). In different screens looking for genes that were induced by DNA damage, and therefore could have protective roles, Growth Arrest and DNA Damage inducible gene GADD45, MyD118 and CR6 (later named as GADD45a, GADD45b and GADD45g respectively) were found (Abdollahi et al., 1991; Fornace, 1992; Fornace et al., 1992; Zhang et al., 1999). All GADD45 proteins encode for a small 18 kDa acidic nuclear protein with a 55-57% amino acid overlap with each other. GADD45a can be induced by DNA damaging agents including UV, ionizing radiation (IR) and MMS, or by growth arresting treatments such as serum depletion from the medium (Fornace et al., 1992; Papathanasiou and Fornace, 1991).

The elucidation of the molecular function (or functions) of GADD45a has been a longstanding puzzle. Based on its structure, very little is revealed about its function. Still, a number of potential roles have been proposed. One is in cell cycle control and apoptosis via its interaction with MEKK4, an upstream activator of the p38/JNK kinases. Binding to the N-terminal domain of MEKK4 by GADD45a stimulates MEKK4 and mediates activation of the p38/JNK pathway (Hildesheim et al., 2002; Takekawa and Saito, 1998; Wang et al., 1999). Another is in DNA repair through interactions with proliferating cell nuclear antigen (PCNA) (Jung et al., 2007; Smith et al., 2000) in both NER and BER as described below.

Nucleotide excision repair (NER) is a versatile DNA repair mechanism in which damage recognition, local opening of the DNA helix around the lesion, damage excision and gap-filling are successive steps (Aboussekhra et al., 1995; de Laat et al., 1999). NER is composed of two sub pathways that differ in the way lesions are recognized. The first NER pathway, which is named transcription coupled repair (TC-NER), starts with the proteins CSB and CSA assisting the polymerase II (RNApolII) in dealing with bulky lesions that provide a steric block to transcription. The precise role of CSA and CSB in this pathway remains elusive, however CSB could act as a chromatin-remodeling factor allowing the other NER factors to access the lesion for repair so transcription can proceed (Citterio et al., 2000; Sarasin and Stary, 2007). Both CSA and CSB bind to the hypophosphorylated RNApolII that is recruited to the transcription initiation complex (Groisman et al., 2003; Rockx et al., 2000). Lesions located anywhere in the genome are removed by the second NER pathway, known as global genome repair (GG-NER) in which the XPC complex carries out the first step by recognizing mutations throughout the genome. The rest of the NER proteins are indispensable for both GG-NER and TC-NER like XPA. Its role is currently described as damage verification, and is thought to be vital for correct alignment of NER proteins like the nucleases ERCC1 and XPG (Hoeijmakers, 2001a). Different mutations in various components of the NER pathway give rise to distinct

clinical syndromes, including xeroderma pigmentosum (XP), Cockayne syndrome (CS), or XP combined with Cockayne syndrome (XPCS), a segmental premature aging or progeroid disease with a high skin cancer risk, and trichothiodystrophy (TTD), another progeroid disorder with additional scaling skin and fragile hair.

GADD45a is not an enzymatic component of the NER pathway and is not required *in vitro* for the reaction to occur. However, GADD45a deficient cells display an enhanced UV sensitivity (Smith et al., 2000) and a reduction in UDS (unscheduled DNA synthesis) following UV irradiation, indicative of a deficiency in GG-NER. One way in which GADD45a may function in DNA repair is via its interaction with PCNA, a protein involved in loading and stimulation of DNA polymerase. In this way GADD45a may participates both in NER as well as long patch base excision repair (BER) (Smith et al., 2000; Xia et al., 2005). Via PCNA it also may participate in the coupling between chromatin assembly and DNA repair (Smith et al., 2000). On the other hand, GADD45a is also found binding to UV-damaged chromatin and improving accessibility of DNA repair proteins to the sites of damage (Carrier et al., 1999; Smith et al., 2000).

Recently a novel role of GADD45 in DNA demethylation was reported. Substrates of GADD45-dependent demethylation include *in vitro* methylated reporter plasmids, promoter-specific CpG islands and global DNA following UV irradiation (Barreto et al 2007). Furthermore, GADD45a interacts genetically and biochemically with components of the NER pathway. Thus although GADD45a itself has no homology to known demethylases, its mechanism of action is proposed to be through recruitment of the NER machinery via repair proteins including XPG and XPB. The methylated cytosines are then excised via the NER cut and patch mechanism and replaced with unmethylated nucleotides. A subsequent study was unable to find any role of GADD45a in DNA demethylation (Jin et al., 2008).

We therefore wanted to test if the NER proteins are in fact required for DNA demethylation in a GADD45-dependent reaction, and if so, what those genetic requirements were and which NER recognition pathway functioned in the demethylation reaction. Our data confirmed a role for essential NER components *Xpa* and *Ercc1* in a GADD45a-dependent demethylation reaction and implicated essential TC-NER components *Csa* and *Csb* as required. Furthermore, our data expand the genetic requirements to *Xpd*, and show that *Xpd* mutations with different disease susceptibilities behave differently with respect to GADD45-dependent DNA demethylation.

Results

To test which components of the NER machinery are required for GADD45-dependent demethylation as reported previously, we made use of cells derived from transgenic mice with deletions of individual components of the NER pathway. Homozygous mutant cell lines (*Xpa* $^{-/-}$, *Ercc1*$^{-/-}$, *Xpc*$^{-/-}$, *Csa* $^{-/-}$ and *Csb* $^{-/-}$) will for simplicity hereafter be referred to as XPA, ERCC1, XPC, CSA and CSB. All experiments were done in spontaneously transformed

MEF cell lines, unless noted otherwise. We first looked at the ability of NER-defcient vs. control cell lines to reactivate an *in vitro* methylated luciferase reporter plasmid. To this end we co-transfected NER deficient and control cell lines with an *in vitro*-methylated plasmid containing a firefly luciferase gene under the control of a viral promoter. A plasmid encoding renilla luciferase under control of the constitutive thymidine kinase promoter was included as a transfection control. GADD45-dependence was tested by transfecting each well either with the murine GADD45a cDNA under control of a constitutive promoter or with the corresponding empty expression vector. Two days after transfection, cells were harvested and the relative light units (RLU) measured (Figure 1). The RLU is depicted as a ratio between the outcome with vs. without the GADD45a plasmid following normalization to the renilla activity. In WT cells we observed a 2.2 fold increase in firefly luciferase upon co-transfection with GADD45 relative to control plasmid, indicative of promoter demethylation and resulting transcriptional activation. In cells deficient in the core NER components XPA and ERCC1 cells, we observed a ratio of RLU around one, indicating that these essential NER genes are also required for GADD45a dependent demethylation (t-test WT with XPA $p = 0.033$, ERCC1 $p = 0.016$).

We next asked if demethylation by GADD45a in this assay is dependent on either of the known NER subpathways, TC-NER or GG-NER, which differ only in the way a lesion is recognized. In XPC cells, which are deficient in GG-NER, the ratio of RLU was similar to WT. However, TC-NER deficient CSA and CSB cells behaved like the other NER deficient cell lines (t-test WT with CSB $p = 0.005$, CSA $p = 0.016$ and XPC $p = 0.806$; t-test XPC with XPA $p = 0.026$, ERCC1 $p = 0.009$, CSB $p = 0.011$, CSA $p = 0.006$). These data confirm the requirement for core NER components in GADD45a dependent methylated plasmid reactivation assay, and further suggest that the initiating event requires TC-NER but not GG-NER. As a control, we also examined the ability of GADD45a to activate the unmethylated plasmid (Figure 1B). Although the magnitude of initial expression from the unmethlated plasmid was higher than the *in vitro* methylated one, it still responded to exogenous GADD45a, likely because unmethylated DNA plasmids become methylated following transient transfection (Escher et al., 2005). Using XPA and CSB cells, we saw similar trends in the unmethlylated as the methylated plasmid.

We next looked at two different mutants of XPD, a core helicase component of the TFIIH complex required for both GG and TC-NER. *XPD* is an interesting gene because mutations in *XPD* can give rise to different diseases. Here we used cells of mice with a R722W mutation that gives rise in mice and humans to TTD a segmental progeria with scaling skin and fragile hair (de Boer et al., 1999). And a G602D mutation, that gives rise in humans, as well as in mice, to the disease XPCS, that is the XP combined with CS, an accelerated segmental progeroid disease with a high risk of early onset of skin cancer (Andressoo et al., 2006). While these cell lines are both mutated in the same gene and the syndromes have overlapping symptoms, the cell lines give very different results (t-test between WT and XPCS cells $p = 0.668$, TTD $p = 0.011$) (Figure 1C). TTD cells showed similar response as XPA cells, indicating that wildtype XPD is required for GADD45a-dependent and NER-dependent DNA demethylation as predicted from the XPA and

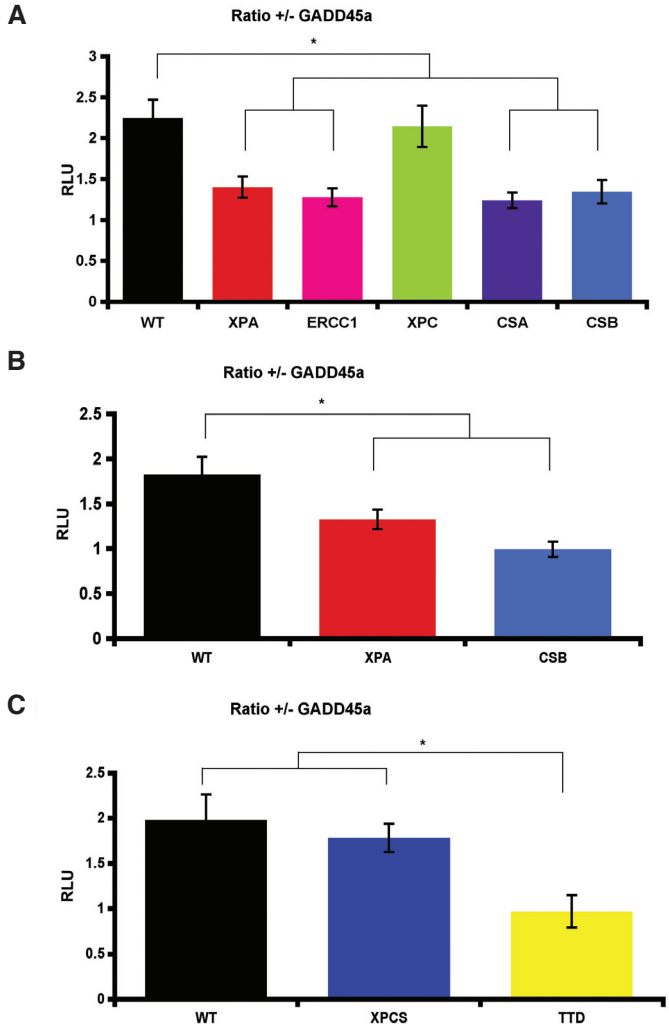


Figure 1, Demethylation by GADD45a involves DNA repair.

A. XPA, ERCC1, CSA and CSB but not XPC are required for GADD45a function in the plasmid reactivation assay. There was no difference between the ratio of relative light units (RLU) in the Luciferase reporter assay in spontaneously transformed cell lines (n = 2 per genotype), except for XPC, that were transiently transfected with methylated CMV promoter in combination with an empty plasmid or plasmid with GADD45a. Error bars indicate standard error of the mean (SEM) (* $p < 0.05$).

B. Transfection with an unmethylated CMV promoter in combination with an empty plasmid or plasmid with GADD45a gave the same result as transfection with a methylated CMV promoter. Error bars indicate SEM (* $p < 0.05$).

C. Different XPD mutants have different requirements for GADD45a function in the plasmid reactivation assay. No difference was observed in RLU in TTD cell lines, but a clear difference in XPCS cell lines (n = 2 per genotype). Error bars indicate SEM (* $p < 0.05$).

ERCC1 results. This is consistent with reduced NER activity in these cells. However, XPCS cells with a different point mutation in *Xpd* gave the same result as XPC and WT cells, inconsistent with the complete lack of NER in these cells.

XPCS cells have the unusual property of high DNA repair synthesis following UV, but without actually repairing the lesions (Andressoo et al., 2006; Berneburg et al., 2000). While in cells completely lacking NER, such as XPA, UV-UDS is reduced to background levels, human XPD-XPCS cells were found to retain a significant percent of their UDS despite their inability to actually remove any of the lesions. A similar phenomenon was observed in cells from the XPCS mouse model dubbed 'phantom' UDS. Whether or not these breaks occur at sites of DNA damage, transcription initiation or both remains controversial (Andressoo et al., 2006; Berneburg et al., 2000). In order to gain further insight into the nature of these breaks, we tested their dependence on GADD45a. To do this, we pursued a knockdown strategy to reduce GADD45a levels in WT and mutant cells and subsequently monitor the GADD45a activity. First, we determined the efficiency of anti-GADD45a siRNA in our various cell lines using the GADD45a-dependent methylated plasmid reactivation assay (Figure 2A). The RLU was significantly decreased to one (WT siRNA Control vs. GADD45a siRNA $p = 0.015$, XPCS siRNA Control vs. siRNA GADD45a $p = 0.030$). Then we checked the lowering of GADD45a expression after transfection with siRNA against GADD45a in primary MEF cell lines via RT-PCR (Figure 2B). In all cell lines (WT, XPA, XPCS, TTD) endogenous GADD45a expression went down significantly compared to the cell lines transfected with control siRNA ($p = 0.032$, 0.005, 0.015, $1{,}37 \times 10^{-5}$, respectively), while levels of GADD45a RNA in untransfected cells were not significantly different among the different cell lines.

Next we performed UV-UDS assays on these cells following anti-GADD45a siRNA treatment to reveal if the unexpected GADD45a-dependent demethylation is linked to 'phantom' UDS found in XPCS cells (Andressoo et al., 2006; Theron et al., 2005). Primary MEF cell lines were co-transfected with GADD45a siRNA or control siRNA together with a green fluorescent indicator (siGLO). Two days later, cells where FACS sorted for the presence of siGLO and plated on cover slips for UV-UDS (Figure 2C). UDS counts measured in WT cells transfected with the control siRNA were set as 100% (Andressoo et al., 2006; de Boer et al., 2002). Note the rather high UDS in both XPCS and TTD. This relative high level of UDS in TTD is linked to partial damage removal (de Boer et al., 2002). However, in XPCS residual UDS is uncoupled to actual UV-lesion removal (Berneburg et al., 2000). This so called 'phantom' UDS decreases significantly in XPCS cells transfected with GADD45a siRNA ($p = 3.95 \times 10^{-10}$), whereas GADD45a reduction in WT and TTD cells does not show a decrease in UDS. Thus a significant fraction of the breaks seen after UV damage in XPCS cells (Theron et al., 2005) are dependent on GADD45a and likely related to DNA demethylation unrelated to the repair of UV lesions.

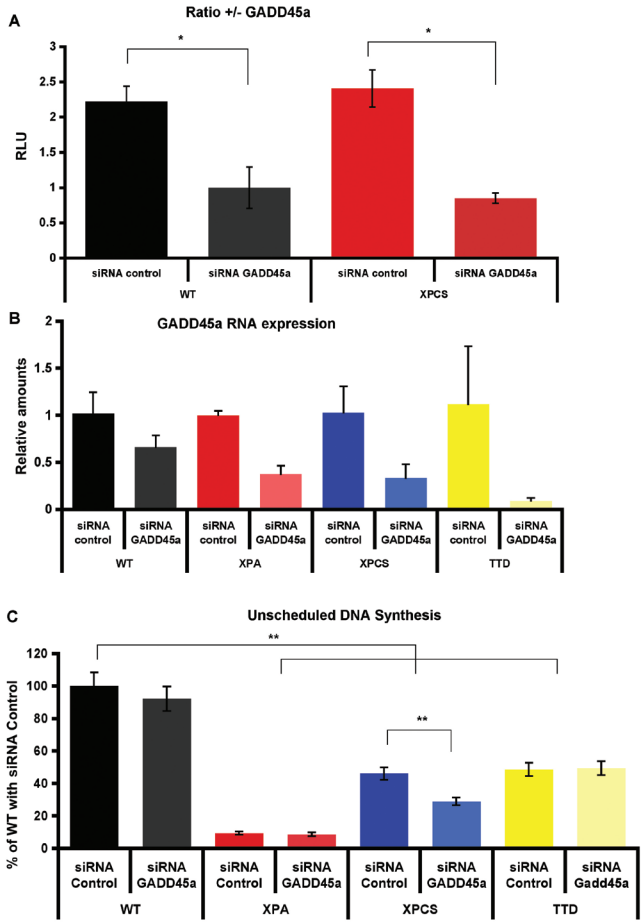


Figure 2. GADD45a down regulation by siRNA and measurements of repair.

A. Reduction of RLU after transfection with siRNA against GADD45a. The ratio of RLU with vs. without GADD45a is back to one if the cells that are transfected with a methylated CMV-luciferase plasmid are co-transfected with siRNA against GADD45a. Error bars indicate SEM (* $p < 0.05$).

B. Lower expression of endogenous GADD45a after transfection with siRNA against GADD45a. Expression of GADD45a RNA is significantly lower ($p < 0.05$) in the different spontaneously transformed cell lines (WT, XPA, XPCS and TTD) after transfection with siRNA against GADD45a compared to the cells transfected with the control siRNA. The amount of endogenous GADD45a RNA expression was not significantly different between the untranfected cell lines (data not shown). Error bars indicate standard deviation (SD).

C. UV-induced DNA repair synthesis capacity in mutant cell lines is considered a measure for GG-NER. UDS is shown in spontaneously transformed MEFs of the different genotypes transfected with Control siRNA or siRNA against GADD45a. UDS was significantly lower in all the mutant cell lines compared to the WT cell line. Transfection with GADD45a siRNA lowered the UDS in the XPCS cell line compared to the one transfected with Control siRNA as well as the WT, although the later did not reach the level of statistical significant ($p = 0.1753$). Error bars indicate SEM (** $p < 0.01$).

Discussion

GADD45a has long been associated with DNA repair via multiple pathways, including NER, but its exact biochemical role in repair has remained a mystery. Early research indicated that GADD45a has functions in DNA repair via BER (Xia et al., 2005), and NER (Smith et al., 1994; Vairapandi et al., 1996). Further research on this subject showed that GADD45a functions in GG-NER, one of the two sub pathways of NER (Amundson et al., 2002; Smith et al., 2000), because reduction of GADD45a protein expression has been found to reduce GG-NER, as measured by the significantly lower GG-NER in GADD45a-null cells have (UDS ~70% of WT). Interestingly, TC-NER as measured by a strand-specific repair assay, was equivalent to WT levels (Smith et al., 1994; Smith et al., 2000). GADD45a can also recognize damaged chromatin and could potentially modify DNA accessibility after UV (Carrier et al., 1999). It has also an association with chromatin assembly after NER via PCNA (Aboussekhra and Wood, 1995; Vairapandi et al., 1996).

More recently, GADD45a and NER have been connected by the process of DNA demethylation. Barreto et al. (2007) found that two proteins that work in NER, XPB and XPG are necessary for GADD45a dependent demethylation of DNA, including at promoters during development and likely in response to stress. This result was contested in a study of Jin et al (2008). Using the same HEK293 cell line, they found no effect of exogenous GADD45a on the expression of a methylated plasmid based reporter gene expression.

In this report we confirmed the data of Barreto et al. (2007) by showing that transfection of a mouse GADD45a construct has an effect on expression of a reporter gene under control of the CmV promoter in an *in vitro* methylated plasmid in WT cells. We further confirmed a role for NER in this GADD45a dependent activity in cells that are defective in NER proteins other than XPB and XPG. In our hands also XPA, CSB, CSA as ERCC1 are necessary for demethylation that is GADD45a dependent, while XPC is not. This suggests that demethylation relevant in the methylated plasmid reporter assay goes via the TC-NER pathway, one of two pathways in which to initiate the NER reaction. Both pathways are essentially the same except for the initial damage recognition step.

Former studies already suggested a model whereby demethylation of CpG rich DNA sequences resembled NER activity such that methylated cytosines are excised and replaced by unmethylated cytosines, the same way damaged nucleotides would be substituted (Barreto et al., 2007). Our data supports this model and shows that DNA promoter demethylation needs TC-NER proteins CSA and CSB, and late NER proteins like ERCC1, but not the GG-NER-specific protein XPC (Model Figure 3A).

In the second experiment we clearly established that the majority of the NER factors participate in GADD45a dependent demethylation, surprisingly however, different mutations in the *Xpd* gene have an entirely differential effect on promoter demethylation that is GADD45a dependent. The TTD causing mutation in *Xpd* gave a similar absence

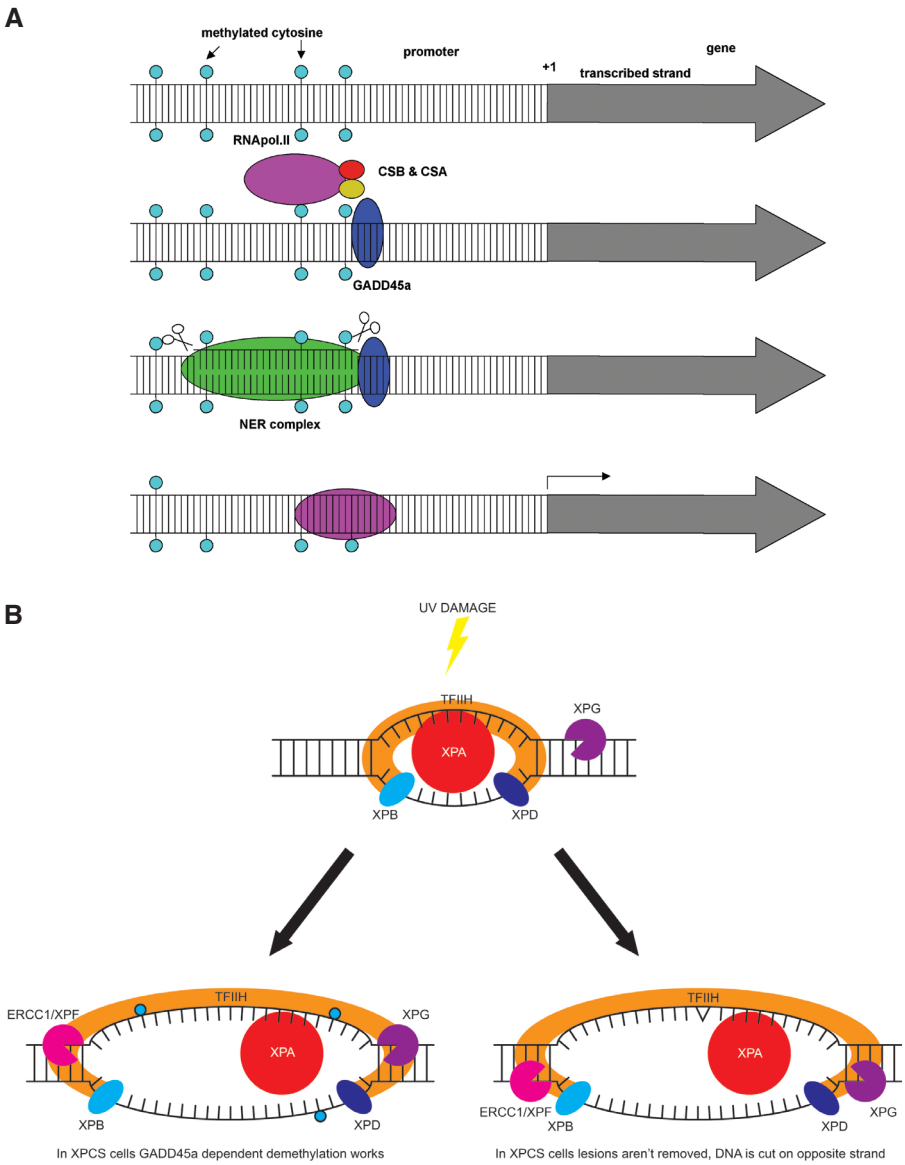


Figure 3. Model

A. Methylated cytosines block transcription by allowing binding of transcriptional repressors, therefore RNA polymerase II can not bind to the DNA. GADD45a recruits CSA and CSB and the rest of the NER complex and excises the methylated cytosine and replace it with unmethylated ones.
B. In XPCS cells GADD45a demethylation works as in WT cells, however removal of lesions doesn't work.

of demethylation as in other NER mutants tested (except for XPC cells). Contrary to our expectations the XPCS causing mutation showed normal GADD45a dependent demethylation. We did not expected this outcome, firstly because XPD is necessary for GADD45a dependent demethylation based on the results in the TTD cell lines, and secondly because the other helicase that unwinds the DNA on the other site of the damage in NER, XPB (Evans et al., 1997) is also necessary in the active demethylation by GADD45a (Barreto et al., 2007). Hence, contrary to our expectations, XPCS cell lines give high levels of RLU, meaning that there is GADD45a dependent demethylation in these cell lines.

Earlier work in our lab and other labs on XPCS cells show that these cells behave differently as expected in other experiments too, like in the experiments that measure repair characteristics of cell lines. XPCS cell lines show 30-40% UDS level compared to WT levels after UV. However there is no evidence for repair, since lesions remained for up to 8 hr following UV treatment (Andressoo et al., 2006; Theron et al., 2005). Thus unlike WT cells, the relative high level of repair synthesis (UDS) observed in XPCS cells does not represent actual lesion removal. Theron et al (2005) even found that these 'repair breaks' are distributed throughout the nucleus after local damage, and that the number of 'breaks' is substantially diminished after treating the cells with transcription inhibitors following UV treatment. They therefore state a model whereby there is induction of breaks by NER close to the sites of transcription initiation in XPCS cell lines. In contrast, we found (Andressoo et al., 2006) that UV-induced 'breaks' were confined to locally UV-induced areas, not expanding to the rest of the nucleus. These findings made us to propose a model in which the NER induced incision is targeted to the wrong (non-damaged) strand, thereby inducing DNA repair synthesis (UDS) without actual damage removal. To investigate if the high level of GADD45a dependent demethylation, that presumably occurs via NER dependent cutting and pasting of nucleotides, could have a correlation with the relative high level of UDS in XPCS cell lines, we first checked γH2AX levels in the different NER deficient MEFs following UV. These levels are indicative of DNA breaks by DNA repair, and were identical to those found previously in MEF cell lines as demonstrated before (Andressoo et al., 2006) (data not shown). Functioning of the siRNA was examined (Figure 2A and B), whereupon the UDS with siRNA against GADD45a could be performed. Surprisingly, the amount of UDS reduces significantly in a XPCS cell line after transfection with GADD45a siRNA. There is still residual UDS ~30% of WT, so the reduction of UDS is only partly. Hence, some of the UDS observed upon UV is not repair of lesions, but likely GADD45-dependent removal of methylated CpGs. This is likely a very small fraction of the observable UDS in WT cells, and therefore also a small, but significant fraction of the so called 'phantom UDS' in XPCS cells ~18%. The rest of the 'phantom' UDS can still be cutting on the opposite strand across from a lesion, because no repair occurs (Model Figure 3B)

In eukaryotes, methylation of DNA is mostly on the cytosines of CpG dinucleotides and it induces and maintains a transcriptional silent state of genes. DNA promoter methylation can directly interfere with transcriptional factors that require contact with cytosine in the major groove of the double helix of promoter DNA (Bird and Wolffe, 1999). However

hypermethylation downstream of promoter regions, in the gene itself, does not block transcription elongation (Gonzalgo and Jones, 1997; Jones, 1999). How DNA methylation occurs is known, but how the demethylation of DNA occurs is a matter of debate. One potential mechanism involves RNA and a possible ribozyme-mediated DNA demethylation (Bhattacharya et al., 1999; Weiss et al., 1996), although this was later contested (Swisher et al., 1998). Later Bhattacharya et al. (1999) found a human protein with DNA demethylase activity MBD2b, although other labs failed to confirm the demethylase activity of this protein (Ng et al., 1999; Wade et al., 1999). Two demethylases, hABH2 and hABH3, are found that repair cytotoxic 1-methyladenie and 1-methylcytosine in DNA and single stranded RNA by methyl group hydroxylation, followed by a retro-aldol reaction (Aas et al., 2003; Duncan et al., 2002). However, a global demethylase that promotes epigenetic gene activation was never found. Therefore it is interesting that Barreto et al. (2007) found that GADD45a is expressed in Xenopus oocytes and required for site specific oct4 demethylation in these cells. Furthermore, GADD45a over expression induces global DNA hypomethylation in HEK293 and 3T3 cells.

In the future it will be essential to determine if NER is also required for global DNA demethylation observed upon acute stress such as UV. Finally, a role for DNA methylation status in the etiology of the various NER disorders must now be considered.

Materials and methods

Cells

Mouse embryonic fibroblasts (MEFs) were cultured in a 50:50 mix of DMEM:Ham's F10 supplemented with 10% fetal bovine serum and antibiotics (pen/strep) in a mixed gas incubator with 5% CO_2 and 3% O_2 at 37°C on TC petridishes (Greiner Bio-One). Cells were trypsinized before they reached 80% confluence.

Transfection

Transient DNA transfections were carried out using FuGENE6 transfection reagent (Roche) following the manufacturers instructions. MEFs were transfected with ON-TARGETplus SMARTpool siRNA against mouse GADD45a (L-042505-00 Dharmacon) or Non-Targeting Control siRNA #5 (D-001210-05-20 Dharmacon, together with siGLO, green transfection indicator (D-001630-01-05 Dharmacon) using DharmaFECT 1 Transfection reagent (T-2001-02 Dharmacon) following the manufacturers instruction. For luciferase reporter assay, DNAs (with or without siRNAs) were transfected 12h after plating the cells.

Luciferase reporter assay

Dual-luciferase reporter assays (E1960 Promega) were performed on spontianiously immortalized MEFs 48 hr following transient transfection of MEFs carried out in 96-well plates with ≈ 10.000 cells per well, containing 40 ng GADD45a cDNA clone in pCMV-SPORT6 vector (IRAVp968FO326D, RZPD) or empty pCMV-SPORT6 vector, 20 ng Tk-firefly luciferace control plasmid and 20 ng Renilla luciferase reporter plasmid (Andressoo et al., 2006) with *in vitro* methylated CmV promoter, using the CpG DNA methylase (M.Ssi methylase), in Optimem. Each sample was performed in two cell lines for the different genotypes at least in triplicate. Each experiment was repeated at least three times.

UDS

UV-induced UDS assays were performed on primary MEFs as described previously (de Waard et al., 2003; Vermeulen et al., 1994). Cells were transfected with siGLO and GADD45a siRNA 24hr later the cells were FACS sorted for green fluorescence and plated back in a 6 wells plate (Greiner Bio-One) with coverslips. UDS was performed 12hr later and repeated twice; each time at least 30 nuclei were counted.

γH2AX

Plates were grown completely full and checked if the primary MEFs stopped dividing by Ki67 staining. If cells stopped they were treated with 10 joules of UV and after 3 hrs washed twice with cold PBS and subsequently fixed and lysed by addition of PBS containing 2% formaldehyde for 15 min. and 2x 10 min. PBS with 0.1% Triton X-100 (Fluka). Cells were washed twice with cold PBS+ Then cells are incubated with the appropriate primary antibodies (diluted 1:1000 in PBS+) for 2 hr at room temperature, and washed three times and two times 10 min. with PBS with 0.1% Triton X-100. Incubation with secondary antibodies (diluted 1:1000 in PBS+) was performed at room temperature for 1 hr followed by three wash steps and 2x 10 min. PBS with 0.1% Triton

X-100. Cells were mounted in mounting medium containing DAPI-Vectashield (50/50). After staining at least 50 cells for each genotype were randomly chosen. Of these, total nuclear intensity of γH2AX signal was measured using Adobe Photoshop.

Quantitative real-time PCR

Total RNA was extracted from the primary MEFs cells from a 6 well plate using TRIzol reagent (Invitrogen) and hexamerprimed cDNA synthesized using SuperScript II (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a Bio-Rad MyIQ Real time PCR machine with Bio-Rad IQ5 program (Bio-Rad, http://www.bio-rad.com) with SYBR Green incorporation. (primers GADD45a 2F: TGGAGGAAGTGCTCAGCAAG, 3R: GGTGAAATGGATCTGCAGAG, GADD45a F: CGAGAACGACATCAACATCC, R: ACTTAAGGCAGGATCCTTCC, Tubg2 F: CAGACCAACC ACTGCTACAT, R: AGGGAATGAAGTTGGCCAGT, mHprt F:CGAAGTGTTGGATACAGGCC, R: GGCAACATCAACAGGACTCC). Relative expression was calculated using the equation (Pfaffl, 2001) ratio = $(E_{target})^{\Delta CPtarget\ (control\text{-}sample)}/ (E_{ref}))^{\Delta CPref\ (control\text{-}sample)}$ where E is calculated by $E = 10^{(-1/slope)}$. Each sample was tested in duplo at least two times.

References

Aas, P.A., Otterlei, M., Falnes, P.O., Vagbo, C.B., Skorpen, F., Akbari, M., Sundheim, O., Bjoras, M., Slupphaug, G., Seeberg, E., et al. (2003). Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. Nature 421, 859-863.

Abdollahi, A., Lord, K.A., Hoffman-Liebermann, B., and Liebermann, D.A. (1991). Sequence and expression of a cDNA encoding MyD118: a novel myeloid differentiation primary response gene induced by multiple cytokines. Oncogene 6, 165-167.

Aboussekhra, A., Biggerstaff, M., Shivji, M.K., Vilpo, J.A., Moncollin, V., Podust, V.N., Protic, M., Hubscher, U., Egly, J.M., and Wood, R.D. (1995). Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell 80, 859-868.

Aboussekhra, A., and Wood, R.D. (1995). Detection of nucleotide excision repair incisions in human fibroblasts by immunostaining for PCNA. Exp Cell Res 221, 326-332.

Amundson, S.A., Patterson, A., Do, K.T., and Fornace, A.J., Jr. (2002). A nucleotide excision repair master-switch: p53 regulated coordinate induction of global genomic repair genes. Cancer Biol Ther 1, 145-149.

Andressoo, J.O., Mitchell, J.R., de Wit, J., Hoogstraten, D., Volker, M., Toussaint, W., Speksnijder, E., Beems, R.B., van Steeg, H., Jans, J., et al. (2006). An Xpd mouse model for the combined xeroderma pigmentosum/Cockayne syndrome exhibiting both cancer predisposition and segmental progeria. Cancer Cell 10, 121-132.

Barreto, G., Schafer, A., Marhold, J., Stach, D., Swaminathan, S.K., Handa, V., Doderlein, G., Maltry, N., Wu, W., Lyko, F., et al. (2007). Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. Nature 445, 671-675.

Berneburg, M., Lowe, J.E., Nardo, T., Araujo, S., Fousteri, M.I., Green, M.H., Krutmann, J., Wood, R.D., Stefanini, M., and Lehmann, A.R. (2000). UV damage causes uncontrolled DNA breakage in cells from patients with combined features of XP-D and Cockayne syndrome. EMBO J 19, 1157-1166.

Bhattacharya, S.K., Ramchandani, S., Cervoni, N., and Szyf, M. (1999). A mammalian protein with specific demethylase activity for mCpG DNA. Nature 397, 579-583.

Bird, A.P., and Wolffe, A.P. (1999). Methylation-induced repression–belts, braces, and chromatin. Cell 99, 451-454.

References

Carrier, F., Georgel, P.T., Pourquier, P., Blake, M., Kontny, H.U., Antinore, M.J., Gariboldi, M., Myers, T.G., Weinstein, J.N., Pommier, Y., et al. (1999). Gadd45, a p53-responsive stress protein, modifies DNA accessibility on damaged chromatin. Mol Cell Biol 19, 1673-1685.

Citterio, E., Van Den Boom, V., Schnitzler, G., Kanaar, R., Bonte, E., Kingston, R.E., Hoeijmakers, J.H., and Vermeulen, W. (2000). ATP-dependent chromatin remodeling by the Cockayne syndrome B DNA repair-transcription-coupling factor. Mol Cell Biol 20, 7643-7653.

de Boer, J., Andressoo, J.O., de Wit, J., Huijmans, J., Beems, R.B., van Steeg, H., Weeda, G., van der Horst, G.T., van Leeuwen, W., Themmen, A.P., et al. (2002). Premature aging in mice deficient in DNA repair and transcription. Science 296, 1276-1279.

de Boer, J., van Steeg, H., Berg, R.J., Garssen, J., de Wit, J., van Oostrum, C.T., Beems, R.B., van der Horst, G.T., van Kreijl, C.F., de Gruijl, F.R., et al. (1999). Mouse model for the DNA repair/basal transcription disorder trichothiodystrophy reveals cancer predisposition. Cancer Res 59, 3489-3494.

de Laat, W.L., Jaspers, N.G., and Hoeijmakers, J.H. (1999). Molecular mechanism of nucleotide excision repair. Genes Dev 13, 768-785.

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

Duncan, T., Trewick, S.C., Koivisto, P., Bates, P.A., Lindahl, T., and Sedgwick, B. (2002). Reversal of DNA alkylation damage by two human dioxygenases. Proc Natl Acad Sci U S A 99, 16660-16665.

Escher, G., Hoang, A., Georges, S., Tchoua, U., El-Osta, A., Krozowski, Z., and Sviridov, D. (2005). Demethylation using the epigenetic modifier, 5-azacytidine, increases the efficiency of transient transfection of macrophages. J Lipid Res 46, 356-365.

Evans, E., Moggs, J.G., Hwang, J.R., Egly, J.M., and Wood, R.D. (1997). Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. EMBO J 16, 6559-6573.

Fornace, A.J., Jr. (1992). Mammalian genes induced by radiation; activation of genes associated with growth control. Annu Rev Genet 26, 507-526.

Fornace, A.J., Jr., Jackman, J., Hollander, M.C., Hoffman-Liebermann, B., and Liebermann, D.A. (1992). Genotoxic-stress-response genes and growth-arrest genes. gadd, MyD, and other genes induced by treatments eliciting growth arrest. Ann N Y Acad Sci 663, 139-153.

References

Gonzalgo, M.L., and Jones, P.A. (1997). Mutagenic and epigenetic effects of DNA methylation. Mutat Res 386, 107-118.

Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A.F., Tanaka, K., and Nakatani, Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell 113, 357-367.

Hartwell, L.H., and Weinert, T.A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science 246, 629-634.

Hildesheim, J., Bulavin, D.V., Anver, M.R., Alvord, W.G., Hollander, M.C., Vardanian, L., and Fornace, A.J., Jr. (2002). Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. Cancer Res 62, 7305-7315.

Hoeijmakers, J.H. (2001a). DNA repair mechanisms. Maturitas 38, 17-22; discussion 22-13.

Hoeijmakers, J.H. (2001b). Genome maintenance mechanisms for preventing cancer. Nature 411, 366-374.

Jin, S.G., Guo, C., and Pfeifer, G.P. (2008). GADD45A does not promote DNA demethylation. PLoS Genet 4, e1000013.

Jones, P.A. (1999). DNA methylation in development of bladder cancer. Adv Exp Med Biol 462, 419-423.

Jung, H.J., Kim, E.H., Mun, J.Y., Park, S., Smith, M.L., Han, S.S., and Seo, Y.R. (2007). Base excision DNA repair defect in Gadd45a-deficient cells. Oncogene 26, 7517-7525.

Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999). MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet 23, 58-61.

Ng, J.M., Vermeulen, W., van der Horst, G.T., Bergink, S., Sugasawa, K., Vrieling, H., and Hoeijmakers, J.H. (2003). A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. Genes Dev 17, 1630-1645.

Papathanasiou, M.A., and Fornace, A.J., Jr. (1991). DNA-damage inducible genes. Cancer Treat Res 57, 13-36.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Rockx, D.A., Mason, R., van Hoffen, A., Barton, M.C., Citterio, E., Bregman, D.B., van Zeeland, A.A., Vrieling, H., and Mullenders, L.H. (2000). UV-induced inhibition of transcription involves repression of

References

transcription initiation and phosphorylation of RNA polymerase II. Proc Natl Acad Sci U S A 97, 10503-10508.

Sarasin, A., and Stary, A. (2007). New insights for understanding the transcription-coupled repair pathway. DNA Repair (Amst) 6, 265-269.

Smith, M.L., Chen, I.T., Zhan, Q., Bae, I., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M., and Fornace, A.J., Jr. (1994). Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266, 1376-1380.

Smith, M.L., Ford, J.M., Hollander, M.C., Bortnick, R.A., Amundson, S.A., Seo, Y.R., Deng, C.X., Hanawalt, P.C., and Fornace, A.J., Jr. (2000). p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol Cell Biol 20, 3705-3714.

Swisher, J.F., Rand, E., Cedar, H., and Marie Pyle, A. (1998). Analysis of putative RNase sensitivity and protease insensitivity of demethylation activity in extracts from rat myoblasts. Nucleic Acids Res 26, 5573-5580.

Takekawa, M., and Saito, H. (1998). A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 95, 521-530.

Theron, T., Fousteri, M.I., Volker, M., Harries, L.W., Botta, E., Stefanini, M., Fujimoto, M., Andressoo, J.O., Mitchell, J., Jaspers, N.G., et al. (2005). Transcription-associated breaks in xeroderma pigmentosum group D cells from patients with combined features of xeroderma pigmentosum and Cockayne syndrome. Mol Cell Biol 25, 8368-8378.

Vairapandi, M., Balliet, A.G., Fornace, A.J., Jr., Hoffman, B., and Liebermann, D.A. (1996). The differentiation primary response gene MyD118, related to GADD45, encodes for a nuclear protein which interacts with PCNA and p21WAF1/CIP1. Oncogene 12, 2579-2594.

Vairapandi, M., Liebermann, D.A., Hoffman, B., and Duker, N.J. (2000). Human DNA-demethylating activity: a glycosylase associated with RNA and PCNA. J Cell Biochem 79, 249-260.

Vermeulen, W., Scott, R.J., Rodgers, S., Muller, H.J., Cole, J., Arlett, C.F., Kleijer, W.J., Bootsma, D., Hoeijmakers, J.H., and Weeda, G. (1994). Clinical heterogeneity within xeroderma pigmentosum associated with mutations in the DNA repair and transcription gene ERCC3. Am J Hum Genet 54, 191-200.

Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F., and Wolffe, A.P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 23, 62-66.

References

Wang, X.W., Zhan, Q., Coursen, J.D., Khan, M.A., Kontny, H.U., Yu, L., Hollander, M.C., O'Connor, P.M., Fornace, A.J., Jr., and Harris, C.C. (1999). GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A 96, 3706-3711.

Weiss, A., Keshet, I., Razin, A., and Cedar, H. (1996). DNA demethylation in vitro: involvement of RNA. Cell 86, 709-718.

Xia, L., Zheng, L., Lee, H.W., Bates, S.E., Federico, L., Shen, B., and O'Connor, T.R. (2005). Human 3-methyladenine-DNA glycosylase: effect of sequence context on excision, association with PCNA, and stimulation by AP endonuclease. J Mol Biol 346, 1259-1274.

Zhang, W., Bae, I., Krishnaraju, K., Azam, N., Fan, W., Smith, K., Hoffman, B., and Liebermann, D.A. (1999). CR6: A third member in the MyD118 and Gadd45 gene family which functions in negative growth control. Oncogene 18, 4899-4907.

8

Nederlandse Samenvatting
List of abbriviations
Portfolio
Curriculum vitae
List of publications
Dankwoord

Nederlandse Samenvatting

DNA, het erfelijke materiaal, oftewel de blauwdruk van het leven genoemd, wordt continu bedreigd door invloeden van buitenaf die schade (mutaties) geven aan het DNA. Deze schade kan leiden tot kanker of veroudering, maar gelukkig beschikken de cellen waaruit een lichaam is opgebouwd over een uitgebreid pakket aan maatregelen om het genetisch materiaal intact te houden. Over één van deze onmisbare maatregelen waarmee de cel haar DNA beschermt gaat dit proefschrift.

Elk levend wezen is opgebouwd uit cellen. Er zijn organismen die uit één of een paar cellen zijn opgebouwd, maar ook organismen die uit vele honderden miljarden cellen zijn opgebouwd, zoals de mens. De mens heeft ongeveer 200 verschillende soorten celtypes (bijvoorbeeld huid-, lever-, bloed-, niercellen). Een groep van verschillende soorten cellen bij elkaar vormt een orgaan dat een bepaalde functie in het lichaam vervult. Elke cel heeft een apart compartiment, de kern genaamd, waarin de informatie ligt die nodig is voor het functioneren van een cel. Deze informatie ligt opgeslagen in codevorm in het DNA. De celkern ligt vol met DNA. Organismen kunnen een verschillend aantal DNA-moleculen per celkern hebben. Die afzonderlijke DNA-moleculen opgerold om eiwitten worden chromosomen genoemd. Een DNA-streng lijkt op een wenteltrap en bestaat uit twee in elkaar gedraaide, antiparallelle polymeren waarvan de ruggengraat gevormd wordt door een keten van afwisselend suiker en fosfaat groepen. De verbinding tussen de twee spiraalvormige ketens wordt gevormd door vier verschillende basen van het DNA: A (adenine), C (cytosine), G (guanine) en T (thymine). C hecht altijd aan G in de tegenoverliggende keten en A hecht aan T in de tegenoverliggende keten. Zo vinden we op het DNA een lange reeks basenparen G-C, A-T, C-G en T-A, in een wisselende volgorde die uniek is voor ieder individu. Tijdens replicatie (verdubbeling van het DNA voor celdeling) ontwindt de dubbele DNA spiraal en hecht zich aan de oude DNA streng een complementaire nucleotide (met behulp van DNA polymerase). Bij elke stap wordt hieraan een nieuwe complementaire nucleotide vastgemaakt, tot de hele streng is afgelezen. Dit gebeurt in beide strengen, zodat er een exacte verdubbeling van het DNA plaatsvindt en de cel zich kan gaan delen. De code in het DNA, geschreven in een vier-letterig alfabet, biedt de mogelijkheid om de erfelijke eigenschappen van een individu vast te leggen. Dit wordt gedaan in de kleinere stukjes van een chromosoom, de genen. De genen bepalen telkens één van de erfelijke eigenschappen van een individu. Een mens heeft ongeveer 20.000 tot 25.000 verschillende genen, gelegen in totaal op 3 miljard basen (2 meter DNA). Elk gen codeert voor één bepaald eiwit. Sommige eiwitten zijn essentieel als structureel element van een cel, terwijl andere betrokken zijn bij biologische processen in de cel. Elke cel in een organisme bevat hetzelfde DNA, maar door het wel of niet actief zijn van verschillende genen kunnen cellen verschillende functies hebben. Actieve genen zijn genen die vertaald worden van gen naar eiwit. Dit gebeurt in twee stappen. Eerst wordt in de kern de DNAcode van het gen afgeschreven oftewel gekopieerd (dit proces heet transcriptie), waarna de kopie in de vorm van een boodschapper (m)RNA molecuul buiten de kern wordt vertaald naar een eiwit (dit heet translatie). Zo schrijft elk celtype verschillende soorten genen af, waardoor de verschillende bouwstenen of processen de cel zijn specifieke eigenschappen geeft.

Het DNA in elke cel wordt continu beschadigd, naar schatting wel zo'n 10.000 keer per dag. Hierbij moet men niet alleen denken aan toxische stoffen en stralingsbronnen uit het milieu (bijvoorbeeld zonlicht, kosmische straling, uitlaatgassen, sigarettenrook of andere chemische middelen), maar ook door stoffen die vrij komen bij chemische processen van het eigen lichaam (zoals vrije zuurstofradicalen, ook wel ROS genoemd). Deze beschadigingen kunnen leiden tot het verhinderen van het afschrijf (kopieer) proces, het stoppen van de celdeling en zelfs celdood, waardoor de weefsel samenstelling wordt verstoord met als consequentie veroudering. Ook kunnen beschadigingen in het DNA leiden tot veranderingen in de DNA code (mutaties), waardoor cellen ongecontroleerd kunnen gaan delen en uiteindelijk kanker kunnen veroorzaken. Als de verkeerde code wordt doorgegeven aan het nageslacht via de geslachtscellen spreekt men van een erfelijke ziekte. Het is duidelijk dat het opruimen van DNA schades van levensbelang is.

Om het DNA in de cel intact te houden is de cel uitgerust met een aantal reparatie mechanismen. De verschillende mechanismen kunnen een bepaalde klasse van DNA beschadiging verwijderen. Gelukkig is er wel overlap tussen de verschillende reparatiesystemen en mocht de reparatie toch falen dan kunnen beschadigde cellen zichzelf opofferen en afsterven (apoptosis), hiermee wordt voorkomen dat de fout wordt doorgegeven of een tumor ontstaat. Één van die herstel mechanismen is Nucleotide Excisie Reparatie ook wel afgekort als NER. Dit mechanisme herstelt schades veroorzaakt door zonlicht en door stoffen uit uitlaatgassen en sigarettenrook. Voor de opsporing van de schades aan DNA gebruikt NER twee subroutes. Het Globaal Genoom NER (GG-NER), dit speurt het hele genoom (al het DNA in een cel) af op zoek naar beschadigingen, of het Transcriptie-gekoppeld NER (TC-NER) dat de schade herkent door het vastlopen van het transcriptieproces. Een team van meer dan 25 eiwitten helpt in beide gevallen met de herkenning en het wegknippen van de schade uit het DNA waarna de genetische code kan worden hersteld aan de hand van de intacte, andere DNA streng. Het hele proces van schadeherstel wordt extra gecompliceerd, als je bedenkt dat de instructie voor het opbouwen van de DNA-schadehersteleiwitten ook gegeven wordt door genen in het DNA. Iedereen kan zich voorstellen dat als de genen beschadigd worden die coderen voor één van die hersteleiwitten, de gevolgen dramatisch zijn en leiden tot een eruptie van ziekteverschijnselen. Het XPD eiwit is één van de componenten van het NER en dit proefschrift gaat over de gevolgen van verschillende mutaties en combinaties daarvan in het XPD eiwit.

In dit proefschrift hebben we naar verschillende mutaties in het XPD eiwit gekeken. Één van die mutaties is de G602D mutatie die zorgt voor xeroderma pigmentosum (XP), een ziekte met een sterk verhoogde kans op huidkanker gecombineerd met de versnelde veroudering van Cockayne syndroom (CS, samen XPCS). Daarnaast hebben we ook gekeken naar de mutatie R722W die lijdt tot trichothiodystrophy (TTD), een ziekte die veel lijkt op de versnelde veroudering van CS al hebben patiënten ook nog een schilferende huid en breekbare haren en nagels. Om deze ziektes goed te bestuderen zijn er muismodellen gegenereerd met dezelfde mutaties in hun DNA. De mutaties in de muis leidden tot vergelijkbare symptomen als in de patiënten.

Van elk gen in je lichaam krijg je een kopie van je vader en een kopie van je moeder. Deze twee kopieën van hetzelfde gen heten allelen. Als een allel niet bijdraagt aan het fenotype (de waarneembare eigenschap) wordt het recessief genoemd en als een allel als enige het fenotype bepaalt wordt het dominant genoemd. De meeste erfelijke aandoeningen zijn recessief, hetgeen betekent dat zowel het allel van je vader als dat van je moeder, gemuteerd moeten zijn. Wij hebben onderzoek gedaan naar muizen met op elk XPD allel een verschillende mutatie: Één allel had een TTD mutatie en de ander een XPCS mutatie. Deze muis noemen we een compound heterozygoot (oftewel CH). De CH muis, met gelijktijdige inactivatie van XPA, een ander belangrijk NER eiwit (ook wel CH|XPA genoemd), leeft een half jaar in vergelijking tot de 3 weken levende XPCS|XPA en TTD|XPA muizen. Bekend is dat veel van de NER deficiënte muimodellen een verlaagt groeihormoon (GH) en insuline gelijkende groeifactor-1 (IGF-1) niveau hebben die lijkt op het niveau van hele oude muizen. Dat is waarom wij in de levercellen van de XPCS|XPA en CH|XPA muis hebben gekeken naar verschillende boodschapper RNA (mRNA) niveaus van GH en IGF-1. We vonden daar een verlaging van de GH/IGF-1 as en een verhoging van de anti-oxidant reactie. Dit genexpressie patroon vind je ook terug in lang levende muizen of muizen die beperkt zijn in hun calorie inname en wordt adaptieve stress reactie genoemd. Deze stress reactie wordt waarschijnlijk aangespoord door ongerepareerde endogene (lichaamseigen) DNA schade en door de dieren gebruikt om reactieve metabolieten, zoals ROS en de extra DNA schade die daardoor ontstaat, te verlagen (Hoofdstukken 2 en 3).

Ook in XPCS en TTD muizen zonder XPA inactivatie vinden we parameters die wijzen op calorie inname beperking, zoals lager lichaamsgewicht, hogere glucose tolerantie en hogere gevoeligheid voor insuline. In de CH dieren lijken deze parameters op die van 'normale' (wildtype) muizen ten opzichte van de XPCS en TTD muizen. Dit duidt erop, dat ook hier de beide recessieve allelen elkaar complementeren en dus samen bereiken wat ze alleen niet kunnen. Deze complementatie vinden we niet terug in UV-gevoeligheid-, carcinogenese- en overlevingstudies in muizen en cellen. Dit leidde tot de conclusie dat beschadigingen in het DNA die ontstaan door UV straling of andere oorzaken niet de enige oorzaak kunnen zijn van het versneld verouderen van de muizen met een beschadigd NER (Hoofdstuk 6).

Een andere vorm van schade die bijdraagt aan het versneld verouderen ontstaat door vrije zuurstof radicalen in het lichaam. Deze ontstaan als je lichaam het voedsel wat je eet omzet. Een snelle manier om veel ROS te laten ontstaan en de reactie van het lichaam op ROS en de bijbehorende acute stress in een orgaan te bestuderen is een nier ischemie reperfusie, oftewel een afklem experiment van de nier. In zo'n experiment wordt de bloedtoevoer naar een orgaan (in dit geval de nier) gestopt en daarna hersteld zoals dit ook gebeurt bij een hartinfarct of een beroerte. In Hoofdstuk 4 laten we zien dat dieren die beperkt worden in hun voedselinname vóór de chirurgische ingreep, door drie dagen vasten of 4 weken 70% van hun normale hoeveelheid voedsel in te nemen, veel beter bestand zijn tegen de acute oxidatieve stress die ontstaat in de nier na de ingreep. Omdat bekend is dat beschadigingen in NER in het lichaam dezelfde reactie oproepen als beperkte voedsel inname hebben we ook naar de oxidatieve stress gevoeligheid van CSA

en CSB (Cockayne syndroom A en B) muizen gekeken. CSA en CSB muizen hebben een orgaan-specifieke bescherming tegen de ontstane oxidatieve stress, waarschijnlijk door hun adaptieve stress reactie (Hoofdstuk 5).

In het laatste hoofdstuk van dit proefschrift (Hoofdstuk 7) heb ik naar genactivatie gekeken die ontstaat door promoter demethylering (het weghalen van de methylgroep van cytosines, zodat het gen overgeschreven kan worden) via het GADD45a eiwit, met als doel te onderzoeken of NER eiwitten hiervoor van belang zijn en welke NER subroute er voor deze GADD45a afhankelijke demethylatie wordt gebruikt. We tonen aan dat TC-NER eiwitten belangrijk zijn voor de GADD45a afhankelijke promoter demethylatie en dat verschillende mutaties in hetzelfde gen (XPD) een andere uitwerking met betrekking tot demethylering hebben.

List of abbreviations

ALS:	acid-labile subunit
APOAIV	apolipoprotein A-IV
AUC	area under the curve
BER	base excision repair
CH	compound heterozygote
CPD	cyclobutane pyrimidine dimer
CR	calorie restriction
CS	Cockayne syndrome
CSA/CSB	Cockayne syndrome A/B (protein)
DDB	DNA damage binding protein
DIO1	deiodinase 1
DNA	deoxyribonucleic acid
DR	dietary restriction
DSB	double strand break
ERCC	human excision repair cross complementing (gene)
FMO3	flavin-containing monoxygenase 3
GADD45a	growth arrest and DNA damage inducible gene 45a
GG-NER	global genome NER
GH	growth hormone
GHR	growth hormone receptor
GHR-KO:	growth hormone receptor knock-out
Gsr	glutathione reductase
HO-1	hemeoxygenase
hHR23B	human homolog of S. cerevisiae repair protein RAD23B
HR	homologous recombination
IF	intermittent fasting
IGF-BP	IGF-1 binding protein
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor receptor
IR	ionizing radiation
IRI	ischemia reperfusion injury
LDH	lactate dehydrogenase
MDF	mouse dermal fibroblast
MED	minimal erythemal dose
MEF	mouse embryonic fibroblast
MMR	mismatch repair
mRNA	messenger RNA
NER	nucleotide excision repair
NHEJ	non-homologous end joining
8-oxodG	8-oxo-2'-deoxyguanosine

PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear factor
PCR	polymerase chain reaction
6-4PP	(6,4)-pyrimidine-primidone photoproduct
POD	post-operative day
RLU	relative light units
RNA	ribonucleic acid
RNApolII	RNA polymerase II
ROS	reactive oxygen species
RPA	replication factor A
RRS	recovery of RNA synthesis
RT-PCR	real-time PCR
SSB	single strand break
SCC	squamous cell carcinoma
SSP	squamous cell papilloma
TC-NER	transcription-coupled NER
TFIIH	transcription factor IIH
TTD	trichothiodystrophy
UDS	unscheduled DNA synthesis
UV	ultraviolet radiation
WT	wild type
XP	xeroderma pigmentosum
XPA-G	xeroderma pigmentosum group A-G (protein)
XPCS	XP combined with CS

Portfolio

Name PhD student: H.W.M van de Ven
Erasmus MC Department: Genetics
Research School: MolMed

PhD period: 2004-2009
Promotor(s): J.H.J Hoeijmakers
Supervisor: J.R. Mitchell

General academic skills	
-Laboratory animal science	2004
-Risk and quality management in laboratories	2004
Research skills	
-Convocal course	2007
In-depth courses (e.g. Research school, Medical Training)	
-Molecular and Cell Biology	2005
-Reading and discussing Literature	2005
-Transgenesis and Gene targeting	2005
-Phylogeny Workshop	2005
-Biomedical English Writing and Communication	2005
Presentations	
- Ride Research Meeting; Parallels between DNA repair-deficient progeria and long-lived dwarf mice	2005
-5th Winterschool of the International Graduierenkolleg GRK767: "Transcriptional Control in Developmental Processes", Kleinwalserthal, Germany; Functionality of the Xpd gene.	2007
-6th Winterschool of the International Graduierenkolleg GRK767: "Transcriptional Control in Developmental Processes", Kleinwalserthal, Germany; GAdd45a demethylation and its function in DNA repair.	2008
-MGC meeting; Fasting offers rapid and robust protection against ischemia reperfusion injury in mice	2008
International conferences	
-MGC/Cancer UK PhD meeting, Liege	2005
-3rd International Conference on functional Genomics of Ageing, Palermo.	2006
-MGC/Cancer UK PhD meeting, Oxford	2007
-95th International Titisee Conference. "Molecular basis of Aging".	2007
-Cold Spring Harbor Labaratory Meeting. "Molecular genetics of Aging".	2008
-Keystone symposia. "Epigenetics, Development and Human Disease", Breckenridge.	2009

Seminars and workshops	
-Lunteren	2005
-Lunteren	2005
-Summerschool KNAW: “Hypothalamic integration of energy metabolism”.	2007
-5th and 6th Winterschool of the International Graduiertenkolleg GRK767: Transcriptional Control in Developmental Processes”, Kleinwalserthal, Germany	2008
-FP6 Integrated Project DNA repair 3rd Annual Review meeting	2008
Didactic skills	
Profielwerkstuk begeleiding 6 VWO: “Veroudering”	2007

Curriculum vitae

Henrica Wilhelmina Maria van de Ven oftewel Marieke werd op 12 mei 1978 geboren te Eindhoven. In 1995 behaalde zij haar Havo diploma en in 1997 haar VWO diploma aan het Heerbeeck Collega te Best. In augustus in hetzelfde jaar is zij begonnen met de studie Medische Biologie aan de Vrije Universiteit van Amsterdam. Haar eerste stage voerde zij uit bij de afdeling Neurofarmacologie van de Vrije Universiteit onder begeleiding van Prof. Dr. A.N.M. Schoffelmeer met als titel: De rol van nicotine receptoren bij de ontwikkeling van sensitisatie van het mesocorticolimbische dopamine systeem en gedrags-hyperresponsiviteit. Haar tweede onderzoeksstage voerde zij uit aan de department of Animal Welfare van de University of Britisch Colombia te Vancouver onder begeleiding van Prof. Dr. D. Fraser en Dr. E.G. Patterson-Kane met als Titel: Rats preferences for comfort materials. In augustus 2002 behaalde zij haar doctoraal diploma. Direct aansluitend begon zij met de opleiding tot eerstegraads docent biologie aan het IDO-VU te Amsterdam tegelijkertijd deed ze een didactische en onderwijskundige stage aan het St. Ignatius Gymnasium te Amsterdam onder begeleiding Dr. Kees Bogert. In mei 2003 deed ze een didactische en onderwijskundige onderzoeksstage bij MASTEP aan de University of Namibia te Windhoek waar ze meehielp aan de ontwikkeling van lesmateriaal voor de opwaardering van het onderwijs niveau. Met als begeleiders Dr. M. Cantrell en Drs. W.J.W. Ottevanger, waarna ze in augustus 2003 haar bevoegdheid kreeg voor eerstegraads biologie leraar. Meteen daarna is ze voor de klas gaan staan eerst op het Zaanlands Oost te Zaandam en later op het Segroek College te den Haag. Tot ze in mei 2004 begon als assistent in opleiding aan de afdeling Celbiologie en Genetica aan de Erasmus Universiteit te Rotterdam onder begeleiding van Prof. Dr. J.H.J. Hoeijmakers en Dr. J.R. Mitchell. De resultaten van dit onderzoek staan in dit proefschrift beschreven.

List of publications

Psychostimulant-induced behavioral sensitization depends on nicotinic recepter activation.Schoffelmeer, A.N.M, De Vries, T.J, Wardeh, G, **van de Ven, H.W.M**, Vanderschuren, L.J.M.J. Journal of Neuroscience. 2002 15;22(8):3269-76

Rats' preferences for corn versus wood-based bedding and nesting materials. Ras, T*, **van de Ven, M***, Patterson-Kane, E.G, Nelson K. Laboratory animals 2002 36(4):420-5

Rescue of progeria in trichothiodystrophy by homozygous lethal Xpd alleles. Andressoo JO, Jans J, de Wit J, Coin F, Hoogstraten D, **van de Ven M**, Toussaint W, Huijmans J, Thio HB, van Leeuwen WJ, de Boer J, Egly JM, Hoeijmakers JH, van der Horst GT, Mitchell JR.PLoS Biol. 2006 Oct;4(10):e322.

Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. **van de Ven M**, Andressoo JO, Holcomb VB, von Lindern M, Jong WM, De Zeeuw CI, Suh Y, Hasty P, Hoeijmakers JH, van der Horst GT, Mitchell JR. PLoS Genet. 2006 Dec 15;2(12):e192.

Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging. **van de Ven M**, Andressoo JO, Holcomb VB, Hasty P, Suh Y, van Steeg H, Garinis GA, Hoeijmakers JH, Mitchell JR. Mech Ageing Dev. 2007 Jan;128(1):58-63.

Congenital DNA repair deficiency results in protection against renal ischemia reperfusion injury in mice. Susa D, Mitchell J.R, Verweij M, **van de Ven M**, Roest H, van den Engel S, Bajema I, Mangundap K, IJzermans J.N.M, Hoeijmakers J.H.J, de Bruin R.W.F. Ageing Cell. 2009 article in press.

Fasting offers rapid and robust protection against ischemia reperfusion injury in mice.Mitchell, J.R, Verweij M, **van de Ven M**, Goemaere N, van den Engel S, Chu T. Forrer F, Müller C, de Jong M, IJzermans J.N.M, Hoeijmakers J.H.J, de Bruin R.W.F. Manuscript in preperation

Different effects of XPD mutations on cancer and ageing in mouse models. **van de Ven M**, Andressoo J-O, van der Horst G.T.J, Hoeijmakers J.H.J, Mitchell J.R. Manuscript in preperation

GADD45a demethylation and its function in DNA repair. **van de Ven M**, Toussaint W, Wijgers N, Vermeulen W, Hoeijmakers J.H.J, Mitchell J.R. Manuscript in preperation

Dankwoord

Eindelijk is het dan zo ver…. de koek is op, de pen is leeg en mijn proefschrift is af! Maar natuurlijk was dit proefschrift niet tot stand gekomen zonder de hulp van een heleboel mensen, die ik dan ook heel erg graag wil bedanken, ook al heb ik helemaal geen zin meer om te schrijven.

Ik heb 5 jaar lang met heel veel plezier op de afdeling Genetica gewerkt en heb door mijn vele interne verhuizingen (van bench naar bench) naast veel verschillende collega's gezeten en plezier met ze gehad. Allereerst wil ik mijn co-promoter en dagelijks begeleider Jay bedanken: **Jay thank you for your support and guidance from day one I entered the** lab. You are the best supervisor I can think of. You inspired and stimulated me in my scientific development, and I admire your tolerance even in the beginning when I had to disturb you for an explanation multiple times. You even stayed patience in the last months during our skype conversations, when I held you from your morning coffee. I will promise you I will never do that again. I also want to thank you, and of course Elisabeth for the great time I had in Boston. It was hard work, but I felt completely at home with you and the kids, thanks for everything!

Natuurlijk wil ik ook mijn promoter Jan Hoeijmakers bedanken voor de mogelijkheden die hij mij heeft geboden. Jan, je was altijd een bron van inspiratie en liet me vrij in het onderzoek dat ik wilde doen. Ook wil ik bij deze Bert en Wim bedanken, ik kon altijd jullie kantoortje binnen lopen voor een vraag of een gesprekje en kreeg dan meestal meteen wat goede tips mee. En natuurlijk ook de rest van de mensen in mijn commissie, dank jullie wel voor het lezen van mijn proefschrift!

Ook wil ik mijn dagelijkse (oud) collega's bedanken: Karin, tja sinds je weg bent is het toch akelig stil in het lab niemand zingt meer mee met de radio. Inêz, Monica, Roel, Lieneke, Joris, Romana en alle andere (oud) lunch partners, het is heerlijk om tijdens de lunch eventjes niet over wetenschap te praten en stoom af te blazen, bedankt voor al de gezellige en grappige hoogdravende gesprekken die we hebben gevoerd boven onze borden en kopjes koffie. Ook wens ik Monica, Eugin en Karl heel veel succes bij hun aankomende promotie, hup jongens het einde is in zicht! Ik wil ook Ingrid en Renate bedanken die me zeker in het begin veel hebben geholpen met de muizen. Dan eventjes naar de andere kant, naar mijn twee reuze teddyberen Arjan en Nils het was heerlijk om eventjes onder het genot van een kopje thee of tijdens jullie hulp met één van mijn experimenten bij te kletsen of plezier te maken om de tijd te doden. Ook Chris en Wendy bedankt voor de gezelligheid op het lab en tijdens de Kleinwalsertal meetings. Wendy ik heb het altijd geweldig gevonden om met jou samen te werken en wens je veel geluk en succes in Gent. Ambra, thanks for all the (scientific) advice and the nice time in NY together with Arjan. Audrey we didn't run as much as we wanted but it was really nice. Dan nog Joris en Jan de Wit, ik ben heel blij dat ik met jullie het kantoortje heb mogen delen, jullie stonden altijd klaar met goede tips, leuke discussies en gesprekken, dank jullie wel! Verder wil ik natuurlijk al mijn (oud) labgenoten van de 7^{e} verdieping

bedanken voor de samenwerking en de gezelligheid. Ook de ondersteuning van de computermannen, dames van de keuken en de dierverzorgers was heel belangrijk. Secretaresses (Marike en Jasperina) hoe vaak heb ik wel niet aan jullie bureau gestaan met een vraag of probleem en het bijzondere was dat het altijd in no-time was opgelost, dank jullie wel. Ook wil ik de mensen van het RIVM bedanken voor de samenwerking en natuurlijk Harry voor de gezelligheid en weetjes over de beste kroegen en pistes op congressen.

Dan wil ik natuurlijk ook mijn vrienden bedanken. Als eerste mijn vrienden en vriendinnen die ik al jaren heb uit Oirschot, dank jullie wel voor jullie steun en natuurlijk ook voor de jarenlange vriendschap, ook al zie ik de meeste van jullie niet heel erg veel, onthoud een ding, jullie zijn speciaal voor me en ik hoop dat we nog heel lang vrienden blijven. Ook mijn vrienden en vriendinnen uit Utrecht die altijd in zijn voor een gezellig feestje, e-mail discussies, een etentje of gewoon een goed gesprek, thanks. En natuurlijk mijn studie vrienden en vrienden en vriendinnen in Amsterdam jullie zijn de laatste tijd erg belangrijk voor me geweest en het was heerlijk om even stoom af te blazen en tegen iemand aan te kletsen tijdens het schrijven van mijn proefschrift. Ook de lol en de goede gesprekken tijdens de vele etentjes, wandelingen, filmavondjes, avondjes stappen, picknicks (ooh, was het maar weer zo ver) en sportactiviteiten zijn me dierbaar.

Ilje mijn tijd in Rotterdam was een stuk saaier geweest zonder al onze DE café dates, succes nu met je eigen onderzoek.

En dan natuurlijk mijn lieve nimfo's Elske en Sam. Elske het grappige is dat we tijdens onze studie niet super veel met elkaar op trokken, maar dat hebben we zeker ingehaald de laatste paar jaren en je bent een echte vriendin voor me gebleken. Het is heerlijk om tegen iemand aan te praten, die echt weet waar je het over hebt en dan ook nog eens met goed advies komt. Je bent een goede sparringpartner zowel in het dagelijks leven als tijdens het kickboxen. Het kost ons de laatste tijd wat moeite om ons te motiveren voor het kickboxen, maar dat is denk ik ook niet zo heel erg vreemd gezien onze workload. Ik wens je heel veel succes met jou promotie en natuurlijk met je nieuwe baan bij NWO. Sam, ik heb je leren kennen tijdens mijn eerste Kleinwalsertal meeting, waar we (soms tot grote irritatie van andere) heel veel plezier hebben gehad. Ook de tweede keer was weer een groot succes en sindsdien ben je een echt maatje van me geworden. Ik geniet van de van de lol en de goede gesprekken die we samen hebben en ik heb echt een super leuke tijd met je gehad in Breckenridge. We gaan zeker nog een keertje werken aan die snowboard techniek van je. Heel veel succes nog met het afronden van jou onderzoek de komende tijd.

Ook wil ik mijn schoonfamilie heel erg bedanken. Wim en Annemarie dank je wel voor de goede gesprekken en de wijze raad, ik voel me altijd erg op mijn gemak bij jullie. Jeroen en Susanne dank je wel voor de gezelligheid bij de vele etentjes.

Dan nog mijn lieve ouders, mijn zusje, twee broers en de schoonzussen. Pap en mam, wat ben ik ontzettend blij met jullie. Jullie hebben me altijd gesteund in elke keuze die ik maakte. Ook al zijn de dingen die ik doe voor jullie vaak een ver-van-je-bed-show en soms moeilijk te begrijpen, jullie blijven liefdevol en geïnteresseerd. Thuis komen in Oirschot voelt voor mij altijd warm en vertrouwd. Op een of andere manier vallen daar de zorgen van me af en kan ik de dingen bekijken vanuit een ander perspectief. Ook Joep, Claudia, Jop, Lena, Maarten, Erica, Suuz, Fien, Marloes en Janneke dank jullie wel voor al de onvoorwaardelijke liefde en steun die ik van jullie heb gehad.

En als laatste, lieve Mattijs ik zou niet eens weten hoe ik jou zou moeten bedanken, je bent echt mijn alles, het allerliefste vriendje en maatje dat ik mezelf zou kunnen wensen. Jij steunt me echt in alles wat ik doe en je hebt zo ontzettend veel geduld. Ik ben zo trots op dit boekje en zo blij dat jij hieraan hebt mee geholpen, wij zijn zo'n goed team! Ik verheug me nu al op onze wel verdiende vakantie.

Zucht!.....nou dat was nog best wel wat schrijfwerk zo'n dankwoord. Ik ben blij dat het nu eindelijk voorbij is, op naar de volgende uitdaging...

Printed by: Wöhrmann Print Service
Design by: Mattijs Arts