Global Genome Responses to DNA-Repair Deficiency Modulate Aging and Stress Response Pathways

Øyvind Fensgård

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Oslo, July 2010

Øyvind Fensgård

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Abbreviations

5-OHC	5-hydroxycytosine
5-OHU	5-hydroxyuracil
8-oxoA	7,8-dihydro-8-oxoadenine
8-oxoG	7,8-dihydro-8-oxoguanine
AID	activation induced cytosine deaminase
APE1	AP endonuclease 1
AP site	apurinic/apyrimidinic site
BER	base excision repair
CAT	catalase
C. elegans	*Caenorhabditis elegans*
CESR	core environmental stress response
CPD	cyclobutane pyrimidine dimer
CR	caloric restriction
CS	Cockayne syndrome
CSR	class-switch recombination
CT	charge-transfer
DDR	DNA damage response
DOG	2-deoxy-D-glucose
dRP	deoxyribose phosphate
DSB	double-strand break
E. coli	*Escherichia coli*
GG-NER	global genome nucleotide excision repair
GH	growth hormone
GO	gene ontology
HNPCC	hereditary non-polyposis colorectal cancer
HR	homologous recombination
ICL	interstrand cross-links
Ig	immunoglobulin
IGF-1	insulin growth factor 1
ILS	insulin-like signaling
IR	ionizing radiation
LP-BER	long-patch base excision repair
MDF	mouse dermal fibroblast
MMR	mismatch repair
MMS	methyl methanesulfonate

msSOD	mitochondrial superoxide dismutase
MNU	N-methyl-N'-nitrosourea
MSI	microsatellite instability
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
nth-1	endonuclease three-like homolog 1
NO	nitric oxide
OGG1	8-oxoguanine DNA glycosylase
PCNA	proliferating cell nuclear antigen
PGL	published gene list
Polβ/δ/ε	polymerase β, δ and ε
RNAi	RNA interference
RNAPII	RNA polymerase II
ROS	reactive oxygen species
rtPCR	reverse transcription polymerase chain reaction
SAM	S-adenosyl-methionine
S. cerevisiae	*Saccharomyces cerevisiae*
SCID	severe combined immunodeficiency
SHM	somatic hypermutation
SOD	superoxide dismutase
SP-BER	short-patch base excision repair
S. pombe	*Schizosaccharomyces pombe*
SSB	single-strand break
TC-NER	transcription-coupled nucleotide excision repair
TCR	transcription-coupled repair
TFIIH	transcription factor IIH
Tg	thymine glycol
TLS	translesion synthesis
TOR	target of rapamycin
TTD	Trichothiodystrophy
UDG	uracil DNA N-glycosylase
ung-1	uracil DNA glycosylase 1
UV	ultraviolet
XP	xeroderma pigmentosum
xpa-1	xeroderma pigmentosum complementing group A
XRCC1	X-ray cross complementing protein 1

Summary

The genomes of all animals are constantly challenged by exogenous and endogenous sources of DNA damaging agents. UV radiation, chemicals, pollutants, and by-products of the cells' own metabolism may damage the genetic material. Such damages are harmful to the animal as they may cause mutations or generate cytotoxic lesions, which in turn may lead to disease, cancer and aging. Protection of the genome is therefore of the utmost importance.

To counteract such potential detrimental effects, all organisms have developed protective mechanisms such as antioxidants and DNA repair mechanisms. DNA excision repair proteins detect lesions in DNA, excise the damaged base and re-insert a correct base, thus maintaining the correct coding properties of the genome. Defects in DNA repair mechanisms may lead to cancer, neurodegeneration, other age-related pathologies or senescence.

The nematode *Caenorhabditis elegans* (*C. elegans*) contains very few DNA glycosylases, which are the lesion-detecting proteins in DNA excision repair, compared to other animals and organisms. Analysis of all transcribed genes in DNA repair-deficient mutants in *C. elegans* revealed a global transcriptional response aimed at minimizing further damage to the genome. This involved a down-regulation of insulin-like signaling and an upregulation of antioxidants and stress response genes, similar to the response seen in both long-lived and old animals. This response seems to be conserved across different species as analysis of comparable mutants in the yeast *Saccharomyces cerevisiae* and mouse showed a similar response.

Pathway reconstruction and literature mining suggests that this response is not elicited only by lack of repair *per se*, but rather from aberrant or attempted processing of lesions by other repair pathways than those normally repairing such lesions. This result in lesions that block the transcription of active genes and signal the transcription of other genes aimed at reducing further damage to DNA.

Analysis of *C. elegans* mutants deficient in two different repair pathways revealed a completely different response with downregulation of Aurora-B and Polo-like kinase 1 signaling networks as well as downregulation of other DNA repair pathways. The mechanism and signaling origin of this response is yet unknown.

Gene expression profiling is emerging as a powerful complementary tool to classical genetics and molecular analysis. By taking a systems biology approach, which takes into account the interplay between many pathways, gene expression profiling may aid in the interpretation of observed phenotypes and assist in the generation of new testable hypotheses.

1. **Introduction**

DNA is the carrier of the genetic information in all living organisms. It is a very dynamic structure and susceptible to a variety of changes induced by a large number of sources. This serves as the basis for evolution, but changes to the genetic material may, at the same time, prove detrimental to the organism. DNA damage includes any change to the chemistry of DNA, as well as chemically correct but inappropriately paired nucleotides. DNA lesions can be fixed as mutations via DNA replication, thus changing the original nucleotide sequence of DNA [1]. A major consequence of mutations is possible loss of function of tumor-suppressor genes and improper activation of oncogenes which in turn may trigger uncontrolled cellular proliferation [2]. Genomic instability is one of the hallmark of all cancers [3].

1.1 Sources of DNA Damage

All biological macromolecules, including DNA, spontaneously degrade. Spontaneous DNA damage can arise under normal cellular conditions and occurs at a frequency of over 10,000 events per human cell per day [4]. One of the most frequent types of endogenous DNA damage is the formation of apurinic/apyrimidinic (AP) sites which arise from hydrolysis of the N-glycosylic bond linking the DNA base with the sugar-phosphate backbone, and 2,000 – 10,000 depurinations are estimated to occur per human genome per day [4, 5]. The loss of pyrimidines is about 5% of this rate [4]. Spontaneous base-loss is one of the most mutagenic events in the genome since they have lost the coding information. Moreover, AP sites are unstable and can rearrange to single-strand breaks (SSBs) and AP sites must therefore be repaired efficiently. The majority of AP sites are cleaved by an AP endonuclease 5' to the abasic site followed by 3'-phosphate elimination by the dRP-lyase activity of DNA polymerase β [5]. AP sites are also generated as intermediates in DNA repair (described below).

The spontaneous removal of an amine group from DNA bases is referred to as hydrolytic deamination. Cytosine, adenine and guanine all contain exocyclic amino groups and their deamination causes the mutagenic lesions uracil, hypoxanthine and xanthine respectively. Even though deamination occurs spontaneously, the process can be greatly enhanced in the

presence of DNA damaging agents [6]. The mechanisms and causes of uracil formation are further described in section 1.1.2.

Alkylating agents are widely present in the environment in food, cigarette smoke, occupational chemicals and chemotherapeutic drugs, but are also formed endogenously [7]. The cellular alkylating agent S-adenosyl-methionine (SAM) [8-10] is a methyl donor in several mammalian methylation reactions such as the methylation of cytosine to give 5-methylcytosine. Methylation occurs at DNA bases as well as the phosphate and deoxyribose moieties [11], and the structure of DNA (single or double stranded) and mode of the chemical reaction (S_N1 or S_N2) influence the degree of alkylation. Methylating agents such as N-methyl-N'-nitrosourea (MNU), are S_N1 type agents and act through a monomolecular mechanism while S_N2 type agents such as methyl methanesulfonate (MMS) function through a bimolecular mechanism [12].

Exogenous sources of DNA damage arise from chemical and physical sources such as ultraviolet (UV) light, ionizing radiation (IR), chemicals, toxins and pollutants [13, 14]. UV radiation gives rise to intrastrand crosslinks between adjacent pyrimidines in the DNA and creates free radicals while ionizing radiation may produce DNA strand breaks and induce the formation of ROS.

The consequences of DNA damage may be deleterious and many lesions interfere with transcription by promoting insertion of a non-cognate base in the mRNA (transcriptional mutagenesis), or by stalling the RNA polymerase [15]. Bypassing of lesions by translesion polymerases may lead to an accumulation of mutations in DNA [16, 17] and a cell that has accumulated a large amount of DNA damage or no longer effectively repairs these can go into senescence, undergo apoptosis or start unregulated cell division which may lead to a cancerous tumor [18, 19].

1.1.1. Oxidative DNA Damage

Oxidative DNA damage comprises oxidation of bases in DNA, the sugar-phosphates, and single- and double-strand breaks [1]. Reactive oxygen species (ROS) can be produced both from endogenous and exogenous sources. Endogenous oxidative DNA damages are caused by ROS, which are generated as byproducts of mitochondrial respiration [20], aerobic

metabolism or inflammatory responses [21, 22] and from the cellular signaling molecule nitric oxide (NO) and its derivatives [23]. It has been estimated that approximately 20,000 lesions arise as a consequence of metabolic processes in each human cell per day [24].

During mitochondrial oxidative metabolism the majority of oxygen is converted to water, but 0.2 – 2 % result in ROS because of transfer of electrons from the electron transport chain directly to oxygen leading to formation of superoxide anions ($\bullet O_2^-$). Other reactive oxygen species include the highly reactive hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen. Superoxide dismutase (SOD) catalyses the dismutation of $\bullet O_2^-$ to H_2O_2 and water, and H_2O_2 is removed rapidly by catalases/peroxidases. Any H_2O_2 that escapes this first line of defense may be partially reduced to the very strong oxidant •OH [25], by reacting with Fe^{2+} through Fenton-like reactions ($H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$) [26]. Since metal ions are present in close proximity to DNA in chromatin, this is biologically relevant as these may catalyze the production of •OH sufficiently close to DNA to oxidize it [27].

Exogenous sources include ionizing radiation (IR), UV light, environmental exposure to chemical oxidants, transition metals, and chemotherapeutic drugs [25]. There are many different mechanisms cells employ to protect against free radicals, for example ROS scavenging agents such as ascorbic acid (vitamin C) and glutathione, ROS protective mechanisms such as the above-mentioned SOD, glutathione peroxidases, glutathione reductase and catalase (CAT). Also, there is a sequestration of free iron ions by transferrin, ferritin and metallothioneins [28] to mention a few.

Oxidative stress has been defined as an imbalance between oxidants and antioxidants, where the level of oxidants exceeds that of antioxidants, resulting in an overall increase in the cellular levels of reactive oxygen species [29]. As ROS can induce base modifications and AP sites in DNA, a high number of lesions can potentially be generated and over 80 different aberrant bases created by ROS have been identified [20].

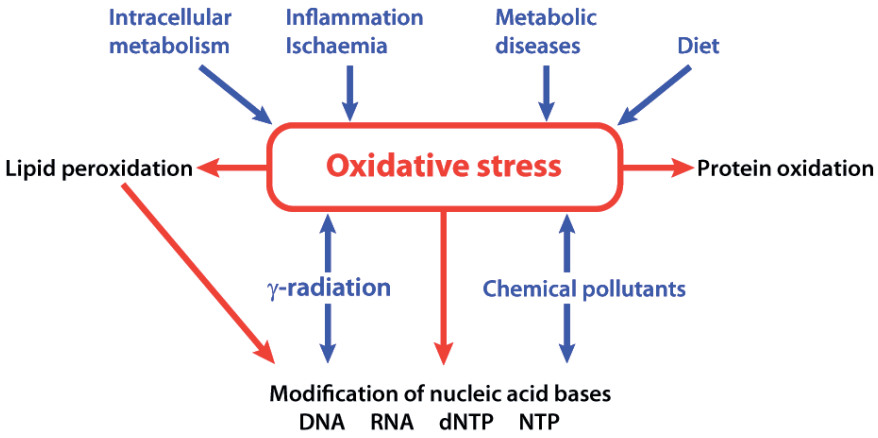

Figure 1: Sources and consequences of oxidative stress. Blue arrows indicate sources and red arrows indicate consequences resulting from oxidative stress.

Guanine is the most readily oxidized base due to its low oxidation potential. The most studied of these is the highly mutagenic 7,8-dihydro-8-oxoguanine (8-oxoG) [30], and the main enzyme for its removal in humans is the 8-oxoguanine DNA glycosylase, hOGG1 [1]. 8-oxoG pairs with adenine as well as cytosine and, if not repaired, can induce GC to TA transversions upon replication by DNA polymerases [31, 32]. The most prevalent damage to pyrimidines results from attack of •OH on the double bond of thymine which generates the cytotoxic 5,6-dihydro-5,6-dihydrothymine (thymine glycol, Tg). A similar attack on cytosine generates cytosine glycol, which is in equilibrium with its dehydrated form, 5-hydroxycytosine (5-OHC) which in turn may deaminate and yield 5-hydroxyuracil (5-OHU). Both of these lesions are mutagenic [33, 34]. Another base injury that can result from ROS is the generation of formamidopyrimidines (faPy) through ring opening of purines. Opening of the adenine ring results in the generation of 4,6-diamino-5-formamidopyrimidine (faPyA), whereas ring opening of guanine generates 2,6-diamino-4-hydroxy-5-formamidopyrimidine (faPyG). These lesions are miscoding *in vitro* and are capable of blocking DNA (and RNA) polymerases [35, 36].

1.1.2. Uracil in DNA

In RNA, uracil is a normal base, but may arise in DNA by utilization of dUTP instead of dTTP for DNA synthesis. Misincorporation of uracil is counteracted by dUTPases, which

keep the concentration of dUMP in the nucleotide pool low [37]. Incorporation of uracil in DNA results in U:A pairs that retain the coding properties, but premutagenic U:G mispairs may arise through spontaneous or enzymatic deamination of cytosine [4]. Deamination of cytosine in DNA, which yields uracil, leads to G:C to A:T transitions upon replication if it is not repaired. Uracil in DNA is the common substrate for uracil-DNA glycosylases (UDGs), which hydrolyze the N-glycosidic bond between the deoxyribose-phosphate backbone and the damaged base. The base is then released resulting in an AP-site in DNA that is a substrate for further processing in the base excision repair pathway [38] (see section 1.2.1.).

Mammalian genomes encode at least two DNA cytosine deaminases: activation induced cytosine deaminase (AID) and APOBEC-3G (also called CEM15). These enzymes are involved in the acquired and innate immune defense, respectively. AID was first identified as a gene expressed only in activated B-cells undergoing class switch recombination (CSR) [39], and it was shown that AID deaminates cytosine in ssDNA *in vitro* [40-43]. Also, overexpression of AID triggers mutations in mammalian cells [43, 44] and in *E. coli* [45].

Both the uracil DNA N-glycosylase UNG2 and AID play an important role in somatic hypermutation (SHM) and CSR in immunoglobulin genes (Ig). In the bone marrow, V(D)J recombination generates the primary repertoire of B-lymphocytes expressing IgM antibody receptors (reviewed in [46]), and when these B-cells encounter foreign antigens, they are activated and migrate to secondary lymphoid organs (lymph nodes, spleen, tonsils) where they proliferate and form germinal centers. These cells then undergo somatic DNA alterations through SHM and CSR. In SHM, affinity maturation is achieved by introducing point mutations in the variable (V) region of the Ig and is followed by positive selection of B-cells that express high affinity Ig for an antigen. In CSR, recombination between two different switch regions upstream from each constant region results in new effector functions of the antibodies. This is also referred to as the isotype switch from IgM to IgG, IgA or IgE.

SHM and CSR is not observed in patients or mouse models with AID deficiency [47]. These patients are susceptible to bacterial but not opportunistic infections, have enlarged secondary lymphoid organs filled with proliferating IgM-expressing B-Cells, and have an autosomal recessive disorder termed hyper IgM syndrome 2 (HIGM2) [48]. A deficiency in UNG2 is also associated with HIGM-like phenotypes in both mice [49] and human patients [50], and it has been shown that Ung-deficient mice develop B-cell lymphomas [51]. In CSR and SHM, then, AID deaminates cytosine into uracil in targeted DNA (immunoglobulin switch- or

variable regions), followed by uracil removal by UNG2. AID-mediated deamination in both donor and switch regions in CSR is assumed to lead to the formation of DNA double-strand breaks (DSBs) followed by deletion of the intervening regions [52].

1.2 DNA Repair

In contrast to modified proteins and lipids, which can be removed by increased cellular turnover, damaged DNA, which may result in cytotoxicity (leading to cell death) or mutations (fixed alterations in DNA), have to be repaired. The present investigation focuses on the effects of DNA repair deficiency in two major pathways, Base Excision Repair (BER) and Nucleotide Excision Repair (NER), which will be discussed in detail in the following sections. Other DNA repair pathways are briefly described below, although their contribution to the effects seen in BER- or NER-deficient strains is not to be underestimated as there is extensive crosstalk between DNA repair pathways [53-56]. Damages to DNA are repaired by several repair mechanisms and six main pathways have been characterized: 1) Direct reversal; 2) mismatch repair (MMR); 3) homologous recombination (HR); 4) non-homologous end-joining (NHEJ); 5) base excision repair (BER) and 6) nucleotide excision repair (NER).

The different pathways have evolved to repair distinct classes of DNA lesions or function at specific timepoints in the cell cycle, although some pathways may have overlapping specificities thus serving as backup systems for each other. In addition to this, cells have evolved damage tolerance mechanisms including special polymerases that allow some damages in DNA to persist during replication and may be involved in lesion bypass by the process of translesion synthesis (TLS).

Direct reversal is the simplest process as it only involves one step where the modified entity on the base is transferred to an enzyme thereby re-establishing the correct chemical structure of the base [57]. In mammals, three enzymes have so far been identified to use this mechanism, the *E. coli* AlkB homologues 2 and 3 (ABH2 and ABH3), and O^6-methylguanine DNA methyltransferase (MGMT or AGT). AlkB is an α-ketoglutarate and iron-dependent oxygenase which hydroxylates the methyl group of 1-meA and 3-meC in DNA [58, 59] and RNA [60] while converting α-ketoglutarate to succinate, releasing the C5 carbon of α-ketoglutarate as CO_2. Three human homologues of AlkB have been identified termed hABH1

(human AlkB homologue 1) [61], hABH2 and hABH3 [60, 62]. In *C. elegans* there are no ABH2 or ABH3 homologues, but homologues for ABH1, 4, 6, 7 and 8 and two MGMT homologues termed *agt-1* and *agt-2* have been identified (table 1).

Table 1: Human genes involved in DNA base repair and their homologs in *C. elegans**

Gene	*H. Sapiens*	*C. elegans*
8-oxoguanine DNA glycosylase	OGG1	-
Nei endonuclease VIII-like 1	NEIL1	-
Nei endonuclease VIII-like 3	NEIL2	-
Nei endonuclease VIII-like 3	NEIL3	-
MutY homolog	MUTYH	-
N-methylpurine-DNA glycosylase	MPG	-
Nth endonuclease III-like 1	NTHL1	*nth-1* (R10E4.5)
Single-strand-selective monofunctional uracil-DNA glycosylase 1	SMUG1	-
Thymine-DNA glycosylase	TDG	-
Uracil-DNA glycosylase	UNG	*ung-1* (Y56A3A.29)
Uracil-DNA glycosylase 2 (cyclin-like)	UNG2	-
Methyl-CpG binding domain protein 4	MBD4	-
AP endonuclease (xth)	APEX1	*exo-3* (R09B3.1)
AP endonuclease-like	APEX2	-
AP endonuclease four-like (nfo)	-	*apn-1* (T05H10.2)
Flap structure-specific endonuclease 1	FEN1	*crn-1* (Y47G6A.8)
Endonuclease V-like	FLJ35220	C08H9.3
Ligase 3	LIG3	K07C5.3
X-ray repair complementing defective repair	XRCC1	-
poly (ADP-ribose) polymerase 1	PARP1	*pme-1* (Y71F9AL.18) *pme-6* (AC8.1)
poly (ADP-ribose) polymerase 2	PARP2	-
polynucleotide kinase 3'-phosphatase	PNKP	F21D5.5
O-6-methylguanine-DNA methyltransferase	MGMT	*agt-1* (Y62E10A.5) *agt-2* (F09E5.13)
AlkB homolog 1	ALKBH	Y51H7C.4
AlkB homolog 2	ALKBH2	-
AlkB homolog 3	ALKBH3	-
AlkB homolog 4	ALKBH4	F09F7.7
AlkB homolog 5	ALKBH5	-
AlkB homolog 6	ALKBH6	B0564.2
AlkB homolog 7	ALKBH7	Y46G5A.35
AlkB homolog 8	ALKBH8	C14B1.10
dUTPase	DUT	*dut-1* (K07A1.2)
Nudix (nucleoside diphosphate linked moiety X)-type motif (8-oxoGTPase)	NUDT1	-
ribonucleotide reductase M2 B (TP53 inducible)	RRM2B	*rnr-2* (C03C10.3) F19G12.2

***** A sequence-based homology search of the whole genome revealed the above *C. elegans* homologs of human base repair genes. Dashes indicate that no obvious homologs were found (T. Rognes, personal communication). See also Table 2 section 1.3.1.

MGMT inactivates itself by transferring the methyl- or other simple alkyl-groups from O^6-alkylguanine or O^4-alkylthymine to an internal cysteine residue (reviewed in [24]). The term "enzyme" in this reaction is somewhat misleading as the protein is stoichiometrically consumed when the alkyl group is transferred, and the proteins are therefore often referred to as "suicide enzymes". Depletion of MGMT sensitize cells to O^6-alkylators and a correlation between the mismatch repair (MMR) system and MGMT levels on the ability to survive treatment with O^6-alkylators have been suggested [63], as MMR proficient cells with low levels of MGMT display cytotoxicity upon low levels of O^6-meG while MMR deficient cells can tolerate high levels of O^6-meG [64, 65]. A proposed mechanism for this phenotype is that during replication, unrepaired O^6-meG in the template strand is mismatched with thymine which triggers the MMR system. However, as the best match for O^6-meG actually is thymine, this initiates a futile repair cycle to repair this mismatch which might lead to cell death [66].

The MMR pathway, as alluded to, removes mismatches, nucleotides that are mispaired by DNA polymerases, and insertion/deletion loops that result from slippage during replication [67]. Poor processing by MMR commonly result in microsatellite instability (MSI), which is often found in hereditary non-polyposis colorectal cancer (HNPCC), and approximately 80% of people carrying germline mutations in one of the central MMR components develop colon cancer, underscoring the importance of this DNA repair pathway [68]. Mammalian MMR involves several protein homologues of the *E. coli* prototype factors MutS and MutL. In human cells, the hMutSα complex (a heterodimer of hMSH2 and hMSH6) recognize single nucleotide mismatches and small insertion/deletion loops. The heterodimer hMutSβ (composed of hMSH2 and hMSH3) also contribute to the repair of small loops during MMR. The eukaryotic MSH2 and MSH3 and bacterial MutS all have intrinsic ATPase activity essential for mismatch repair [69], and structural studies of *E. coli* and *Thermus aquaticus* (Taq) MutS indicate that the mismatched DNA is detected by kinking the DNA towards the major groove [70, 71]. Heterodimeric complexes of the MutL-like proteins hMLH1/hPMS2 (hMutLα) and hMLH1/hPMS1 (hMutLβ) interact with replication factors and MSH complexes. hMLH1 also interacts with MLH3 (forming MutLγ), MSH4, MBD4 and C-MYC [72-74].

The MMR system is required for activation of the S-phase checkpoint in response to ionizing radiation, as cells deficient in MLH1 and MSH2 show radioresistant DNA synthesis after IR

and restoration of normal MMR function was shown to restore normal S-phase checkpoint function [75]. A certain level of MSH2 has also been demonstrated to be required for G2/M checkpoint activation [76]. Several proteins are involved in the excision of the damaged strand and resynthesis, such as Polδ/ε, RPA, PCNA, RFC, EXO1 and FEN1 and MMR components functionally interact with proteins involved in recombination and NER [77].

DSBs are thought to mainly arise from IR, free radicals, chemicals and during repair of single strand breaks (SSBs), and it is estimated that ~50 DSBs are produced during each cell cycle [78]. Repair of double strand breaks are predominantly mediated by either the NHEJ or HR pathways [79, 80]. In mammalian cells, NHEJ is thought to be the predominant repair pathway for DSBs. However, in late S/G2 phase when the sister chromatids are close to each other and available for exchange, HR and NHEJ may compete for repair [81], and HR may actually be the major mechanism for DSB repair [82]. After replication, in S or G2 phase of the cell cycle when a second identical DNA copy is available, HR seems to be preferred while NHEJ is especially active in the G1 phase [83].

NHEJ is initiated by binding of the heterodimer Ku70/Ku86 and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the broken DNA ends. DNA-PKcs then phosphorylates and forms a complex with the protein Artemis, and this complex acts as an endonuclease that trims both 5'- and 3'-ends at the DSB and ligation is performed by the XRCC4-DNA ligase IV complex [84]. NHEJ is also required for the development of T- and B-cell repertoires through T-cell receptor α/β and Ig V(D)J recombination and inactivation of ARTEMIS in humans results in severe combined immunodeficiency (SCID) in humans and the same phenotype is seen in DNA-PKcs-null mice [85, 86]. Other phenotypes associated with defects in NHEJ are radiosensitivity, genomic instability, growth retardation, impaired embryonic development and cancer predisposition [56].

HR uses an intact sister chromatid as the template and the 5'-ends at the DSB are resected by a 5' to 3' exonuclease which produces 3' overhangs that are subsequently used as substrates for homologous pairing and strand invasion [87]. This resection has been proposed to be executed by the Rad50-Mre11-NBS1 complex [88] and the resected ends are protected by RPA until displaced by Rad51 [87]. Strand invasion and single-strand annealing is performed by the combined action of Rad52 and Rad54 (reviewed in [89]) and DNA synthesis is initiated and extended past the original DSB site. By resolving the Holliday junction and extending the ends beyond the original DSB site, error-free repair is achieved.

Defects in either repair pathway can have severe consequences and the human syndromes AT (ataxia telangiectasia) like disorder (ATLD) and Nijmegen Breakage Syndrome (NBS) are caused by defects in DSB repair. ATLD patients show neurodegeneration and immunodeficiency whereas NBS patients are characterized by growth retardation, immunodeficiency, microcephaly and cancer predispositions [90]. Mutations in genes encoding RecQ helicases, which are required for efficient HR, are associated with rare syndromes such as Werner syndrome (WS), Rothmund-Thomson syndrome (RTS) and Bloom syndrome (BS) [91].

The distinction between the different pathways is not as clear cut as their classifications may imply due to the extensive crosstalk between them and some DNA repair proteins have been shown to be involved in more than one pathway [53-56]. In the present study, BER and NER has been the focus of two of the papers and these pathways will be discussed in more detail below.

1.2.1. Base Excision Repair

The main mechanism for removal of endogenously generated DNA base damage, including oxidative lesions, small alkylation products and different kinds of single-strand breaks is the base excision repair (BER) pathway [4, 87]. BER is initiated by DNA glycosylases that recognize and excise groups of related lesions [92]. At least 12 different mammalian DNA glycosylases exist, of which 7 have been reported to have overlapping specificities towards oxidative DNA damage [21, 93]. Glycosylases are classified as either mono- or bifunctional.

Monofunctional glycosylases are hydrolases and use an activated water molecule to attack the N-glycosylic bond that links the base and the DNA backbone. The 5'-phosphodiester linkage of the AP site is then cleaved hydrolytically by a Mg^{2+}-dependent AP-endonuclease (APE1), leaving 3'-hydroxyl and 5'-deoxyribophosphate (5'-dRP) termini. Polymerase β (Pol β) then removes the abasic residue with its associated dRP-lyase activity and inserts the correct base. Bifunctional glycosylases on the other hand, use an active site amine nucleophile instead of water to remove the damaged base. This amine serves as a Schiff-base electron sink to facilitate the subsequent β-elimination reaction in which the 3'-phosphate of the abasic nucleotide is expelled.

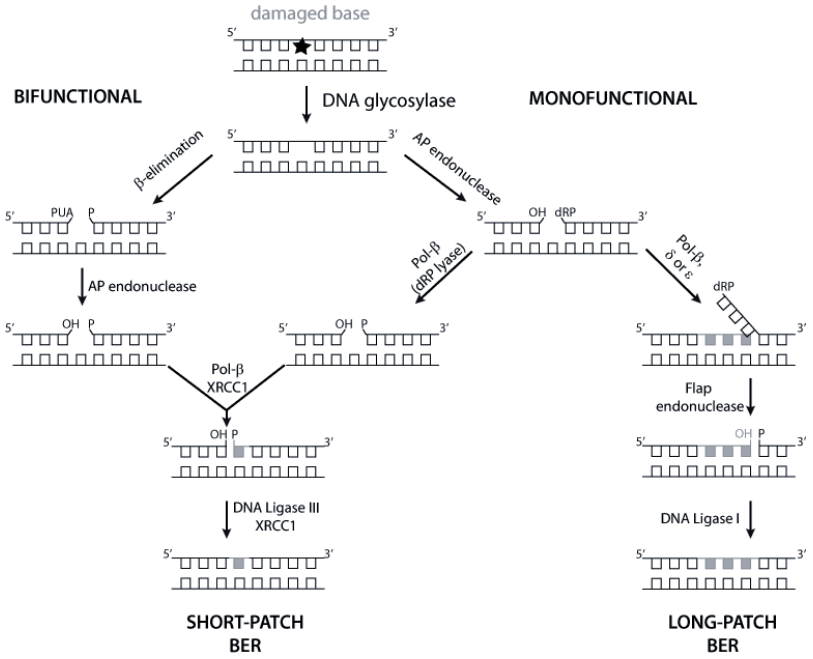

Figure 2: A simplified schematic of the main steps in Base Excision Repair. The damaged base (indicated by a star) is recognized and removed by a bifunctional or monofunctional DNA glycosylase. After incision of the DNA strand and end processing, the intermediate is directed either into LP-BER or SP-BER for DNA synthesis and strand annealing. For details, see text. Image adapted and modified from [94]

Abasic sites are both mutagenic and cytotoxic [95]. It is believed that most mammalian DNA glycosylases remain bound to the abasic site after incision [96, 97] and that APE1 replaces the glycosylase at the AP site [98].

BER is further divided into two subpathways: the short-patch (SP-BER) which inserts a single nucleotide, or long-patch (LP-BER) which inserts a stretch of 2-12 nucleotides. In SP-BER, after insertion of the correct base by Pol β, the backbone is sealed by a DNA ligase and X-ray repair cross complementing protein 1 (XRCC1). XRCC1 has no detected enzymatic activity, and has been proposed to play a central role in coordinating the activities of enzymes participating in BER [99]. The importance of this protein is underscored by the observation that cells lacking XRCC1 are hypersensitive to IR [100], oxidizing and alkylating agents [101, 102], and display elevated spontaneous frequency of chromosomal aberrations and deletions [103].

In LP-BER, the strand containing a 5'-dRP at the incised AP site is replaced by several nucleotides. Strand displacement synthesis can be performed by Pol β, Pol δ or Pol ε [104]. Pol δ and Pol ε are believed to be the main polymerases involved in LP-BER, as Pol β knockout cells are deficient in SP-BER, but proficient in LP-BER [105]. The action of DNA polymerases create a 5' flap that is removed by the flap endonuclease FEN1 [104], and the resulting DNA break is ligated by DNA ligase I. Throughout the process, proliferating cell nuclear antigen (PCNA) interacts with Pol δ/ε, FEN1 and DNA ligase I and supports its functions [106] as a processivity factor.

The choice of pathway may be determined by the type of damage, the glycosylase, the DNA polymerase involved and the cell cycle. Lesions excised by bifunctional DNA glycosylases are predominantly repaired via the short patch pathway, while lesions excised by monofunctional DNA glycosylases are repaired by either pathway [107].

1.2.2. Nucleotide Excision Repair

Nucleotide excision repair (NER) primarily eliminates helix-distorting lesions that interfere with base pairing and obstruct transcription and replication [77, 108]. NER is comprised of four sequential steps: 1) damaged DNA is recognized; 2) the damaged base, embedded in an oligonucleotide 24-32 nucleotides long, is excised by dual incision of the damaged strand on both sides of the base damage; 3) the resulting gap is filled by one or more DNA polymerases; and 4) the nick is ligated [109].

NER is divided into two subpathways: transcription-coupled repair (TC-NER) and global genome repair (GG-NER). The difference between these is in the damage recognition mechanisms. TC-NER targets lesions that block transcription [110, 111], and it is believed that arrest of RNA polymerase II at bulky lesions is a signal for recruitment of DNA repair factors [112]. GG-NER, on the other hand, surveys the entire genome and is therefore more involved in repairing damages at non-transcribed regions of the genome [113]. TC-NER is a rapid process compared to GG-NER and the level of compaction of DNA is important for this rate, illustrated by Philip Hannawalt and colleagues (reviewed in Hanawalt, 2001) as they found that actively transcribed regions are repaired faster than transcriptionally silent regions. Exactly how damage is recognized in transcriptionally silent DNA is not fully understood,

although the remodeling of chromatin that accompanies transcription may make it passively more accessible for GG- NER factors. The detection of transcription blocks have also been proposed to enhance global lesion detection as a result of the global chromatin relaxation seen by p53-mediated histone acetylation [114].

Some of the proteins that take part in the initial steps of binding to the lesion are known, such as XPC and RAD23 [112]. Purified XPC have been shown to preferentially bind to DNA with base damages that are substrates for NER and is tightly complexed with hRAD23B (a human homologue of RAD23) in human cells , [115, 116]. XPC is not required for damage recognition in TC-NER, as humans and mice defective in XPC show no deficiency in this branch of NER and it is therefore believed that this complex is responsible for damage recognition mainly in GG-NER [117]. On the other hand, a deficiency in either CSA or CSB, which are part of the normal transcription machinery, results in loss of TC-NER implicating these proteins as part of the transcriptional branch of NER.

In mammalian cells, there are two homologues of RAD23, hRAD23A and hRAD23B, and knockouts in mice of either one give a NER phenotype. The double knockout is embryonic lethal, indicating an indispensable function during development [112]. The expression of XPC is induced by p53 following UV-light exposure [118], and the stability of XPC is increased by both homologues of RAD23 and by binding to DNA damage [119].

After recognition of the lesions, the two NER branches feed into a common pathway. In GG-NER, the core transcription factor IIH (TFIIH) is recruited directly after damage recognition by XPC and RAD23. In TC-NER, binding of TFIIH happens after displacement of the stalled RNA polymerase by CSA/CSB. TFIIH consists of six subunits and is a part of the RNA polymerase II basal transcription complex which is required for initiation of transcription. Two of the subunits of TFIIH, XPB and XPG, are DNA helicases which unwind the DNA both during transcription and NER. The XPA protein binds to the lesion, verifies the damage, then anchors the ERCC1-XPF nuclease and activates the nuclease activity of XPG [120]. The bubble structure created by TFIIH is important for correct incisions by the endonuclease XPG and the heterodimer ERCC1-XPF [121]. These cleave the oligonucleotide backbone 3' and 5' to the lesion respectively, and release the fragment of 24-32 nucleotides. The exact length of this fragment and where the base damage is positioned within it depends on the type of damage. The resulting gap is filled by DNA polymerase δ and ε (pol δ/ε) and the nick is ligated by DNA ligase I.

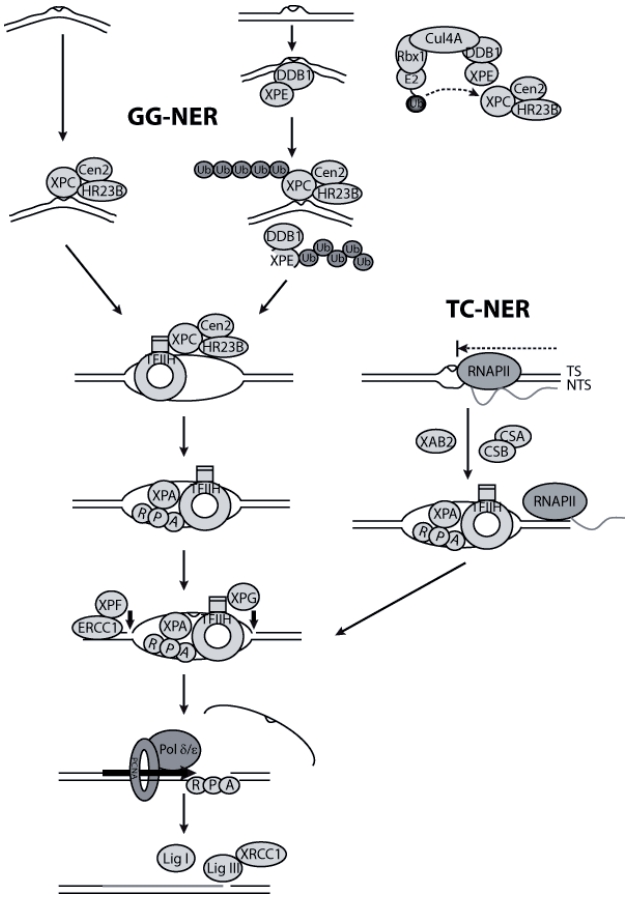

Figure 3: Mechanisms of Nucleotide Excision Repair. In GG-NER, the lesion is recognized by the XPC complex and the core transcription factor TFIIH is recruited. XPA verifies the damage and anchors the ERCC1-XPF nuclease for incision of the DNA backbone, the gap is filled by pol δ/ε. In TC-NER RNA polymerase II is stalled by lesions in the transcribed strand (TS) and recruits NER factors, and repair proceeds as in GG-NER. Ubiquitination of XPC increases affinity, while ubiquitination of XPE leads to degradation. For details, see the text. Adapted and modified from [122].

The importance of lesion types, their recognition, signaling and repair are illustrated by the syndromes associated with mutations in different components of the NER pathway. *Xeroderma pigmentosum* (XP) is an autosomal recessive disorder characterized by a strong predisposition to various skin cancers, mainly squamous cell carcinomas and basal cell carcinomas and to a smaller extent, melanomas. Mutations in any of the seven NER genes XPA-XPG have been linked to the development of XP [123], and patients are thus deficient in

either GG-NER or the common pathway. Cockayne syndrome (CS) on the other hand, is caused by mutations in either CSA or CSB which recognize damages and initiate TC-NER, and patients are UV-sensitive but not cancer prone after exposure to UV-light [111]. Many, but not all patients diagnosed with *trichothiodystrophy* (TTD) have mutations in XPB or XPD and these patients are also UV-sensitive but not skin-cancer prone [56, 113, 124].

The biochemically phenotypic difference in cancer predisposition in XP patients compared with CS or TTD patients could be that defective TC-NER in CS and NER in TTD is still able to signal induction of apoptosis, thus giving some protection against UV-light induced cancer and underscoring the importance of the DNA damage response [124, 125].

Another of the clinically observed phenotypes seen in the above-mentioned syndromes is a progressive neurological abnormality which is not directly explained by defects in NER, as NER primarily resolves helix-distorting lesions. It has been proposed that neurodegeneration may be caused by ineffective repair of oxidative lesions as neurons consume great amounts of molecular oxygen. The major DNA lesions produced by oxidative damage are non-bulky lesions (although there are some oxidative lesions, like cyclobutyl-adducts, that are bulky) and are thus substrates primarily for the base excision repair pathway [126]. However, reconstituted human NER proteins have been shown to repair 8-oxoG and Tg *in vitro* [127]. The authors speculate that the level and activity of glycosylases in neurons are insufficient to deal with the large amount of oxidative damage in these cells and that NER plays a major role in defending neural cells against oxidative damage. Defective repair of non-bulky oxidative lesions in XP patients may therefore be the cause of the observed neurodegeneration. Indeed, in *Saccharomyces cerevisiae* (*S. cerevisiae*), a mutant defective in both BER and NER showed greatly enhanced sensitivity to the oxidizing agents menadione and H_2O_2 compared to only BER or NER mutants alone [128].

Here the authors investigated the overlapping specificities of four different repair pathways and created mutants deficient in BER, NER, TLS and HR. BER mutants deficient in the Endonuclease III homologues Ntg1p and Ntg2p showed no increased sensitivity to oxidizing agents either singly or in combination. A triple mutant including the major AP endonuclease Apn1 (which incises abasic sites created by N-glycosylases) did not show any sensitivity either, ruling out the possibility of backup excision activity by other glycosylases and suggesting either involvement of other DNA repair pathways or increased tolerance to DNA damages (bypassing of lesions during replication). When this triple mutant was additionally

depleted of RAD1, which is responsible for the 5' incision of damages in the Rad1-Rad10 protein complex in the NER pathway, however, a greatly enhanced sensitivity to both menadione and H_2O_2 was observed. When the BER triple mutant was depleted of REV3, the catalytic subunit of pol ζ and involved in translesion synthesis, it remained resistant to oxidizing agents. However, disrupting RAD52, a component of the HR pathway, in either BER or TLS mutants gave a sensitive phenotype to oxidative damage. This experiment showed the importance and consequences of the types of lesions that are induced as disrupting BER and NER gave highest sensitivity to H_2O_2, while disrupting BER and recombination or TLS and recombination gave highest sensitivity to menadione [128]. Both of these agents are oxidizing, but menadione may also produce strand breaks which may explain the sensitivity in the recombination mutants, but also indicates that NER is involved in the repair of oxidative lesions and has overlapping substrate specificity with BER.

S. cerevisiae is a very powerful genetic model organism for elucidating the overlapping specificities and cross-talk between different pathways for DNA repair. However, it is a single-cell organism and does not display the complexity of different tissues or cell-cell communications present in higher order animals. On the other hand, this complexity of higher order animals like mice and humans also renders genetic analyses more complex. This makes *C. elegans* very well suited for genetic investigations in higher order animals as it is genetically tractable, has a high conservation rate of its genes during evolution, contains different tissues and is easily manipulated.

1.3 *C. elegans* as a Model System

Caenorhabditis elegans (*C. elegans*) is a small (about 1 mm in length) free-living nematode commonly found in many parts of the world – if not all. It is easily maintained in the laboratory on agar plates or in liquid culture as it feeds primarily on bacteria. Under optimal conditions, *C. elegans* reproduces with a life cycle of about 3 days. There are two sexes, hermaphrodites and males, which have the same length, but differ in appearance. Hermaphrodites can reproduce by self-fertilization as they produce both oocytes and sperm. Males arise spontaneously by X-chromosome non-disjunction, comprise about 0.05 % of the total population and can fertilize hermaphrodites. Hermaphrodites cannot cross-fertilize. A self-fertilized hermaphrodite lays about 300 eggs during its reproductive life span. Hatched

worms develop through 4 larval stages separated by molts. The mature adult is fertile for about 4 days and lives for an additional 10-15 days at 20°C in the laboratory [129].

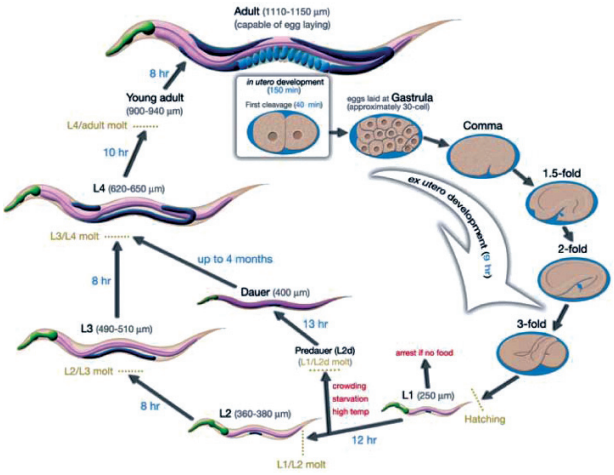

Figure 4: Life cycle of the nematode *Caenorhabditis elegans*. Image adapted from www.wormatlas.org

C. elegans is one of the simplest organisms with a nervous system and every single synaptic connection in the worm is known (a complete wiring diagram of the neuronal system is available) [130]. *C. elegans* was also the first multicellular organism to have its genome completely sequenced and published in 1998 [131], and currently about 20,000 genes have been identified.

The animals are transparent throughout their life cycle and the development can be studied at the cellular level with the aid of a light microscope [129]. Unlike any other animal, the entire life cycle of the nematode is understood at a single cellular resolution [130]. This enables researchers to study the contribution of progenitor cells to development, directly target certain cells in order to elucidate cell-cell communications or specifically ablate cells to investigate development or communication between different tissues.

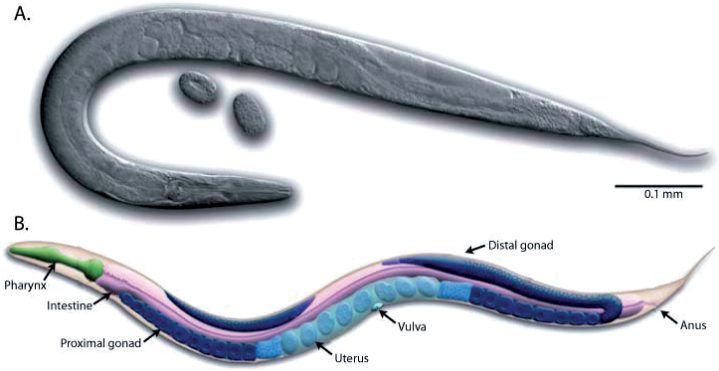

Figure 5: Anatomy of an adult hermaphrodite *C. elegans*. A) DIC image left lateral side; B) Schematic drawing of the anatomical structure. Image adapted and modified from www.wormatlas.org

Research using *C. elegans* was begun in 1974 by Sidney Brenner and is now an established model organism [132]. *C. elegans* has five pairs of autosomes and one pair of sex-chromosomes (hermaphrodites having a matched pair, XX, and males having a single copy, XO). The hermaphrodite has 959 somatic cells and the male 1031, and all cell fates have been mapped out in their entirety [133, 134]. A large number of cells (131 in the hermaphrodite) are eliminated by programmed cell death, which have aided the study of the physiological apoptotic response [135] and led to the Nobel prize in 2002 to Sidney Brenner, H. Robert Horvitz and John Sulston.

The core apoptotic machinery consists of four proteins that act sequentially in the killing phase of programmed cell death. Four of the genes in the apoptotic pathway are "death-promoting" in that strong loss-of-function mutations in *ced-13, egl-1*, *ced-4* or *ced-3* result in the survival of essentially all cells that undergo programmed cell death during development [136-138], and these genes act within dying cells to promote apoptosis [136, 139]. Another member of the core apoptotic pathway, *ced-9*, protects cells from undergoing programmed cell death during development, and loss of *ced-9* cause embryonic lethality [140]. The *ced-9* gene encodes an ortholog of the human Bcl-2 [141] which prevents apoptosis in mammals in a similar mode to that of CED-9 [142]. EGL-1 and CED-13 contain a BH3 motif found in all pro-apoptotic members of the Bcl-2 gene family and mediates direct binding of these to anti-apoptotic Bcl-2 members [136, 143]. CED-3 encodes the founding member of the caspase

family [144, 145], and CED-4 encodes a protein similar to the human activator of caspase-9, Apaf-1 [146, 147].

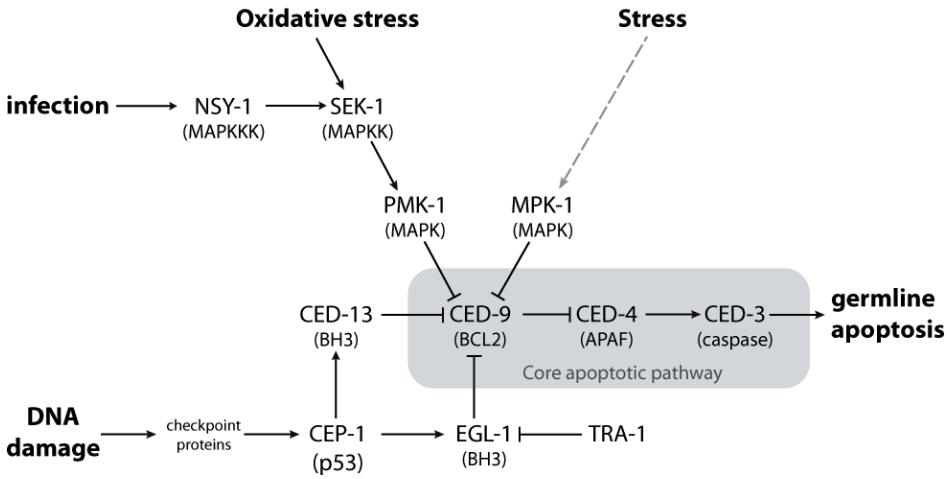

Figure 6: Genetic pathways feeding into the core apoptotic pathway (grey box). Image modified from [148].

RNA interference (RNAi) is a powerful method to transiently silence gene expression and was first characterized in *C. elegans* [149]. This discovery led to the Nobel Prize in physiology or medicine in 2006 to Andrew Z. Fire and Craig C. Mello. In this method, double-stranded RNA (dsRNA) is introduced into the animal and results in the specific silencing of genes with a complementary sequence [150]. Delivery of the dsRNA is easily administered to the nematode as the bacteria it feeds on can be made to express the desired dsRNA on plasmids. The bacteria will then be ingested by the nematode and the RNA taken up in the intestinal tract. Other methods are also used such as soaking the worms in a dsRNA solution or injecting dsRNA with a needle.

Another discovery that has lead to a Nobel Prize (in chemistry, 2008) was the discovery and utilization of green fluorescent protein, GFP. The discovery and cloning of GFP from the jellyfish *Aequorea victoria*, along with the chemical synthesis of many other colors, was attributed to Osamu Shimomura and Roger Tsien. Martin Chalfie spliced the GFP sequence into DNA under the control of different promoters which control protein expression which allows researchers to follow proteins tagged with GFP under the microscope [151].

All of these features make *C. elegans* a powerful model organism in which to study many different diseases pertaining to humans and in the elucidation of genetic mechanisms.

1.3.1. DNA Repair in *C. elegans*

Although more studies on DNA repair in *C.* elegans are emerging, the main focus up till now has been to study DNA damage induced activation of intracellular signaling pathways, the so-called DNA damage response (DDR). Mitotic germline nuclei arrest proliferation in response to DNA damage and cells with DNA damage are removed by apoptosis before oogenesis in the meiotic region (Figure 7) [152]. DNA damage-induced apoptosis occurs in addition to physiological germ cell death which is thought to aid in maintaining germline homeostasis. Physiological germ cell death is induced independently of the BH3-only domain protein EGL-1, which is involved in other apoptosis in *C.* elegans [153], and is also independent of the transcription factor p53 (CEP-1), which is required for radiation-induced germ cell apoptosis [154, 155]. The DNA damage-induced germ cell death requires CEP-1 and acts partially through EGL-1 and CED-13 [154-157]. In addition to the physiological and DNA damage-induced germ cell apoptosis, this has also been observed in response to pathogen infections [158, 159]. All of these pathways converge on the conserved apoptotic machinery [157] (Figure 6).

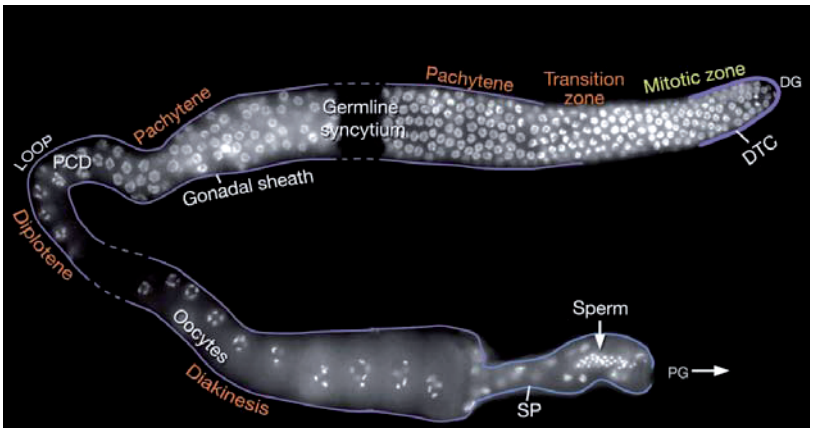

Figure 7: The C. elegans germline. Image of a DAPI-stained dissected adult hermaphrodite *C. elegans* gonad with the different regions indicated. Meiosis is indicated in orange and prophase I starts at the transition zone. PCD: programmed cell death. DTC: distal tip cells. SP: spermatheca. PG/DG: proximal/distal gonad. Magnification 400x. Adapted from www.wormatlas.org

DNA damage response signaling is best described following ionizing or UV-radiation and the mechanism seem to be similar to that observed in human cells. Briefly, DNA damage sensors (e.g. the 9-1-1 complex) detect damage, recruit transducers (ATM-1, ATL-1, CLK-2) that activate signaling cascades (CHK-1, CHK-2) that activate the effectors (e.g. CEP-1) to induce cell cycle arrest or apoptosis [160]. Overexpression of human CLK2 in *C. elegans* have been shown to result in hypersensitivity to apoptosis triggered by oxidative stress or DNA replication blockage [161] and abrogation of *clk-2* have been shown to eliminate cell-cycle arrest and apoptosis after dUTPase depletion [162]. This demonstrates that endogenous DNA damage is able to signal to the DDR. However, this is dependent on processing by BER to generate DNA repair intermediates. No obvious DNA damage-induced checkpoint has so far been described in somatic cells in *C. elegans*, but DNA repair and DNA damage response checkpoint proteins are known to exist and function here ([163] and T. SenGupta, H. Nilsen unpublished results).

Most of the DNA repair mechanisms appear to be evolutionary conserved and this is seen in *C. elegans* and all six main repair pathways described above are present.

Table 2: DNA repair genes belonging to different repair pathways identified in the *C. elegans* genome *

Pathway	Gene	Cosmid	Reference
BER	*nth-1*	R10E4.5	[164]
	ung-1	Y56A3A.29	[165]
NER	*xpa-1*	K07G5.2	[166]
	XPB	Y66D12A.15	-
	XPC	Y76B12C.2	[167]
	XPD	Y50D7A.2	-
	XPE	M18.5	[168]
	XPF	C47D12.8	[169]
	XPG	F57B10.6	[170]
	ercc-1	F10G8.7	[171]

	csb-1	F53H4.1	[172]
MMR	*msh-2*	Y47G6A.11	[173]
	msh-6	H26D21.2	[174]
	mlh-1	T28A8.7	[175]
	pms-2	H12C20.2	[175]
NHEJ	*cku-70*	Y47D3A.4	[176]
	cku-80	R07E5.8	[177]
	lig-4	C07H6.1	[177]
HR	*rad-54*	W06D4.6	[177]
	rad-50	T04H1.4	[178]
	rad-51	Y43C5A.6	[179]
	top-3	Y56A3A.27	[180]
	dna-2	F43G6.1	[181]
Direct reversal	*agt-1*	Y62E10A.5	[182]
	agt-2	F09E5.13	[182]
	ALKBH	Y51H7C.4	(T.Rognes, personal communication)
	AlkB homolog 4	F09F7.7	(T. Rognes, personal communication)
	AlkB homolog 6	B0564.2	(T. Rognes, personal communication)
	AlkB homolog 7	Y46G5A.35	(T. Rognes, personal communication)
	AlkB homolog 8	C14B1.10	(T. Rognes, personal communication)

*The selection is adapted and modified from [152]

The first study of repair functions in *C. elegans* was performed by Hartman and Herman in 1982, who identified nine radiation-sensitive (Rad-) mutants that were hypersensitive to ultraviolet light during embryogenesis [183]. Many of these mutants have since then been mapped, including the *rad-3* mutant which is defective for *xpa-1* [166]. Reverse genetic techniques such as RNAi and deletion mutants have subsequently been used to investigate the role of different DNA damage response pathways [166, 169, 172].

Homologues of the four MMR proteins MSH2, MSH6, MLH1 and PMS2 have been identified in *C. elegans* and transient depletion of *msh-2*, *msh-6*, *mlh-1* and *pms-2* by RNAi have been shown to result in a mutator phenotype as measured by monitoring *in vivo* reversions back into frame of an out-of-frame lacZ transgene reporter gene [173]. Loss of *msh-2* has also been shown to result in increased microsatellite instability [174] as is also observed in humans with defects in MMR (section 1.2.). Homologues for all of the seven XP

genes in the NER pathway in addition to *ercc-1* and *csb-1* are also found in the *C. elegans* genome [152]. A recent paper demonstrated that eukaryotic NER is well conserved in *C. elegans* and that the involvement of the two subpathways in response to UV varies with the developmental stage; GG-NER is the major pathway in germ cells and early embryos, while TC-NER predominates in later stages [171]. The authors confirm previous observations that apoptosis is not induced after irradiation in pachytene cells lacking functional XPA-1 [167] and speculate that this might be due to lack of necessary signaling of DNA damage to the apoptotic machinery by active NER, possibly via NER-generated intermediates such as ssDNA [171].

A sequence-based homology search for genes known to be involved in BER, however, revealed a striking underrepresentation of DNA glycosylases, most notably a lack of glycosylases that recognize and excise oxidized purines (Table 1). This apparent lack of glycosylases other than UNG-1 [165, 184] and NTH-1 [164] is remarkable. Uracil-DNA glycosylase activity in *C. elegans* was first described by Shatilla and Ramotar that showed uracil-excising activity in embryonic extracts and that this activity was inhibited by Ugi, a *Bacillus subtilis* bacteriophage PBS2 peptide inhibitor of UDG [165]. The UNG-1 enzyme was later purified and showed to have activity against uracil in both single- and double-stranded DNA but with a higher substrate specificity for uracil in dsDNA than ssDNA [184] which is unlike other UNG-family UDGs which have a preference for ssDNA [185]. This was later confirmed in our laboratory and an additional Ugi-inhibited uracil-excising activity was also detected in *ung-1* mutants [148]. Lack of UNG-1 rescues the lethality associated with increased uracil in DNA by depletion of dUTPase [162], and leads to increased number of apoptotic bodies after IR implicating UNG-1 in the repair of IR lesions [148]. The NTH-1 enzyme has also been purified and shown to have activity against Tg, 5-formyluracil (5-foU), 5-hydroxymethyluracil (5-hmU) and a weak ability to excise 8-oxoG paired with guanine [164] and *nth-1* mutants show a 17-fold increased mutation rate [186].

The lifespan of *ung-1* and *nth-1* mutants are normal ([148, 184, 186] and H. Kassahun, H. Nilsen unpublished) while there are conflicting reports on the lifespan of *xpa-1* mutants ranging from near-normal [183, 187] to a maximum of 15 days compared to 25 days in the wildtype [188]. In Paper II the *xpa-1* mutant was shown to have a reduced mean lifespan of 3 days [189]. The contribution of DNA damage and repair to lifespan remains to be resolved.

1.4. Aging

Why do organisms age? This question has for quite obvious reasons been debated throughout history and many have tried to find the elusive "fountain of youth". Molecular biologists have also tried to answer this question in a scientific manner and several theories have been launched. The most prominent of these is Harman's oxidative theory of aging. This theory proposes that aging is a result of accumulated damage to the genome [190] and was inspired by two sources: the "rate of living" theory proposed by Max Rubner in 1908 [191] (formalized by Raymond Pearl [192]), and observations made by Gerschman and Gilbert [193].

The rate of living theory postulates that lifespan is the inverse relationship of an organism's metabolic rate and was based on observations which suggested that animals with slower metabolic rates tended to have longer lives. The other finding that led to Harman's theory was the observation by Rebeca Gerschman *et al* that oxygen poisoning and radiation toxicity were both caused by ROS [193]. Harman combined these two sources and argued that ROS produced during normal respiration would lead to damage accumulation, organismal loss of functionality and finally death. Harman later modified his free radical theory [194] to emphasize the importance of mitochondria as these generate most of the ROS found in cells through the electron transport chain. The immediate implication of this theory is that increasing concentrations of ROS would lead to a shorter lifespan and, conversely, a reduction would increase longevity.

Work done in the fruit fly *Drosophila melanogaster* (*D. melanogaster*) in the 1990s demonstrated a relationship between the level of mitochondrial superoxide dismutase (MnSOD) and lifespan. SOD is a detoxification enzyme which converts superoxide to hydrogen peroxide, which then can be converted to water via the action of catalases [195] (Section 1.1.1.), and is therefore a good enzyme to manipulate in order to determine the relationship between ROS produced in mitochondria and lifespan. Artificial selection for longevity over 25 generations in *D. melanogaster* showed an increase in content and activity of cytoplasmic copper-zinc superoxide dismutase (CuZnSOD) and increased transcription of CuZnSOD, MnSOD and CAT mRNA [196]. An accompanying phenotype of these long-lived flies was increased resistance to the free-radical generator paraquat [197], and reversed

selection in this strain resulted in simultaneous reduction in lifespan and paraquat resistance [198] suggesting a correlation between increased antioxidant defenses and lifespan.

Constitutive expression of the cytoplasmic form of superoxide dismutase, *Sod1*, has given conflicting results ranging from extension of lifespan [199] to no change or reduction [200]. Combined overexpression of both cytoplasmic (*Sod1*) and mitochondrial (*Sod2*) forms, however, was shown to have an additive effect in that the extension of lifespan was proportional to the level of SOD overexpression [201]. Concomitant overexpression of SOD and CAT also showed phenotypes with an increase in both average and maximum lifespan [202].

A *Sod1* null mutant displays an 80% reduction in lifespan [203] and this has been attributed to accelerated aging as an acceleration of the temporal-age pattern of expression of the *wingless* gene, a marker for aging, was observed [204]. Knocking down *sod2* via RNAi resulted in postnatal lethality (10 days after birth) [205] and a complete knockout resulted in death within 36 hours after hatching [206], showing the importance of superoxide dismutases for viability of fruit flies.

This seems to be the case also in *S. cerevisiae* as knocking out *Sod1* here results in a decrease in the clonal and replicative lifespan (the number of daughter cells generated by a single mother) [207, 208] and an extension of chronological aging (survival of a population of nondividing cells) [209]. Knockout of the mitochondrial form, *Sod2,* also gave decreased chronological and replicative lifespan [208, 209].

Mice lacking *Sod1* display high levels of oxidative stress and acceleration of age-related pathologies. Tissue-wide lipid peroxidation was elevated 2- to 3-fold as measured by the levels of plasma F_2-isoprostanes [210]. An increase in DNA mutation frequency in the liver [211] and an increase in oxidative damage to DNA and protein carbonyls was reported in *Sod1*$^{-/-}$ mice along with a 30% reduction in lifespan [212]. *Sod2* knockout mice have a ~4-fold increase in oxidative DNA damage as measured by the levels of 8-oxoG, 8-oxoA and 5-OHC [213], they are very sensitive to hyperoxia [214, 215] and suffer from neonatal or perinatal lethality [216, 217]. The third type of superoxide dismutases, the extracellular SOD (SOD3), is present in plasma and most abundantly in the lung [218]. Deletion of *Sod3* in mice was not reported to impact lifespan although they displayed an increased sensitivity to hyperoxia [219].

The above results obtained in flies, yeast and mice thus correlate with Harman's oxidative theory of aging with respect to the involvement of superoxide dismutases. In *C. elegans*, on the other hand, only a mild decrease in lifespan was observed with RNAi knockdown of *sod-1*, and no effect was reported for knockdown of *sod-2*, although both knockdowns resulted in an increased sensitivity to paraquat and an increase in oxidized proteins, as measured by Oxyblot [220]. RNA interference does not completely abolish the translation of the targeted gene and the remaining SOD activity could be sufficient for normal lifespan, as has been reported in *D. melanogaster* by Rogina et al where only 5% remaining activity of *Sod1* was enough to maintain normal life span [204]. However, mutants in any of the *sod* genes in *C. elegans* do not display decreased lifespan thus corroborating the observations made with RNAi [221].

C. elegans is unusual in that it encodes five SODs [222], as opposed to three in most organisms: one cytoplasmic, one mitochondrial and one extracellular termed *sod-1*, *sod-2* and *sod-4* in the worm respectively [223-226]. In addition to these, *sod-3* and *sod-5*, are expressed in the mitochondrial matrix [227] and the cytoplasm [228], respectively, totaling two mitochondrial, two cytoplasmic and one extracellular SOD in the worm. This overlap in SODs could therefore be the underlying cause of the lack of decreased lifespan seen in *sod-1* or *sod-2* mutants. Also, a small increase (2-fold or less) in mRNA of other *sod* genes was seen in single mutants [221] and could potentially compensate for the diminished activity. However, neither double mutants for *sod-1* and *sod-2* as well as *sod-3;sod*-5 nor triple mutants (*sod-1;sod-3;sod-5, sod-2;sod-3;sod-5* and *sod-1;sod-2;sod-4*) showed decreased lifespan. A surprising finding, however, was that the *sod-2* mutant actually showed an *increase* in lifespan, contrary to that seen in flies, yeast and mice and even more surprising was that double and triple mutants with *sod-2* also displayed increased lifespan [221]. These results indicate that concentrating on isolated processes or enzymes may not be sufficient to explain any correlation between ROS and lifespan.

The observation that lack of *sod-2* in *C. elegans* increases lifespan suggests that reduction of antioxidant activity (i.e. increase in ROS) may invoke other mechanisms that prove to be beneficial to the organism in dealing with increased oxidative stress. This is in accordance with the concept of hormesis: a short-lasting and nonlethal stressor that induces stress response mechanisms in an organism and thus increases both stress resistance and overall life expectancy [229, 230]. In short, a low amount of transient stress will induce stress resistance mechanisms which help to protect the animal from further stressors. A hormesis effect has

been attributed to underly the life-extending mechanisms of regular exercise as observed in humans [231, 232], although physical activity increases ROS formation [233].

Treatment with 2-deoxy-D-glucose (DOG) in *C. elegans* shifts the metabolic activity from glycolytic to mitochondrial metabolism. DOG is phosphorylated by hexokinase but cannot be further metabolized and this leads to a specific inhibition of glycolysis and glucose metabolism while breakdown of other nutrients remain unaffected [234]. The organism compensates for this impairment by inducing mitochondrial respiration, which is also seen in glucose restriction of *S. cerevisiae* [235]. This results in an increased production of endogenous ROS as a byproduct of increased electron-transfer within the respiratory chain [236, 237] and should, according to the free radical theory, result in a decreased lifespan. However, the opposite was seen as the animals displayed an increased maximum lifespan of ~25% [237] and ~15% [236] extension of mean lifespan compared with untreated worms. The increase in ROS production after treatment with DOG for 48 hours was approximately 3-fold compared to non-treated as quantified by DCF fluorescence [238]. No upregulation of SOD or glutathione peroxidases were seen, but a significant increase in catalase was seen after 6 days of treatment (but not after 2, 24 or 48 hours) leading the authors to speculate that the increased ROS production leads to a secondary increase in CAT and that increased antioxidant defenses is a consequence of increased ROS formation [238]. The DOG treated animals also showed increased survival rates when exposed to paraquat demonstrating an increased stress resistance in spite of the elevated respiratory activity.

Pre-treating the animals with the antioxidant N-acetylcysteine (NAC), a membrane-permeable glutathione precursor, significantly decreased the ROS formation in DOG-treated nematodes as expected and also diminished the increased resistance to paraquat hinting at a hormetic effect of the increased ROS production. Most importantly, however, was that pre-treatment with NAC and other antioxidants such as ascorbic acid (vitamin C) and the α-tocopherol (vitamin E) derivative trolox completely abrogated the extension of lifespan observed in DOG-treated nematodes [238].

This is in opposition to the prevailing notion that antioxidants administered through the diet will increase the health and well-being of an individual by reducing ROS. In fact, it has recently been suggested that dietary supplements of antioxidants may actually decrease lifespan in humans [239]. In mice, overexpression of CuZnSOD does not increase lifespan [240], although overexpression of antioxidant enzymes provide protection against oxidative

stress in mice cell culture models [241] and overexpression of *Sod1* in *Drosophila* results in an extension of lifespan [242]. The conflicting reports on these theories makes it premature to conclude which is the dominating mechanism, and most probably it is a combination of these or a context dependency that determines which is beneficial or detrimental.

Only two interventions have consistently been reported to increase lifespan in mice: caloric restriction (CR) [243] and modulation of the growth hormone (GH)/insulin growth factor 1 (IGF-1) axis [244-246].

Restricting the food intake of rats was shown already in 1935 to have a life extending effect [247]. The mechanisms behind this extension of lifespan are still not completely understood, but several explanations have been postulated. In the original study by McCay et al. the authors speculate that the increase in longevity was due to a retardation of growth – the animal simply developed slower with a lowered caloric intake and thus reached old age slower [247]. However, rats fed on a normal diet until the end of their rapid growth period (at 26 weeks) and then fed on a CR diet showed similar extension of mean and top 10^{th} percentile maximum lifespan as rats fed on CR from 2 weeks post weaning [248].

As body fat is associated with premature death in humans, CR was proposed to increase longevity by reducing the body fat content [249]. CR decreases body fat in rats and mice [250, 251], particularly visceral fat [252], and obese (*ob/ob*) mice fed on a normal diet show a shorter lifespan than lean mice on the same diet. However, *ob/ob* mice on a CR diet live longer than lean mice on a normal diet despite a 48% fat content compared to 22% in the lean mice, and *ob/ob* mice and lean mice fed on a CR diet have the same lifespan [251]. A sirtuin deacetylase protein SIR2 has been shown to be required for extension of the replicative life span of *S. cerevisiae* [253] and this has also been observed in *D. melanogaster*, *C. elegans* [254] and in mammals [255]. Sirtuin deacetylase activity decreases fat deposition and increases fat mobilization which together decrease fat mass in white adipose tissue [255] and may provide a molecular mechanism linking longevity and reduction of body fat.

Caloric restriction has been shown by several methods to reduce the age-associated accumulation of oxidized molecules in rodents (reviewed in [243]) and is also seen in monkeys [256]. The mechanisms for this reduction could be linked to decreased rate of ROS generation, increased efficiency of protective mechanisms, increase in repair activity or a combination of these processes. CR has been shown to increase non-enzymatic antioxidant defenses such as reduced glutathione in the rat liver [257] and the age-associated decline in

protein turnover rates have also been shown to be abolished in CR rats, replacing damaged proteins faster at old age [258]. In rat hepatocytes exposed to UV, the NER capacity was found to be comparable in cells from young mice fed a normal diet and in old rats on CR, while old rats fed a control diet displayed a 40% decrease in NER capacity [259]. CR seems therefore to protect rodents from oxidative damage, and as described above, a deletion of *Sod1* or *Sod2* in mice leads to reduction of life span. However, overexpressing SOD1 in mice has no additional effect on lifespan [240], and increasing ROS production in *C. elegans* actually leads to increased lifespan [238], making the effects of CR on oxidative damage inconclusive, although CR has been associated with hormesis as CR rodents are less sensitive to toxic drugs [260, 261]

Caloric restriction has also been shown to reduce the IGF-1 signaling in mammalian models that display an increased lifespan [262]. Insulin increases the glucose uptake in cells and serves as the primary regulator of blood glucose levels [263]. It has also been revealed that insulin has a central role in regulating lifespan and aging in *C. elegans* [264]. This role of insulin signaling is not only restricted to *C. elegans*, as inactivation of IGF-1in mice has been shown to extend lifespan and increase the resistance to ROS [265]. Much work on the effects of insulin has been carried out in *C. elegans* and the elucidation of the insulin pathway arose from studies of the dauer pathway, which can be considered as a life-extending mechanism as nematodes may maintain this state of hibernation for up to 3 months. In addition to regulating the entry into dauer [130], the insulin/IGF-1 system also regulates reproduction [266, 267] and lipid metabolism [268].

Animals that carry mutations in the insulin/IGF-1 receptor DAF-2 [268] live twice as long as the wild type and are youthful throughout their lifespan [266]. Strong mutations in *daf-2* however, result in a constitutive dauer formation [130] (Figure 4). The DAF-2 pathway controls dauer formation during development, but acts exclusively to influence aging in the adult [269]. These two processes in addition to reproduction and lipid metabolism have been shown to be regulated independently of each other by the DAF-2 pathway [270-272]. The effects of DAF-2 on these processes depend on the activity of DAF-16 [266, 273], a FOXO-family transcription factor [274]. The *daf-2* pathway promotes phosphorylation of DAF-16 and prevents its nuclear translocation through the actions of a conserved phosphatidylinositol 3-OH kinase (AGE-1) which activates a kinase cascade involving protein kinase D and AKT-1/2 [264, 275]. Mutations in DAF-16 abolishes the effects of AGE-1 or strong DAF-2 mutations [276].

The downstream effects of *daf-2* are shown to work cell non-autonomously as removing *daf-2* activity from subsets of cells cause the entire animal to enter dauer or become long-lived [277], indicating that these effects may be due to hormonal signaling. This is corroborated by studies done in Laron mice which have less than 10% of normal IGF-1 levels as well as other hormonal deficiencies and live 40-55% longer than wildtype [246]. *C. elegans* mutants with reduced DAF-2 activity show greater resistance to heat and oxidative stress [278, 279], and heterozygous IGF-1 mutant mice show greater resistance to oxidative stress [265] suggesting again that the ability to prevent or repair oxidative damage is a determinant of aging.

Murphy et al used microarray analysis of *C. elegans daf-2* and *age-1* mutants along with double mutants of *daf-2;daf-16* and RNAi of *daf-2* and *daf-16* to extend the knowledge of gene regulation downstream of DAF-16. These genes were classified into two categories with genes expected to extend lifespan (class 1) and genes expected to shorten lifespan (class 2) [280]. The results showed that a wide variety of different mechanisms were involved in the lifespan extending mechanism of insulin signaling. A positive feedback loop with *ins-7* was identified in this screen. *Ins-7* was downregulated in animals with repressed *daf-2* activity and reducing *ins-7* in *daf-2* (+) animals increased lifespan and formation of dauers in accordance with the behavior of a DAF-2 agonist. Other genes that encode proteins involved in synthesis of steroid or lipid-soluble hormones such as cytochrome P450s, estradiol-17-β-dehydrogenases, alcohol/short-chain dehydrogenases, UDP-glucoronosyltransferases and *fat* genes involved in fatty-acid desaturation were identified as class 1 genes. RNAi against most of these genes resulted in lifespan reduction of up to 20% supporting a function in lifespan determination. In addition to upregulation of *sod-3* and *mtl-1*, the catalase genes *ctl-1/2*, the glutathione S-transferase *gst-4* and small heat shock proteins were induced in *daf-2* animals suggesting that a broad-based stress response is elicited. As inhibition of these genes by RNAi resulted in decreased lifespan of *daf-2* animals, these genes contribute to the longevity effect along with the antibacterial lysosymes *lys-7* and *lys-8* and the saposin-like gene *spp-1*. The turnover rate of specific proteins or metabolites may also be affected in the IGF-1 mediated longevity pathways as several proteases and metabolic genes, as well as several F-box/cullin/Skp proteins, associated with ubiquitin-mediated protein degradation were found to be regulated. Also, *gei-7*, which is a glyoxylate gene, was found upregulated in the *daf-2* mutants [280] suggesting that this alternative metabolic pathway contributes to longevity as inhibition reduced lifespan and previous reports have shown upregulation of this gene in hibernating mammals [281] and dauers [282].

The insulin/IGF-1 system therefore seem to encompass many of the above mentioned theories to extend lifespan, although some paradoxes still remain such as how reduction of insulin may extend lifespan while insulin resistance leads to diabetes type II. That is, low insulin levels are associated with good health, but low insulin responsiveness is associated with bad health [283]. One possible explanation for this discrepancy has been proposed and that is that the insulin system works in conjunction with TOR (target of rapamycin) signaling, and TOR activity has been shown in many organisms to be involved in aging [284-286]. The hypothesis set forth is that a decrease in IGF-1/insulin signaling decreases TOR activity , and overactive TOR leads to insulin resistance unifying the two seemingly paradoxical outcomes of similar mechanisms [287].

The question of why we age is therefore still not understood, but it seems certain that not one pathway, but a combination of many different mechanisms are involved. Key questions are to which extent does damage to DNA contribute to aging and does DNA repair contribute to lifespan regulation?

1.4.1. Aging and DNA Repair

Although the experimental observations outlined above point to a combination of different mechanisms that contribute to aging, maintaining genomic integrity remains important for cell viability over time. DNA encodes the blueprint for all proteins/RNA in the cell and accumulation of defective cell components will ultimately cause cytotoxicity, uncontrolled cell growth, cellular senescence or apoptosis. DNA repair is clearly important for animal development, as germline deletions of any gene essential for BER is embryonic lethal in mice [288]. To date, only 3 studies have measured the impact of aging on BER capacity, and this was found on average to be reduced by 40-50 % in old animals [289], indicating that the ability to repair damages to the genome diminishes with age and an imbalance in BER has been shown to drive genomic instability [290-293]. NER has also been shown to be reduced with age as old rat hepatocytes display a 40 % reduction in NER compared to young [259].

Several segmental progeroid syndromes such as Cockayne syndrome, Werner syndrome, ataxia telangiectasia and trichothiodystrophy are caused by defects in the cellular response to DNA damage suggesting that defective genome maintenance contributes to aging [294]. For

example, mutations in the DNA helicase XPD are found in the human disorder trichothiodystrophy (TTD) (section 1.2.2.), and TTD mice carrying an XPD point mutation found in TTD patients ($R^{722}W$) exhibit many symptoms of premature aging such as osteoporosis and kyphosis, early graying, cachexia, infertility and reduced lifespan [295]. The average lifespan of TTD mice was less than 12 months compared with more than 2 years in the wildtype. When crossed with mice carrying an XPA null allele [296], the animals displayed extreme cachexia and had a severely shortened lifespan of only 22 days, suggesting that unrepaired endogenous DNA lesions aggravate the TTD symptoms as these mice were completely devoid of NER activity [295]. However, XPA patients and *xpa*$^{-/-}$ mice, which are completely NER deficient, are highly cancer-prone, but do not exhibit accelerated aging [296]. One interesting observation though is that CSB mutant mice show a small increase in cancer susceptibility but a pronounced exacerbation of the accelerated aging symptoms when crossed to XPA mutant mice [297].

The study of segmental progeroid syndromes for understanding normal aging have been criticized as symptoms are tissue-specific [298], but this could also just reflect the stochastic nature of the generation of DNA damages and that each tissue have different requirements for the various repair mechanisms [283]. Nevertheless, the highly significant similar transcriptomic changes seen in the livers of old mice, *Ercc1*$^{-/-}$ mutants and the severely progeroid *Xpa*$^{-/-}$;*Csb*$^{m/m}$ mutants do not overlap with those of young or non-progeroid *Xpa*$^{-/-}$ or *Csb*$^{m/m}$ single mutants [299] and provide a good indication that age-related changes in normal aging can be studied in DNA repair deficient strains.

The studies on the *Ercc1*$^{-/-}$ mice were prompted by the characterization of a patient with a unique combination of progeroid symptoms, and complementation analyses revealed that the patient had a homozygous mutation in XPF in a region frequently involved in protein interactions [300]. Subsequent immunodetection revealed that this patient showed low levels of ERCC1 and that this resulted in a high sensitivity to interstrand cross-links (ICL), distinct from that in pure NER syndromes [299]. *Ercc1*$^{-/-}$ mice model this progeroid syndrome (termed "XFE") as they are viable [301, 302] but display a wide array of progeroid symptoms and typically have a lifespan of only 4 weeks. In addition, *Ercc1*$^{-/-}$ cells are more sensitive to ICL similar to that of XFE, and XPF is not detectable in *Ercc1*$^{-/-}$ mouse tissue. An additional finding in these mice was that the GH/IGF-1 hormonal axis was downregulated and that this was also seen in wildtype mice chronically exposed to subtoxic doses of the crosslinking

agent mitomycin C [299]. A similar response is also observed in aging mammals including humans [303].

Niedernhofer et al promote a model of how DNA damage contributes to aging in that incomplete DNA repair or accumulation of lesions from endogenous or exogenous sources trigger a stress response either directly or via interference with transcription or replication [299]. This response includes a systemic dampening of the GH/IGF-1 hormonal axis through a conserved, but unknown mechanism [283, 304]. This leads to metabolic changes that shift the energy usage from proliferation and growth to protective maintenance aimed at minimizing further damage, but contributing to apoptosis [163]. A continued accumulation of damage will lead to degenerative processes and ultimately death. This model reconciles the hypothesis that aging is genetically regulated [304] and that aging is a consequence of accumulated DNA damage [283] as damage drives the functional decline associated with aging and that the longevity assurance mechanism mediated by the IGF-1/insulin pathway determines the rapidity of damage accumulation and loss of function [299].

The role of transcription blocking lesions in eliciting the attenuation of the GH/IGF-1 hormonal axis (termed the "somatotroph axis") and upregulation of antioxidant and stress responses was recently investigated [305]. The authors first analyzed gene expression profiles of UV-exposed primary mouse dermal fibroblasts (MDFs) and compared these to profiles of long-lived Ames and Snell mice as well as CR mice. The expression profiles showed an increase in genes associated with stress resistance and UV treatment before exposure to H_2O_2 resulted in a dose-dependent increase in survival to oxidative stress. The canonical DNA damage checkpoint genes were not affecting the GH/IGF1 expression as a p53 deletion did not rescue the progeroid phenotype of $Csb^{m/m};Xpa^{-/-}$ or $Ercc1^{-/-}$ mice, and attenuation of the somatotroph axis was not seen in ATM or ATR deficient primary MDFs. An interesting observation was that wildtype cells showed a transient repression of GH/IGF1 after UV exposure, while the DNA-repair deficient $Csb^{m/m};Xpa^{-/-}$ cells showed a continuous attenuation. Also, non-progeroid GG-NER deficient $Xpc^{-/-}$ primary MDFs showed the same transient GH/IGF1 repression as wildtype cells. This suggests that persistent lesions outside of actively transcribed genes do not induce GH/IGF1 attenuation and that progeroid TC-NER defects lead to prolonged attenuation, while cancer-prone GG-NER defects do not.

Treatment of primary MDFs with H_2O_2 did not induce significant GH/IGF1 attenuation suggesting that these damages do not elicit the same response as more persistent damages. In

contrast to UV-damages, oxidative damages are transient and quickly repaired by BER. UV-induced cyclobutane dimers (CPDs), however, can persist for days and even be carried through replication [306, 307], and are only eliminated via transcription-coupled repair (TCR) when leading to stalling of transcriptional elongation [308, 309]. Administration of illudin S, which produces transcription-blocking lesions [310], caused attenuation of the GH/IGF1 axis in quiescent fibroblasts and neurons [305].

A mechanistic link between attenuation of the GH/IGF1 axis and persistent transcription-blocking lesions were investigated by isolating elongating RNA polymerase II (RNAPII) complexes from the chromatin of UV-irradiated chondrocytes of wild-type and progeroid $Csb^{m/m};Xpa^{-/-}$ mice, by *in vivo* crosslinking and chromatin immunoprecipitation. Slot-blot analysis revealed an enrichment of CPDs in the chromatin of $Csb^{m/m};Xpa^{-/-}$ cells but not wild-type cells 48 hours after UV treatment. These results indicate that transcription-blocking lesions or signaling of these contribute to the GH/IGF1 attenuation and activation of antioxidant responses as removal of CPDs by photolyases reversed the UV-induced resistance to oxidative stress [305].

1.5. Microarray Technology

The wealth of information now available through the sequenced genomes enables researchers to study the expression and function of every gene in an organism. One of the ways in which to analyze all transcripts in an organism is through microarray technology. The use of gene expression profiling was first published in 1995 [311], and is now being used in a variety of different research areas. A microarray is a miniaturized gene hybridization array, and individual microarray assays are measured in microns from as small as 5 µm up to 200 µm depending on the type of array. Each element consists of a DNA sequence from one gene and is used to measure the expression of its corresponding messenger RNA (mRNA) in a sample. There are many types of microarray platforms available, but the two most common approaches are complementary DNA arrays and short oligonucleotide arrays.

In complementary DNA (cDNA) arrays, full-length cDNA clones are spotted either on filters or on specially coated glass slides. These arrays require simultaneous analysis of two biological samples, a test and a control, where each is labeled with a different fluorescent dye

and the signal intensities between the two are used to measure the gene expression. Full-length cDNA clones enable stringent hybridization conditions and lowers cross-hybridization of related genes, although closely related gene families may be able to anneal to some extent. The major advantage with this method is the low cost in comparison to the commercial Affymetrix high-density short oligonucleotide technology.

The short oligonucleotide array technology was first reported in early 1990 [312] and has been commercially available for several years. Some oligonucleotide arrays like the GeneChips® from Affymetrix use a technology that takes advantage of the hybridization specificity of short oligonucleotides. Any potential problems with cross-hybridization of oligonucleotides to unrelated probes in complex eukaryotic genomes, due to the short length of the target sequence, are dealt with by including several oligonucleotides per gene or transcript. It also makes use of the so-called "one sample, one chip" approach in contrast to the cDNA arrays which combine sample and control in the same array.

The Affymetrix GeneChip® arrays [313, 314] are high-density short oligonucleotide arrays. In contrast to cDNA arrays where 100-2000 bases are used in the oligonucleotide probes, the GeneChip® arrays use 25mers, which confers high specificity. Eleven to twenty probe pairs are routinely used for each expression measurement and this ensures high sensitivity and reproducibility. Advantages of this system include the comparatively smaller feature size which permits for the assaying of larger numbers of transcripts in a single experiment, and designer probe sequences allow for uniform hybridization behavior and the ability to distinguish closely related sequences thus enriching transcript quantification. The *C. elegans* whole genome GeneChip represents 22,500 transcripts.

The Affymetrix microarray is a silicon slide with the 25mer oligonucleotide sequences "built" directly onto the surface. Total RNA is first isolated from the sample to be analyzed and is then used as a template to create double-stranded cDNA through linear reverse transcriptase polymerase chain reaction (PCR) using poly-T primers containing a T7 RNA polymerase promoter sequence. The cDNA is then transcribed and labeled with biotin using the T7 RNA polymerase. The biotin-labeled RNA is then hybridized to the array which is subsequently stained with phycoerythrin-conjugated streptavidin prior to scanning. A grid is laid over the array image and the intensities of each probe pair are then used to make expression measurements.

1.6. Systems Biology

The origin of systems biology dates back to at least 1969 with the description of systems theory by Ludwig von Bertalanffy [315], but there is currently no unified definition of systems biology. However, the main goal of systems biology is the study of an organism, viewed as an *integrated* and *interacting network* of genes, proteins and biochemical reactions which give rise to life. The ultimate goal of systems biology therefore is to understand entire biological systems by elucidating, modeling and predicting the behavior of all components and interactions. Not a small feat to accomplish.

Another feature of systems biology is to uncover the emergent properties of a system. Emergent properties are properties and functions that arise from the interacting parts of a system that are not evident by just looking at the individual parts and in this regard may be non-intuitive [316]. Such properties are therefore also termed "irreducible", as they cannot be reduced to their individual parts and studied one at a time with the expectation of understanding the emergent properties of the system, and systems with such unpredictable properties are referred to as "complex systems" [315].

Thus, a central step towards a systems-level understanding of biology is to take a *holist* approach instead of a *reductionist* approach (top-down approach instead of bottom-up) [317]. A *holist* approach is to take a snapshot of all elements at a certain level (genes, transcripts, proteins, etc), instead of looking at one element and then move on to its connections, roles and mechanisms of action as in a *reductionist* approach. Many experimental techniques are available and used routinely in this endeavor. The entire set of components of one kind is described with terms ending in *–ome* (genome, proteome), and the techniques to identify these sets ends with *–omics* (genomics, proteomics) [315]. The genomes of many organisms have been sequenced, beginning with *E. coli* in 1997 (*C. elegans* in 1998, as mentioned above) and have now reached 1,257 completely sequenced genomes [318].

These high-throughput experimental techniques generate huge amounts of data and scientists are faced with the problem of how to make sense out of this wealth of information. One solution is network analysis presented below.

1.6.1 Network Analysis

A network is an informal description of a set of elements with interactions or connections between them, like a protein interaction network. Such networks are modeled in the way of graphs, and once a biological process is represented in a network its structural properties can be systematically characterized. The analysis of this network "topology" can then uncover the functional organization, underlying design principles and unknown organizing principles of cellular systems. These organizing principles of empirical networks can reflect crucial system properties such as robustness, redundancy or other functional interdependencies between network elements. Quantitative analyses of large-scale characteristics of complex networks thus contribute to a better understanding of the organization of cellular functions [315].

Gene regulation networks (or transcriptional regulation networks) control the gene expression in cells, and the expression of one gene can be controlled by the expression of another. It is therefore possible to model these networks where genes are vertices (nodes) and directed edges (the connection between the nodes) represent control. This modeling is achieved by using graph theory which help to provide at least two types of insights: 1) an overview of the global organization of biochemical networks, and 2) results from multivariate experiments such as microarrays can infer regulation of known pathways and networks when combined with prior knowledge [319]. Advances in data collection and analysis has made it possible to elucidate large-scale gene regulation networks such as in *S. cerevisiae* [320]. A limitation of graph theory for this modeling is the static nature of graphs. Biochemical networks are dynamic as nodes and edges change with time, the abstraction of these in graphs may mask temporal aspects of information flow. However, such static representations of a system is a prerequisite for building detailed dynamic models [321] and must therefore be continuously updated as new information is uncovered.

In 1998, such different entities as the *C. elegans* neuronal network, the power grid of the United States and the collaboration graph of film actors were compared and found to have similar properties: they are highly clustered , yet they have small characteristic path lengths [322]. The authors of this observation named this phenomenon *small world networks*, by analogy with the small-world phenomenon described by Milgram in 1967 [323], which states that any person on the planet is connected to any other person by on average six

acquaintances. A simple model for these networks were described a year later by Barabási and Albert and was found to follow a scale-free power-law distribution and aptly named them *scale-free networks* [324]. This connectivity distribution is important because it shows that many vertices have few links, while some are highly connected. As a result, scale-free networks are very robust against failure, such as the removal of arbitrary elements in the network [325], but are also very vulnerable if a "hub" with many vertices is affected. The tumor suppressor protein p53 is such a hub with many connections and plays a central role in the signaling of DNA damage which, with an active protein, will lead to apoptosis. A mutation in p53 will therefore be crucial to the development of cancer as this cell will not undergo programmed cell death, but may keep on dividing with a defective control mechanism.

The robustness of scale-free networks are seen in biological systems where the removal of one gene with few connections changes the dynamics of the network and results in compensatory signaling pathways (very much like if an airplane cannot land in one airport, you are still able to get to your destination via other transport possibilities).

Software programs are very useful to investigate interactions between sets of genes. The online Functional Interactions Browser FunCoup [326] for example, uses Bayesian probability to assign functional interactions between genes. Bayesian probability interprets the concept of probability as a "measure of the state of knowledge" meaning that it measures the probability of a hypothesis (a connection in this case) by specifying some prior probability and is then updated as new relevant data is entered. This is then the basis for combining data from different species to strengthen conserved interactions and inferring new or probable connections.

1.6.2. Gene Ontology

Looking at the entire network of an organism's transcriptional state is not a feasible option until all connections have been mapped out in detail and modeled, so other approaches must be used. Using databases where genes are grouped according to their function or localization is therefore one way to narrow down the dataset to find the components that are most likely to be involved in a certain experimental question, such as antioxidant properties for example.

Gene Ontology (GO) is a way of describing gene products in different databases [327]. The Gene Ontology project uses defined terms to represent gene product properties. The ontology covers three domains: *cellular component*, *molecular function*, and *biological process*.

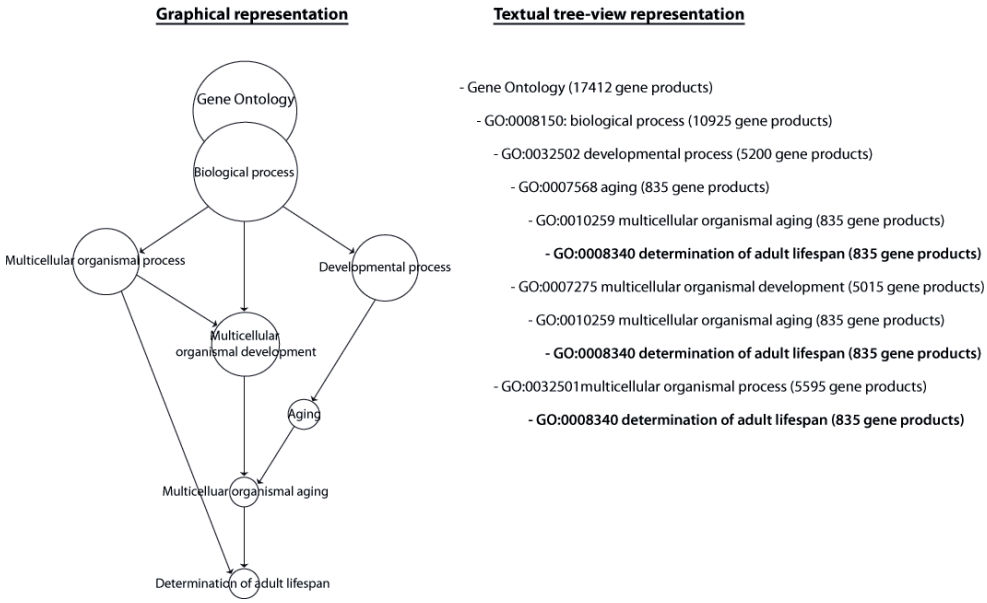

Figure 8: Different representations of Gene Ontology relationships. Gene ontology relationships may be represented in different ways such as a "tree-view" which is textual, or graphically such as performed in Cytoscape [328] with the BinGO plugin [329]. Here, the different views are shown on the relationship to the biological process "determination of adult lifespan" which contains few steps to illustrate the two ways of sorting gene ontology relationships.

The Gene Ontology vocabulary is designed to be species-neutral and the files are not static so additions, corrections and alterations can be suggested by members of the research community which is then reviewed by ontology editors and implemented where appropriate. This makes it a continuously evolving way of describing gene functions which in turn aid in the classification of processes. However, it is important to note that the GO database does not describe protein domains, structural features or protein interactions and individual curators of the database evaluate data differently. This implies that some of the genes entered could be classified in inappropriate groups and that some genes are missing from other groups. As no other comprehensive cross-species database is available, this is still the best database to search with a controlled vocabulary.

Combining functional interactions and gene ontology classifications in a network analysis of a given microarray dataset for example, provide the researcher with a better possibility of finding emergent properties of a system, compensatory mechanisms or new hypotheses.

2. Present Investigation

2.1. Aims of the Study

The work performed in this thesis is part of a long-term research goal to investigate the effects of endogenous oxidative DNA damage to age-associated phenotypes such as neurodegeneration. This investigation was initiated by the observation of the apparent underrepresentation of DNA repair enzymes in the nematode *Caenorhabditis elegans* and consisted of three main goals:

- Characterize *C. elegans* mutants deficient in DNA repair systems to investigate the mechanism of DNA repair in this model organism.

- Perform whole-genome microarray analyses on unstressed DNA repair mutants in order to elucidate the gene expression changes elicited in these mutants and uncover compensatory responses to lack of DNA repair.

- Establish the analysis of complex datasets as a complementary method for interpreting biological data.

2.2. Summary of Papers

Paper I: As a first step in elucidating the mechanisms of BER in *C. elegans*, one of the two known DNA glycosylases, UNG-1, was characterized in this paper. The UNG-1 protein was shown to be an active uracil-DNA glycosylase that recognized and excises uracil paired with either adenine or guanine in double-stranded DNA and uracil from single-stranded DNA. UNG-1 had about 2-fold higher activity on substrates containing U:G compared with ssU. The *C. elegans* UNG-1 did not have a preference for single-stranded over double-stranded substrates as other UDG enzymes have been shown to have under our conditions. An important aspect discussed in this paper is the food source of the worm, especially when studying highly active enzymes that are also present in bacteria, which the nematode feeds on. We show that enzymes expressed in bacteria are a potential source of contamination that needs to be taken into account when measuring activity from extracts. The extracts used here were checked for UDG contamination through Mass Spectrometry analyses, and other nuclease activity was excluded through use of HPLC to verify release of uracil. The *ung-1* mutant had a reduced ability to repair uracil-containing DNA, but an alternative Ugi-inhibited activity was shown to be present. *ung-1* mutants showed altered levels of apoptotic cell corpses formed in response to DNA damaging agents. The increased apoptosis in response to IR suggested that UNG-1 contributes to repair of IR-lesions consistent with an *in vivo* role for repair of DNA oxidation products formed after IR. The reduced apoptosis seen in response to paraquat is hypothesized to be a result of modulation of stress-response signals and not DNA repair related *per se*, as microarray analyses showed regulation of genes classified to respond to oxidative stress, downregulation of insulin-like signaling and regulation of genes known or predicted to determine adult lifespan. A compensatory transcriptomic shift that modulates the oxidative stress response was seen as a consequence of lack of *ung-1*. The results presented illustrate the intricate and complex network interactions in biological systems.

Paper II: The transcriptional profile of three different *C. elegans* mutants in DNA repair, *nth-1*, *xpa-1*, and *nth-1;xpa-1,* were recorded and compared to the wild type profile. Lowering the DNA repair capacity revealed a striking modulation of the transcriptome. Notably, in the *nth-1* mutant, genes that respond to oxidative stress were found to be induced and genes involved in insulin/IGF-1 signaling were reduced. Both of these processes have

been shown to be involved in the protection of the genome. In addition, genes that are known to respond to exogenous stressors were found to be negatively regulated suggesting that the response emanates from within the organism. A similar, but more pronounced response was found in the *xpa-1* mutant. Phenotypic analysis also revealed a shorter lifespan of the *xpa-1* mutant. The upregulation of oxidative stress genes was confirmed *in vivo* by fluorescence microscopy in a GFP::GST-4 reporter strain, as an increase in expression was seen after depletion of NTH-1 or XPA-1 by RNAi. The impact of downregulation of genes responding to exogenous stress was confirmed by all mutants showing mild sensitivity to heat shock.

In the double mutant, *nth-1;xpa-1*, the transcriptional signature was markedly different from those in the single mutants. DNA repair genes were found to be enriched, but contrary to expectations, these were all downregulated. In addition, genes that are validated interactors of Polo-like kinase 1 (PLK-1) and Aurora B kinase (AIR-2) were found to be regulated and modulation of these point at somatic preservation. Phenotypic analysis of the double mutant revealed a restoration of normal lifespan. The transcriptional signatures of the single and double mutants lead to a hypothesis of a two-tiered compensatory response to DNA repair in *C. elegans*. Lack of either base excision repair (BER) or nucleotide excision repair (NER) results in activation of genes responding to endogenous stressors and suppression of insulin-like signaling (ILS), lack of both BER and NER shifts the transcriptional response to somatic preservation and a reduction of proliferation.

To illustrate the generality of this hypothesis, we re-analyzed previously published microarray datasets on BER, NER and BER/NER deficient *Saccharomyces cerevisiae* strains. This meta-analysis revealed that i) BER mutants show transcriptional changes, ii) loss of NER induces more substantial changes than BER and iii) loss of BER in the NER mutant shifts the response to regulate processes that maintain DNA integrity. This indicates that the responses elucidated from our *C. elegans* arrays are conserved mechanisms across species to protect the genome from damages in the face of diminished DNA repair capacity.

We hypothesize that the transcriptomic changes seen are signaled by transcription blocking lesions, in agreement with reports indicating that such lesions cause attenuation of ILS and activation of oxidative stress responses. Attempted, but inefficient, processing of NER lesions by the BER may be the cause of the stronger response seen in the *xpa-1* mutant and the restoration of lifespan in the double mutant. The response seen in the BER mutant indicates that this response may be a general strategy for survival in repair mutants.

Paper III: The model organism *Schizosaccharomyces pombe* (*S. pombe*) was used here in order to investigate whether a transcriptional response was induced after irradiation with ultraviolet light (UV) in the G1 phase. UV is known to induce a G1/S checkpoint and the goal was to decipher the global transcriptional response to this insult. A surprisingly weak transcriptional response was detected which is unlike the marked changes detected after some other types of treatments and in several other checkpoints. The alterations seen were not similar to those observed after ionizing radiation or oxidative stress. A weak response was therefore detected in *S. pombe* in response to UV at the transcriptional level. No genes were found regulated that are likely to be involved in the G1/S checkpoint mechanism, suggesting that the checkpoint is not dependent upon transcriptional regulation. The network analysis indicated that pathways involved in recovery after DNA damage are induced after 90 minutes, and that these processes probably reflect the importance of the translation machinery in recovery after UVC treatment. Lack of good *S. pombe* databases was solved by using *Saccharomyces cerevisiae* homologs.

3. Discussion and Future Perspectives

Aging is a fundamental process of life, although death by old age is rarely observed in nature as most species commonly die by predation, accidents, succumb to lethal infections or from age-related diseases such as atherosclerosis, cancer, diabetes etc [287]. Several theories have been proposed to explain the aging process, and the most prominent of these is Harman's oxidative theory of aging [190] which states that accumulation of DNA damages lead to cytotoxicity resulting in decreased cell viability. Two interventions have been shown to reliably extend the lifespan of animals: 1) modulation of the growth hormone (GH)/insulin growth factor 1 (IGF-1) axis [244-246], as have been described in long-lived *C. elegans* mutants [266] and also reported in other long-lived animals [299], and 2) caloric restriction (CR) [243]. Both strategies involve the downregulation of insulin signaling and upregulation of antioxidant defenses [257, 262] and thus point at a common feature of increasing cellular defenses against damaging agents. Reduction of insulin-like signaling (ILS) causes the transcription of genes aimed at reducing damages to the genome, promote stress resistance and longevity [280].

Animals with short lifespans have also been studied and such progeroid syndromes have been described in both mice and humans. These often show a deficiency in DNA repair, which suggests a link between responses to DNA damage and aging. The transcriptional profiles of progeroid and old animals have been compared to that of long-lived animals and show a surprising similarity. For example, the profile of the progeroid mouse models $Csb^{m/m};Xpa^{-/-}$ and $Ercc1^{-/-}$ have been compared to that of long-lived Ames and Snell dwarf mice and calorically restricted mice and they all showed a suppression of the GH/IGF1 somatotroph axis and oxidative metabolism along with upregulated stress responses [330]. The similar responses seen in both progeroid syndromes and old animals as well as in long-lived individuals, presents an apparent paradox in that processes aimed at preventing damages to DNA and increase longevity are similar to those seen in already old animals. This could of course indicate that DNA-repair deficient animals just age faster and that the transcriptional profile simply is an "old profile". However, several lines of evidence suggest that this is not the case.

Firstly, inducing these processes in healthy young animals do not make them "old" or result in the display of age-related symptoms or early death. Rather, inducing these processes

lengthens the lifespan of these animals and they remain healthy and viable as seen in DAF-2 *C. elegans* mutants [266] and in CR animals [243].

Secondly, there is a critical difference between accumulated damages and inability to repair damages. If a lack of repair is signaled early in life and the compensatory mechanisms are employed early, this could be expected to reduce the genomic insults over time and thus have a more pronounced effect on lifespan than if initiated late in life. At old age in repair proficient animals, however, the response is more likely elicited by already accumulated damages which will remain in the genome and cause cytotoxicity, and result in aging and eventually death. In fact, it can be argued that these compensatory mechanisms may actually ensure that animals stay alive longer than they would have without this response.

So why not just employ these mechanisms all the time in addition to a well-functioning DNA repair system? Initiation of these mechanisms does not in itself ensure long life, as shown by the reduced lifespan of *xpa-1* and progeroid animals. These responses could rather be interpreted as a concerted response to prevent further damages to accumulate and allow other DNA repair mechanisms time to repair already produced lesions (such as reducing additional damages to a minimum until TCR can be employed in S-phase in dividing cells for example). This is indicated by transient induction of these responses to UV radiation for example, and that cells return to "normal" states after the insult is repaired [305]. Oxidants are also used intracellularly as signaling molecules [331, 332] and increasing the concentrations of antioxidants further as an additional response to inevitable damages to DNA may interfere with the normal metabolism of the cell.

In this thesis, *C. elegans* mutants deficient in BER, NER and both BER and NER have been analyzed with respect to phenotypes and gene expression profiles. The BER-deficient *nth-1* and *ung-1* mutants show normal lifespan and brood sizes. Gene expression profiles showed a reduced expression of genes involved in ILS and an upregulation of antioxidant defense genes. The NER deficient *xpa-1* mutant also displayed this altered gene regulation, although in a more pronounced manner, but in addition showed a reduced lifespan. These observations support the hypothesis that DNA damage contribute to transcriptomic shifts and that these shifts are compensatory responses seeking to limit the deleterious consequences of DNA repair deficiency. The response seen in the *nth-1* mutant appears to be sufficient to maintain normal lifespan, while the response in the *xpa-1* mutant fails to do so. The more pronounced

compensatory response recorded in the *xpa-1* mutant suggests that the degree of challenge to the genomic integrity can be read out from the level of compensatory responses employed.

The transcriptional profiles show that lack of DNA repair, and thus increased lesion load, elicits compensatory responses, but the question remains as to how these responses are signaled. The results presented in Paper II suggest that the severe aging phenotypes often seen in NER-deficient animals may not necessarily be caused by bulky lesions repaired by NER *per se*, but from attempted processing of these lesions by other pathways (such as BER) resulting in stalling of transcription or replication resulting in cytotoxicity and aging. Induction of transcription blocking lesions has recently been shown to induce attenuation of ILS in mouse fibroblasts and neurons [305]. This lends support to the damage-accumulation theory of aging and transcription blocking lesions as signaling origins of compensatory responses, but also hints at the signaling and mechanism behind the initiation of these processes.

3.1 Lesion recognition and signaling

Glycosylases recognize and bind to lesions in DNA and initiate repair through the BER pathway. Lesion recognition is proposed to be the rate-limiting step of BER within the cell [333], and current models propose that repair proteins detect lesions by sliding along the DNA strand, flipping or "tapping" bases to investigate base properties or finding transiently opened sites [334]. However, glycosylases are often found in low copy numbers and the *E. coli* homolog of NTH-1, EndoIII, is calculated to be present at only ~500 copies per cell [335], and scanning the entire genome by this method is a formidable task.

A mechanism for efficient scanning of the genome by DNA charge transfer (CT) has been proposed by Barton and co-workers based on the observation that the overlapping π system of stacked DNA bases can mediate the transfer of an electrical charge over long distances [336], and that DNA CT is very sensitive to perturbations in the base pair stack such as lesions or mismatches [337]. Glycosylases such as NTH-1 contain an iron-sulfur ([4Fe-4S]) cluster [164] which can exist in an oxidized or reduced state and when not bound to DNA the proteins are found in the reduced $[4Fe\text{-}4S]^{2+}$ state [338]. Binding to DNA oxidizes this cluster to the $[4Fe\text{-}4S]^{3+}$ state and increases the DNA affinity ≥1,000-fold compared to the

reduced state [339]. The electron released in this oxidation reaction travels along DNA on the π system of stacked DNA bases until it encounters a damaged base or another oxidized [4Fe-4S] cluster protein (Figure 9). Such a distally bound $[4Fe\text{-}4S]^{3+}$ cluster protein is then reduced back to $[4Fe\text{-}4S]^{2+}$ and subsequently dissociates from DNA due to its lower affinity. If a lesion is encountered between the two proteins, the CT reaction cannot be completed and both proteins remain bound to DNA in the vicinity of the lesion and only need to slide across a small region to bind to the lesion [340]. This allows for fast searching across long stretches of DNA without physically scanning all bases. When bound to DNA, the proteins scan for damaged bases in a processive manner and bind upon detection to flip out the damaged base and initiate repair.

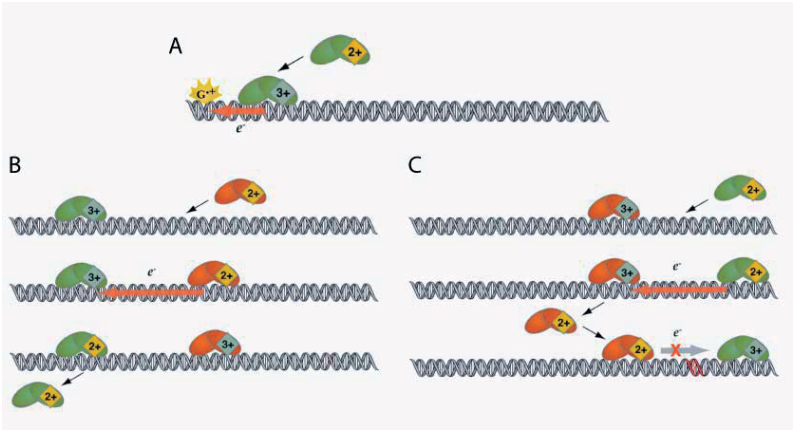

Figure 9: Mechanism of DNA Charge Transfer. A. DNA repair proteins containing $[4Fe4S]^{2+}$ clusters bind to DNA, are oxidized to the $[4Fe4S]^{3+}$ state and stabilized. The proteins processively scan DNA for lesions. The electron released in the oxidation reaction travels along the DNA base pair stack. **B.** If the intervening DNA between two distally bound proteins is undamaged, the released election will reduce the receptor protein, diminish its affinity to DNA and the protein dissociates. **C.** If a lesion is present between two proteins, the DNA-mediated CT step is inhibited and the oxidized proteins remains bound and continue scanning the DNA. Adapted and modified from [341].

Transcription blocking lesions are proposed to elicit the signaling responses seen in the single mutants, and it is tempting to speculate that DNA CT detection of lesions by NTH-1 may contribute to their generation and the different levels of responses. The DNA CT detection of lesions by NTH-1 would make this enzyme very effective in detecting damages. As CT is very sensitive to alterations in DNA, NTH-1 would presumably be recruited to all sites of damage and bind to the damage and either attempt repair or be displaced by other repair

proteins. In the *xpa-1* mutant then, lesions normally repaired by NER would instead recruit NTH-1 through CT scanning and result in an increase in attempted processing of such lesions by BER. This would in turn result in an increase in transcription blocking lesions and a stronger compensatory response as observed in the gene expression profile of *xpa-1*. The response seen in the *nth-1* mutant would thus be due to attempted processing by NER, as postulated in Paper II [189], but at a slower rate, as XPA-1 does not contain an [4Fe-4S] cluster and use less efficient methods to scan DNA, resulting in less transcription blocking lesions and a less pronounced compensatory response. In the *ung-1* mutant, however, NTH-1 would presumably also bind to lesions normally repaired by UNG-1, but either more of these lesions can be resolved by NER or by the backup activity detected in Paper I [148] resulting in a similar response as in the *nth-1* mutant. The nature of the lesions and how they are signaled should therefore be examined more closely in future studies. In the double mutant, *nth-1;xpa-1*, there is no lesion recognition or repair by NTH-1 or XPA-1 which would result in the creation of less transcription blocking lesions, but presumably more persistent lesions. A different transcriptional signature is detected and how this is signaled should also be a focus of future studies. It would also be very interesting to investigate the long-term fitness of these mutants, especially in a competitive environment with other mutants or wild type animals.

3.2 Microarray and pathway analyses

Microarray studies have been widely used since its introduction over a decade ago [311] and has found extensive use in the fields of functional genomics and molecular genetics. However, a lack of reproducibility across experiments have been reported [342] and microarray studies have been characterized as being very noisy due to many imperfections inherent in the technology. This could be the underlying rationale for the use of rtPCR to verify microarray data (although both methods measure mRNA levels) and that results from microarrays have a lower significance score in Bayesian models such as FunCoup [326]. However, a controlled study where the biological variability was eliminated by only using technical replicates, showed that this effect was very small and in fact negligible in causing effects on the results of statistical inference, at least for the Affymetrix GeneChip® arrays [343]. The variability across samples was attributed to the biological variability, small sample sizes or in the statistical methods used rather than the technical noise. The authors

investigated only the Affymetrix GeneChip® arrays and a greater technical variability may therefore become more pronounced when data from different platforms, or data arrayed years apart are combined, as the technology is constantly improving. The lack of reproducibility have also been shown to be attributed to improper analysis or variation, inadequate reporting of findings or insufficient control of false positives [344, 345], rather than the arrays themselves. Quality control of experimental conditions and samples are therefore of utmost importance and during a microarray run, internal controls and physical inspection of the arrays along with quality control of the fragmented RNA by Bioanalyzer should be conducted.

A large body of microarray datasets performed on *C. elegans* is available for a wide variety of mutants and conditions. For example, global transcriptional profiles elicited by pathogen infection, such as by *Pseudomonas aeruginosa* and *Bacillus thuringiensis* among others, are available [346-349]. These datasets were used in our internal quality control in order to ensure that the changes we observed were not merely a response to infection, but a response to the mutation. Published gene lists (PGLs) from these studies were collected and a simple overlap function was performed against our gene lists in a spreadsheet to identify gene signatures that resemble those elicited upon pathogen infection. However, hits in these lists must be met with a biological understanding of the processes involved and such comparisons should not be performed without a critical investigation of the results. Several pathways involved in pathogen recognition and resistance also have other biological roles, such as the p38 and JNK pathways for example and their involvement must be seen in a "holist" perspective.

The microarrays analyzed in Paper III were performed on spotted glass DNA arrays and the datasets obtained illustrate some technical aspects relating to such arrays. In the time-course experiment, on average 36% of the genes displayed missing values, and the quality of the data was somewhat reduced as data pertaining to genes where too many data points were missing were excluded.

The pathway analysis was conducted on the PGL by first employing GO enrichment analysis to identify processes that were enriched in both the restrictive temperature and the time-course experiment. The nature of the PGLs were such that genes identified as members of the core environmental stress response (CESR) were filtered out in order to identify unique genes for the response to UVC, resulting in a limited number of genes available for analysis. As there

are currently no comprehensive databases available for inclusion in functional interaction software for *S. pombe*, the identified genes in enriched GO groups were converted to *S. cerevisiae* orthologs for which such databases exist. Direct protein-protein interactions were then determined based on the *S. cerevisiae* orthologs. It is likely that stringent statistical methods (such as correcting for false positives in both identification of differentially expressed genes and profile analysis) and the exclusion of CESR genes could mask UVC-relevant gene expression changes.

However, the pathway analysis did indicate that the induction of ribosomal genes as well as genes involved in rRNA biosynthesis and ribosome assembly were induced after 90 minutes after the insult and suggests that the translation machinery is important in the recovery process, as has been shown in *D. melanogaster* after UV radiation [350]. Also, the pathway analysis performed illustrate the use of higher-order process analysis (such as GO enrichment) and functional interaction tools to complement and expand the interpretation of gene lists and provide added information to explain observed phenotypes.

3.3 Data and Statistical Considerations

The transcription profiles that are analyzed and presented in this thesis are so-called two-class comparisons in which two genotypes within one experiment have been analyzed. When more complex analyses between several genotypes, several treatment regimes, within different experiments and incorporation of previously published datasets are to be performed, we will quickly be faced with the current limitations of genetic analysis of expression profile changes such as different technology platforms employed and the biological variability of the strains used. So far, this problem has generally been "solved" by researchers not utilizing datasets other than those performed themselves within one experiment. This approach, however, greatly limits the information extracted from gene expression profiling projects and is very costly. Analyzing high-throughput data in a manner that circumvents this problem will greatly enhance the data available and may uncover emergent properties in a more cost-effective manner across disciplines.

In Paper II, we conducted a meta-analysis on the level of Gene Ontology, which represents the variables in the datasets on a biological process rather than on a gene-to-gene level, to

reveal common gene expression patterns in comparable DNA repair deficient mutants of *C. elegans*, *S. cerevisiae* and mouse [189]. This method proved to be effective when comparing gene expression patterns across species when the experimental setups are similar such as deficiencies in DNA repair only. However, the results presented in Paper II warrant new experiments in which DNA repair deficient animals are challenged by treatment with an oxidizing agent like paraquat or juglone for example (as the upregulation of antioxidant defenses seen do not confer resistance to such agents) and the transcriptional changes induced are recorded and analyzed. This presents us with a different experimental setup than the problems usually described in the literature: a "disease vs normal" setup. Rather, this setup will have to consider both a mutation effect as well as a treatment effect that can be considered a multi-class issue. Recently, Lu and co-workers [351] published a paper describing statistical methods for combining multi-class studies and the authors successfully use the *p*-values from a one-tailed t-test as test statistics in contrast to fold change or signal intensity. Using a one-tailed rather than two-tailed t-test gives a starting point for generating directionality of the gene expression changes, which is important for pathway reconstruction and modeling. This will allow for separation of mutational and treatment effects and also allow for comparison with datasets from different platforms and conditions.

3.4 Hypothesis-free approaches

Hypotheses-free approaches are investigations that are not aimed at testing specific hypotheses, but to generate new knowledge and from these data explain phenotypes and prompt formulation of testable hypotheses. Such approaches are very useful in basic and translational research as they have the potential of generating new and exciting views on mechanisms behind observed phenotypes in a non-biased way. This approach was utilized in Paper I and II of this thesis and illustrates how hypothesis-free transcriptomics may function as a complementary method to classical genetics and molecular analysis. For example, a striking phenotype observed in the *ung-1* mutant was that it was unable to induce apoptosis in response to the oxidizing agent paraquat. This phenotype could be erroneously interpreted to support that the UNG-1 enzyme is required for processing of oxidative DNA damage induced by paraquat, which in turn would lead to activation of DDR culminating in apoptosis as was previously described for a different type of DNA damage [162]. Instead, the gene expression

profiling and pathway analyses performed on the *ung-1* microarray dataset revealed that the phenotype results from suppression of input to the p38 MAPK and JNK-1 stress response pathways [148].

The hypothesis-free approach is also valuable in translational research and can be used to uncover new mechanisms in previously described phenotypes. For example, in all our single mutants we have detected a large upregulation of the aquaporin *aqp-1*. The aquaporins have been implicated as part of the life-extending mechanisms in *C. elegans* [352] and are part of a feedback-loop in the insulin-signaling pathway. Blackwell et al have suggested that the upregulation of aquaporins is due to oxidative stress triggering the reduction of insulin signaling [353]. Our hypothesis-free approach provides results that may combine these two observations and prompt new investigations into whether the downregulation of insulin-signaling in response to increased oxidative stress is actually signaled through the activity of DNA repair proteins.

3.5 Concluding remarks

Repair of DNA and genome maintenance is essential for the viability of cells. The calculated amount of lesions that are generated in cells each day [4, 5] exemplify the need for robust DNA repair mechanisms to maintain genomic integrity. Animals with deficiencies in the Base Excision Repair pathway have few phenotypes associated with them [354] and this is often attributed to backup systems or redundant substrate specificities of the lesion-recognizing glycosylases. Nucleotide Excision Repair deficient animals have more severe symptoms associated with them and many of these show increased neurodegeneration and accelerated aging phenotypes [294, 295, 355].

The gene expression signatures recorded for DNA repair mutants show that mutants are not just wild types minus a gene but that extensive global transcriptional changes occur to compensate for the loss of a gene. This system-level response is important in interpreting data from mutants as all responses seen are not necessarily caused only by the missing gene, but from the compensatory response. Gene expression profiling is a powerful complementary method to classical genetics and molecular analysis and aid in the interpretation of data as

shown in Paper I, where pathway reconstruction from microarrays provided additional information to explain the observed phenotype.

Gene expression profiling of *nth-1*, *xpa-1* and *ung-1* single mutants revealed a compensatory response to DNA damage involving upregulation of antioxidant genes and a downregulation of ILS. This mechanism seems to be conserved across species as similar responses were also seen in comparable mutants in *S. cerevisiae* and mice by performing gene expression analysis on higher order processes instead of gene-to-gene comparisons. This approach allows for the use of previous datasets to expand our current knowledge.

The responses recorded seem to be the result of transcription blocking lesions generated by aberrant or attempted processing of lesions by other repair pathways than those normally repairing such lesions. The absence of this response in the double mutant *nth-1;xpa-1* indicate that increased lesion load due to diminished DNA repair can be compensated for successfully by more than one strategy. To date, there is no hypothesis on how this is signaled and this should be part of the investigation in future studies.

4. References

1. Akbari, M. and H.E. Krokan (2008). Cytotoxicity and mutagenicity of endogenous DNA base lesions as potential cause of human aging. Mech Ageing Dev. **129:** 353-65.
2. Kinzler, K.W. and B. Vogelstein (1997). Cancer-susceptibility genes. Gatekeepers and caretakers. Nature. **386:** 761, 763.
3. Hanahan, D. and R.A. Weinberg (2000). The hallmarks of cancer. Cell. **100:** 57-70.
4. Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature. **362:** 709-15.
5. Nakamura, J., V.E. Walker, P.B. Upton, S.Y. Chiang, Y.W. Kow, and J.A. Swenberg (1998). Highly sensitive apurinic/apyrimidinic site assay can detect spontaneous and chemically induced depurination under physiological conditions. Cancer Res. **58:** 222-5.
6. Wink, D.A., K.S. Kasprzak, C.M. Maragos, R.K. Elespuru, M. Misra, T.M. Dunams, T.A. Cebula, W.H. Koch, A.W. Andrews, J.S. Allen, and et al. (1991). DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science. **254:** 1001-3.
7. Sedgwick, B. (2004). Repairing DNA-methylation damage. Nat Rev Mol Cell Biol. **5:** 148-57.
8. Rydberg, B. and T. Lindahl (1982). Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. Embo J. **1:** 211-6.
9. Barrows, L.R. and P.N. Magee (1982). Nonenzymatic methylation of DNA by S-adenosylmethionine in vitro. Carcinogenesis. **3:** 349-51.
10. Naslund, M., D. Segerback, and A. Kolman (1983). S-adenosylmethionine, an endogenous alkylating agent. Mutat Res. **119:** 229-32.
11. Falnes, P.O. and T. Rognes (2003). DNA repair by bacterial AlkB proteins. Res Microbiol. **154:** 531-8.
12. Drablos, F., E. Feyzi, P.A. Aas, C.B. Vaagbo, B. Kavli, M.S. Bratlie, J. Pena-Diaz, M. Otterlei, G. Slupphaug, and H.E. Krokan (2004). Alkylation damage in DNA and RNA--repair mechanisms and medical significance. DNA Repair (Amst). **3:** 1389-407.
13. Fry, R.C., T.J. Begley, and L.D. Samson (2005). Genome-wide responses to DNA-damaging agents. Annu Rev Microbiol. **59:** 357-77.
14. Mena, S., A. Ortega, and J.M. Estrela (2009). Oxidative stress in environmental-induced carcinogenesis. Mutat Res. **674:** 36-44.
15. Nouspikel, T. (2007). DNA repair in differentiated cells: some new answers to old questions. Neuroscience. **145:** 1213-21.
16. Goodman, M.F. and B. Tippin (2000). Sloppier copier DNA polymerases involved in genome repair. Curr Opin Genet Dev. **10:** 162-8.
17. Kunkel, T.A. and K. Bebenek (2000). DNA replication fidelity. Annu Rev Biochem. **69:** 497-529.
18. Bartek, J. and J. Lukas (2007). DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol. **19:** 238-45.
19. Schmitt, E., C. Paquet, M. Beauchemin, and R. Bertrand (2007). DNA-damage response network at the crossroads of cell-cycle checkpoints, cellular senescence and apoptosis. J Zhejiang Univ Sci B. **8:** 377-97.
20. Bjelland, S. and E. Seeberg (2003). Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. Mutat Res. **531:** 37-80.
21. Barnes, D.E. and T. Lindahl (2004). Repair and genetic consequences of endogenous DNA base damage in mammalian cells. Annu Rev Genet. **38:** 445-76.
22. De Bont, R. and N. van Larebeke (2004). Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis. **19:** 169-85.
23. Burney, S., J.L. Caulfield, J.C. Niles, J.S. Wishnok, and S.R. Tannenbaum (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. Mutat Res. **424:** 37-49.

24. Friedberg, E.C., G.C. Walker, W. Siede, R.D. Wood, R.A. Schultz, and T. Ellenberger (2006). DNA Repair and Mutagenesis. Washington, D. C., ASM Press.
25. van Loon, B., E. Markkanen, and U. Hubscher (2010). Oxygen as a friend and enemy: How to combat the mutational potential of 8-oxo-guanine. DNA Repair (Amst).
26. Gutteridge, J.M. and B. Halliwell (1989). Iron toxicity and oxygen radicals. Baillieres Clin Haematol. **2:** 195-256.
27. Andronikashvili, E.L., L.M. Mosulishvili, A.I. Belokobilski, N.E. Kharabadze, T.K. Tevzieva, and E.Y. Efremova (1974). Content of some trace elements in sarcoma M-1 DNA in dynamics of malignant growth. Cancer Res. **34:** 271-4.
28. Salganik, R.I. (2001). The benefits and hazards of antioxidants: controlling apoptosis and other protective mechanisms in cancer patients and the human population. J Am Coll Nutr. **20:** 464S-472S; discussion 473S-475S.
29. Klaunig, J.E. and L.M. Kamendulis (2004). The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol. **44:** 239-67.
30. Klungland, A. and S. Bjelland (2007). Oxidative damage to purines in DNA: role of mammalian Ogg1. DNA Repair (Amst). **6:** 481-8.
31. Shibutani, S., M. Takeshita, and A.P. Grollman (1991). Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature. **349:** 431-4.
32. Cheng, K.C., D.S. Cahill, H. Kasai, S. Nishimura, and L.A. Loeb (1992). 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. J Biol Chem. **267:** 166-72.
33. Arashidani, K., N. Iwamoto-Tanaka, M. Muraoka, and H. Kasai (1998). Genotoxicity of ribo- and deoxyribonucleosides of 8-hydroxyguanine, 5-hydroxycytosine, and 2-hydroxyadenine: induction of SCE in human lymphocytes and mutagenicity in Salmonella typhimurium TA 100. Mutat Res. **403:** 223-7.
34. Kreutzer, D.A. and J.M. Essigmann (1998). Oxidized, deaminated cytosines are a source of C --> T transitions in vivo. Proc Natl Acad Sci U S A. **95:** 3578-82.
35. Delaney, M.O., C.J. Wiederholt, and M.M. Greenberg (2002). Fapy.dA induces nucleotide misincorporation translesionally by a DNA polymerase. Angew Chem Int Ed Engl. **41:** 771-3.
36. Wiederholt, C.J. and M.M. Greenberg (2002). Fapy.dG instructs Klenow exo(-) to misincorporate deoxyadenosine. J Am Chem Soc. **124:** 7278-9.
37. Tye, B.K. and I.R. Lehman (1977). Excision repair of uracil incorporated in DNA as a result of a defect in dUTPase. J Mol Biol. **117:** 293-306.
38. Krokan, H.E., F. Drablos, and G. Slupphaug (2002). Uracil in DNA--occurrence, consequences and repair. Oncogene. **21:** 8935-48.
39. Muramatsu, M., V.S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N.O. Davidson, and T. Honjo (1999). Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. J Biol Chem. **274:** 18470-6.
40. Bransteitter, R., P. Pham, M.D. Scharff, and M.F. Goodman (2003). Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. Proc Natl Acad Sci U S A. **100:** 4102-7.
41. Chaudhuri, J., M. Tian, C. Khuong, K. Chua, E. Pinaud, and F.W. Alt (2003). Transcription-targeted DNA deamination by the AID antibody diversification enzyme. Nature. **422:** 726-30.
42. Ramiro, A.R., P. Stavropoulos, M. Jankovic, and M.C. Nussenzweig (2003). Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. Nat Immunol. **4:** 452-6.
43. Martin, A., P.D. Bardwell, C.J. Woo, M. Fan, M.J. Shulman, and M.D. Scharff (2002). Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. Nature. **415:** 802-6.

44. Yoshikawa, K., I.M. Okazaki, T. Eto, K. Kinoshita, M. Muramatsu, H. Nagaoka, and T. Honjo (2002). AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. Science. **296:** 2033-6.
45. Petersen-Mahrt, S.K., R.S. Harris, and M.S. Neuberger (2002). AID mutates E. coli suggesting a DNA deamination mechanism for antibody diversification. Nature. **418:** 99-103.
46. Bassing, C.H., W. Swat, and F.W. Alt (2002). The mechanism and regulation of chromosomal V(D)J recombination. Cell. **109 Suppl:** S45-55.
47. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo (2000). Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. **102:** 553-63.
48. Revy, P., T. Muto, Y. Levy, F. Geissmann, A. Plebani, O. Sanal, N. Catalan, M. Forveille, R. Dufourcq-Labelouse, A. Gennery, I. Tezcan, F. Ersoy, H. Kayserili, A.G. Ugazio, N. Brousse, M. Muramatsu, L.D. Notarangelo, K. Kinoshita, T. Honjo, A. Fischer, and A. Durandy (2000). Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). Cell. **102:** 565-75.
49. Rada, C., G.T. Williams, H. Nilsen, D.E. Barnes, T. Lindahl, and M.S. Neuberger (2002). Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in UNG-deficient mice. Curr Biol. **12:** 1748-55.
50. Imai, K., G. Slupphaug, W.I. Lee, P. Revy, S. Nonoyama, N. Catalan, L. Yel, M. Forveille, B. Kavli, H.E. Krokan, H.D. Ochs, A. Fischer, and A. Durandy (2003). Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. Nat Immunol. **4:** 1023-8.
51. Nilsen, H., G. Stamp, S. Andersen, G. Hrivnak, H.E. Krokan, T. Lindahl, and D.E. Barnes (2003). Gene-targeted mice lacking the Ung uracil-DNA glycosylase develop B-cell lymphomas. Oncogene. **22:** 5381-6.
52. Klein, U. and R. Dalla-Favera (2008). Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol. **8:** 22-33.
53. Tuo, J., C. Chen, X. Zeng, M. Christiansen, and V.A. Bohr (2002). Functional crosstalk between hOgg1 and the helicase domain of Cockayne syndrome group B protein. DNA Repair (Amst). **1:** 913-27.
54. Risinger, M.A. and J. Groden (2004). Crosslinks and crosstalk: human cancer syndromes and DNA repair defects. Cancer Cell. **6:** 539-45.
55. Kovtun, I.V. and C.T. McMurray (2007). Crosstalk of DNA glycosylases with pathways other than base excision repair. DNA Repair (Amst). **6:** 517-29.
56. Hakem, R. (2008). DNA-damage repair; the good, the bad, and the ugly. Embo J. **27:** 589-605.
57. Sedgwick, B., P.A. Bates, J. Paik, S.C. Jacobs, and T. Lindahl (2007). Repair of alkylated DNA: recent advances. DNA Repair (Amst). **6:** 429-42.
58. Falnes, P.O., R.F. Johansen, and E. Seeberg (2002). AlkB-mediated oxidative demethylation reverses DNA damage in Escherichia coli. Nature. **419:** 178-82.
59. Trewick, S.C., T.F. Henshaw, R.P. Hausinger, T. Lindahl, and B. Sedgwick (2002). Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature. **419:** 174-8.
60. Aas, P.A., M. Otterlei, P.O. Falnes, C.B. Vagbo, F. Skorpen, M. Akbari, O. Sundheim, M. Bjoras, G. Slupphaug, E. Seeberg, and H.E. Krokan (2003). Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. Nature. **421:** 859-63.
61. Wei, Y.F., K.C. Carter, R.P. Wang, and B.K. Shell (1996). Molecular cloning and functional analysis of a human cDNA encoding an Escherichia coli AlkB homolog, a protein involved in DNA alkylation damage repair. Nucleic Acids Res. **24:** 931-37.
62. Duncan, T., S.C. Trewick, P. Koivisto, P.A. Bates, T. Lindahl, and B. Sedgwick (2002). Reversal of DNA alkylation damage by two human dioxygenases. Proc Natl Acad Sci U S A. **99:** 16660-5.

63. Pepponi, R., G. Marra, M.P. Fuggetta, S. Falcinelli, E. Pagani, E. Bonmassar, J. Jiricny, and S. D'Atri (2003). The effect of O6-alkylguanine-DNA alkyltransferase and mismatch repair activities on the sensitivity of human melanoma cells to temozolomide, 1,3-bis(2-chloroethyl)1-nitrosourea, and cisplatin. J Pharmacol Exp Ther. **304:** 661-8.
64. Branch, P., G. Aquilina, M. Bignami, and P. Karran (1993). Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. Nature. **362:** 652-4.
65. Jiricny, J. (1998). Eukaryotic mismatch repair: an update. Mutat Res. **409:** 107-21.
66. Karran, P. (2001). Mechanisms of tolerance to DNA damaging therapeutic drugs. Carcinogenesis. **22:** 1931-7.
67. Jiricny, J. (2006). The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol. **7:** 335-46.
68. Vasen, H.F. (2007). Review article: The Lynch syndrome (hereditary nonpolyposis colorectal cancer). Aliment Pharmacol Ther. **26 Suppl 2:** 113-26.
69. Drotschmann, K., W. Yang, and T.A. Kunkel (2002). Evidence for sequential action of two ATPase active sites in yeast Msh2-Msh6. DNA Repair (Amst). **1:** 743-53.
70. Obmolova, G., C. Ban, P. Hsieh, and W. Yang (2000). Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. Nature. **407:** 703-10.
71. Lamers, M.H., A. Perrakis, J.H. Enzlin, H.H. Winterwerp, N. de Wind, and T.K. Sixma (2000). The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch. Nature. **407:** 711-7.
72. Santucci-Darmanin, S., D. Walpita, F. Lespinasse, C. Desnuelle, T. Ashley, and V. Paquis-Flucklinger (2000). MSH4 acts in conjunction with MLH1 during mammalian meiosis. Faseb J. **14:** 1539-47.
73. Petronzelli, F., A. Riccio, G.D. Markham, S.H. Seeholzer, J. Stoerker, M. Genuardi, A.T. Yeung, Y. Matsumoto, and A. Bellacosa (2000). Biphasic kinetics of the human DNA repair protein MED1 (MBD4), a mismatch-specific DNA N-glycosylase. J Biol Chem. **275:** 32422-9.
74. Mac Partlin, M., E. Homer, H. Robinson, C.J. McCormick, D.H. Crouch, S.T. Durant, E.C. Matheson, A.G. Hall, D.A. Gillespie, and R. Brown (2003). Interactions of the DNA mismatch repair proteins MLH1 and MSH2 with c-MYC and MAX. Oncogene. **22:** 819-25.
75. Brown, K.D., A. Rathi, R. Kamath, D.I. Beardsley, Q. Zhan, J.L. Mannino, and R. Baskaran (2003). The mismatch repair system is required for S-phase checkpoint activation. Nat Genet. **33:** 80-4.
76. Claij, N. and H. Te Riele (2002). Methylation tolerance in mismatch repair proficient cells with low MSH2 protein level. Oncogene. **21:** 2873-9.
77. Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. Nature. **411:** 366-74.
78. Vilenchik, M.M. and A.G. Knudson (2003). Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. Proc Natl Acad Sci U S A. **100:** 12871-6.
79. Kanaar, R., C. Wyman, and R. Rothstein (2008). Quality control of DNA break metabolism: in the 'end', it's a good thing. Embo J. **27:** 581-8.
80. Shrivastav, M., L.P. De Haro, and J.A. Nickoloff (2008). Regulation of DNA double-strand break repair pathway choice. Cell Res. **18:** 134-47.
81. Allen, C., J. Halbrook, and J.A. Nickoloff (2003). Interactive competition between homologous recombination and non-homologous end joining. Mol Cancer Res. **1:** 913-20.
82. Rothkamm, K., I. Kruger, L.H. Thompson, and M. Lobrich (2003). Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol. **23:** 5706-15.
83. Delacote, F. and B.S. Lopez (2008). Importance of the cell cycle phase for the choice of the appropriate DSB repair pathway, for genome stability maintenance: the trans-S double-strand break repair model. Cell Cycle. **7:** 33-8.
84. Lieber, M.R., Y. Ma, U. Pannicke, and K. Schwarz (2003). Mechanism and regulation of human non-homologous DNA end-joining. Nat Rev Mol Cell Biol. **4:** 712-20.
85. Bosma, G.C., R.P. Custer, and M.J. Bosma (1983). A severe combined immunodeficiency mutation in the mouse. Nature. **301:** 527-30.

86. Blunt, T., N.J. Finnie, G.E. Taccioli, G.C. Smith, J. Demengeot, T.M. Gottlieb, R. Mizuta, A.J. Varghese, F.W. Alt, P.A. Jeggo, and et al. (1995). Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. Cell. **80:** 813-23.
87. Slupphaug, G., B. Kavli, and H.E. Krokan (2003). The interacting pathways for prevention and repair of oxidative DNA damage. Mutat Res. **531:** 231-51.
88. Tauchi, H., J. Kobayashi, K. Morishima, D.C. van Gent, T. Shiraishi, N.S. Verkaik, D. vanHeems, E. Ito, A. Nakamura, E. Sonoda, M. Takata, S. Takeda, S. Matsuura, and K. Komatsu (2002). Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells. Nature. **420:** 93-8.
89. West, S.C. (2003). Molecular views of recombination proteins and their control. Nat Rev Mol Cell Biol. **4:** 435-45.
90. Thompson, L.H. and D. Schild (2002). Recombinational DNA repair and human disease. Mutat Res. **509:** 49-78.
91. Singh, D.K., B. Ahn, and V.A. Bohr (2009). Roles of RECQ helicases in recombination based DNA repair, genomic stability and aging. Biogerontology. **10:** 235-52.
92. Krokan, H.E., H. Nilsen, F. Skorpen, M. Otterlei, and G. Slupphaug (2000). Base excision repair of DNA in mammalian cells. FEBS Lett. **476:** 73-7.
93. Arczewska, K.D., K. Michalickova, I.M. Donaldson, and H. Nilsen (2008). The contribution of DNA base damage to human cancer is modulated by the base excision repair interaction network. Crit Rev Oncog. **14:** 217-73.
94. Baute, J. and A. Depicker (2008). Base excision repair and its role in maintaining genome stability. Crit Rev Biochem Mol Biol. **43:** 239-76.
95. Otterlei, M., B. Kavli, R. Standal, C. Skjelbred, S. Bharati, and H.E. Krokan (2000). Repair of chromosomal abasic sites in vivo involves at least three different repair pathways. Embo J. **19:** 5542-51.
96. Waters, T.R. and P.F. Swann (1998). Kinetics of the action of thymine DNA glycosylase. J Biol Chem. **273:** 20007-14.
97. Parikh, S.S., C.D. Mol, G. Slupphaug, S. Bharati, H.E. Krokan, and J.A. Tainer (1998). Base excision repair initiation revealed by crystal structures and binding kinetics of human uracil-DNA glycosylase with DNA. Embo J. **17:** 5214-26.
98. Waters, T.R., P. Gallinari, J. Jiricny, and P.F. Swann (1999). Human thymine DNA glycosylase binds to apurinic sites in DNA but is displaced by human apurinic endonuclease 1. J Biol Chem. **274:** 67-74.
99. Marsin, S., A.E. Vidal, M. Sossou, J. Menissier-de Murcia, F. Le Page, S. Boiteux, G. de Murcia, and J.P. Radicella (2003). Role of XRCC1 in the coordination and stimulation of oxidative DNA damage repair initiated by the DNA glycosylase hOGG1. J Biol Chem. **278:** 44068-74.
100. Thompson, L.H., K.W. Brookman, N.J. Jones, S.A. Allen, and A.V. Carrano (1990). Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Mol Cell Biol. **10:** 6160-71.
101. Cantoni, O., D. Murray, and R.E. Meyn (1987). Induction and repair of DNA single-strand breaks in EM9 mutant CHO cells treated with hydrogen peroxide. Chem Biol Interact. **63:** 29-38.
102. Thompson, L.H., K.W. Brookman, L.E. Dillehay, A.V. Carrano, J.A. Mazrimas, C.L. Mooney, and J.L. Minkler (1982). A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. Mutat Res. **95:** 427-40.
103. Thompson, L.H. and M.G. West (2000). XRCC1 keeps DNA from getting stranded. Mutat Res. **459:** 1-18.
104. Klungland, A. and T. Lindahl (1997). Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). Embo J. **16:** 3341-8.

105. Sobol, R.W., J.K. Horton, R. Kuhn, H. Gu, R.K. Singhal, R. Prasad, K. Rajewsky, and S.H. Wilson (1996). Requirement of mammalian DNA polymerase-beta in base-excision repair. Nature. **379:** 183-6.
106. Gary, R., K. Kim, H.L. Cornelius, M.S. Park, and Y. Matsumoto (1999). Proliferating cell nuclear antigen facilitates excision in long-patch base excision repair. J Biol Chem. **274:** 4354-63.
107. Akbari, M., M. Otterlei, J. Pena-Diaz, P.A. Aas, B. Kavli, N.B. Liabakk, L. Hagen, K. Imai, A. Durandy, G. Slupphaug, and H.E. Krokan (2004). Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells. Nucleic Acids Res. **32:** 5486-98.
108. Leibeling, D., P. Laspe, and S. Emmert (2006). Nucleotide excision repair and cancer. J Mol Histol. **37:** 225-38.
109. Ura, K. and J.J. Hayes (2002). Nucleotide excision repair and chromatin remodeling. Eur J Biochem. **269:** 2288-93.
110. Hanawalt, P.C. (2002). Subpathways of nucleotide excision repair and their regulation. Oncogene. **21:** 8949-56.
111. Fousteri, M. and L.H. Mullenders (2008). Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. Cell Res. **18:** 73-84.
112. Friedberg, E.C. (2001). How nucleotide excision repair protects against cancer. Nat Rev Cancer. **1:** 22-33.
113. Cleaver, J.E. (2005). Cancer in xeroderma pigmentosum and related disorders of DNA repair. Nat Rev Cancer. **5:** 564-73.
114. Rubbi, C.P. and J. Milner (2003). p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage. Embo J. **22:** 975-86.
115. Sugasawa, K., T. Okamoto, Y. Shimizu, C. Masutani, S. Iwai, and F. Hanaoka (2001). A multistep damage recognition mechanism for global genomic nucleotide excision repair. Genes Dev. **15:** 507-21.
116. Wood, R.D. (1999). DNA damage recognition during nucleotide excision repair in mammalian cells. Biochimie. **81:** 39-44.
117. Sugasawa, K., J.M. Ng, C. Masutani, S. Iwai, P.J. van der Spek, A.P. Eker, F. Hanaoka, D. Bootsma, and J.H. Hoeijmakers (1998). Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Mol Cell. **2:** 223-32.
118. Adimoolam, S. and J.M. Ford (2003). p53 and regulation of DNA damage recognition during nucleotide excision repair. DNA Repair (Amst). **2:** 947-54.
119. Ng, J.M., W. Vermeulen, G.T. van der Horst, S. Bergink, K. Sugasawa, H. Vrieling, and J.H. Hoeijmakers (2003). A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. Genes Dev. **17:** 1630-45.
120. Riedl, T., F. Hanaoka, and J.M. Egly (2003). The comings and goings of nucleotide excision repair factors on damaged DNA. Embo J. **22:** 5293-303.
121. Ito, S., I. Kuraoka, P. Chymkowitch, E. Compe, A. Takedachi, C. Ishigami, F. Coin, J.M. Egly, and K. Tanaka (2007). XPG stabilizes TFIIH, allowing transactivation of nuclear receptors: implications for Cockayne syndrome in XP-G/CS patients. Mol Cell. **26:** 231-43.
122. Nouspikel, T. (2009). DNA repair in mammalian cells : Nucleotide excision repair: variations on versatility. Cell Mol Life Sci. **66:** 994-1009.
123. Dworaczek, H. and W. Xiao (2007). Xeroderma pigmentosum: a glimpse into nucleotide excision repair, genetic instability, and cancer. Crit Rev Oncog. **13:** 159-77.
124. Thoms, K.M., C. Kuschal, and S. Emmert (2007). Lessons learned from DNA repair defective syndromes. Exp Dermatol. **16:** 532-44.
125. Parris, C.N. and K.H. Kraemer (1993). Ultraviolet-induced mutations in Cockayne syndrome cells are primarily caused by cyclobutane dimer photoproducts while repair of other photoproducts is normal. Proc Natl Acad Sci U S A. **90:** 7260-4.
126. Lindahl, T. and R.D. Wood (1999). Quality control by DNA repair. Science. **286:** 1897-905.

127. Reardon, J.T., T. Bessho, H.C. Kung, P.H. Bolton, and A. Sancar (1997). In vitro repair of oxidative DNA damage by human nucleotide excision repair system: possible explanation for neurodegeneration in xeroderma pigmentosum patients. Proc Natl Acad Sci U S A. **94:** 9463-8.
128. Swanson, R.L., N.J. Morey, P.W. Doetsch, and S. Jinks-Robertson (1999). Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae. Mol Cell Biol. **19:** 2929-35.
129. Wood, W.B. (1988). "Chapter 1: Introduction to C. elegans Biology". The Nematode *Caenorhabditis elegans*. W.B. Wood, Cold Spring Harbor Laboratory Press.
130. Riddle, D.L., T. Blumenthal, B.J. Meyer, and J.R. Priess, Eds. (1997). *C. elegans II*. New York, Cold Spring Harbor Laboratory Press.
131. C._elegans_sequencing_consortium (1998). Genome sequence of the nematode C. elegans: a platform for investigating biology. Science. **282:** 2012-8.
132. Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics. **77:** 71-94.
133. Sulston, J.E. and H.R. Horvitz (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol. **56:** 110-56.
134. Kimble, J. and D. Hirsh (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. Dev Biol. **70:** 396-417.
135. Hengartner, M.O. (1997). Cell death. *C. elegans II*. D.L. Riddle, et al. New York, Cold Spring Laboratory Press.
136. Conradt, B. and H.R. Horvitz (1998). The C. elegans protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell. **93:** 519-29.
137. Ellis, R.E. and H.R. Horvitz (1991). Two C. elegans genes control the programmed deaths of specific cells in the pharynx. Development. **112:** 591-603.
138. Greiss, S., B. Schumacher, K. Grandien, J. Rothblatt, and A. Gartner (2008). Transcriptional profiling in C. elegans suggests DNA damage dependent apoptosis as an ancient function of the p53 family. BMC Genomics. **9:** 334.
139. Shaham, S. and H.R. Horvitz (1996). Developing Caenorhabditis elegans neurons may contain both cell-death protective and killer activities. Genes Dev. **10:** 578-91.
140. Hengartner, M.O., R.E. Ellis, and H.R. Horvitz (1992). Caenorhabditis elegans gene ced-9 protects cells from programmed cell death. Nature. **356:** 494-9.
141. Hengartner, M.O. and H.R. Horvitz (1994). C. elegans cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell. **76:** 665-76.
142. Adams, J.M. and S. Cory (2001). Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci. **26:** 61-6.
143. Bouillet, P. and A. Strasser (2002). BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J Cell Sci. **115:** 1567-74.
144. Alnemri, E.S., D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, and J. Yuan (1996). Human ICE/CED-3 protease nomenclature. Cell. **87:** 171.
145. Yuan, J., S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell. **75:** 641-52.
146. Yuan, J.Y. and H.R. Horvitz (1990). The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. Dev Biol. **138:** 33-41.
147. Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. **90:** 405-13.
148. Skjeldam, H.K., H. Kassahun, O. Fensgard, T. Sengupta, E. Babaie, J.M. Lindvall, K. Arczewska, and H. Nilsen (2010). Loss of Caenorhabditis elegans UNG-1 uracil-DNA glycosylase affects apoptosis in response to DNA damaging agents. DNA Repair (Amst).

149. Fire, A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature. **391:** 806-11.
150. Fortunato, A. and A.G. Fraser (2005). Uncover genetic interactions in Caenorhabditis elegans by RNA interference. Biosci Rep. **25:** 299-307.
151. Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher (1994). Green fluorescent protein as a marker for gene expression. Science. **263:** 802-5.
152. O'Neill, N. and A. Rose, DNA repair. WormBook.
153. Gumienny, T.L., E. Lambie, E. Hartwieg, H.R. Horvitz, and M.O. Hengartner (1999). Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline. Development. **126:** 1011-22.
154. Derry, W.B., A.P. Putzke, and J.H. Rothman (2001). Caenorhabditis elegans p53: role in apoptosis, meiosis, and stress resistance. Science. **294:** 591-5.
155. Schumacher, B., K. Hofmann, S. Boulton, and A. Gartner (2001). The C. elegans homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. Curr Biol. **11:** 1722-7.
156. Schumacher, B., C. Schertel, N. Wittenburg, S. Tuck, S. Mitani, A. Gartner, B. Conradt, and S. Shaham (2005). C. elegans ced-13 can promote apoptosis and is induced in response to DNA damage. Cell Death Differ. **12:** 153-61.
157. Gartner, A., S. Milstein, S. Ahmed, J. Hodgkin, and M.O. Hengartner (2000). A conserved checkpoint pathway mediates DNA damage--induced apoptosis and cell cycle arrest in C. elegans. Mol Cell. **5:** 435-43.
158. Salinas, L.S., E. Maldonado, and R.E. Navarro (2006). Stress-induced germ cell apoptosis by a p53 independent pathway in Caenorhabditis elegans. Cell Death Differ. **13:** 2129-39.
159. Aballay, A. and F.M. Ausubel (2001). Programmed cell death mediated by ced-3 and ced-4 protects Caenorhabditis elegans from Salmonella typhimurium-mediated killing. Proc Natl Acad Sci U S A. **98:** 2735-9.
160. Stergiou, L. and M.O. Hengartner (2004). Death and more: DNA damage response pathways in the nematode C. elegans. Cell Death Differ. **11:** 21-8.
161. Jiang, N., C.Y. Benard, H. Kebir, E.A. Shoubridge, and S. Hekimi (2003). Human CLK2 links cell cycle progression, apoptosis, and telomere length regulation. J Biol Chem. **278:** 21678-84.
162. Dengg, M., T. Garcia-Muse, S.G. Gill, N. Ashcroft, S.J. Boulton, and H. Nilsen (2006). Abrogation of the CLK-2 checkpoint leads to tolerance to base-excision repair intermediates. EMBO Rep. **7:** 1046-51.
163. Pinkston, J.M., D. Garigan, M. Hansen, and C. Kenyon (2006). Mutations that increase the life span of C. elegans inhibit tumor growth. Science. **313:** 971-5.
164. Morinaga, H., S.I. Yonekura, N. Nakamura, H. Sugiyama, S. Yonei, and Q.M. Zhang-Akiyama (2009). Purification and characterization of Caenorhabditis elegans NTH, a homolog of human endonuclease III: Essential role of N-terminal region. DNA Repair (Amst).
165. Shatilla, A. and D. Ramotar (2002). Embryonic extracts derived from the nematode Caenorhabditis elegans remove uracil from DNA by the sequential action of uracil-DNA glycosylase and AP (apurinic/apyrimidinic) endonuclease. Biochem J. **365:** 547-53.
166. Park, H.K., J.S. Yook, H.S. Koo, I.S. Choi, and B. Ahn (2002). The Caenorhabditis elegans XPA homolog of human XPA. Mol Cells. **14:** 50-5.
167. Stergiou, L., K. Doukoumetzidis, A. Sendoel, and M.O. Hengartner (2007). The nucleotide excision repair pathway is required for UV-C-induced apoptosis in Caenorhabditis elegans. Cell Death Differ. **14:** 1129-38.
168. Kamath, R.S., A.G. Fraser, Y. Dong, G. Poulin, R. Durbin, M. Gotta, A. Kanapin, N. Le Bot, S. Moreno, M. Sohrmann, D.P. Welchman, P. Zipperlen, and J. Ahringer (2003). Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature. **421:** 231-7.
169. Park, H.K., D. Suh, M. Hyun, H.S. Koo, and B. Ahn (2004). A DNA repair gene of Caenorhabditis elegans: a homolog of human XPF. DNA Repair (Amst). **3:** 1375-83.

170. Piano, F., A.J. Schetter, D.G. Morton, K.C. Gunsalus, V. Reinke, S.K. Kim, and K.J. Kemphues (2002). Gene clustering based on RNAi phenotypes of ovary-enriched genes in C. elegans. Curr Biol. **12:** 1959-64.
171. Lans, H., J.A. Marteijn, B. Schumacher, J.H. Hoeijmakers, G. Jansen, and W. Vermeulen (2010). Involvement of global genome repair, transcription coupled repair, and chromatin remodeling in UV DNA damage response changes during development. PLoS Genet. **6:** e1000941.
172. Lee, M.H., B. Ahn, I.S. Choi, and H.S. Koo (2002). The gene expression and deficiency phenotypes of Cockayne syndrome B protein in Caenorhabditis elegans. FEBS Lett. **522:** 47-51.
173. Tijsterman, M., J. Pothof, and R.H. Plasterk (2002). Frequent germline mutations and somatic repeat instability in DNA mismatch-repair-deficient Caenorhabditis elegans. Genetics. **161:** 651-60.
174. Degtyareva, N.P., P. Greenwell, E.R. Hofmann, M.O. Hengartner, L. Zhang, J.G. Culotti, and T.D. Petes (2002). Caenorhabditis elegans DNA mismatch repair gene msh-2 is required for microsatellite stability and maintenance of genome integrity. Proc Natl Acad Sci U S A. **99:** 2158-63.
175. Pothof, J., G. van Haaften, K. Thijssen, R.S. Kamath, A.G. Fraser, J. Ahringer, R.H. Plasterk, and M. Tijsterman (2003). Identification of genes that protect the C. elegans genome against mutations by genome-wide RNAi. Genes Dev. **17:** 443-8.
176. McColl, G., M.C. Vantipalli, and G.J. Lithgow (2005). The C. elegans ortholog of mammalian Ku70, interacts with insulin-like signaling to modulate stress resistance and life span. Faseb J. **19:** 1716-8.
177. Boulton, S.J., A. Gartner, J. Reboul, P. Vaglio, N. Dyson, D.E. Hill, and M. Vidal (2002). Combined functional genomic maps of the C. elegans DNA damage response. Science. **295:** 127-31.
178. Hayashi, M., G.M. Chin, and A.M. Villeneuve (2007). C. elegans germ cells switch between distinct modes of double-strand break repair during meiotic prophase progression. PLoS Genet. **3:** e191.
179. Takanami, T., S. Sato, T. Ishihara, I. Katsura, H. Takahashi, and A. Higashitani (1998). Characterization of a Caenorhabditis elegans recA-like gene Ce-rdh-1 involved in meiotic recombination. DNA Res. **5:** 373-7.
180. Wicky, C., A. Alpi, M. Passannante, A. Rose, A. Gartner, and F. Muller (2004). Multiple genetic pathways involving the Caenorhabditis elegans Bloom's syndrome genes him-6, rad-51, and top-3 are needed to maintain genome stability in the germ line. Mol Cell Biol. **24:** 5016-27.
181. Lee, M.H., S.M. Han, J.W. Han, Y.M. Kim, J. Ahnn, and H.S. Koo (2003). Caenorhabditis elegans dna-2 is involved in DNA repair and is essential for germ-line development. FEBS Lett. **555:** 250-6.
182. Kanugula, S. and A.E. Pegg (2001). Novel DNA repair alkyltransferase from Caenorhabditis elegans. Environ Mol Mutagen. **38:** 235-43.
183. Hartman, P.S. and R.K. Herman (1982). Radiation-sensitive mutants of Caenorhabditis elegans. Genetics. **102:** 159-78.
184. Nakamura, N., H. Morinaga, M. Kikuchi, S.I. Yonekura, N. Ishii, K. Yamamoto, S. Yonei, and Q.M. Zhang (2008). Cloning and characterization of uracil-DNA glycosylase and the biological consequences of the loss of its function in the nematode Caenorhabditis elegans. Mutagenesis.
185. Kavli, B., O. Sundheim, M. Akbari, M. Otterlei, H. Nilsen, F. Skorpen, P.A. Aas, L. Hagen, H.E. Krokan, and G. Slupphaug (2002). hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup. J Biol Chem. **277:** 39926-36.

186. Denver, D.R., S. Feinberg, C. Steding, M. Durbin, and M. Lynch (2006). The relative roles of three DNA repair pathways in preventing Caenorhabditis elegans mutation accumulation. Genetics. **174:** 57-65.
187. Astin, J.W., N.J. O'Neil, and P.E. Kuwabara (2008). Nucleotide excision repair and the degradation of RNA pol II by the Caenorhabditis elegans XPA and Rsp5 orthologues, RAD-3 and WWP-1. DNA Repair (Amst). **7:** 267-80.
188. Hyun, M., J. Lee, K. Lee, A. May, V.A. Bohr, and B. Ahn (2008). Longevity and resistance to stress correlate with DNA repair capacity in Caenorhabditis elegans. Nucleic Acids Res. **36:** 1380-9.
189. Fensgard, O., H. Kassahun, I. Bombik, T. Rognes, J.M. Lindvall, and H. Nilsen (2010). A Two-tiered compensatory response to loss of DNA repair modulates aging and stress response pathways. Aging (Albany NY). **2**.
190. Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. J Gerontol. **11:** 298-300.
191. Rubner, M. (1908). Das Problem der Lebensdauer und seine Beziehungen sum Wachstum und Ernahrung. Munic, Oldenburg.
192. Pearl, R. (1928). The Rate of Living, Being an Account of Some Experimental Studies on the Biology of Life Duration. New York, Alfred A. Knopf.
193. Gerschman, R., D.L. Gilbert, S.W. Nye, P. Dwyer, and W.O. Fenn (1954). Oxygen poisoning and x-irradiation: a mechanism in common. Science. **119:** 623-6.
194. Harman, D. (1972). The biologic clock: the mitochondria? J Am Geriatr Soc. **20:** 145-7.
195. Fridovich, I. (1995). Superoxide radical and superoxide dismutases. Annu Rev Biochem. **64:** 97-112.
196. Arking, R. (1987). Successful selection for increased longevity in Drosophila: analysis of the survival data and presentation of a hypothesis on the genetic regulation of longevity. Exp Gerontol. **22:** 199-220.
197. Arking, R., S. Buck, A. Berrios, S. Dwyer, and G.T. Baker, 3rd (1991). Elevated paraquat resistance can be used as a bioassay for longevity in a genetically based long-lived strain of Drosophila. Dev Genet. **12:** 362-70.
198. Arking, R., V. Burde, K. Graves, R. Hari, E. Feldman, A. Zeevi, S. Soliman, A. Saraiya, S. Buck, J. Vettraino, K. Sathrasala, N. Wehr, and R.L. Levine (2000). Forward and reverse selection for longevity in Drosophila is characterized by alteration of antioxidant gene expression and oxidative damage patterns. Exp Gerontol. **35:** 167-85.
199. Reveillaud, I., A. Niedzwiecki, K.G. Bensch, and J.E. Fleming (1991). Expression of bovine superoxide dismutase in Drosophila melanogaster augments resistance of oxidative stress. Mol Cell Biol. **11:** 632-40.
200. Seto, N.O., S. Hayashi, and G.M. Tener (1990). Overexpression of Cu-Zn superoxide dismutase in Drosophila does not affect life-span. Proc Natl Acad Sci U S A. **87:** 4270-4.
201. Sun, J., J. Molitor, and J. Tower (2004). Effects of simultaneous over-expression of Cu/ZnSOD and MnSOD on Drosophila melanogaster life span. Mech Ageing Dev. **125:** 341-9.
202. Sohal, R.S., A. Agarwal, S. Agarwal, and W.C. Orr (1995). Simultaneous overexpression of copper- and zinc-containing superoxide dismutase and catalase retards age-related oxidative damage and increases metabolic potential in Drosophila melanogaster. J Biol Chem. **270:** 15671-4.
203. Reveillaud, I., J. Phillips, B. Duyf, A. Hilliker, A. Kongpachith, and J.E. Fleming (1994). Phenotypic rescue by a bovine transgene in a Cu/Zn superoxide dismutase-null mutant of Drosophila melanogaster. Mol Cell Biol. **14:** 1302-7.
204. Rogina, B. and S.L. Helfand (2000). Cu, Zn superoxide dismutase deficiency accelerates the time course of an age-related marker in Drosophila melanogaster. Biogerontology. **1:** 163-9.
205. Kirby, K., J. Hu, A.J. Hilliker, and J.P. Phillips (2002). RNA interference-mediated silencing of Sod2 in Drosophila leads to early adult-onset mortality and elevated endogenous oxidative stress. Proc Natl Acad Sci U S A. **99:** 16162-7.

206. Duttaroy, A., A. Paul, M. Kundu, and A. Belton (2003). A Sod2 null mutation confers severely reduced adult life span in Drosophila. Genetics. **165:** 2295-9.
207. Wawryn, J., A. Krzepilko, A. Myszka, and T. Bilinski (1999). Deficiency in superoxide dismutases shortens life span of yeast cells. Acta Biochim Pol. **46:** 249-53.
208. Unlu, E.S. and A. Koc (2007). Effects of deleting mitochondrial antioxidant genes on life span. Ann N Y Acad Sci. **1100:** 505-9.
209. Longo, V.D., L.L. Liou, J.S. Valentine, and E.B. Gralla (1999). Mitochondrial superoxide decreases yeast survival in stationary phase. Arch Biochem Biophys. **365:** 131-42.
210. Muller, F.L., W. Song, Y. Liu, A. Chaudhuri, S. Pieke-Dahl, R. Strong, T.T. Huang, C.J. Epstein, L.J. Roberts, 2nd, M. Csete, J.A. Faulkner, and H. Van Remmen (2006). Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy. Free Radic Biol Med. **40:** 1993-2004.
211. Busuttil, R.A., A.M. Garcia, C. Cabrera, A. Rodriguez, Y. Suh, W.H. Kim, T.T. Huang, and J. Vijg (2005). Organ-specific increase in mutation accumulation and apoptosis rate in CuZn-superoxide dismutase-deficient mice. Cancer Res. **65:** 11271-5.
212. Elchuri, S., T.D. Oberley, W. Qi, R.S. Eisenstein, L. Jackson Roberts, H. Van Remmen, C.J. Epstein, and T.T. Huang (2005). CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. Oncogene. **24:** 367-80.
213. Melov, S., P. Coskun, M. Patel, R. Tuinstra, B. Cottrell, A.S. Jun, T.H. Zastawny, M. Dizdaroglu, S.I. Goodman, T.T. Huang, H. Miziorko, C.J. Epstein, and D.C. Wallace (1999). Mitochondrial disease in superoxide dismutase 2 mutant mice. Proc Natl Acad Sci U S A. **96:** 846-51.
214. Asikainen, T.M., T.T. Huang, E. Taskinen, A.L. Levonen, E. Carlson, R. Lapatto, C.J. Epstein, and K.O. Raivio (2002). Increased sensitivity of homozygous Sod2 mutant mice to oxygen toxicity. Free Radic Biol Med. **32:** 175-86.
215. Tsan, M.F., J.E. White, B. Caska, C.J. Epstein, and C.Y. Lee (1998). Susceptibility of heterozygous MnSOD gene-knockout mice to oxygen toxicity. Am J Respir Cell Mol Biol. **19:** 114-20.
216. Lebovitz, R.M., H. Zhang, H. Vogel, J. Cartwright, Jr., L. Dionne, N. Lu, S. Huang, and M.M. Matzuk (1996). Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Natl Acad Sci U S A. **93:** 9782-7.
217. Li, Y., T.T. Huang, E.J. Carlson, S. Melov, P.C. Ursell, J.L. Olson, L.J. Noble, M.P. Yoshimura, C. Berger, P.H. Chan, D.C. Wallace, and C.J. Epstein (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat Genet. **11:** 376-81.
218. Marklund, S.L. (1982). Human copper-containing superoxide dismutase of high molecular weight. Proc Natl Acad Sci U S A. **79:** 7634-8.
219. Carlsson, L.M., J. Jonsson, T. Edlund, and S.L. Marklund (1995). Mice lacking extracellular superoxide dismutase are more sensitive to hyperoxia. Proc Natl Acad Sci U S A. **92:** 6264-8.
220. Yang, W., J. Li, and S. Hekimi (2007). A Measurable increase in oxidative damage due to reduction in superoxide detoxification fails to shorten the life span of long-lived mitochondrial mutants of Caenorhabditis elegans. Genetics. **177:** 2063-74.
221. Van Raamsdonk, J.M. and S. Hekimi (2009). Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans. PLoS Genet. **5:** e1000361.
222. Landis, G.N. and J. Tower (2005). Superoxide dismutase evolution and life span regulation. Mech Ageing Dev. **126:** 365-79.
223. Giglio, A.M., T. Hunter, J.V. Bannister, W.H. Bannister, and G.J. Hunter (1994). The copper/zinc superoxide dismutase gene of Caenorhabditis elegans. Biochem Mol Biol Int. **33:** 41-4.
224. Giglio, M.P., T. Hunter, J.V. Bannister, W.H. Bannister, and G.J. Hunter (1994). The manganese superoxide dismutase gene of Caenorhabditis elegans. Biochem Mol Biol Int. **33:** 37-40.

225. Suzuki, N., K. Inokuma, K. Yasuda, and N. Ishii (1996). Cloning, sequencing and mapping of a manganese superoxide dismutase gene of the nematode Caenorhabditis elegans. DNA Res. **3:** 171-4.
226. Fujii, M., N. Ishii, A. Joguchi, K. Yasuda, and D. Ayusawa (1998). A novel superoxide dismutase gene encoding membrane-bound and extracellular isoforms by alternative splicing in Caenorhabditis elegans. DNA Res. **5:** 25-30.
227. Hunter, T., W.H. Bannister, and G.J. Hunter (1997). Cloning, expression, and characterization of two manganese superoxide dismutases from Caenorhabditis elegans. J Biol Chem. **272:** 28652-9.
228. Jensen, L.T. and V.C. Culotta (2005). Activation of CuZn superoxide dismutases from Caenorhabditis elegans does not require the copper chaperone CCS. J Biol Chem. **280:** 41373-9.
229. Masoro, E.J. (2006). Dietary restriction-induced life extension: a broadly based biological phenomenon. Biogerontology. **7:** 153-5.
230. Rattan, S.I. (2001). Applying hormesis in aging research and therapy. Hum Exp Toxicol. **20:** 281-5; discussion 293-4.
231. Lindsted, K.D., S. Tonstad, and J.W. Kuzma (1991). Self-report of physical activity and patterns of mortality in Seventh-Day Adventist men. J Clin Epidemiol. **44:** 355-64.
232. Manini, T.M., J.E. Everhart, K.V. Patel, D.A. Schoeller, L.H. Colbert, M. Visser, F. Tylavsky, D.C. Bauer, B.H. Goodpaster, and T.B. Harris (2006). Daily activity energy expenditure and mortality among older adults. Jama. **296:** 171-9.
233. Chevion, S., D.S. Moran, Y. Heled, Y. Shani, G. Regev, B. Abbou, E. Berenshtein, E.R. Stadtman, and Y. Epstein (2003). Plasma antioxidant status and cell injury after severe physical exercise. Proc Natl Acad Sci U S A. **100:** 5119-23.
234. Sols, A. and R.K. Crane (1954). Substrate specificity of brain hexokinase. J Biol Chem. **210:** 581-95.
235. Lin, S.J., M. Kaeberlein, A.A. Andalis, L.A. Sturtz, P.A. Defossez, V.C. Culotta, G.R. Fink, and L. Guarente (2002). Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature. **418:** 344-8.
236. Rea, S. and T.E. Johnson (2003). A metabolic model for life span determination in Caenorhabditis elegans. Dev Cell. **5:** 197-203.
237. Droge, W. (2002). Free radicals in the physiological control of cell function. Physiol Rev. **82:** 47-95.
238. Schulz, T.J., K. Zarse, A. Voigt, N. Urban, M. Birringer, and M. Ristow (2007). Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab. **6:** 280-93.
239. Bjelakovic, G., D. Nikolova, L.L. Gluud, R.G. Simonetti, and C. Gluud (2007). Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. Jama. **297:** 842-57.
240. Huang, T.T., E.J. Carlson, A.M. Gillespie, Y. Shi, and C.J. Epstein (2000). Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. J Gerontol A Biol Sci Med Sci. **55:** B5-9.
241. Mele, J., H. Van Remmen, J. Vijg, and A. Richardson (2006). Characterization of transgenic mice that overexpress both copper zinc superoxide dismutase and catalase. Antioxid Redox Signal. **8:** 628-38.
242. Sun, J., D. Folk, T.J. Bradley, and J. Tower (2002). Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics. **161:** 661-72.
243. Yu, B.P. (1996). Aging and oxidative stress: modulation by dietary restriction. Free Radic Biol Med. **21:** 651-68.

244. Flurkey, K., J. Papaconstantinou, R.A. Miller, and D.E. Harrison (2001). Lifespan extension and delayed immune and collagen aging in mutant mice with defects in growth hormone production. Proc Natl Acad Sci U S A. **98:** 6736-41.
245. Rollo, C.D. (2002). Growth negatively impacts the life span of mammals. Evol Dev. **4:** 55-61.
246. Coschigano, K.T., D. Clemmons, L.L. Bellush, and J.J. Kopchick (2000). Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. Endocrinology. **141:** 2608-13.
247. McCay, C., M. Crowell, and L. Maynard (1935). The effect of retarded growth upon the length of life and upon the ultimate body size. J. Nutr. **10:** 63-79.
248. Yu, B.P., E.J. Masoro, I. Murata, H.A. Bertrand, and F.T. Lynd (1982). Life span study of SPF Fischer 344 male rats fed ad libitum or restricted diets: longevity, growth, lean body mass and disease. J Gerontol. **37:** 130-41.
249. Berg, B.N. and H.S. Simms (1960). Nutrition and longevity in the rat. II. Longevity and onset of disease with different levels of food intake. J Nutr. **71:** 255-63.
250. Garthwaite, S.M., H. Cheng, J.E. Bryan, B.W. Craig, and J.O. Holloszy (1986). Ageing, exercise and food restriction: effects on body composition. Mech Ageing Dev. **36:** 187-96.
251. Harrison, D.E., J.R. Archer, and C.M. Astle (1984). Effects of food restriction on aging: separation of food intake and adiposity. Proc Natl Acad Sci U S A. **81:** 1835-8.
252. Barzilai, N. and G. Gupta (1999). Revisiting the role of fat mass in the life extension induced by caloric restriction. J Gerontol A Biol Sci Med Sci. **54:** B89-96; discussion B97-8.
253. Lin, S.J., P.A. Defossez, and L. Guarente (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science. **289:** 2126-8.
254. Wood, J.G., B. Rogina, S. Lavu, K. Howitz, S.L. Helfand, M. Tatar, and D. Sinclair (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature. **430:** 686-9.
255. Picard, F., M. Kurtev, N. Chung, A. Topark-Ngarm, T. Senawong, R. Machado De Oliveira, M. Leid, M.W. McBurney, and L. Guarente (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature. **429:** 771-6.
256. Zainal, T.A., T.D. Oberley, D.B. Allison, L.I. Szweda, and R. Weindruch (2000). Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. Faseb J. **14:** 1825-36.
257. Armeni, T., C. Pieri, M. Marra, F. Saccucci, and G. Principato (1998). Studies on the life prolonging effect of food restriction: glutathione levels and glyoxalase enzymes in rat liver. Mech Ageing Dev. **101:** 101-10.
258. Lewis, S.E., D.F. Goldspink, J.G. Phillips, B.J. Merry, and A.M. Holehan (1985). The effects of aging and chronic dietary restriction on whole body growth and protein turnover in the rat. Exp Gerontol. **20:** 253-63.
259. Guo, Z., A. Heydari, and A. Richardson (1998). Nucleotide excision repair of actively transcribed versus nontranscribed DNA in rat hepatocytes: effect of age and dietary restriction. Exp Cell Res. **245:** 228-38.
260. Keenan, K.P., G.C. Ballam, R. Dixit, K.A. Soper, P. Laroque, B.A. Mattson, S.P. Adams, and J.B. Coleman (1997). The effects of diet, overfeeding and moderate dietary restriction on Sprague-Dawley rat survival, disease and toxicology. J Nutr. **127:** 851S-856S.
261. Berg, T.F., P.J. Breen, R.J. Feuers, E.T. Oriaku, F.X. Chen, and R.W. Hart (1994). Acute toxicity of ganciclovir: effect of dietary restriction and chronobiology. Food Chem Toxicol. **32:** 45-50.
262. D'Costa, A.P., J.E. Lenham, R.L. Ingram, and W.E. Sonntag (1993). Moderate caloric restriction increases type 1 IGF receptors and protein synthesis in aging rats. Mech Ageing Dev. **71:** 59-71.
263. Saltiel, A.R. and C.R. Kahn (2001). Insulin signalling and the regulation of glucose and lipid metabolism. Nature. **414:** 799-806.
264. Nelson, D.W. and R.W. Padgett (2003). Insulin worms its way into the spotlight. Genes Dev. **17:** 813-8.

265. Holzenberger, M., J. Dupont, B. Ducos, P. Leneuve, A. Geloen, P.C. Even, P. Cervera, and Y. Le Bouc (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature. **421:** 182-7.
266. Kenyon, C., J. Chang, E. Gensch, A. Rudner, and R. Tabtiang (1993). A C. elegans mutant that lives twice as long as wild type. Nature. **366:** 461-4.
267. Larsen, P.L., P.S. Albert, and D.L. Riddle (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics. **139:** 1567-83.
268. Kimura, K.D., H.A. Tissenbaum, Y. Liu, and G. Ruvkun (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science. **277:** 942-6.
269. Dillin, A., D.K. Crawford, and C. Kenyon (2002). Timing requirements for insulin/IGF-1 signaling in C. elegans. Science. **298:** 830-4.
270. Guarente, L. and C. Kenyon (2000). Genetic pathways that regulate ageing in model organisms. Nature. **408:** 255-62.
271. Gems, D. and L. Partridge (2001). Insulin/IGF signalling and ageing: seeing the bigger picture. Curr Opin Genet Dev. **11:** 287-92.
272. Wolkow, C.A., K.D. Kimura, M.S. Lee, and G. Ruvkun (2000). Regulation of C. elegans life-span by insulinlike signaling in the nervous system. Science. **290:** 147-50.
273. Tissenbaum, H.A. and G. Ruvkun (1998). An insulin-like signaling pathway affects both longevity and reproduction in Caenorhabditis elegans. Genetics. **148:** 703-17.
274. Lin, K., J.B. Dorman, A. Rodan, and C. Kenyon (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science. **278:** 1319-22.
275. Tatar, M., A. Bartke, and A. Antebi (2003). The endocrine regulation of aging by insulin-like signals. Science. **299:** 1346-51.
276. Ogg, S., S. Paradis, S. Gottlieb, G.I. Patterson, L. Lee, H.A. Tissenbaum, and G. Ruvkun (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature. **389:** 994-9.
277. Apfeld, J. and C. Kenyon (1998). Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. Cell. **95:** 199-210.
278. Larsen, P.L. (1993). Aging and resistance to oxidative damage in Caenorhabditis elegans. Proc Natl Acad Sci U S A. **90:** 8905-9.
279. Lithgow, G.J., T.M. White, D.A. Hinerfeld, and T.E. Johnson (1994). Thermotolerance of a long-lived mutant of Caenorhabditis elegans. J Gerontol. **49:** B270-6.
280. Murphy, C.T., S.A. McCarroll, C.I. Bargmann, A. Fraser, R.S. Kamath, J. Ahringer, H. Li, and C. Kenyon (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature. **424:** 277-83.
281. Davis, W.L., D.B. Goodman, L.A. Crawford, O.J. Cooper, and J.L. Matthews (1990). Hibernation activates glyoxylate cycle and gluconeogenesis in black bear brown adipose tissue. Biochim Biophys Acta. **1051:** 276-8.
282. Wang, J. and S.K. Kim (2003). Global analysis of dauer gene expression in Caenorhabditis elegans. Development. **130:** 1621-34.
283. Kirkwood, T.B. (2005). Understanding the odd science of aging. Cell. **120:** 437-47.
284. Vellai, T., K. Takacs-Vellai, Y. Zhang, A.L. Kovacs, L. Orosz, and F. Muller (2003). Genetics: influence of TOR kinase on lifespan in C. elegans. Nature. **426:** 620.
285. Kapahi, P., B.M. Zid, T. Harper, D. Koslover, V. Sapin, and S. Benzer (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. **14:** 885-90.
286. Kaeberlein, M., R.W. Powers, 3rd, K.K. Steffen, E.A. Westman, D. Hu, N. Dang, E.O. Kerr, K.T. Kirkland, S. Fields, and B.K. Kennedy (2005). Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science. **310:** 1193-6.
287. Blagosklonny, M.V. (2007). Paradoxes of aging. Cell Cycle. **6:** 2997-3003.

288. Tebbs, R.S., M.L. Flannery, J.J. Meneses, A. Hartmann, J.D. Tucker, L.H. Thompson, J.E. Cleaver, and R.A. Pedersen (1999). Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. Dev Biol. **208:** 513-29.
289. Cabelof, D.C. (2007). Aging and base excision repair: in need of a comprehensive approach. DNA Repair (Amst). **6:** 1399-402.
290. Cabelof, D.C., J.J. Raffoul, J. Nakamura, D. Kapoor, H. Abdalla, and A.R. Heydari (2004). Imbalanced base excision repair in response to folate deficiency is accelerated by polymerase beta haploinsufficiency. J Biol Chem. **279:** 36504-13.
291. Pascucci, B., M.T. Russo, M. Crescenzi, M. Bignami, and E. Dogliotti (2005). The accumulation of MMS-induced single strand breaks in G1 phase is recombinogenic in DNA polymerase beta defective mammalian cells. Nucleic Acids Res. **33:** 280-8.
292. Sobol, R.W., M. Kartalou, K.H. Almeida, D.F. Joyce, B.P. Engelward, J.K. Horton, R. Prasad, L.D. Samson, and S.H. Wilson (2003). Base excision repair intermediates induce p53-independent cytotoxic and genotoxic responses. J Biol Chem. **278:** 39951-9.
293. Trivedi, R.N., K.H. Almeida, J.L. Fornsaglio, S. Schamus, and R.W. Sobol (2005). The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. Cancer Res. **65:** 6394-400.
294. Hasty, P., J. Campisi, J. Hoeijmakers, H. van Steeg, and J. Vijg (2003). Aging and genome maintenance: lessons from the mouse? Science. **299:** 1355-9.
295. de Boer, J., J.O. Andressoo, J. de Wit, J. Huijmans, R.B. Beems, H. van Steeg, G. Weeda, G.T. van der Horst, W. van Leeuwen, A.P. Themmen, M. Meradji, and J.H. Hoeijmakers (2002). Premature aging in mice deficient in DNA repair and transcription. Science. **296:** 1276-9.
296. de Vries, A., C.T. van Oostrom, F.M. Hofhuis, P.M. Dortant, R.J. Berg, F.R. de Gruijl, P.W. Wester, C.F. van Kreijl, P.J. Capel, H. van Steeg, and et al. (1995). Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. Nature. **377:** 169-73.
297. Murai, M., Y. Enokido, N. Inamura, M. Yoshino, Y. Nakatsu, G.T. van der Horst, J.H. Hoeijmakers, K. Tanaka, and H. Hatanaka (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.
298. Kipling, D., T. Davis, E.L. Ostler, and R.G. Faragher (2004). What can progeroid syndromes tell us about human aging? Science. **305:** 1426-31.
299. 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.
300. Tsodikov, O.V., J.H. Enzlin, O.D. Scharer, and T. Ellenberger (2005). Crystal structure and DNA binding functions of ERCC1, a subunit of the DNA structure-specific endonuclease XPF-ERCC1. Proc Natl Acad Sci U S A. **102:** 11236-41.
301. McWhir, J., J. Selfridge, D.J. Harrison, S. Squires, and D.W. Melton (1993). Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. Nat Genet. **5:** 217-24.
302. Weeda, G., I. Donker, J. de Wit, H. Morreau, R. Janssens, C.J. Vissers, A. Nigg, H. van Steeg, D. Bootsma, and J.H. Hoeijmakers (1997). Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. Curr Biol. **7:** 427-39.
303. Lombardi, G., C. Di Somma, F. Rota, and A. Colao (2005). Associated hormonal decline in aging: is there a role for GH therapy in aging men? J Endocrinol Invest. **28:** 99-108.
304. Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. Cell. **120:** 449-60.
305. Garinis, G.A., L.M. Uittenboogaard, H. Stachelscheid, M. Fousteri, W. van Ijcken, T.M. Breit, H. van Steeg, L.H. Mullenders, G.T. van der Horst, J.C. Bruning, C.M. Niessen, J.H.

Hoeijmakers, and B. Schumacher (2009). Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. Nat Cell Biol. **11:** 604-15.
306. Garinis, G.A., J.R. Mitchell, M.J. Moorhouse, K. Hanada, H. de Waard, D. Vandeputte, J. Jans, K. Brand, M. Smid, P.J. van der Spek, J.H. Hoeijmakers, R. Kanaar, and G.T. van der Horst (2005). Transcriptome analysis reveals cyclobutane pyrimidine dimers as a major source of UV-induced DNA breaks. Embo J. **24:** 3952-62.
307. You, Y.H., D.H. Lee, J.H. Yoon, S. Nakajima, A. Yasui, and G.P. Pfeifer (2001). Cyclobutane pyrimidine dimers are responsible for the vast majority of mutations induced by UVB irradiation in mammalian cells. J Biol Chem. **276:** 44688-94.
308. Mellon, I., G. Spivak, and P.C. Hanawalt (1987). Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell. **51:** 241-9.
309. Bohr, V.A., C.A. Smith, D.S. Okumoto, and P.C. Hanawalt (1985). DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell. **40:** 359-69.
310. Jaspers, N.G., A. Raams, M.J. Kelner, J.M. Ng, Y.M. Yamashita, S. Takeda, T.C. McMorris, and J.H. Hoeijmakers (2002). Anti-tumour compounds illudin S and Irofulven induce DNA lesions ignored by global repair and exclusively processed by transcription- and replication-coupled repair pathways. DNA Repair (Amst). **1:** 1027-38.
311. Schena, M., D. Shalon, R.W. Davis, and P.O. Brown (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. **270:** 467-70.
312. Fodor, S.P., J.L. Read, M.C. Pirrung, L. Stryer, A.T. Lu, and D. Solas (1991). Light-directed, spatially addressable parallel chemical synthesis. Science. **251:** 767-73.
313. Lipshutz, R.J., S.P. Fodor, T.R. Gingeras, and D.J. Lockhart (1999). High density synthetic oligonucleotide arrays. Nat Genet. **21:** 20-4.
314. Lockhart, D.J., H. Dong, M.C. Byrne, M.T. Follettie, M.V. Gallo, M.S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton, and E.L. Brown (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol. **14:** 1675-80.
315. Junker, B.H. and F. Schreiber, Eds. (2008). Analysis of Biological networks. Hoboken, New Jersey, John Wiley & Sons.
316. Bhalla, U.S. and R. Iyengar (1999). Emergent properties of networks of biological signaling pathways. Science. **283:** 381-7.
317. Katagiri, F. (2003). Attacking complex problems with the power of systems biology. Plant Physiol. **132:** 417-9.
318. GOLD (2010). GOLD - genomes online database http://www.genomesonline.org.
319. Ma'ayan, A. (2009). Insights into the organization of biochemical regulatory networks using graph theory analyses. J Biol Chem. **284:** 5451-5.
320. Lee, T.I., N.J. Rinaldi, F. Robert, D.T. Odom, Z. Bar-Joseph, G.K. Gerber, N.M. Hannett, C.T. Harbison, C.M. Thompson, I. Simon, J. Zeitlinger, E.G. Jennings, H.L. Murray, D.B. Gordon, B. Ren, J.J. Wyrick, J.B. Tagne, T.L. Volkert, E. Fraenkel, D.K. Gifford, and R.A. Young (2002). Transcriptional regulatory networks in Saccharomyces cerevisiae. Science. **298:** 799-804.
321. Eungdamrong, N.J. and R. Iyengar (2004). Computational approaches for modeling regulatory cellular networks. Trends Cell Biol. **14:** 661-9.
322. Watts, D.J. and S.H. Strogatz (1998). Collective dynamics of 'small-world' networks. Nature. **393:** 440-2.
323. Milgram, S. (1967). The small-world problem. Psychology Today. **1:** 61-67.
324. Barabasi, A.L. and R. Albert (1999). Emergence of scaling in random networks. Science. **286:** 509-12.
325. Albert, R., H. Jeong, and A.L. Barabasi (2000). Error and attack tolerance of complex networks. Nature. **406:** 378-82.
326. Alexeyenko, A. and E.L. Sonnhammer (2009). Global networks of functional coupling in eukaryotes from comprehensive data integration. Genome Res. **19:** 1107-16.

327. Ashburner, M., C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig, M.A. Harris, D.P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J.C. Matese, J.E. Richardson, M. Ringwald, G.M. Rubin, and G. Sherlock (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. **25:** 25-9.
328. Shannon, P., A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. **13:** 2498-504.
329. Maere, S., K. Heymans, and M. Kuiper (2005). BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics. **21:** 3448-9.
330. 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.
331. Finkel, T. (2003). Oxidant signals and oxidative stress. Curr Opin Cell Biol. **15:** 247-54.
332. Aguirre, J., M. Rios-Momberg, D. Hewitt, and W. Hansberg (2005). Reactive oxygen species and development in microbial eukaryotes. Trends Microbiol. **13:** 111-8.
333. Livingston, A.L., V.L. O'Shea, T. Kim, E.T. Kool, and S.S. David (2008). Unnatural substrates reveal the importance of 8-oxoguanine for in vivo mismatch repair by MutY. Nat Chem Biol. **4:** 51-8.
334. Parker, J.B., M.A. Bianchet, D.J. Krosky, J.I. Friedman, L.M. Amzel, and J.T. Stivers (2007). Enzymatic capture of an extrahelical thymine in the search for uracil in DNA. Nature. **449:** 433-7.
335. Demple, B. and L. Harrison (1994). Repair of oxidative damage to DNA: enzymology and biology. Annu Rev Biochem. **63:** 915-48.
336. Boon, E.M., A.L. Livingston, N.H. Chmiel, S.S. David, and J.K. Barton (2003). DNA-mediated charge transport for DNA repair. Proc Natl Acad Sci U S A. **100:** 12543-7.
337. Boal, A.K. and J.K. Barton (2005). Electrochemical detection of lesions in DNA. Bioconjug Chem. **16:** 312-21.
338. Cunningham, R.P., H. Asahara, J.F. Bank, C.P. Scholes, J.C. Salerno, K. Surerus, E. Munck, J. McCracken, J. Peisach, and M.H. Emptage (1989). Endonuclease III is an iron-sulfur protein. Biochemistry. **28:** 4450-5.
339. Gorodetsky, A.A., A.K. Boal, and J.K. Barton (2006). Direct electrochemistry of endonuclease III in the presence and absence of DNA. J Am Chem Soc. **128:** 12082-3.
340. Boal, A.K., E. Yavin, and J.K. Barton (2007). DNA repair glycosylases with a [4Fe-4S] cluster: a redox cofactor for DNA-mediated charge transport? J Inorg Biochem. **101:** 1913-21.
341. Boal, A.K., J.C. Genereux, P.A. Sontz, J.A. Gralnick, D.K. Newman, and J.K. Barton (2009). Redox signaling between DNA repair proteins for efficient lesion detection. Proc Natl Acad Sci U S A. **106:** 15237-42.
342. Ntzani, E.E. and J.P. Ioannidis (2003). Predictive ability of DNA microarrays for cancer outcomes and correlates: an empirical assessment. Lancet. **362:** 1439-44.
343. Klebanov, L. and A. Yakovlev (2007). How high is the level of technical noise in microarray data? Biol Direct. **2:** 9.
344. Jafari, P. and F. Azuaje (2006). An assessment of recently published gene expression data analyses: reporting experimental design and statistical factors. BMC Med Inform Decis Mak. **6:** 27.
345. Dupuy, A. and R.M. Simon (2007). Critical review of published microarray studies for cancer outcome and guidelines on statistical analysis and reporting. J Natl Cancer Inst. **99:** 147-57.
346. O'Rourke, D., D. Baban, M. Demidova, R. Mott, and J. Hodgkin (2006). Genomic clusters, putative pathogen recognition molecules, and antimicrobial genes are induced by infection of C. elegans with M. nematophilum. Genome Res. **16:** 1005-16.

347. Shapira, M., B.J. Hamlin, J. Rong, K. Chen, M. Ronen, and M.W. Tan (2006). A conserved role for a GATA transcription factor in regulating epithelial innate immune responses. Proc Natl Acad Sci U S A. **103:** 14086-91.
348. Huffman, D.L., L. Abrami, R. Sasik, J. Corbeil, F.G. van der Goot, and R.V. Aroian (2004). Mitogen-activated protein kinase pathways defend against bacterial pore-forming toxins. Proc Natl Acad Sci U S A. **101:** 10995-1000.
349. Schulenburg, H., M.P. Hoeppner, J. Weiner, 3rd, and E. Bornberg-Bauer (2008). Specificity of the innate immune system and diversity of C-type lectin domain (CTLD) proteins in the nematode Caenorhabditis elegans. Immunobiology. **213:** 237-50.
350. Adelman, K., M.A. Kennedy, S. Nechaev, D.A. Gilchrist, G.W. Muse, Y. Chinenov, and I. Rogatsky (2009). Immediate mediators of the inflammatory response are poised for gene activation through RNA polymerase II stalling. Proc Natl Acad Sci U S A. **106:** 18207-12.
351. Lu, S., J. Li, C. Song, K. Shen, and G.C. Tseng (2009). Biomarker detection in the integration of multiple multi-class genomic studies. Bioinformatics. **26:** 333-40.
352. Lee, S.J., C.T. Murphy, and C. Kenyon (2009). Glucose shortens the life span of C. elegans by downregulating DAF-16/FOXO activity and aquaporin gene expression. Cell Metab. **10:** 379-91.
353. Abate, J.P. and T.K. Blackwell (2009). Life is short, if sweet. Cell Metab. **10:** 338-9.
354. Friedberg, E.C. and L.B. Meira (2006). Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage Version 7. DNA Repair (Amst). **5:** 189-209.
355. Niedernhofer, L.J. (2008). Tissue-specific accelerated aging in nucleotide excision repair deficiency. Mech Ageing Dev. **129:** 408-15.

I

II

Research Paper

A Two-tiered compensatory response to loss of DNA repair modulates aging and stress response pathways

Øyvind Fensgård[1], Henok Kassahun[1], Izabela Bombik[1], Torbjørn Rognes[2], Jessica Margareta Lindvall[2] and Hilde Nilsen[1]

[1] University of Oslo, The Biotechnology Centre, P.O. Box 1125 Blindern, 0317 Oslo, Norway.
[2] University of Oslo, Department of Informatics, P.O. Box 1080 Blindern, NO-0316 Oslo, Norway

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Correspondence: *Hilde Nilsen, PhD, University of Oslo, The Biotechnology Centre, PO Box 1125 Blindern, 0317 Oslo, Norway*
Received: *03/10/10; accepted: 03/27/10; published on line: 03/29/10*
E-mail: *hilde.nilsen@biotek.uio.no*

Abstract: Activation of oxidative stress-responses and downregulation of insulin-like signaling (ILS) is seen in Nucleotide Excision Repair (NER) deficient segmental progeroid mice. Evidence suggests that this is a survival response to persistent transcription-blocking DNA damage, although the relevant lesions have not been identified. Here we show that loss of NTH-1, the only Base Excision Repair (BER) enzyme known to initiate repair of oxidative DNA damage in *C. elegans*, restores normal lifespan of the short-lived NER deficient *xpa-1* mutant. Loss of NTH-1 leads to oxidative stress and global expression profile changes that involve upregulation of genes responding to endogenous stress and downregulation of ILS. A similar, but more extensive, transcriptomic shift is observed in the *xpa-1* mutant whereas loss of both NTH-1 and XPA-1 elicits a different profile with downregulation of Aurora-B and Polo-like kinase 1 signaling networks as well as DNA repair and DNA damage response genes. The restoration of normal lifespan and absence oxidative stress responses in *nth-1;xpa-1* indicate that BER contributes to generate transcription blocking lesions from oxidative DNA damage. Hence, our data strongly suggests that the DNA lesions relevant for aging are repair intermediates resulting from aberrant or attempted processing by BER of lesions normally repaired by NER.

INTRODUCTION

The Base excision repair (BER) pathway is the main mechanism for removal of endogenously generated DNA base damage [1]. BER is initiated by DNA glycosylases that recognise and excise groups of related lesions [2]. There are at least 12 different mammalian DNA glycosylases, of which at least 7 have overlapping specificities towards oxidative DNA damage [3, 4]. *Caenorhabditis elegans* (*C. elegans*) is a multicellular animal that encodes only two DNA-glycosylases: UNG-1 [5, 6] and NTH-1 [7]. *C. elegans* is therefore an attractive system in which to study consequences of BER-deficiency in animals. Furthermore, the strong genetic and mechanistic correlation between stress resistance and longevity in *C. elegans* [8], allows us to probe the contribution of DNA damage, in particular oxidative DNA damage, and its repair to phenotypes associated with oxidative stress in large populations over the entire lifespan.

C. elegans NTH-1, a homolog of *E. coli nth*, was recently shown to have activity against oxidized pyrimidines [7]. A deletion mutant lacking exons 2 through 4, *nth-1(ok724),* is expected to be a null mutant and has elevated mutant rate [9] but no hypersensitivity to oxidizing agents [7]. The absence of a DNA-glycosylase with specificity towards oxidized purines in

C. elegans is puzzling. Although *C. elegans* NTH-1 appears to have a weak ability to excise one of the major purine oxidation products (8-hydroxyguanine) [7], it seems likely that other DNA repair pathways such as Nucleotide Excision Repair (NER) might contribute to repair of oxidised purines in *C. elegans* as has been shown *in vitro* [10] and in *vivo* in *Saccharomyces cerevisiae* [11]. Genetic studies in *S. cerevisiae* show that NER is the preferred repair pathway for oxidative DNA damage in the absence of BER [12]. The NER pathway is highly conserved and orthologs of the core NER proteins are present in *C. elegans* [13]. XPA is required for formation of the preincision complex [14]. *C. elegans xpa-1* mutants are UV-sensitive [15, 16] and the *xpa-1 (ok698)* mutant has reduced capacity to repair UV-induced DNA damage [13, 17].

Expression profiling in NER-defective mice has revealed gene expression changes associated with segmental progeroid phenotypes [18-20]. For example, the NER-defective *Csbm/m/Xpa*$^{-/-}$ mice show suppression of signaling through the growth hormone (GH)/insulin growth factor 1 (IGF1) pathways and increased antioxidant responses. Similar changes could be induced in wild type mice through chronic administration of a reactive oxygen species (ROS) - inducing agent, suggesting that the transcriptional responses result from defects in transcription-coupled repair of oxidative DNA damage [21].

Although ROS are believed to be a main contributor to the stochastic endogenous DNA damage accumulating with increase age, and BER is the preferred pathway for repair of oxidative DNA damage, similar expression profiling has not been performed in BER defective animals. However, studies in *S. cerevisiae* suggest that mutants in BER as well as NER show global expression profile changes originating from unrepaired oxidative DNA damage after treatment with oxidizing agents [22, 23].

Mutants in DNA glycosylases generally show very mild phenotypes, which has been attributed to the existence of backup enzymes with overlapping substrate specificities. Here we show that compensatory transcriptional responses contribute to maintain wild type phenotypes including lifespan, in the presence of endogenous oxidative stress in DNA repair mutants.

RESULTS

The transcriptional signatures of mixed populations of wild type N2 as well as *nth-1*, *xpa-1*, and *nth-1;xpa-1* mutants were measured using Affymetrix GeneChip *C. elegans* Genome Arrays in well fed animals cultured on plates to avoid stressful growth conditions.

Oxidative stress response and reduced insulin/IGF-1 signaling in *nth-1(ok724)*

Since DNA damage responses often show small changes on the transcriptional level [24], we analysed the gene expression signatures using a fold-change cut-off criterion ≥1.8. We found a high number of differentially expressed transcripts between the N2 reference strain and the *nth-1* mutant considering the unstressed conditions of the animals: 2074 probe sets were differentially expressed ≥1.8 fold (Supplemental Table SI). The low number of transcripts regulated ≥4-fold (185 probe sets) suggests that there is a focused transcriptomic response to loss of the NTH-1 enzyme.

Gene ontology (GO) enrichment analysis revealed that genes involved in determining adult lifespan ($p < 0.007$) were enriched among the regulated genes in the *nth-1* mutant (Figure 1). Of these, 17 are known to act through the insulin/IGF-1 signaling (ILS) pathway. Reduced signaling through the canonical ILS pathway leads to nuclear localisation of the FOXO transcription factor DAF-16 [25]. A total of 84 genes previously identified as downstream targets of DAF-16 (*dod*) [26, 27], were differentially regulated in *nth-1*, of which 67 were not assigned to the aging cluster based on present GO annotation. However, some confirmed targets of DAF-16 (e.g. *hsf-1, hsp-90, hsp-70*) were not differentially regulated, and there was no significant overlap between our dataset and the previously reported *daf-16* dataset [27] (data not shown). Moreover *dao-6*, which is positively regulated by DAF-16 and negatively regulated by DAF-2, was downregulated by 7.7-fold. Thus, the transcriptional changes in the *nth-1* mutant appear not to be dominated by DAF-16. The downregulation of *ins-1* and *ins-7* (2.17 and 3-fold, respectively), two DAF-2 agonists whose expression are repressed by DAF-16, likely reflects negative feedback inhibition of ILS rather than sensory neuronal input to the ILS pathway.

Previous genetic and genomic studies have demonstrated that there is a close interconnection between the ILS and stress-response pathways in *C. elegans* [8, 28]. This is reflected in the *nth-1* dataset: The genes in the aging cluster, as well as individual genes regulated more than 4-fold (Supplemental Table SI), indicate that oxidative stress responses are activated. SOD-3 is a mitochondrial Mn-containing superoxide dismutase [29] and increased expression of *sod-3* has been reported in response to oxidative stress

[30]. *sod-3* is a target of DAF-16, and the well established inverse regulation between *ins-7* and *sod-3* [31] is observed in *nth-1* (-3 and 1.84-fold, respectively). Activation of an oxidative stress response in *nth-1* is further suggested by the upregulation of *gst-4* (2.31-fold), a regulator of SKN-1 which is a transcription factor mediating transcriptional responses to oxidative stress [32]. Regulation of steroid signaling and stress responses are also reflected in the second GO enriched cluster, proteolysis ($p < 0.01$) (Figure 1).

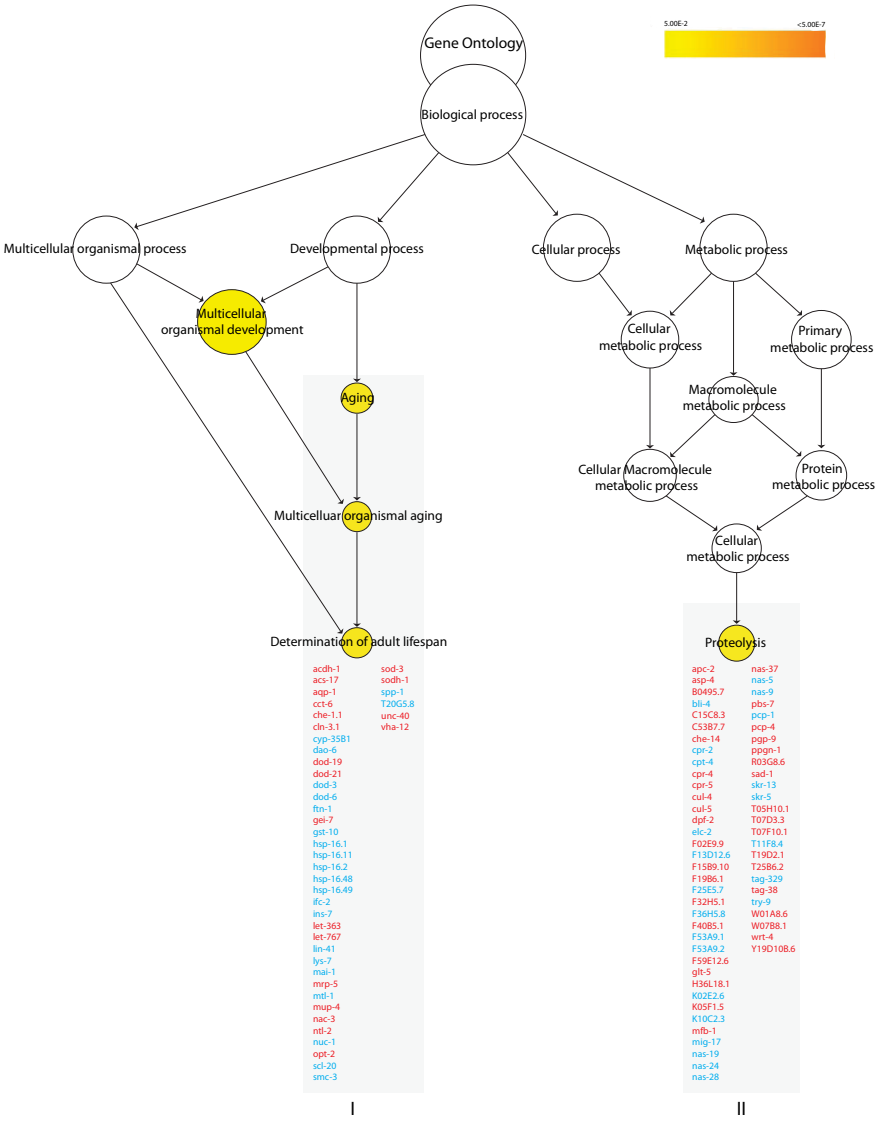

Figure 1. Overrepresented biological processes in N2 vs. *nth-1*. Enriched biological processes in *nth-1* vs. N2 are Aging *($p < 0.007$)* and Proteolysis *($p < 0.01$)*. The Aging cluster contains 17 genes involved in ILS signaling, including *ins-7* and *sod-3*. Genes responding to stress and steroid signaling are found in the Proteolysis cluster. Genes in red and blue are found to be upregulated and downregulated, respectively.

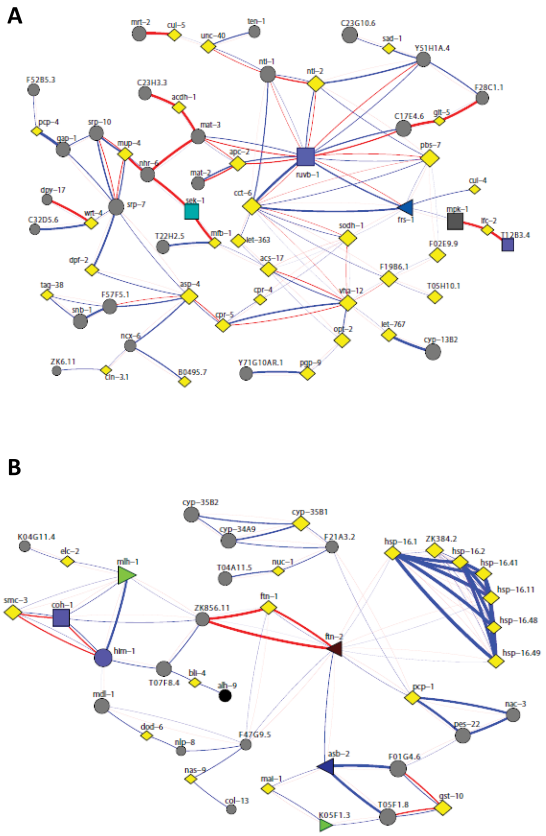

Figure 2. Network analysis revealed a close interconnection between the two enriched clusters in *nth-1*. (**A**) Functional interactions among upregulated genes in the two clusters was analysed using FunCoup [54]. A network of 97 most probable links between 95 genes was returned, involving 31 of the 58 regulated genes (12 and 19 from cluster I and II, respectively). (**B**) Network analysis of the 45 downregulated genes resulted in a network of 79 most probable links between 71 genes from both clusters.

A search for functional interactions among upregulated genes in the two clusters using the online functional interaction browser FunCoup revealed a close interconnection between the two enriched clusters involving 31 of the 58 regulated genes (12 and 19 from cluster I and II, respectively) (Figure 2A). The expression of the CeTOR (let-363) kinase is upregulated in *nth-1* (2.33-fold), possibly indicating activation of a survival response to stress. The TOR pathway controls protein homeostasis and contributes to longevity, and the network analysis indicates that TOR might connect the two clusters via the AAA+ ATPase homolog RUVB-1, a component of the TOR pathway [33]. Direct protein-protein interactions involving RUVB-1 have been demonstrated with several upregulated genes in both enriched GO clusters.

The protein-interaction network (Figure 2A) suggests that the transcriptional changes may also involve regulation of the redundant activities of the conserved p38 and JNK stress-activated protein kinase pathways: MFB-1, for example, directly interacts with SEK-1, a MAPK kinase required for germline stress-induced cell death independent of the CEP-1 (*C. elegans* p53) DNA damage response [34]. SEK-1 is also required for nuclear localisation of DAF-16 in response to oxidative stress [35]. It was suggested that oxidative stress mediates regulation of DAF-16 through activating the p38 signal transduction pathway upstream of DAF-16. Therefore, regulation of DAF-16 target genes in *nth-1* is consistent with activation of an oxidative stress response. Alternatively, the regulation of DAF-16 targets could be secondary to *aqp-1* upregulation. Aquaporin-1, a glycerol channel protein, was recently demonstrated to modulate expression of DAF-16-regulated genes and suggested to act as a feedback regulator in the ILS pathway [36]. There is a strong upregulation of *aqp-1* in *nth-1* (31-fold). Moreover, 6 out of 7 genes negatively regulated by AQP-1 are repressed in *nth-1* (Supplemental Table SII).

Network analysis of the 45 downregulated genes resulted in a network involving 71 genes from both clusters (Figure 2B). The pronounced downregulation of genes specifically responding to exogenous oxidative and heat stress (such as the *hsp-16* family, *ftn-1* and *gst-10*, *lys-7*, *mtl-1*) and anti-microbial immunity (several c-lectins, *cpr-2, ilys-3, abf-2, cnc-7*) in the *nth-1* mutant suggests that a specific response to endogenous stressors is triggered. Hence, loss of BER in *C. elegans* appears to induce transcriptional responses involving similar pathways as those regulated in mammalian NER mutants [19].

Shared transcriptional responses in *nth-1* and *xpa-1* mutants

To experimentally validate whether there is similarity between the transcriptional programs associated with loss of BER and NER capacity in *C. elegans*, we collected the expression profile of the *xpa-1(ok698)* mutant. In *xpa-1*, we identified 2815 differentially expressed transcripts having a fold-change of ≥1.8 (Supplemental Table SIII).

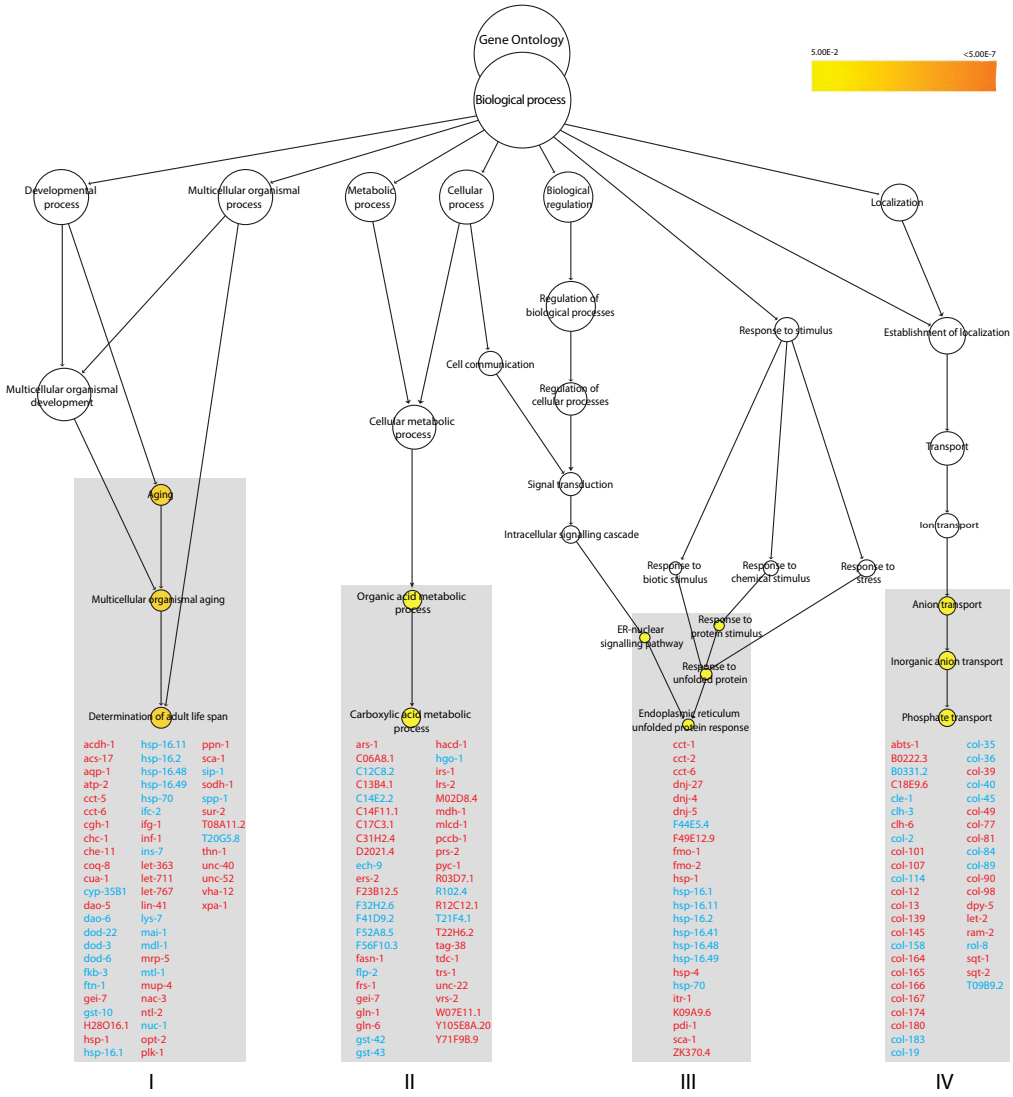

Figure 3. GO enrichment clusters in *xpa-1*. Genes that respond to oxidative stress and redox homeostasis are found in the enriched biological processes in *xpa-1* vs. N2. Aging *($p < 0.0007$)*, regulation of carboxylic acid metabolism ($p < 0.03$), ER unfolded protein response ($p < 0.05$) and phosphate transport ($p < 0.02$). Genes in red and blue are found to be upregulated and downregulated, respectively.

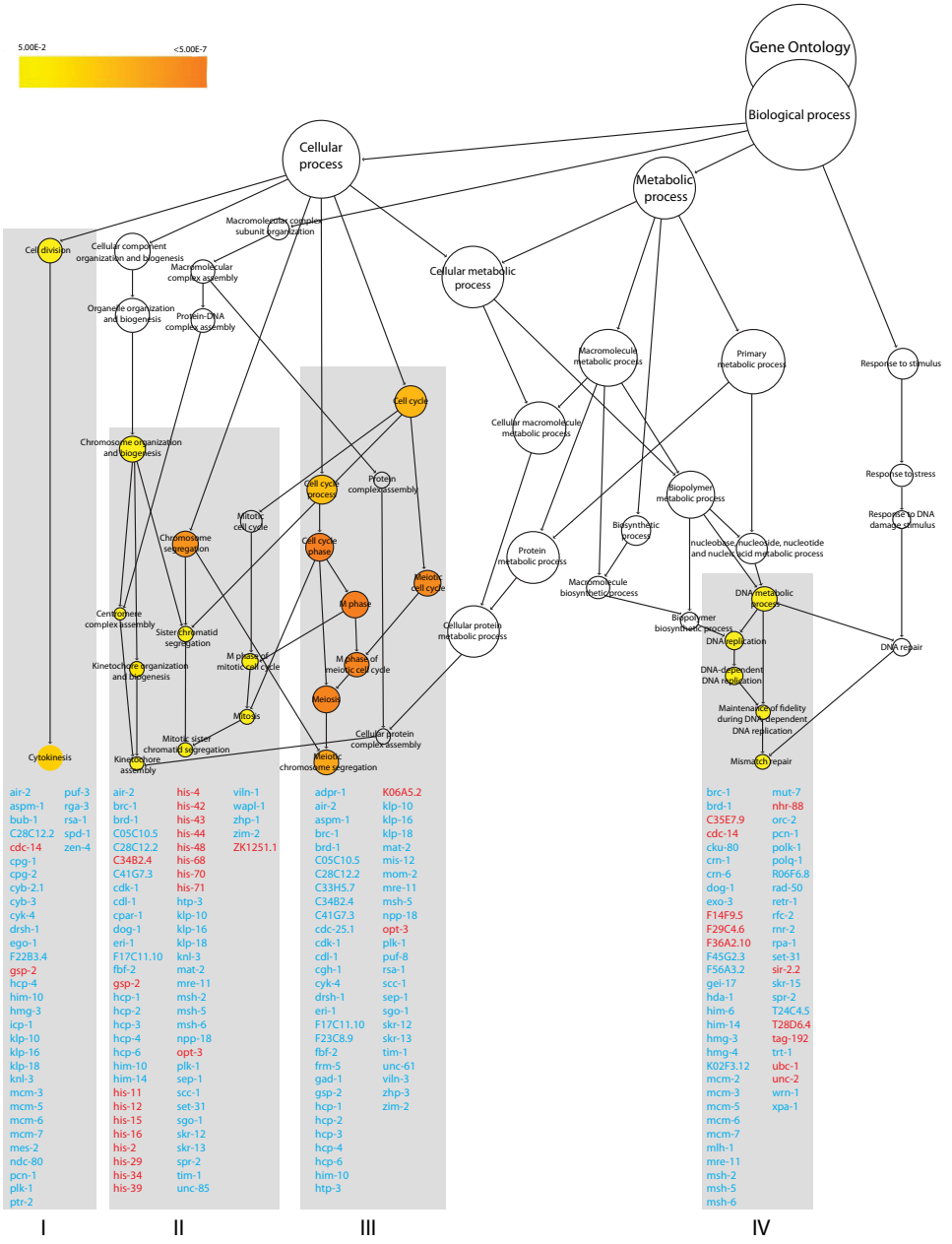

Figure 4. GO enrichment clusters in *nth-1;xpa-1*. The transcriptional response in the double mutant *nth-1;xpa-1* is dominated by genes involved in cell-cycle regulation (clusters I-III) and DNA repair (cluster IV). Cluster I (*p < 0.003*) and II (*p < 0.01*) contain genes that function in mitosis-related processes. Cluster III (*p < 0.00001*) reflect regulation of progression through meiosis. Genes involved in DNA repair and DNA damage checkpoint pathways in cluster IV *(p < 0.02)* are downregulated. Genes in red and blue are found to be upregulated and downregulated, respectively.

GO enrichment analysis revealed four significantly regulated clusters in *xpa-1* (Figure 3). The GO process determination of adult lifespan ($p < 0.0007$) was shared with *nth-1* (Figure 1), and 67% (28 out of 42) of the individual genes in this cluster in *nth-1* were shared with *xpa-1*. In *xpa-1*, genes that respond to oxidative stress and redox homeostasis are not only represented in the "aging" cluster, but are also found in the clusters containing genes involved in the ER unfolded protein response ($p < 0.05$) and regulation of carboxylic acid metabolism ($p < 0.03$). Network analysis of the 57 downregulated genes within the enriched clusters resulted in a network resembling that of nth-1 (Supplemental Figure S1). Thus, qualitatively similar responses were activated to compensate for the loss of NTH-1 or XPA-1. Regression analysis using the 1.8-fold-change data confirmed the similarity of the *nth-1* and expression profiles ($R_2 = 0.96$). However, there is stronger modulation of gene expression in *xpa-1* compared to *nth-1*, with an increased number of transcripts with higher fold-change; e.g. expression of *ins-7* (8.8-and 3-fold), *aqp-1* (34.8- and 31-fold, respectively), and *hsp-16.49* (-15.99 and -5.58- fold) in *xpa-1* and *nth-1*, respectively (Supplemental Tables SI and SIII).

Somatic preservation in *nth-1;xpa-1*

Genes regulating adult lifespan were not among the four enriched GO processes identified from the 2787 regulated probe sets with fold-change ≥1.8 (1225 up and 1562 down) in *nth-1;xpa-1* (Figure 4 and Supplemental Table SIV). Instead, the transcriptional response was dominated by genes involved in cell-cycle regulation (clusters I-III) and DNA repair (cluster IV). Cluster I ($p < 0.003$) and II ($p < 0.01$) contain genes that function in mitosis-related processes such as chromosome segregation, mitotic spindle assembly and stability, and replication licensing. Only 2 out of 36 genes in cluster I are upregulated. In contrast, 15 of the 64 genes present in cluster II are upregulated and most encode histone genes. Cluster III ($p < 0.00001$) share many genes with cluster I and II but reflect regulation of progression through meiosis.

Genes involved in DNA repair and DNA damage checkpoint pathways are enriched in Cluster IV ($p < 0.02$). Naively, it could be expected that the double mutant would compensate for loss of integrity of two DNA repair pathways by upregulating alternative DNA repair modes. However, the opposite seems to be the case. Several mismatch repair, homologous recombination (HR), non-homologous end-joining (NHEJ), and DNA damage checkpoint genes, such as the *C. elegans* homolog of BRCA1 (*brc-1*) and its associated proteins, *brd-1* and *dog-1*, are downregulated (Table 1). Many uncharacterized genes that have previously been identified in screens for genes that result in mutator phenotypes when depleted by RNAi [37, 38] were also suppressed in the *nth-1;xpa-1* mutant. Network analyses illustrate close interrelation of clusters I through IV also on protein level returning protein-protein interactions between 157 of the 212 genes in all clusters (data not shown).

Suppression of the Aurora-B kinase and Polo-like kinase 1 regulatory network in *nth-1;xpa-1*

The GO analysis suggests that the double mutant differs from either single mutant. Linear regression analysis comparing the overlapping transcripts in the ≥1.8-fold-change lists from *nth-1;xpa-1* and *xpa-1* confirmed this difference (R2 = 0.12) whereas the single mutants show significantly stronger correlation ($R_2 = 0.94$). Principal Component Analysis (PCA) on the entire dataset (Figure 5A) confirms that the overall expression profiles of the single mutants cluster together and therefore resemble each other but, although *nth-1;xpa-1* clusters separately from the wild type, it seems to be in closer proximity to it than to either single mutant. Hierarchical clustering confirmed the closer relationship between *nth-1;xpa-1* and the wild type (Supplemental Figure S2). Hierarchical clustering of the mutants only revealed even more clearly that the single mutants are more similar to each other than either are to the double mutant. Several transcripts have opposite regulation, most notably in *xpa-1* and *nth-1;xpa-1* (Figure 5B). DNA repair and DNA damage response genes are prominent among the genes regulated in an opposite direction (a selection is presented in Table 1).

Polo-like kinase 1 (PLK-1), which is upregulated in *xpa-1* (1.97-fold) but repressed in *nth-1;xpa-1* (-2.11-fold), has emerged as an important modulator of DNA damage checkpoints [39, 40]. Moreover, Aurora B kinase (*air-2*) is downregulated (-1.83-fold), and an inhibitor of AIR-2 activation, gsp-2, is one of the few upregulated genes in *nth-1;xpa-1*. Several other components of AIR-2 and PLK-1 networks are represented in the enriched GO clusters in the double mutant (Figure 4). Moreover, the transcriptional changes observed in *nth-1;xpa-1* involved several genes that are validated interactors of AIR-2 and PLK-1. The direction of the expression changes suggests that there is a concerted response that suppresses AIR-2 and PLK-1 signaling networks in the double mutant (Figure 6) that are consistent with published literature evidence: Plk-1 stimulates the activation of Cdk-1, several cyclin

B proteins and a G2/M specific cyclin A through Cdc-25.1 [40]. The inner centromere protein (INCENP), ICP-1, coordinates cytokinesis and mitotic processes in the cell and integrates the PLK-1 and AIR-2 signaling at kinetochores. AIR-2 and PLK-1 regulate mitosis and cytokinesis through CYK-4 and ZEN-4 [41]. Downregulation of MCM2-7 could prevent firing of dormant replication origins which are often used when the transcriptional machinery is blocked or otherwise impaired [42]. Hence, the suppression of DNA metabolism suggested by the transcriptional signature reflects a concerted response. In summary, there seems to be a two-tiered compensatory response to loss of DNA repair in *C. elegans*: While lack of either BER or NER results in activation of genes responding to endogenous stressors and suppression of ILS, lack of both BER and NER shifts the transcriptional response to reduction of proliferation and somatic preservation through modulation of AIR-2 and PLK-1 signaling networks.

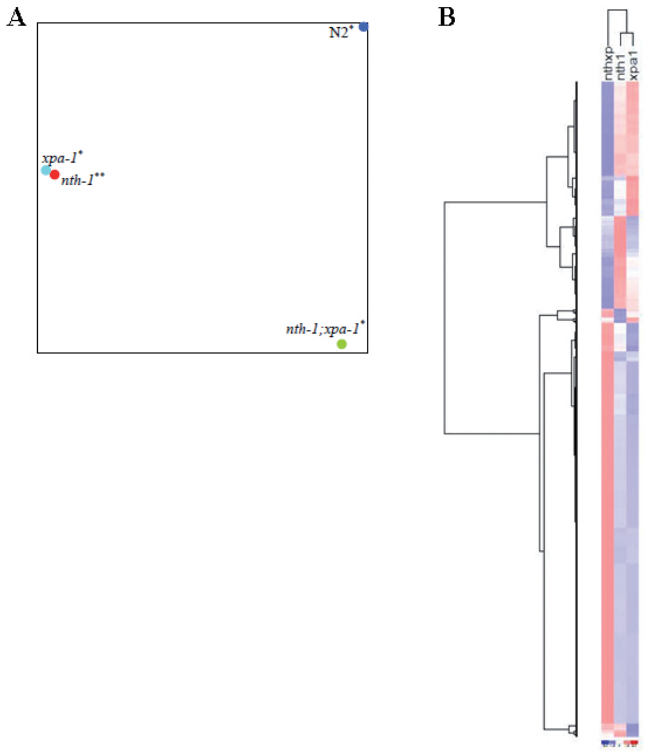

Figure 5. Comparative analyses of transcriptomes in DNA repair mutants. (**A**) The distance between respective mutants denoting the similarities or dissimilarities between *nth-1* (red circle), *xpa-1* (light blue circle), *nth-1;xpa-1* (green circle) and wild-type (blue circle) is shown using PCA. (**B**) Separation of the different mutant sample groups using Hierachical clustering.

Table 1. Regulation of DNA repair and DNA damage response genes in DNA repair mutants*

pathway		Gene[#]	Fold-change[+]		
			nth-1	*xpa-1*	*nth-1;xpa-1*
DNA repair	BER/NER	*exo-3*			-2,38
	MMR	*exo-1*			-2,03
		mlh-1		2,83	-2,23
		msh-2		1,83	-1,94
		msh-6		2,33	-1,9
	HR	*dna-2*		1,85	-2,43
		rad-50			-1,85
	NHEJ	*mre-11*			-1,94
		cku-80			-2,1
	DSB	*cnb-1*			2,19
		crn-1			-1,93
		polk-1			-2,21
		polq-1		2,45	-2,61
	Helicases	*dog-1*		1,89	-1,91
		him-6		2,95	-2,15
		wrn-1			-1,97
	Other	*rpa-1*			-2,03
		pcn-1			-2,24
		dpl-1		2,06	-2,01
		rfc-2			-1,97
DNA Damage Response/Cell Cycle		*air-2*		2,09	-1,9
		ani-2		1,82	-2,21
		brc-1			-1,85
		brd-1			-1,99
		C16C8.14		1,94	2,16
		cdc-14			-2,16
		cdc-25.1			-1,88
		gst-5			-2,26
		hil-1		-2,08	2,47
		hsr-9		1,83	-1,98
		K08F4.2			-2,19
		lin-35			-1,9
		mdf-1			-1,96
		pme-5	1,96	2,69	-1,95

*Gene classifications were determined based on previous analyses in references [54] and from information presented in Wormbase (www.wormbase.org)

[#] A selection of DNA repair and DNA damage response genes regulated in *nth-1;xpa-1*

[+] Fold-changes calculated from the comparative analyses presented in Supplemental Tables SI, SIII and SIV

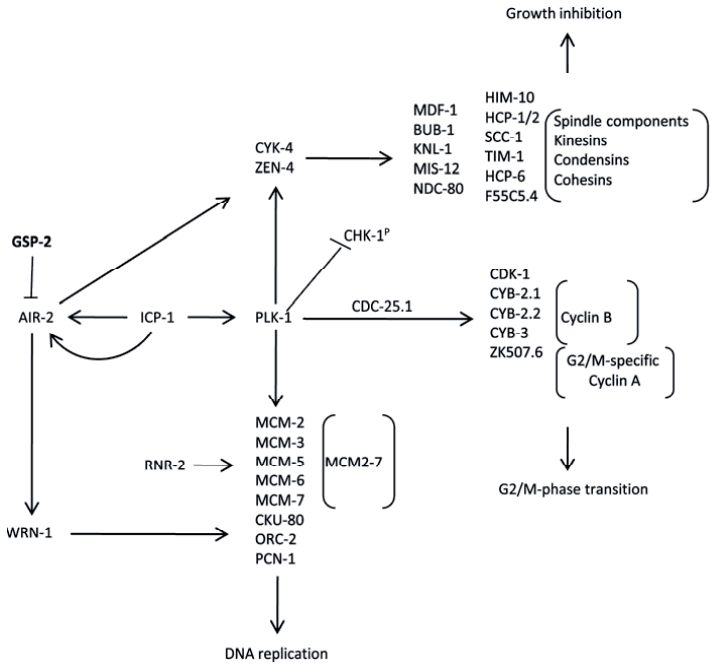

Figure 6. Somatic preservation through modulation of AIR-2 and PLK-1 signaling networks in *nth-1;xpa-1*. Genes encoding proteins known to stimulate AIR-2 and PLK-1 signaling are downregulated in *nth-1;xpa-1*: Plk-1 is known to stimulate activation of CDK-1, several cyclin B proteins and a G2/M specific cyclin A through CDC-25.1. Furthermore, PLK-1 and AIR-2 signaling coordinates cytokinesis and mitotic signaling at kinetochores via in the inner centromere ICP-1, regulates mitosis and cytokinesis through CYK-4 and ZEN-4, and could prevent firing of dormant replication origins via downregulation of MCM2-7. An inhibitor of AIR-2 activation, GSP-2, is one of the few upregulated genes.

Depletion of NTH-1 and XPA-1 induces oxidative stress responses

Transcriptomic profiling strongly indicates that the *nth-1* and *xpa-1* mutants experience oxidative stress. To experimentally validate whether loss of NTH-1 and XPA-1 induces oxidative stress, we took advantage of the established reporter strain CL2166, which expresses green fluorescent protein (GFP) under the control of the glutathione-S-transferase GST-4 promoter [32]. *gst-4* expression is upregulated in both *nth-1* and in *xpa-1* (2.31, and 2.01-fold respectively). Whereas GFP is normally expressed in hypodermal muscle, GFP-fluorescence increases in the body wall muscles and translocates to the intestinal nuclei upon oxidative stress

As expected, paraquat, which generates superoxide *in vivo*, increases the average number of GFP positive intestinal nuclei up to 46 compared to 15 in untreated animals ($p < 0.001$). Depletion of NTH-1 or XPA-1 by RNAi significantly increased the number of foci to 26 and 25, respectively ($p < 0.001$) (Figure 7), thus demonstrating that even transient depletion of NTH-1 or XPA-1 induces oxidative stress responses. Co-depletion of NTH-1 and XPA-1 did not increase the number of intestinal GFP-positive foci. The *gst-4*::GFP reporter assay therefore experimentally validated the high throughput genomic results and confirmed that loss of NTH-1 or XPA-1, but not both, leads to oxidative stress and activation of oxidative stress responses.

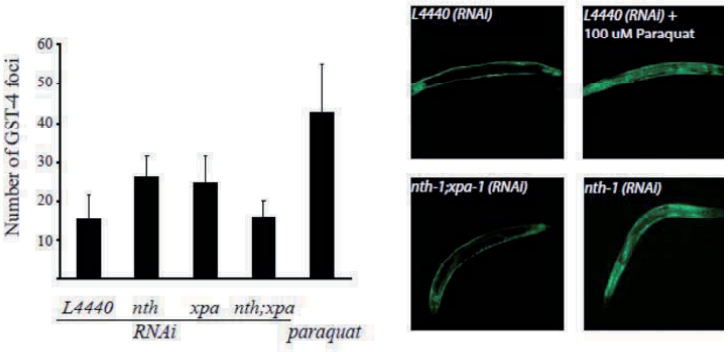

Figure 7. Oxidative stress is induced upon depletion of NTH-1 or XPA-1. The CL2166 reporter strain harbouring a GFP under the control of the *gst-4* promoter was used to determine whether reduction of BER or NER, via *nth-1(RNAi)* and *xpa-1(RNAi)* respectively, or both pathways induces oxidative stress. A significant increase in GST-4 foci compared to the empty vector control (*L4440* (n = 97)) was observed in animals treated with RNAi against NTH-1 (n = 57) or XPA-1 (n = 95) *(p<0.0001)* using Student's *t*-test). Co-depletion of NTH-1 and XPA-1 (n= 46) did not give more GST-4 positive foci (*p* = 0,507). GST-4 positive foci induced by paraquat (100 μM) was included as a positive control (n = 50).

The transcriptional changes do not protect against exogenous acute stress

The expression profiling indicated that the transcriptional responses in the single-mutants are aimed at compensating for oxidative stress resulting from DNA-repair deficiency. The responses appear to be selectively tuned to compensate for endogenous stress. The down-regulation of other stress induced factors, such as the *hsp-16* family, may serve to prevent unsolicited activation of a full-blown stress response. Thus, we would not expect the DNA repair mutants to show resistance to oxidizing agents which is correlated with reduced ILS in *C. elegans* [8, 30]. In agreement with previous reports, neither *xpa-1* [43], nth-1 [7] nor *nth-1;xpa-1* were hypersensitive to paraquat (data not shown). However, all mutants showed mild sensitivity to an acute exposure to another superoxide generating agent, juglone (Figure 8A) and mild heat-shock (data not shown). Hence, the upregulation of oxidative stress responses do not confer resistance to acute exogenous stress. These phenotypes are consistent with downregulation of genes responding to exogenous stressors as observed.

Loss of NTH-1 restores normal lifespan in *xpa-1*

Reduced ILS induces longevity in *C. elegans* [44], but reduced ILS is also seen in segmental progeroid NER defective mice [21]. This apparent paradox can be interpreted as the reduced ILS in the DNA repair defective mice is part of a compensatory attempt to extend lifespan in organisms suffering from DNA damage associated stress. Thus, we were interested to test whether the reduced ILS in *nth-1* and *xpa-1* observed here was accompanied by reduced lifespan – or whether the compensatory response was sufficient to sustain normal lifespan. The lifespan of nth-1 was indistinguishable from the wild type, as was recently shown [7], whereas the *xpa-1* mutant displayed reduced lifespan compared to the wild type (mean survival of 14.5 and 17.3 days, respectively) (Figure 8B). In *C. elegans* therefore, as in mice, the challenges that loss of NER poses to the organism is more severe than loss of a single DNA-glycosylase. Our results demonstrate that this difference in challenge can be read out as a stronger activation of the antioxidant defense and reduction in ILS.

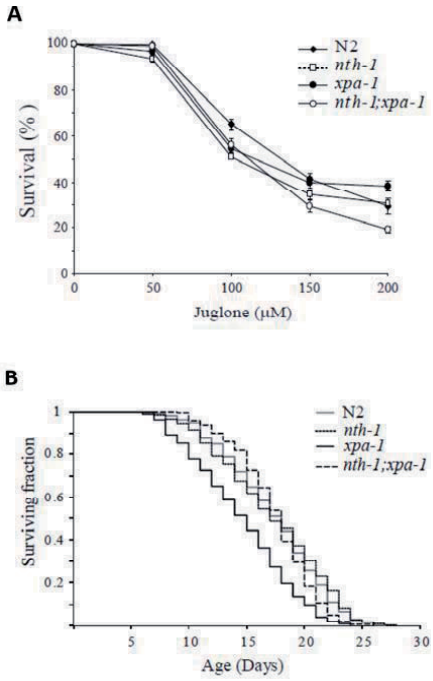

Figure 8. Compensatory responses specific for endogenous stressors. (**A**) Increase in the oxidative stress response do not confer resistance to juglone. Viability was scored as touch-provoked movement after 24 hour recovery from one hour exposure of young adults to juglone. Mean survival (+/- standard error of the mean) relative to untreated control was calculated from five independent experiments comprising a total of 250-350 animals. (**B**) Lack of *nth-1* rescues the lifespan of an *xpa-1* mutant. Synchronized L4 larvae were placed on NGM plates at t = 0, incubated at 20°C, and transferred daily to fresh plates during the egg-laying period. The worms were monitored daily for touched-provoked movement; animals that failed to respond were considered dead. The *xpa-1* mutant shows a reduced lifespan compared to *nth-1* and *nth-1;xpa-1* and wild type, N2.

The *nth-1;xpa-1* double mutant has a more profound DNA repair defect and is expected to be unable to repair a much wider spectrum of DNA lesions. If the accumulation of DNA damage itself is the bigger lifespan reducing challenge in xpa-1, we would expect nth-1;xpa-1to be more severely affected. Interestingly, normal lifespan was restored in *nth-1;xpa-1* with a mean survival of 17.4 days. One possible interpretation of these results is that the oxidative lesions most relevant for aging are those that are normally repaired by NER, but are attempted processed by BER in the absence of the preferred repair pathway.

DISCUSSION

Mutants in DNA glycosylases generally have weak phenotypes. This has been explained by the existence of backup enzymes with overlapping substrate specificities. Here we present data that reveal additional explanations to how wild type phenotypes and lifespan are maintained in animals that lack a DNA glycosylase.

Oxidative stress induced in DNA repair mutants

Here we present the first comprehensive report describing compensatory transcriptional responses to loss of base excision repair genes in animals. Using a well established transgenic reporter assay, we show that transient depletion of NTH-1 and XPA-1 by RNAi induces oxidative stress, thus it seems likely that this initiates transcriptome-modulation in the mutants. We show that lack of the NTH-1 and XPA-1 enzymes are accompanied by upregulation of oxidative stress responses tuned towards endogenous stressors. This is in agreement with identification of a focused compensatory response to BER intermediates (AP-sites and strand breaks) previously shown in *S. cerevisiae*, where the transcriptional responses differed from the common environmental stress response or the DNA damage signature [45]. A DNA-damage dependent ROS response to unrepaired oxidative DNA damage was previously demonstrated in *S. cerevisiae* BER and NER mutants [46]. Interestingly, no indication of oxidative stress or increased expression of oxidative stress response genes was observed in a mutant lacking both NTH-1 and XPA-1. This transcriptomic shift argues against a DNA-base damage dependent activation of oxidative stress responses, but instead indicates that the DNA repair enzymes mediate signaling to activate stress response pathways. Although the upstream signaling events in the *nth-1;xpa-1* double mutant remain to be elucidated, the modulation of AIR-2 and PLK-1 interaction networks may be a consequence of absence of DNA repair enzyme-mediated signaling of transcription blocking lesions. Alternatively, the extensive new synthesis of histone genes suggests that signaling involves chromatin dynamics in the absence of the global genome damage binding proteins, NTH-1 and XPA-1.

The biological significance of transcriptome modulation seen here is confirmed by the DNA repair mutants showing a mild sensitivity to oxidizing agents. It seems likely that the downregulation of the *hsp-16* family and *ftn-1* contributes to the higher sensitivity to juglone in *xpa-1* and *nth-1*, particularly as it is unlikely that a short acute exposure to oxidizing agents (or heat-stress) may

lead to DNA-damage mediated toxicity on organismal level.

Conserved compensatory responses to BER and NER deficiency

Few systematic studies have been performed to look at transcriptomic changes in BER mutant animals and none, to the best of our knowledge, have compared mutants in BER and NER.

A study on gene expression profiling in BER- or NER-defective mutant *S. cerevisiae* showed transcriptional changes in mutants defective in both pathways after treatment with hydrogen peroxide [22], but not in the double mutant. Instead, transcriptome changes in the BER/NER defective strain were already elicited from unrepaired spontaneous DNA damage [23]. To test the generality of our finding, we re-analyzed the baseline data sets from *S. cerevisiae* BER (Ntg1, Ntg2, and Apn1 deficient), NER (Rad1 deficient) and BER/NER defective mutants. We performed GO enrichment analysis on expressed transcripts in the individual strains (Supplemental Table SV). This analysis showed that BER-defective cells had few expressed transcripts and only one enriched GO process, DNA replication ($p < 0.05$). Informative enriched GO processes found only in the NER defective strain include transcription regulation, ubiquitin dependent protein degradation, sister chromatid segregation, and cell communication ($p < 0.01$). The BER/NER and NER defective cells share many enriched GO clusters and show 38% overlap of individual expressed transcripts. Enriched GO processes found only in the BER/NER mutant include DNA repair, DNA packaging, response to DNA damage stimulus, and cell-cycle checkpoint. Regulation of RNA polymerase II transcription was not enriched in the BER/NER mutant. Therefore, the main conclusions drawn here from BER, NER and BER/NER deficient *C. elegans*, resemble those previously seen in *S. cerevisiae* [22, 23, 45]: i) BER mutants show transcriptomic changes. ii) Loss of NER induces more substantial transcriptional responses than BER involving modulation of RNA metabolism and regulation of transcription iii) Loss of BER in the NER mutant shifts the response to regulate processes that maintain DNA integrity.

Reduced mean lifespan in *xpa-1(ok698)*

The first *xpa-1* mutant identified, *rad-3(mn159)*, was reported to have a near-normal lifespan [15] but there are conflicting reports on the lifespan of *xpa-1(ok 698)* allele ranging from normal [43] to a maximum lifespan of 15 days compared to 25 days in the wild type [17]. Here, we show a moderate reduction of lifespan in *xpa-1*. We did therefore not anticipate that the XPA-1 mutant would display a transcriptional profile resembling that of the segmental progeroid NER defective mice [19]. Nevertheless, the reduced lifespan is entirely consistent with the transcriptional changes observed. A recent transcriptomic signature of $Xpa^{-/-}$ mouse dermal fibroblasts shows that suppression of ILS and activation of oxidative stress responses is also seen in DNA repair mutants that do not exhibit accelerated aging [47]. In support of this, GO enrichment analysis, performed as part of the present study, on the differentially expressed genes in $Xpa^{-/-}$ mice [21] showed enrichment of genes that regulate lifespan. Hence, the transcriptomic changes in NER mutants are conserved.

However, the consequences of loss of XPA-1 appear more severe in *C. elegans* compared to mice, both with respect to transcriptional regulation and lifespan. This might indicate that *C. elegans* XPA-1 contributes to repair of spontaneous DNA damage. The 13-fold elevated mutation accumulation rate in *xpa-1* compared to 7-fold in *nth-1* [9], supports this possibility.

CONCLUDING REMARKS

Based on a large body of evidence indicating that persistent transcription-blocking DNA damage cause attenuation of ILS and activation of oxidative stress responses [18-20, 47], it is reasonable to speculate that the transcriptome modulation in the *xpa-1* mutant reflects accumulation of transcription blocking DNA lesions. That similar changes are seen in the *nth-1* mutant suggests that such transcriptomic shifts may be a general strategy for survival in DNA repair mutants. Since BER is the main pathway for repair of endogenous oxidative lesions, this lends further support to the notion that oxidative DNA damage contributes to these phenotypes. However, few known BER substrates are recognized as being transcription blocking and cyclopurines, that are often mentioned in this context [47], are NER substrates [48]. The qualitatively different responses in the double mutant support a model where the NTH-1 and XPA-1 enzymes themselves take part in the signaling events that result in activation of responses tunes to compensate for endogenous stress and suppression of ILS. We hypothesize that binding or inefficient processing of oxidative damage relevant to aging in the absence of the preferred repair pathway leads to formation of transcription blocking structures or signaling inter-mediates. The restoration of normal lifespan upon

deletion of NTH-1 supports this hypothesis and strongly suggests that inefficient BER-mediated processing of lesions normally repaired by NER, results in intermediates that pose a lifespan-reducing challenge.

MATERIALS AND METHODS

Strains and culture conditions. All strains were maintained at 20°C as described [49]. The wild-type Bristol N2, *nth-1(ok724)*, *xpa-1(ok698)* and the transgenic strain CL2166 (*dvIs19[pAF15(gst-4::GFP::NLS*) were all kindly provided by the Caenorhabditis Genetic Center (University of Minnesota, St Paul, MN, USA). The double mutant *nth-1(ok724)*; *xpa-1(ok698)* was generated for this work. All strains were backcrossed 3-4 times immediately ahead of the experiments.

RNA isolation and microarray processing. Mixed stage populations of N2, *xpa-1*, *nth-1* and *nth1;xpa-1* were reared at 20°C on HT115(DE3)-seeded on NGM plates (30 plates per replicate, 3 replicates per strain) until the nematodes had cleared the plates of food. Worms were washed off with S-medium, left to digest remaining food in the gut, and washed 3 times before pelleting and suspended in TRIZOL and frozen at -80°C. Total RNA isolation was then performed by standard procedures (Invitrogen). Synthesis of double stranded cDNA and Biotin-labeled cRNA was performed according to manufacturer's instructions (Affymetrix, Santa Clara, CA, US). Fragmented cRNA preparations were hybridized to the Affymetrix GeneChip *C. elegans* Genome Arrays on an Affymetrix Fluidics station 450. Data deposit footnote: GSE16405.

Data and statistical analysis. The processing and primary data analysis was performed in DNA-Chip Analyzer (dChip) (http://biosun1.harvard.edu/complab/dchip/) where normalization (invariant set), model-based expression correction (PM-only model), comparative analysis, PCA and Hierachical clustering was conducted. XLStat (Excel) was used for linear regression analysis. Enriched GO clusters were analysed using Cytoscape [50], in conjunction with the plug-in system BiNGO [51] in addition to DAVID (http://niaid.abcc.ncifcrf.gov) [52, 53]. The Hyper-geometric Test with Benjamini-Hochberg False Discovery Rate Correction was chosen for both the analyses [51]. Functional interaction networks were generated using the online browser FunCoup [54].

gst-4::GFP expression. RNAi feeding constructs in the pL4440 vector harbouring the NTH-1 and XPA-1 open reading frames were generated by Gateway Technology and transformed into *E. coli* HT115(DE3). NGM plates containing 2 mM IPTG seeded with bacteria expressing the empty vector control L4440 or *nth-1(RNAi), xpa-1(RNAi)* individually or in combination were activated at 37°C for one hour and left to cool to room temp before the CL2166 reporter strain was added. Plates containing 100 μM paraquat (Sigma) were used as positive control. All plates were incubated at 20°C for 2 days before quantification of GST-4 foci on a Nikon eclipse Ti microscope.

Sensitivity to oxidising agents. The sensitivity to the superoxide-generating compound juglone (Sigma) was performed as previously described [55]. Briefly, young adults were exposed to juglone dissolved in M9 buffer for 1 hour in liquid culture. Viability was scored as touch-provoked movement after a 24h recovery period at 20°C on NGM plates seeded with OP50.

Lifespan determination. Assessment of lifespan was performed essentially as described [56]. Briefly, synchronized L4 larvae were placed on NGM plates at t = 0, incubated at 20°C, and transferred daily to fresh plates during the egg-laying period. The worms were monitored daily for touched-provoked movement. Triplicates comprising 10 plates containing at least 10 worms per plate were performed for each strain. Kaplan-Meier survival distributions were generated and Wilcoxon's log rank test was used to assess significance.

Comparisons with published microarray and Real-Time PCR data. Our datasets were compared to data from van der Pluijm et al. [21]: Significantly differentially expressed transcripts found in $Xpa^{-/-}$ compared to wild type mice were extracted and translated into corresponding *C. elegans* orthologs (using NetAffx, http://www.affymetrix.com/analysis/index.affx). These orthologous set of genes were analysed using Cytoscape [50] to find enriched GO Biological Processes.

Next, we compared our results to datasets generated by Evert et al. from untreated wild type, BER, NER and BER/NER *S. cerevisiae* mutants [51]. Cytoscape was used in order to get a comprehensive overview of enriched Biological Processes in each individual sample group. Also, using dChip, expressed transcrips from each sample group were re-analysed in a comparative analysis giving a list of differentially expressed transcripts with a fold-change ≥2 between wild type and mutant cells. In dChip, replicates were combined and a mean signal value was calculated prior to the comparative analysis. These fold-change lists were then imported into Cytoscape for GO enrichment analysis.

Finally, we extracted genes found to be significantly differentially expressed in the *aqp-1* compared to the wild type in a Real-Time PCR data set from a recent paper by Lee et al. [51] and compared these to our data set.

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CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interest to declare.

REFERENCES

1. Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993; 362:709-715.

2. Krokan HE et al. Base excision repair of DNA in mammalian cells. FEBS Lett. 2000; 476:73-77.

3. Arczewska KD et al. The contribution of DNA base damage to human cancer is modulated by the base excision repair interaction network. Crit Rev Oncog. 2008; 14:217-273.

4. Barnes DE and Lindahl T. Repair and genetic consequences of endogenous DNA base damage in mammalian cells. Annu Rev Genet. 2004; 38:445-476.

5. Shatilla A and Ramotar D. Embryonic extracts derived from the nematode Caenorhabditis elegans remove uracil from DNA by the sequential action of uracil-DNA glycosylase and AP (apurinic/apyrimidinic) endonuclease. Biochem J. 2002; 365:547-553.

6. Nakamura N et al. Cloning and characterization of uracil-DNA glycosylase and the biological consequences of the loss of its function in the nematode Caenorhabditis elegans. Mutagenesis. 2008; 23:407-413.

7. Morinaga H et al. Purification and characterization of Caenorhabditis elegans NTH, a homolog of human endonuclease III: essential role of N-terminal region. DNA Repair (Amst). 2009; 8:844-851.

8. Olsen A, Vantipalli MC, and Lithgow GJ. Using Caenorhabditis elegans as a model for aging and age-related diseases. Ann N Y Acad Sci. 2006; 1067:120-128.

9. Denver DR et al. The relative roles of three DNA repair pathways in preventing Caenorhabditis elegans mutation accumulation. Genetics. 2006; 174:57-65.

10. Reardon JT et al. In vitro repair of oxidative DNA damage by human nucleotide excision repair system: possible explanation for neurodegeneration in xeroderma pigmentosum patients. Proc Natl Acad Sci U S A. 1997; 94:9463-9468.

11. Swanson R et al. Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathways for DNA base damage in Saccharomyces cerevisiae. Mol Cell Biol. 1999; 19:2929-2935.

12. Boiteux S, Gellon L, and Guibourt N. Repair of 8-oxoguanine in Saccharomyces cerevisiae: interplay of DNA repair and replication mechanisms. Free Radical Biology and Medicine. 2002; 32:1244-1253.

13. Meyer JN et al. Decline of nucleotide excision repair capacity in aging Caenorhabditis elegans. Genome Biol. 2007; 8:R70.

14. Aboussekhra A et al. Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell. 1995; 80:859-868.

15. Hartman PS and Herman RK Radiation-sensitive mutants of Caenorhabditis elegans. Genetics. 1982; 102:159-178.

16. Hartman PS et al. Excision repair of UV radiation-induced DNA damage in Caenorhabditis elegans. Genetics. 1989; 122:379-385.

17. Hyun M et al. Longevity and resistance to stress correlate with DNA repair capacity in Caenorhabditis elegans. Nucleic Acids Res. 2008; 36:1380-1389.

18. Kirkwood TB. Understanding the odd science of aging. Cell. 2005; 120:437-447.

19. Garinis GA et al. DNA damage and ageing: new-age ideas for an age-old problem. Nat Cell Biol. 2008; 10:1241-1247.

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

21. van der Pluijm I et al. Impaired genome maintenance suppresses the growth hormone--insulin-like growth factor 1 axis in mice with Cockayne syndrome. PLoS Biol. 2007; 5: e2.

22. Evert BA et al. Spontaneous DNA damage in Saccharomyces cerevisiae elicits phenotypic properties similar to cancer cells. J Biol Chem. 2004; 279:22585-22594.

23. Salmon TB et al. Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. Nucleic Acids Res. 2004; 32:3712-3723.

24. Greiss S et al. Transcriptional profiling in C. elegans suggests DNA damage dependent apoptosis as an ancient function of the p53 family. BMC Genomics. 2008; 9:334.

25. Antebi A. Genetics of aging in Caenorhabditis elegans. PLoS Genet. 2007; 3:1565-1571.

26. Murphy CT et al. Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature. 2003; 424:277-283.

27. McElwee J, Bubb K, and Thomas JH. Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. Aging Cell. 2003; 2:111-121**.**

28. Johnson TE et al. Longevity genes in the nematode Caenorhabditis elegans also mediate increased resistance to stress and prevent disease. J Inherit Metab Dis. 2002; 25:197-206.

29. Wolf M et al. The MAP kinase JNK-1 of Caenorhabditis elegans: location, activation, and influences over temperature-dependent insulin-like signaling, stress responses, and fitness. J Cell Physiol. 2008; 214:721-729.

30. Honda Y and Honda S. Oxidative stress and life span determination in the nematode Caenorhabditis elegans. Ann N Y Acad Sci. 2002; 959:466-474.

31. Murphy CT, Lee SJ, and Kenyon C. Tissue entrainment by feedback regulation of insulin gene expression in the endoderm of Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2007; 104: 19046-19050.
32. Kahn NW et al. Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. Biochem J. 2008; 409:205-213.
33. Sheaffer KL, Updike DL, and Mango SE. The Target of Rapamycin pathway antagonizes pha-4/FoxA to control development and aging. Curr Biol. 2008; 18:1355-1364.
34. Salinas LS, Maldonado E, and Navarro RE. Stress-induced germ cell apoptosis by a p53 independent pathway in Caenorhabditis elegans. Cell Death Differ. 2006; 13:2129-2139.
35. Kondo M et al. The p38 signal transduction pathway participates in the oxidative stress-mediated translocation of DAF-16 to Caenorhabditis elegans nuclei. Mech Ageing Dev. 2005; 126:642-647.
36. Lee SJ, Murphy CT, and Kenyon C. Glucose shortens the life span of C. elegans by downregulating DAF-16/FOXO activity and aquaporin gene expression. Cell Metab. 2009; 10: 379-391.
37. Pothof J et al. Identification of genes that protect the C. elegans genome against mutations by genome-wide RNAi. Genes Dev. 2003; 17:443-448.
38. van Haaften G et al. Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality. Proc Natl Acad Sci U S A. 2004; 101:12992-12996.
39. Trenz K, Errico A, and Costanzo V. Plx1 is required for chromosomal DNA replication under stressful conditions. Embo J. 2008; 27:876-885.
40. Takaki T et al. Polo-like kinase 1 reaches beyond mitosis--cytokinesis, DNA damage response, and development. Curr Opin Cell Biol. 2008; 20:650-660.
41. Mishima M, Kaitna S., and Glotzer M. Central spindle assembly and cytokinesis require a kinesin-like protein/RhoGAP complex with microtubule bundling activity. Dev Cell. 2002; 2:41-54.
42. Woodward AM et al. Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress. J Cell Biol. 2006; 173:673-683.
43. Astin JW, O'Neil NJ, and Kuwabara PE. Nucleotide excision repair and the degradation of RNA pol II by the Caenorhabditis elegans XPA and Rsp5 orthologues, RAD-3 and WWP-1. DNA Repair (Amst) 2008; 7:267-280.
44. Kenyon C. The plasticity of aging: insights from long-lived mutants. Cell 2005; 120:449-460.
45. Rusyn I et al. Transcriptional networks in S. cerevisiae linked to an accumulation of base excision repair intermediates. PLoS ONE 2007; 2:e1252.
46. Rowe LA, Degtyareva N. and Doetsch PW. DNA damage-induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae. Free Radic Biol Med. 2008; 45:1167-1177.
47. Garinis GA et al. Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. Nat Cell Biol. 2009; 11:604-615.
48. Kuraoka I et al. Removal of oxygen free-radical-induced 5',8-purine cyclodeoxynucleosides from DNA by the nucleotide excision-repair pathway in human cells. Proc Natl Acad Sci U S A 2000; 97: 3832-3837.
49. Brenner S. The genetics of Caenorhabditis elegans. Genetics. 1974; 77:71-94.
50. Shannon P et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003; 13:2498-2504.
51. Maere S, Heymans K. and Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics. 2005; 21:3448-3849.
52. Dennis G Jr. et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003; 4:P3.
53. Huang da W, Sherman BT, and Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009; 4:44-57.
54. Alexeyenko A and Sonnhammer EL. Global networks of functional coupling in eukaryotes from comprehensive data integration. Genome Res. 2009; 19:1107-1116.
55. Przybysz AJ et al. Increased age reduces DAF-16 and SKN-1 signaling and the hormetic response of Caenorhabditis elegans to the xenobiotic juglone. Mech Ageing Dev. 2009; 130: 357-**369.**
56. Kenyon C et al. A C. elegans mutant that lives twice as long as wild type. Nature 1993; 366:461-464.

III

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Research article

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Global transcriptional response after exposure of fission yeast cells to ultraviolet light

Henriette C Skjølberg[1,2], Øyvind Fensgård[3], Hilde Nilsen[3], Beáta Grallert[1,2] and Erik Boye*[1,2]

Address: [1]Department of Cell Biology, Institute for Cancer Research, Oslo University Hospital, Radiumhospitalet, Montebello, 0310 Oslo, Norway, [2]Institute for Molecular Biosciences, University of Oslo, Blindernveien 31, 0371 Oslo, Norway and [3]The Biotechnology Center, University of Oslo, Gaustadalleen 21, 0349 Oslo, Norway

Email: Henriette C Skjølberg - henriette.c.skjolberg@rr-research.no; Øyvind Fensgård - oyvind.fensgard@biotek.uio.no; Hilde Nilsen - hilde.nilsen@biotek.uio.no; Beáta Grallert - beata.grallert@rr-research.no; Erik Boye* - eboye@rr-research.no

* Corresponding author

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Abstract

Background: In many cell types, including the fission yeast *Schizosaccharomyces pombe*, a set of checkpoints are induced by perturbations of the cell cycle or by DNA damage. Many of the checkpoint responses include a substantial change of the transcriptional pattern. As part of characterising a novel G1/S checkpoint in fission yeast we have investigated whether a transcriptional response is induced after irradiation with ultraviolet light.

Results: Microarray analyses were used to measure the global transcription levels of all open reading frames of fission yeast after 254 nm ultraviolet irradiation, which is known to induce a G1/S checkpoint. We discovered a surprisingly weak transcriptional response, which is quite unlike the marked changes detected after some other types of treatment and in several other checkpoints. Interestingly, the alterations in gene expression after ultraviolet irradiation were not similar to those observed after ionising radiation or oxidative stress. Pathway analysis suggests that there is little systematic transcriptional response to the irradiation by ultraviolet light, but a marked, coordinated transcriptional response was noted on progression of the cells from G1 to S phase.

Conclusion: There is little response in fission yeast to ultraviolet light at the transcriptional level. Amongst the genes induced or repressed after ultraviolet irradiation we found none that are likely to be involved in the G1/S checkpoint mechanism, suggesting that the checkpoint is not dependent upon transcriptional regulation.

Background

Cell cycle progression is fundamental for all proliferation. Transition from one cell-cycle phase to the next is often brought about by a changed transcriptional pattern: repression of specific genes and/or expression of new genes promote progression from one phase into the next. The regulation of transcriptional patterns during the cell cycle is conserved from yeast to humans [1,2], although the actual genes and transcriptional factors involved are not necessarily conserved. In addition to transcriptional

regulation the key components of the cell cycle are also frequently regulated at the translational and the post-translational levels.

Regulation of transcription is important in the cellular response to environmental stress. Exposure to radiation, toxic chemicals, fluctuations in temperature, osmolarity or nutrient availability profoundly affect cell growth and the genomic expression programme is adjusted to adapt to the different challenges. Microarray technology has been used to characterise global gene expression profiles for several different stress conditions in the model organism *Schizosaccharomyces pombe* [3,4]. A common set of genes responding to many different forms of stress has been identified in both fission and budding yeast. These genes are known as core environmental stress response genes, CESR [3] in *S. pombe* and environmental stress response genes [5] or the common environmental response genes [6] in *Saccharomyces cerevisiae*. In addition to this common pattern there are genes that are specifically expressed in response to each individual stress treatment.

In general, stress represents a threat to genome stability. Depending on the type of damage inflicted and the position in the cell cycle different strategies are used for handling a stress situation. Checkpoint mechanisms delay the cell cycle to allow the cells to repair DNA damage and to ensure stable inheritance of the genome. Several checkpoint pathways target the transcription machinery to ensure the appropriate expression levels of genes involved in the response to the insult. In *S. pombe* there are separate checkpoints that inhibit mitosis when the DNA is damaged (the G2/M checkpoint) or when S phase has not been completed (the S/M checkpoint) and that inhibits DNA replication when the DNA is damaged (the intra-S checkpoint) [7]. These checkpoints have at least two features in common: they all operate through the five so-called checkpoint Rad proteins and they all bring about the cell-cycle delay via inhibition of the Cdc2 protein kinase, the key regulator of cell-cycle progression [8]. In addition, they also include Rad3-dependent transcriptional responses [4,9,10].

In G1 phase the cell decides whether to commit to a new round of the cell cycle or to enter stationary phase or meiosis. The G1/S DNA damage checkpoint regulates the transition into S phase [11], and insensitivity to growth-inhibitory signals, especially in the G1 phase, is one of the hallmarks of cancer [12]. We have recently discovered and partly characterised a novel checkpoint mechanism in *S. pombe* which delays S-phase entry after UVC irradiation in a Gcn2-dependent manner [13]. In the present work we have investigated whether the response to UVC in G1 phase involves a specific transcriptional response and searched for possible genes to be involved in the G1/S checkpoint. Furthermore, we compare the genes differentially expressed after UVC irradiation with the transcriptional response to oxidative stress (H_2O_2) and ionising radiation (IR).

Results

We have performed genome-wide expression analyses of UVC-irradiated G1-phase fission yeast cells to further characterise the G1/S checkpoint [13,14]. We have investigated what kind of transcriptional response UVC irradiation imposes on the cells and searched for potential candidate genes involved in the regulatory process of the G1/S checkpoint. The cells were synchronised by employing a temperature-sensitive version of Cdc10, a transcription factor required for progression from G1 into S phase. This method gives good synchrony and allows convenient detection of the G1/S checkpoint. During a four-hour shift to 36°C the cells were arrested in G1 phase and could be released synchronously into the cell cycle or kept in G1 phase. Total RNA was isolated from both UVC-irradiated cells and unirradiated control cells. The RNA was subjected to total genomic microarray analysis.

The two experiments

Two distinct experiments were performed (see also Additional file 1): First, G1-phase cells were irradiated and kept at the restrictive temperature (hereafter called the "restrictive-temperature experiment"). Thirty minutes after the time of irradiation the UVC-irradiated (UV30) and unirradiated control (C30) cells were collected. These cells were still in early G1 phase due to the continued inactivation of Cdc10. Second, G1-cells were released into the cell cycle after synchronisation by reducing the temperature to 25°C, thus reactivating Cdc10, allowing the cells to continue in the cell cycle. Cell samples were collected at 0, 30 and 90 min after irradiation (hereafter called the "time-course experiment"). Samples of irradiated and unirradiated cells were analyzed on microarrays and compared to a common reference pool (see Methods). Flow cytometry (Additional file 2) demonstrated that the control cells had entered S phase by 60 minutes after release, whereas the UVC-irradiated cells delayed in G1 phase and moved from G1 to S phase around 90 minutes after release, in agreement with previous data [13].

We have previously shown the existence of the G1/S checkpoint using several synchronisation methods [14], but none of the other methods provided good enough synchrony to perform similar analyses on global transcription. We have looked at our data for all known Cdc10 targets in the 12 arrays from the time-course experiment and observed no trend showing that UVC delays the occurrence of these transcripts. This is consistent with our RNA blots of the two selected transcripts *cdc18* and *cig2*,

which are not delayed by UVC in block-and-release experiments [Fig. 4D in [14]].

The restrictive-temperature experiment

In this experiment we searched for genes that altered their expression more than twofold as a consequence of UVC irradiation in G1 phase. Of almost 5000 genes represented on the microarrays as few as 74 genes were induced twofold or more and 43 of these were non-CESR genes (Fig. 1A). Most of the 74 genes were induced two- to threefold, and only three genes were induced more than fivefold. Most of the induced non-CESR genes are likely to be UVC-specific and not cell-cycle related, since the cells did not move into S phase during the time of the experiment. No non-CESR genes were found to be induced by both UVC and H_2O_2 [3], but two genes, *SPCC132.04c* and *SPBC16A3.17c* were induced after both UVC (this work) and IR treatment [4] (see Table 1). We categorised the 43 non-CESR genes into eight different groups according to the functions of their products (Table 1). These genes are involved in a variety of functions such as signalling and stress response, ribosome biogenesis and translation, DNA/RNA binding, and as many as 15 of the genes are involved in transport mechanisms. There were no obvious candidates for genes involved in the G1/S checkpoint amongst the 74 upregulated genes. Surprisingly, only one of the induced genes, *rhp4b*, encoding a nucleotide excision repair factor, is involved in DNA repair of UVC-induced lesions, strongly suggesting that the capacity to perform DNA repair is not regulated at the transcriptional level. Another possibly interesting induced gene is *pyp1*, a protein-tyrosine phosphatase that acts on Sty1, the MAPK (mitogen-activated protein kinase) that regulates various stress responses in *S. pombe* [15]. We have shown that the G1/S checkpoint does not require Sty1 [13], but it is possible that Pyp1 has additional targets in the cell besides Sty1.

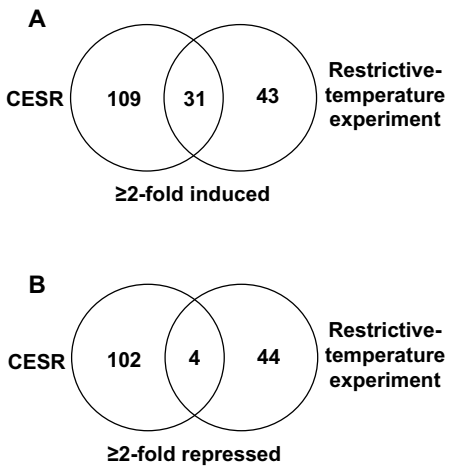

Figure 1
Comparison of induced and repressed genes from the restrictive-temperature experiment and CESR genes. The number of genes induced (A) and repressed (B) more than two-fold in the restrictive-temperature experiment illustrated in a Venn diagram. The numbers of genes common for the restrictive-temperature experiment and the previously identified CESR are shown within the overlapping regions.

We also identified 44 genes that were repressed more than twofold and almost all of them (40) were non-CESR genes (Fig. 1B). These 40 genes were categorised according to the function of their products (Additional file 3), and, like for the proteins encoded by the induced genes, were known to be involved in a wide variety of activities including transport, metabolism, mating and mitochondrial activities. Amongst the repressed genes no obvious candidate genes for checkpoint regulators were found.

The time-course experiment

In the unirradiated control cells only 53 genes from the whole dataset were upregulated (more than twofold) and 29 were downregulated in either the C30 and/or the C90 sample relative to the situation in cells at the start of the experiment (C0) (Table 2). In comparison, altogether 41 genes were upregulated and 35 downregulated in either the UV0, UV30 or the UV90 sample relative to C0 during the time-course. Like in the restrictive-temperature experiment, most of the induced genes were only upregulated two- or threefold. Only four genes in either C or UV were induced more than fivefold in both repeats of the experiment and this was only found for the C90 sample. It is striking that only about 1% of around 5000 genes has a changed expression after cell cycle progression and UVC irradiation.

Furthermore, we searched for genes differentially expressed between 0/30, 30/90 and 0/90 minutes in both the C and UV samples. To this end we used moderated t-statistics with a P-value cut-off of 0.05 (for details see Methods). In these experiments each gene could potentially be assigned 12 expression values (three time points, C and UV, two repeats). About 35% of the values were missing in the entire dataset. The reasons for the missing values will be discussed below. We decided to remove the data for a gene if four or more of the 12 possible data values were missing. This action reduced the dataset from 5266 to 2836 genes, and data for the resulting 54% of the genes was considered more reliable and was used for further analysis.

Table 1: UV-induced genes not present in the CESR

UV-induced genes not present in the CESR at UV30:	
Gene name	**Annotation**
gst2	Glutathione S-transferase, similarity to Gst1p, induced by oxidative stress. Also induced by IR
SPBC1A4.07c	Sof1-like domain containing family and contains 7 WD domains, similarity to *S. cerevisiae* Sof1p
rrn3	Involved in initiation of transcription of rDNA promoter
scw1	Involved in negative regulation of cell wall integrity and septum formation
SPBC19F5.02c	Protein containing six WD domains, similarity to S. cerevisiae Utp4p
SPCC584.07c	Pseudogene
SPAC17A2.02c	Protein of unknown function, similarity to uncharacterized C. albicans Ipf15301p
SPBC8E4.02c	Protein of unknown function
fip1	Iron permease FTR1 family. Also indused after IR and between C30 and C90

Gene annotations are from GeneDB http://www.genedb.org/genedb/pombe/index.jsp.

Cell-cycle-regulated genes

The RNA samples from irradiated cells reflect gene expression changes that occur for two separate reasons: first, the cells are progressing through the cell cycle and will necessarily change gene expression [16] and, second, the cells have been exposed to UVC light and will display stress-related and UVC-specific changes. In contrast, differences between the samples in the restrictive-temperature experiment (above) should reflect only the stress-related and UVC-specific changes. In the present analysis, we attempt to identify the genes specifically affected by UVC exposure. For this reason we identified the genes in unirradiated control cells whose expression varied after release into the cell cycle and these genes were classified as not

Table 2: Differentially expressed genes in the time-course experiment

The time course experiment:		
Differentially expressed genes		
	induced ≥2-fold	**repressed ≥2-fold**
UV0	0	0
UV30	12	3
UV90	35	34
Total	41	35
C0		
C30	13	8
C90	41	28
Total	53	29
Differentially expressed genes when comparing the different timepoints		
Comparison	**induced (P ≤ 0.05)**	**induced ≥2-fold**
UV0-UV30	11	0
UV30-UV90	0	0
UV0-UV90	241	12
Total	241	12
CO-C30	4	0
C30-C90	47	14
CO-C90	129	30
Total	143	20

specifically responding to UVC. In the unirradiated cells altogether 143 genes were found to be differentially expressed between G1 phase (C0 or C30) and S/G2 phase (C90) (Fig. 2A and Table 2). Only 4 genes (3+1) were differentially expressed at 30 minutes after release into the cell cycle (C0 compared to C30). In comparing C30 (late G1 phase) with C90 (S/G2 phase) 47 genes (32+14+1) were found to be differentially expressed. Finally, 129 genes (93+32+1) had a changed transcriptional level when comparing C0 (early G1 phase) to C90 (S/G2 phase). This means that, not surprisingly, most of the induced genes were found in cells entering S phase (the comparison of C0 with C90 and C30 with C90). Well-known G1-specific genes, like *cdc18*, *cig2*, *cdc22* and *cdt2*, were induced normally during G1 phase (C30) in our study (data not shown), serving as convenient controls.

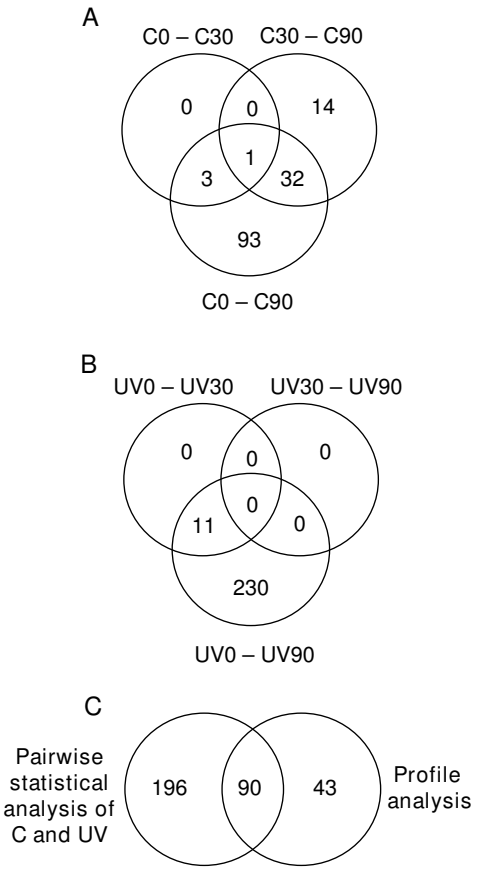

Figure 2
Differentially expressed genes during the time-course experiment. Venn diagram comparison of differentially expressed genes in control cells when comparing C0 to C30, C30 to C90 and C0 to C90 (A), and in UVC-irradiated cells when comparing UV0 to UV30, UV30 to UV90 and UV0 to UV90 (B). The numbers of differentially expressed genes in either the control [C] or irradiated [UVC] cells identified by the pairwise statistical and the profile analysis are illustrated in a Venn diagram (C). The numbers of common genes are shown within the overlapping regions.

UVC-regulated genes

Using the P-value cut-off of 0.05, we identified as many as 241 genes in total that were differentially expressed in the UVC-irradiated cells after release into the cell cycle (Fig. 2B and Table 2). Of all the 241 genes only 11 were different between UV0 and UV30, so little was happening at the transcriptional level during the first 30 minutes after irradiation. No genes were determined to be differentially expressed when comparing UV30 (late G1 phase) and UV90 (S phase). However, as many as 230 genes were defined to be different between UV0 (early G1 phase) and UV90 (S phase). The apparent discrepancy between the numbers of regulated genes between UV0/UV30 and UV30/UV90 on the one hand and between UV0/UV90 on the other can be explained as follows: Expression of a number of genes is different between UV0 and UV30, but only 11 genes were significantly different ($P < 0.05$). Similarly, a number of genes changed their expression between UV30 and UV90, but no difference passed the threshold we had set. However, when comparing UV0 and UV90, a number of genes (230) had altered their expression sufficiently during the total interval. It follows that the level of change for all of these genes was low.

Some of the 241 genes that were up- or downregulated after UVC in this experiment are regulated as a consequence of the cell-cycle progression and not of the UVC irradiation. To identify the UVC-specific transcripts, we excluded the 143 cell-cycle-regulated genes identified above (see *Cell-cycle-regulated genes*), resulting in 172 UVC-regulated genes (Additional file 4), of which 162 are non-CESR genes. Of these 162 genes as many as 26 genes are dedicated to the translational machinery. Nine of the 162 genes were specifically upregulated in UV30 (Table 3), which is at a time when cells are arrested in G1 phase by the G1/S checkpoint. These 9 genes were compared to the set of non-CESR genes induced by H_2O_2 and IR [3,4]. No genes were found to be induced by both UVC and H_2O_2 treatment. Only two genes, *gst2* and *fip1*, were induced after both UVC (this work) and IR treatment. None of the 9 UVC-specific genes are likely to be regulators of the G1/S checkpoint, judging from their annotations.

Table 3: UV-induced genes not present in the CESR at UV30

UV-induced genes not present in the CESR:	
Gene name	**Annotation**
Repair and DNA metabolism	
rhp4b	Nucleotide excision repair factor involved in the repair of UV damaged DNA
mus81	Holliday junction resolvase subunit that associates with Eme1p
Metabolism	
SPCC132.04c	Similarity to S. cerevisiae Gdh2p, a glutamate dehydrogenase
Transort	
SPAC328.09	Similarity to 2-oxodicarboxylate transporter (S. cerevisiae Odc2p
SPCC794.04c	Sugar (and other) transporter family and the major facilitator superfamily
str1	Probable ferrichrome-iron transporter
SPCPB1C11.01	Similarity to S. cerevisiae Mep2p, ammonium transporter family of membrane transporters
SPAC1002.16c	Sugar (and other) transporter family, and the major facilitator superfamily
SPBC36.02c	Similarity to C. albicans Flu1p, a membrane transporter, major facilitator superfamily
SPBC530.15c	Similarity to S. cerevisiae Tpo3p, a polyamine transport protein
SPBC409.08	Similarity to S. cerevisiae Tpo2p, a polyamine transport protein
SPAC8C9.12c	Similarity to mitochondrial RNA splicing protein 3 (S. cerevisiae Mrs3p), mitochondrial carrier protein family
ptr2-a;ptr2	Similarity to S. cerevisiae Ptr2p, a peptide permease nitrogen-repressible transporter
SPCC569.05c	Major facilitator superfamily, similarity to C. albicans Flu1p
SPBC16A3.17c	Major facilitator superfamily, similarity to S. pombe Fnx1p, transporter required for long-term survival in N starved cells
SPCC2H8.02	Major facilitator superfamily and the sugar (and other) transporter family
SPCC2H8.00	Major facilitator superfamily and the sugar (and other) transporter family, similarity to S. cerevisiae Pho84p
SPCC1183.11	Mechanosensitive ion channel family
Signaling and stress response	
SPAC9B6.03	Protein containing a FYVE zinc finger domain, which bind phosphatidylinositol 3-phosphate
gaf2	Iron-sensing transcription factor that binds GATA elements to regulate iron transporter gene transcription
rst2	Transcriptional activator that positively regulates the transcription of ste11 and fbp2
SPBC19C2.13c	Similarity to S. cerevisiae Ncs2p, involved in pseudohyphal growth and cellular response to starvation
pyp1	Protein-tyrosine phosphatase that acts on Sty1p and negatively regulates mitosis
(isa1)	Protein that binds ferredoxin (Etp1p) and contains iron-sulfur clusters
(cta3)	Probable Ca2+-ATPase, transcription is induced under high salt conditions
Ribosome biogenesis and translation	
rrn2	Protein involved in initiation of transcription of rDNA promoter
SPCP1E11.06	Similarity to S. cerevisiae Nsa2p, a nuclear protein involved in ribosome biogenesis, part of the small ribosomal subunit
DNA/RNA binding	
SPBP8B7.15c	Zinc knuckle domain, which can bind RNA or DNA in eukaryotes, similarity to S. cerevisiae Mpe1p
cbh2	DNA binding protein, may be involved in chromosome segregation
Others	
SPAC323.07c	Member of the MatE family, which are integral membrane proteins
ppr1	Resistance to the L-proline analog AZC, catalyzes acetylation of AZC, homolog of S. cerevisiae Mpr1p
SPAC3H8.09c	Containing an RNA recognition motif, similarity to S. cerevisiae Nab3p
SPBC1773.17c	Containing D-isomer specific 2-hydroxyacid dehydrogenase NAD binding and catalytic domains
SPAPB18E9.04c	Similarity to S. cerevisiae Pry3p, may have a role in mating efficiency
Protein of unknown function	
SPAPB18E9.03c	*SPNCRNA.101 SPAC18G6.09c SPAPB1A10.06 SPBC19C7.04c SPAC17A5.8 SPAC23H3.15c SPCC2H8.01 SPAC8C9.10c*

Gene annotations are from GeneDB http://www.genedb.org/genedb/pombe/index.jsp.

Profile analyses

To further analyse the kinetics of gene expression in the time-course experiment we applied the software package maSigPro [17] on the filtered data, which allowed a multiple comparison of all three time points. There were 133 genes that significantly changed their expression levels during the time-course ($P \leq 0.05$), and the expression of almost all (105 genes) was changed in both control and UVC-irradiated cells. Fifteen genes were identified as specific for the irradiated cells. There was a good correlation between the genes identified in this profile analysis and the differentially expressed genes determined in the statistical analysis above (Fig. 2C).

The expression levels of the 133 genes with expression that varied during the time-course were subjected to a clustering analysis, forming a two-dimensional map (Fig. 3). Genes being up- or downregulated at the particular

time point are represented by different colours, green indicating induction and blue repression. The map shows that analysis of the two biological replicates revealed that they were indeed similar and assembled into the closest branches of the cluster. This confirms a good reproducibility of the experiments. Furthermore, the profile analysis is also consistent with the conclusions of the pairwise statistical analyses (above), showing that the expression patterns in UV30 and UV90 were similar. The map shows little difference between control and irradiated samples at all time points, and the least closely related branches of the cluster refer to changes occurring during progression in the cell cycle. Thus, this analysis corroborates our conclusions from the above pairwise comparisons, that the cell cycle progression affects transcription profiles more than the UVC treatment and there is only a weak transcriptional response to UVC irradiation.

Pathway analyses

Gene ontology (GO) enrichment analysis was performed on the 43 UVC-induced genes in the restrictive-temperature experiment (Table 1) using the DAVID software after ID-conversion. Ten unique genes (20%) were members of enriched GO groups, suggesting upregulation of genes involved in ion transport or ion homeostasis in the restrictive-temperature experiment (Additional file 5). Analysis of over-represented GO annotations amongst the 172 upregulated genes in the time-course experiment (Table 2) showed that 38 unique genes (22%) are members of the enriched GO groups. The nature of these groups indicates regulation of genes that affect protein biosynthesis or structural components of ribosomes (Table 4). Further network analysis shows a concerted response involving direct protein-protein interactions between 20 of the 172 induced gene products. Thus, the analyses suggest a coordinated induction of rRNA biogenesis, ribosome assembly and components of the 60S and 40S ribosomal subunits in the time-course experiment (Fig. 4). (Additional file 7)

Confirmation of the microarray data

RNA blotting and hybridisation was used to confirm our microarray results for four selected transcripts. The induction of *SPAC2E1P3.05c*, *fip1* and *gst2* found in the time-course experiment was verified (Fig. 5A). Fip1, an iron permease, and Gst2, a glutathione S-transferase, have also been shown to be induced after IR [4]. On the other hand, *rhp4b* had no values in any of the 12 arrays in our time-course experiments and was also not detected after RNA blotting and hybridisation. However, in the restrictive-temperature experiment, *rhp4b* transcription was found to be induced by UVC irradiation and this finding was also confirmed by RNA blotting (Fig. 5B). Therefore, RNA-blotting experiments with all four selected genes verified the results from the microarray experiments.

UVC does not induce the unfolded protein response

The unfolded protein response (UPR) is activated by the accumulation of unfolded proteins in the endoplasmic reticulum (ER). The UPR triggers a transcriptional response which serves to induce the production of ER components and to increase the degradation capacity to dispose of the unfolded proteins [18]. It is possible that UVC irradiation might stress the ER and thus activate the UPR. Furthermore, GCN2 has been shown to be required for the induction of a majority of UPR target genes during ER stress in *S. cerevisiae* [19]. We therefore used the present data from both the restrictive-temperature and time-course experiments to investigate whether UPR genes are induced by UVC in fission yeast. We identified UPR-genes in fission yeast as the homologues of the UPR-induced budding yeast genes [20]. There was little, if any, induction of the UPR-genes identified by this method (Additional file 6). The lack of transcriptional response of these genes after UVC strongly argues that the G1/S checkpoint is not a manifestation of the UPR.

Discussion

Here we have investigated gene expression in *S. pombe* cells traversing the G1/S border in a synchronous manner, both UVC-irradiated and unirradiated cells. The transcriptional response after UVC-irradiation in G1 phase was surprisingly weak. The vast majority of genes did not change their transcription pattern appreciably and the few that did increased or decreased their expression levels only two- to three-fold.

Comparison of the data from the two experiments

162 genes were identified as specifically UVC-regulated in the time-course experiment and 43 in the restrictive-temperature experiment. Surprisingly, as few as 4 genes (see Additional file 4) were found to be common for the two datasets, and these 4 genes were not differently expressed in UV0 and UV30 in the time-course experiment (Table 3), which is the time period when the irradiated cells were arrested in the G1/S checkpoint. The UVC irradiation elicits a quite weak transcriptional response on the cells both when considering the number of genes affected and the level of the response for the affected genes. Thus, our assay must be considered to be rather sensitive and even a small change in the experimental setup might bring the marginal levels of gene expression over or under our threshold, which could be one reason for the poor overlap between the two experiments. This further underlines our conclusion that UVC has only a marginal effect on gene transcription when given in G1 phase. It is possible that the biological differences between the cells in our two types of experiments is dominating and that the dissimilar sets of regulated genes reflect a biological difference rather than an artefact of our data analysis. In the restrictive-temperature experiment cells were arrested in G1 phase by the

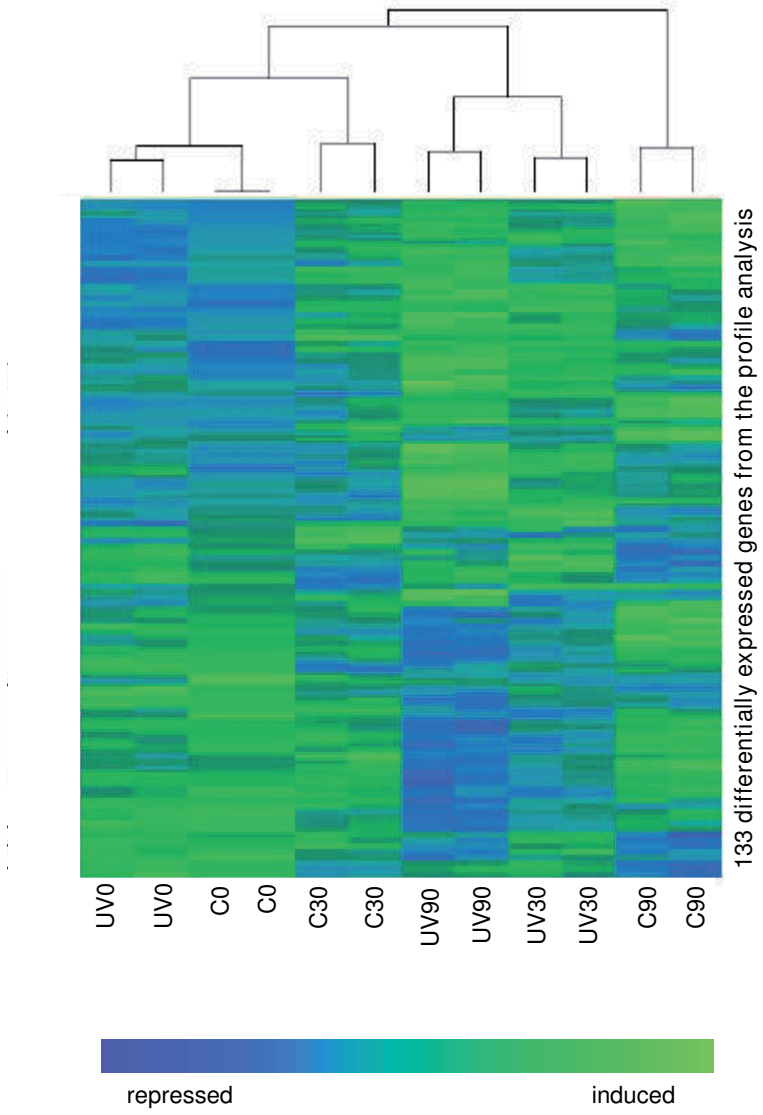

Figure 3
Regulation of gene expression during the time-course experiment in control and UVC-irradiated cells. The expression pattern of 133 genes whose expression changed significantly ($P \leq 0.05$) during the time course are shown. The columns represent samples taken after 0, 30 or 90 min in control and UVC-irradiated cells. Hierarchical clustering was performed as described in Methods, pairing the 133 genes in the different samples according to their expression level. The changes in transcription level are colour coded with induced genes as green and repressed genes as blue.

Table 4: Enriched Gene Ontology Groups in the restrictive-temperature experiment

Category	GO number	Term	Count*	%	P-value
GOTERM_Cellular Component	GO:0005887	integral to plasma membrane	3	8.11	0,024
	GO:0044459	plasma membrane part	4	10.81	0,035
	GO:0031226	intrinsic to plasma membrane	3	8.11	0,038
GOTERM_Biological Process	GO:0015674	di-, tri-valent inorganic cation transport	4	10.81	0,001
	GO:0006812	cation transport	5	13.51	0,007
	GO:0030001	metal ion transport	4	10.81	0,008
	GO:0030003	cellular cation homeostasis	4	10.81	0,018
	GO:0055082	cellular chemical homeostasis	4	10.81	0,020
	GO:0050801	ion homeostasis	4	10.81	0,022
GOTERM_Molecular Function	GO:0008324	cation transmembrane transporter activity	5	13.51	0,011
	GO:0046873	metal ion transmembrane transporter activity	3	8.11	0,042

* Count denotes the number of genes in our dataset in each cluster. Percent coverage of these genes relative to the numbers on the gene ontology clusters were calculated.

inactivation of Cdc10, which means that cell-cycle dependent responses were inhibited. Thus, this experiment reveals transcriptional regulation exclusively due to UVC irradiation, at a specific stage in the cell cycle. In contrast, in the time-course experiment cells were allowed to progress into the cell cycle. Even though we have subtracted the cell-cycle-regulated genes when identifying the UVC-regulated genes, the cell cycle stage in which the cellular response is analysed was still different from that of cells held in the *cdc10* block. Consistent with this line of reasoning, we have shown that little happened at the transcriptional level during the first 30 minutes after irradiation in the time-course experiment (only 11 regulated genes when comparing UV0 to UV30), and most of the 172 genes changed their transcription enough to satisfy the criteria of our analysis only by a later time point, as the cells entered S phase. Therefore these genes have not been identified as regulated in the restrictive-temperature experiment.

Missing values

In the data for the time-course experiment values were missing for many data points. This can be attributed to at least two reasons: lack of expression of the relevant open reading frames and technical problems with the microarrays. At any one time there are genes that are poorly expressed, so it was expected that there would be some missing values in our datasets. Low expression is the most likely reason for the absent microarray signal for the *rhp4b* transcript, which could also not be detected after RNA blotting. However, lack of expression cannot explain all the missing values. The 12 microarrays used in the time-course experiment comprised from 18% to 55% missing values (on average 35%), arguing that there were technical problems with at least some of the microarrays. Such a high level and difference in the number of missing values was not found for the two repeats of the restrictive-temperature experiment, which both had about 6% missing values, further suggesting technical problems with the arrays used in the time-course experiment. It should be noted that the microarrays used for this experiment came from a different batch/production than those used for the restrictive-temperature experiment. This problem did reduce the quality of the data for some of the genes in the time-course experiment, and we decided to remove all the data pertaining to genes where too many data points were missing (detailed in Results). However, the stringency of our analysis allows us to draw conclusions in spite of the missing data. Furthermore, any technical problem would have affected a random set of genes, and a strong transcriptional response would have been obvious even from the time-course experiment.

Comparisons with other organisms

This is the first report about the global transcriptional response after UVC irradiation in fission *S. pombe* and there are only a few reports about similar experiments in other organisms. The available data indicate that the weak transcriptional response to UVC stress we observed in fission yeast might be a conserved feature. For example, in human cells exposed to UVC only 155 of more than 7500 genes investigated changed their expression more than 2-fold [21]. An early microarray-report using *Escherichia coli* cells identified several differentially expressed genes after UVC-irradiation, but the response was generally not more than two-fold [22].

Many checkpoints, both in fission yeast and in other organisms, involve transcriptional regulation. For instance, in multicellular organisms one of the best characterised checkpoint targets is p53, a transcription factor which is mutated in over half of human cancers. p53 stimulates transcription of cell-cycle inhibitors such as p21 [23] and is essential for a persistent G1 arrest. Another

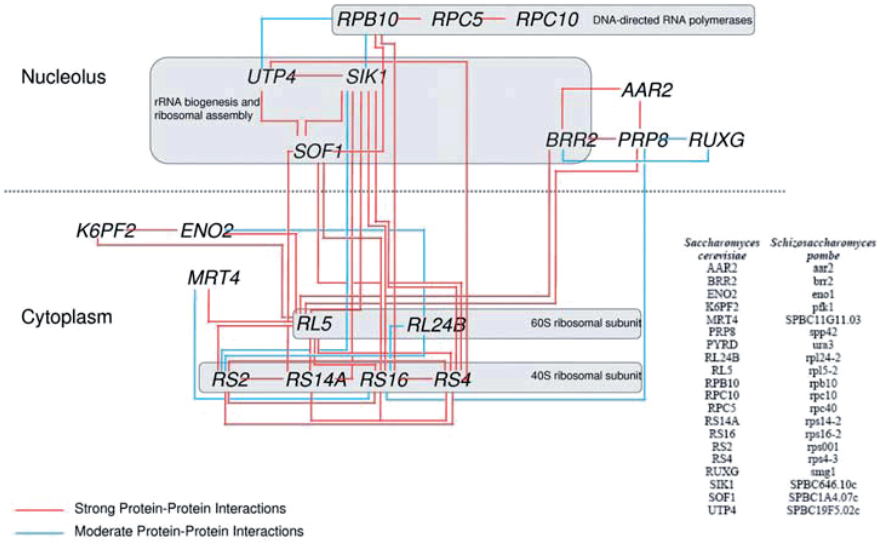

Figure 4
Protein-protein interaction network. Gene products of the regulated genes from the time-course experiment form an interconnected network involving translation and transcription. Protein-protein interactions were analyzed in FunCoup using the corresponding *S. cerevisiae* orthologues (presented in the table on the right). Strong (red lines) and moderate (blue lines) interactions are shown. DNA-directed RNA polymerases, 60S and 40S ribosomal subunits and genes involved in rRNA biogenesis and ribosomal assembly are indicated by grey boxes. The full list of interactions is found in Additional file 7.

tumour suppressor, pRb, targets the E2F-driven transcriptional programme in the G1/S checkpoint [23,24]. In budding yeast activation of the G1/S checkpoint also impinges on transcriptional regulation in that the transcription factor SWI6 is phosphorylated and thereby inactivated by the checkpoint protein RAD53, leading to delayed transcription of CLN1 and CLN2 and a delayed entry into S phase [25]. Recent data show that in fission yeast the transcriptional response is important in the intra-S checkpoint after exposure to hydroxyurea [10,26].

Comparisons with other stress agents

We have observed no obvious change of the transcriptional programme that could be responsible for the G1/S checkpoint and also no strong induction of genes involved in DNA repair or checkpoint function. It is interesting to note that other DNA-damaging agents, such as H_2O_2 and IR, also do not lead totranscriptional induction of many DNA repair- or checkpoint-related genes [3,4]. Therefore, it is likely that there is no need to specifically induce transcription of DNA repair-related genes, suggesting that the DNA repair capacity is high already before the UVC exposure, as the case is for budding yeast [27].

We have compared our data from synchronised cells to data based on H_2O_2- and IR-treated asynchronous cells [3,4], and there is little overlap in the spectrum of non-CESR genes differentially expressed after exposure to the three agents. As discussed above, it seems that the cell-cycle position is important for the transcriptional profile obtained when exposing the cells to a stress treatment. However, when comparing the differentially expressed genes in asynchronously growing cells exposed to UVC (our unpublished observations) to cells exposed to H_2O_2 [3] and IR [4] there is little overlap.

Pathway analyses

DNA-damaging agents, heat and other forms of stress give overlapping responses, described as the CESR, that involves 14% of the genome in *S. cerevisiae* [5]. Few UVC-specific expression changes have been reported [21] and the fold-changes observed are low [28]. It is therefore possible that transcriptional responses to heat stress combined with stringent statistical analyses and exclusion of the CESR-genes mask UVC-relevant gene expression changes in the restrictive-temperature experiment. Similarly, the absence of significant gene-expression changes

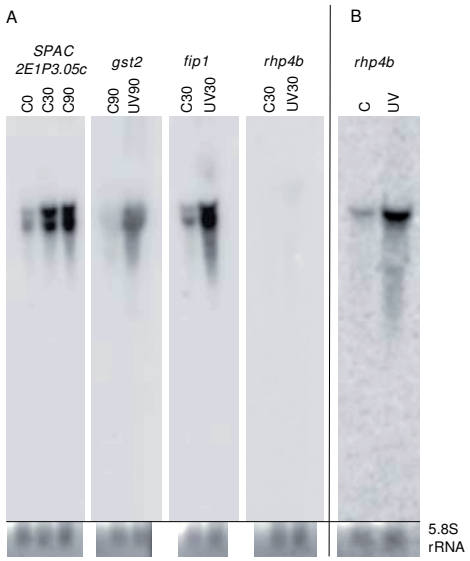

Figure 5
Detection of selected transcripts by RNA blotting and hybridisation. Four different transcripts from different time points and treatments in the time-course experiment (A) and one transcript from the restrictive-temperature experiment (B) were detected as described in Methods. The 5.8S rRNA from the ethidium bromide stained agarose gel before blotting was used as a loading control.

after 30 minutes recovery in the time-course experiment indicates that UVC-relevant transcriptional responses are masked by the small fold-changes and extensive overlap between the UVC-specific response and the CESR. This is supported by appearance of differences on the transcriptional level first after 90 minutes. Our network analyses of gene-expression changes indicate that pathways involved in recovery after DNA damage are induced after 90 minutes (Fig. 4). The processes involved probably reflect the importance of the translation machinery in recovery after the insult. These recovery processes are likely to be important for survival after UVC treatment, as was shown in MMS-treated *S. cerevisiae* [29], although they may not be components of the G1/S checkpoint *per se*.

Regulation of the G1/S checkpoint

In the G1/S checkpoint the Gcn2 kinase is activated to phosphorylate the eukaryotic initiation factor 2α, eIF2α, thereby inhibiting translation [13]. There is a good correlation between eIF2α phosphorylation and checkpoint activation [30], but it is still unclear whether and, if so, how the checkpoint is dependent upon this phosphorylation and on the ensuing downregulation of translation. Gcn2 is best known for its role in the starvation response, where eIF2α phosphorylation leads to induction of the transcription factor GCN4 both in budding yeast and higher eukaryotes. Fission yeast does not have a GCN4 homologue, and it remains to be seen whether the GCN2-dependent G1/S checkpoint in budding yeast [31] involves activation of GCN4. One might expect a transcriptional response after Gcn2 activation also in *S. pombe*, but the finding that no such transcriptional response could be identified argues that in fission yeast the G1/S checkpoint does not operate like it does in budding yeast or higher eukaryotes and is not dependent upon the strong transcriptional induction of one or a set of genes. We have shown that the G1/S checkpoint in *S. pombe* is associated with a strong downregulation of translation and it might be relevant that among the few genes that are affected at the transcription level, 15% affect the translation machinery. Importantly, amongst the transcriptionally regulated genes none were detected that are likely to be directly involved in the G1/S checkpoint according to their annotations.

Induction or inhibition of transcription is a fairly slow response and regulation of the cell cycle in *S. pombe* should preferably occur rapidly in order to be efficient and meaningful. Therefore, it intuitively makes sense that the present data suggest that the G1/S checkpoint is regulated at the level of protein modification and/or translation, which is rapid, rather than a slower regulation of gene expression.

Conclusions

The transcriptional response to UVC irradiation of fission yeast cells in G1 phase was shown to be weak. We conclude that the novel G1/S checkpoint is not regulated by changing the transcriptional programme. This is supported by an examination of the few genes that are induced or repressed by UVC, and none of them appears to have any relationship to cell-cycle regulation.

Methods

Yeast strains and cell growth

The *cdc10-M17* strain is a derivative of the L972 strain [32]. The basic growth media were as described [33]. The temperature-sensitive *cdc10* cells were grown exponentially in EMM to an optical density (595 nm) of 0.15 (about 3×10^6 cells/ml), before they were synchronised by a four-hour temperature shift to 36°C and irradiated with 1100 J/m^2 UVC (254 nm), giving a cell survival of ~15% [14]. Samples of 25 ml of control or UVC-irradiated cells were harvested at different time points by centrifugation and snap-frozen in liquid nitrogen.

RNA isolation

Total RNA was extracted using a hot phenol method [34]. RNA concentrations and qualities were measured in a

NanoDrop (NanoDrop Technologies) and in a 2100 Bioanalyzer (Agilent Technologies).

Microarray hybridization and data acquisition

Total RNA was reverse transcribed (GibcoBRL) in the presence of Cy3- or Cy5-labelled dCTP. The cDNA was hybridised onto glass DNA microarrays containing duplicate probes for 99.3% of all known and predicted open reading frames in the fission yeast genome (for details on protocols and microarrays, see Lyne et al. 2003 and http://www.sanger.ac.uk/PostGenomics/S_pombe). A GenePix 4000 B laser scanner was used for scanning the microarrays before analysis with GenePix Pro software (Axon Instruments). A Perl script was used for removing unreliable signals and normalization of the data [34]. All raw data are available under accession number A-MEXP-1666 and A-MEXP-1667 from ArrayExpress.

Experimental design

Two different types of experiment were performed (see Results) and for both types the RNA from two biological repeats were analysed with a dye swap. Samples from all three time points of the "time-course experiment" were hybridised individually against a reference pool containing equal amounts of RNA from the unirradiated cells, at all the three time points (0, 30 and 90 min). After normalisation (for details see Lyne et al. 2003), the ratio of the values for the actual sample and for the reference pool for each gene was divided by the corresponding ratio for untreated cells at time 0 (0 min control/reference pool). Samples from UVC-irradiated cells kept at 36°C, the "restrictive temperature experiment", were hybridized to the arrays against RNA from unirradiated cells.

Pathway analyses

Genes found differentially expressed in the time-course experiment (Table 4) were analyzed for gene ontology-enriched clusters using DAVID (Database for Annotation, Visualization and Integrated Discovery) http://niaid.abcc.ncifcrf.gov[35,36]. As *Schizosaccharomyces pombe* gene names are not recognized, the gene names were converted to UniProt accession numbers using the YOGY (eukarYotic OrtholoGY) software http://www.bahlerlab.info/YOGY/[37]. The YOGY software was also used to find *Saccharomyces cerevisiae* orthologues for use in the pathway analyses. When several orthologues were found, the *S. pombe* sequence was used as template for a BLAST search and the best hit in *S. cerevisiae* used. As there are very few resources available to investigate functional interactions in *S. pombe*, the *S. cerevisiae* orthologues were used to map protein-protein interactions. These interactions were processed using FunCoup (networks of functional coupling) http://funcoup.sbc.su.se/ [38].

Data evaluation

In both types of experiment genes were classified as differentially expressed when expression values were changed more than twofold (linear values) in both of the two biological repeats. Before statistically analysing the time-course experiment, a filtering of the data was performed. This filtering excluded all genes in the dataset that did not have a value in two-thirds of the arrays (i.e. missing more than 3 out of 12 values). The normalised expression values (see paragraph above) for the remaining genes were transformed from linear values to log2 values. Moderated t-statistics with a P-value cut-off of 0.05 was used to identify genes differently expressed during the time-course [39]. Benjamini and Hochberg's method was used to calculate adjusted P-values and to statistically correct for the occurrence of false positives [40]. The statistical analysis was performed using the programme R and Bioconductor [41]. The Bioconductor package maSigPro was used to perform profile analysis of the time-course experiment to show how gene expression changed with time. Benjamini and Hochberg's method was also used in the profile analysis and the P-value cut-off was set to 0.05.

RNA blots

Total RNA was run on agarose gels in formaldehyde, blotted onto Hybond-XL membranes (Amersham Bioscience) and cross-linked by UVC. Probes were prepared by PCR of genomic DNA and labelled with ^{32}P-d-CTP (Rediprime II Random prime labelling system, Amersham Bioscience). Phosphoimager screens were exposed to the washed blots and analyzed by a Pharos FX scanner (BioRad).

Authors' contributions

HCS carried out all the experiments and drafted the manuscript. ØF and HN performed the pathway and GO analyses. BG and EB contributed in planning the project, in designing the experiments and in writing the manuscript. All authors have read and approved the final version of this manuscript.

Additional material

Additional file 1

A schematic presentation of the experimental design. *Exponentially growing cells were synchronised by a four-hour temperature shift to 36°C. For the time-course experiment cells were UVC-irradiated when shifted back to the permissive temperature and control or irradiated cells were harvested at the time points indicated (black dots). For the restrictive-temperature experiment cells were UVC-irradiated at 36°C after synchronisation, held at the restrictive temperature and control or irradiated cells were harvested at the time point indicated (black dot).*

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[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S1.PDF]

Additional file 2

Flow cytometry histogram from the time-course experiment. *Flow cytometry histograms of control (C) and UVC-irradiated (UVC) G1-synchronised cells incubated for the times indicated (in minutes) after exposure.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S2.PDF]

Additional file 3

UV-repressed genes that are not CESR genes. *44 genes that were repressed more than twofold were indentified and almost all of them (40) were non-CESR genes. These 40 genes were categorised according to the function of their products.*
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[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S3.PDF]

Additional file 4

172 genes changed in the time-course experiment by UV-irradiation. *The cell-cycle-regulated genes were excluded from the 241 genes that were up- or downregulated after UVC, in order to identify the UVC-specific transcripts, resulting in the 172 UVC-regulated genes shown here.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S4.PDF]

Additional file 5

Enriched gene ontology groups in the restrictive-temperature experiment. *Gene ontology (GO) enrichment analysis was performed on the 43 UVC-induced genes in the restrictive-temperature experiment (Table 1) using the DAVID software after ID-conversion. Ten unique genes (20%) were members of enriched GO groups.*
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[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S5.PDF]

Additional file 6

Fission yeast homologues of UPR-induced budding yeast genes. *We used the data from both the restrictive-temperature and timecourse experiments to investigate whether UPR genes are induced by UVC in fission yeast. We identified UPR-genes in fission yeast as the homologues of the UPR-induced budding yeast genes.*
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[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S6.PDF]

Additional file 7

Genes induced in the time-course experiment: protein-protein interactions. *Gene products of the regulated genes from the timecourse experiment form an interconnected network involving translation and transcription. Protein-protein interactions were analyzed in FunCoup using the corresponding* S. cerevisiae *orthologues.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2121-10-87-S7.PDF]

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References

1. Breeden LL: **Periodic transcription: a cycle within a cycle.** *CurrBiol* 2003, **13(1):**R31-R38.
2. Cho RJ, Huang M, Campbell MJ, Dong H, Steinmetz L, Sapinoso L, Hampton G, Elledge SJ, Davis RW, Lockhart DJ: **Transcriptional regulation and function during the human cell cycle.** *NatGenet* 2001, **27(1):**48-54.
3. Chen D, Toone WM, Mata J, Lyne R, Burns G, Kivinen K, Brazma A, Jones N, Bahler J: **Global transcriptional responses of fission yeast to environmental stress.** *Molecular Biology of the Cell* 2003, **14(1):**214-229.
4. Watson A, Mata J, Bahler J, Carr A, Humphrey T: **Global gene expression responses of fission yeast to ionizing radiation.** *Molecular Biology of the Cell* 2004, **15(2):**851-860.
5. Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO: **Genomic expression programs in the response of yeast cells to environmental changes.** *Molecular Biology of the Cell* 2000, **11(12):**4241-4257.
6. Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA: **Remodeling of yeast genome expression in response to environmental changes.** *Molecular Biology of the Cell* 2001, **12(2):**323-337.
7. Caspari T, Carr AM: **DNA structure checkpoint pathways in Schizosaccharomyces pombe.** *Biochimie* 1999, **81(1-2):**173-181.
8. Murakami H, Nurse P: **DNA replication and damage checkpoints and meiotic cell cycle controls in the fission and budding yeasts.** *BiochemJ* 2000, **349(Pt 1):**1-12.
9. de Bruin RA, Kalashnikova TI, Aslanian A, Wohlschlegel J, Chahwan C, Yates JR, Russell P, Wittenberg C: **DNA replication checkpoint promotes G1-S transcription by inactivating the MBF repressor Nrm1.** *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105(32):**11230-11235.
10. Dutta C, Patel PK, Rosebrock A, Oliva A, Leatherwood J, Rhind N: **The DNA replication checkpoint directly regulates MBF-dependent G1/S transcription.** *Mol Cell Biol* 2008, **28(19):**5977-5985.
11. Grallert B, Boye E: **The Gcn2 kinase as a cell cycle regulator.** *Cell cycle (Georgetown, Tex)* 2007, **6(22):**2768-2772.
12. Hanahan D, Weinberg RA: **The hallmarks of cancer.** *Cell* 2000, **100(1):**57-70.
13. Tvegård T, Soltani H, Skjølberg HC, Krohn M, Nilssen EA, Kearsey SE, Grallert B, Boye E: **A novel checkpoint mechanism regulating the G1/S transition.** *Genes and Development* 2007, **21(6):**649-654.
14. Nilssen EA, Synnes M, Kleckner N, Grallert B, Boye E: **Intra-G1 arrest in response to UV irradiation in fission yeast.** *Proceedings of the National Academy of Sciences* 2003, **100(19):**10758-10763.
15. Alao JP, Sunnerhagen P: **Rad3 and Sty1 function in Schizosaccharomyces pombe: an integrated response to DNA damage and environmental stress?** *Molecular microbiology* 2008, **68(2):**246-254.
16. Rustici G, Mata J, Kivinen K, Lio P, Penkett CJ, Burns G, Hayles J, Brazma A, Nurse P, Bahler J: **Periodic gene expression program of the fission yeast cell cycle.** *NatGenet* 2004, **36(8):**809-817.
17. Conesa A, Nueda MJ, Ferrer A, Talon M: **maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments.** *Bioinformatics (Oxford, England)* 2006, **22(9):**1096-1102.
18. Bernales S, Papa FR, Walter P: **Intracellular signaling by the unfolded protein response.** *Annual review of cell and developmental biology* 2006, **22:**487-508.
19. Patil CK, Li H, Walter P: **Gcn4p and novel upstream activating sequences regulate targets of the unfolded protein response.** *PLoS biology* 2004, **2(8):**E246.
20. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P: **Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation.** *Cell* 2000, **101(3):**249-258.

21. Koch-Paiz CA, Amundson SA, Bittner ML, Meltzer PS, Fornace AJ Jr: **Functional genomics of UV radiation responses in human cells.** *Mutation research* 2004, **549(1-2):**65-78.
22. Courcelle J, Khodursky A, Peter B, Brown PO, Hanawalt PC: **Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient Escherichia coli.** *Genetics* 2001, **158(1):**41-64.
23. Bartek J, Lukas J: **Mammalian G1- and S-phase checkpoints in response to DNA damage.** *Curr Opin Cell Biol* 2001, **13(6):**738-747.
24. Lukas J, Lukas C, Bartek J: **Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time.** *DNA repair* 2004, **3(8-9):**997-1007.
25. Sidorova JM, Breeden LL: **Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae.** *Genes & development* 1997, **11(22):**3032-3045.
26. Chu Z, Li J, Eshaghi M, Peng X, Karuturi RK, Liu J: **Modulation of cell cycle-specific gene expressions at the onset of S phase arrest contributes to the robust DNA replication checkpoint response in fission yeast.** *Mol Biol Cell* 2007, **18(5):**1756-1767.
27. Birrell GW, Brown JA, Wu HI, Giaever G, Chu AM, Davis RW, Brown JM: **Transcriptional response of Saccharomyces cerevisiae to DNA-damaging agents does not identify the genes that protect against these agents.** *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99(13):**8778-8783.
28. Heinloth AN, Shackelford RE, Innes CL, Bennett L, Li L, Amin RP, Sieber SO, Flores KG, Bushel PR, Paules RS: **Identification of distinct and common gene expression changes after oxidative stress and gamma and ultraviolet radiation.** *Mol Carcinog* 2003, **37(2):**65-82.
29. Rooney JP, George AD, Patil A, Begley U, Bessette E, Zappala MR, Huang X, Conklin DS, Cunningham RP, Begley TJ: **Systems based mapping demonstrates that recovery from alkylation damage requires DNA repair, RNA processing, and translation associated networks.** *Genomics* 2009, **93(1):**42-51.
30. Krohn M, Skjølberg HC, Soltani H, Grallert B, Boye E: **The G1-S checkpoint in fission yeast is not a general DNA damage checkpoint.** *J Cell Sci* 2008, **121(Pt 24):**4047-54.
31. Menacho-Marquez M, Perez-Valle J, Arino J, Gadea J, Murguia JR: **Gcn2p regulates a G1/S cell cycle checkpoint in response to DNA damage.** *Cell cycle (Georgetown, Tex)* 2007, **6(18):**2302-2305.
32. Kim SM, Huberman JA: **Regulation of replication timing in fission yeast.** *Embo J* 2001, **20(21):**6115-6126.
33. Moreno S, Klar A, Nurse P: **Molecular genetic analysis of fission yeast Schizosaccharomyces pombe.** *Methods Enzymol* 1991, **194:**795-823.
34. Lyne R, Burns G, Mata J, Penkett CJ, Rustici G, Chen D, Langford C, Vetrie D, Bahler J: **Whole-genome microarrays of fission yeast: characteristics, accuracy, reproducibility, and processing of array data.** *BMCGenomics* 2003, **4(1):**27.
35. Huang da W, Sherman BT, Lempicki RA: **Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.** *Nature protocols* 2009, **4(1):**44-57.
36. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA: **DAVID: Database for Annotation, Visualization, and Integrated Discovery.** *Genome biology* 2003, **4(5):**P3.
37. Penkett CJ, Morris JA, Wood V, Bahler J: **YOGY: a web-based, integrated database to retrieve protein orthologs and associated Gene Ontology terms.** *Nucleic Acids Res* 2006:W330-334.
38. Alexeyenko A, Sonnhammer EL: **Global networks of functional coupling in eukaryotes from comprehensive data integration.** *Genome Res* 2009, **19(6):**1107-1116.
39. Smyth GK: **Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments.** *Applications in Genetics and Molecular Biology* 2004, **3(1):**.
40. Benjamini Y, Hochberg : **Yosef Controlling the false discovery rate: a practical and powerful approach to multiple testing.** *Journal of the Royal Statistical Society Series B* 1995, **57(1):**289-300.
41. Gentleman RCCV, Huber W, Irizarry RA, Dudoit S: **Bioinformatics and Computational Biology Solutions Using R and Bioconductor.** Springer, New York; 2005:397-420.