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The role of DNA exonucleases in protecting genome stability and their impact on ageing

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Abstract Exonucleases are key enzymes involved in many aspects of cellular metabolism and maintenance and are essential to genome stability, acting to cleave DNA from free ends. Exonucleases can act as proofreaders during DNA polymerisation in DNA replication, to remove unusual DNA structures that arise from problems with DNA replication fork progression, and they can be directly involved in repairing damaged DNA. Several exonucleases have been recently discovered, with potentially critical roles in genome stability and ageing. Here we discuss how both intrinsic and extrinsic exonuclease activities contribute to the fidelity of DNA polymerases in DNA replication. The action of exonucleases in processing DNA intermediates during normal and aberrant DNA replication is then assessed, as is the importance of exonucleases in repair of double-strand breaks and interstrand crosslinks. Finally we examine how exonucleases are involved in maintenance of mitochondrial genome stability. Throughout the review, we assess how nuclease mutation or loss predisposes to a range of clinical diseases and particularly ageing.

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Introduction

Maintenance of an intact genome is a key requisite both for evolutionary fitness and for health of the individual organism. While cells have evolved protective DNA packaging, such as eukaryotic chromatin, DNA is an active molecule involved in replication and transcription, and it can be modified by direct covalent changes such as CpG methylation during epigenetic regulation. It is also subject to mutation from both internal and external sources. Highly conserved surveillance and repair mechanisms have therefore evolved to keep pace with the daily insults that the genome undergoes. Most, if not all, of these mechanisms require cleavage of the DNA's sugar–phosphate backbone in a controlled and accurate way by enzymes collectively called nucleases. Nucleases act in multiple pathways of DNA metabolism, including during the normal course of DNA replication, providing proofreading capacity to enhance polymerase fidelity, in mismatch repair (MMR), in tackling ends and unusual structures at stalled DNA replication forks, during repair of damaged bases (BER) or larger lesions (NER and recombinational repair) and in telomere maintenance (Fig. 1). Here, we describe the action of exonucleases that are most likely to be

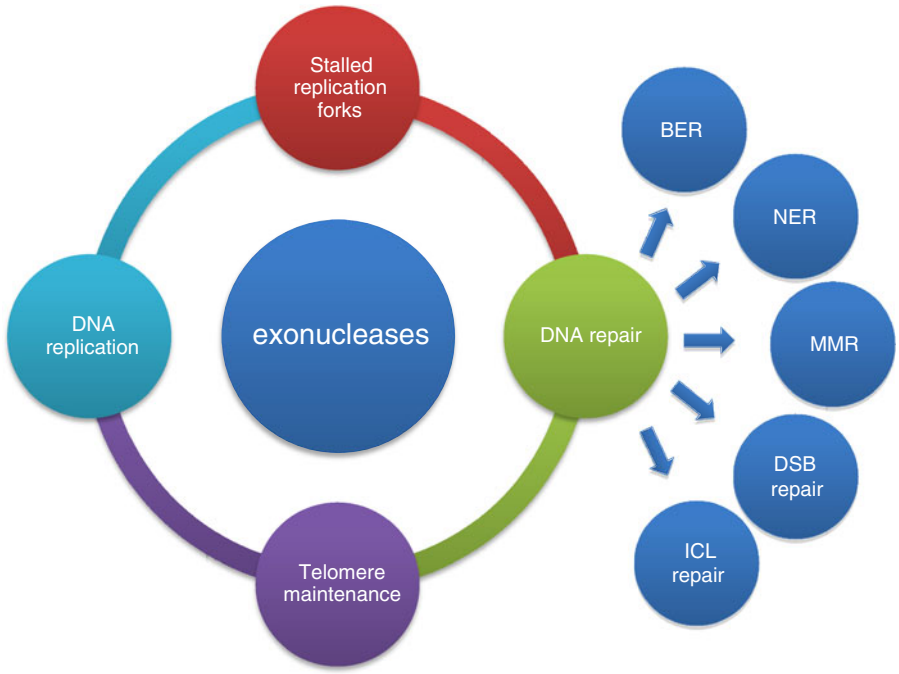

Fig. 1 DNA transactions involving exonucleases. Exonucleases are central to many processes of DNA metabolism. They are important in DNA replication in providing proofreading capacity both intrinsic to the replicative DNA polymerases δ and ε and extrinsic (e.g. ExoN) or autonomous proofreaders (e.g. TREX1 and 2). During the process of Okazaki fragment processing on the lagging strand, nucleases FEN1 and Dna2 (with RNaseH) are important for cleaving the primer and iDNA. Where replication forks stall or collapse, exonucleases are important in resolving aberrant DNA structures to permit fork restart. Such nucleases include the progeroid WRN protein and EXO1. Telomere maintenance also requires the action of exonucleases, possibly also using the same nucleases WRN and EXO1. DNA repair takes many forms depending on the lesion (on one or both strands); nucleases are involved in base excision repair (BER) (e.g. FEN1), in nucleotide excision repair, mismatch repair (e.g. EXO1) and in repair of DNA double-strand breaks (e.g. Mre11). Finally, repair of interstrand crosslinks (ICL) requires the action of a newly discovered nuclease FAN1, which is thought to be recruited by association with the Fanconi anaemia protein complex FANC ID (see "FAN1")

associated with DNA metabolism important in preventing premature ageing (Table 1).

Nucleases are highly evolutionarily conserved and can be classified into families based on both sequence and functional homology. Of particular relevance to this review are the 5′-3′ exo C-terminal domain (CTD) superfamily and the RNaseH domain superfamily, the latter of which includes the DnaQ-like family and other 3′-5 exonucleases (Table 2). While endonucleases cleave DNA internally by cutting the phosphodiester backbone, exonucleases act biochemically to catalyse the removal of a single nucleotide monophosphate (dNMP) from the end of one strand of DNA. A critical characteristic of such nucleases is thus their ability to bind to the relevant DNA substrate, and most exonucleases do so in a non-sequence specific manner, though they generally show some degree of structure specificity. For example, the 5′-3′ exo CTD superfamily share a common helix hairpin helix (HhH) motif (Doherty et al. 1996; Thayer et al. 1995) that permits broad specificity DNA binding, probably through hydrogen bonding between the sugar–phosphate backbone of DNA and the protein backbone.

The functions of exonucleases are many and varied, reflected in the large number of independent nucleases found in many species. For example, of the DnaQ-like family (Table 2), humans possess 25 different family members, mice have 23, and other experimentally relevant organisms also have multiple DnaQ-like exonucleases (seven in fruit flies, eight in

Table 1 Exonucleases discussed in this article

Exonuclease	Polarity	Metabolic pathway	References
Polymerase δ, ε, γ	3′-5′	Replication (proofreading)	Hubscher et al. 2002; Tran et al. 1999; Longley et al. 2001
ExoN	3′-5′	Unknown—proofreading?	Brown et al. 2002
TREX1/TREX2	3′-5′	Aberrant ssDNA removal/proofreading?	Chen et al. 2007; Yang et al. 2007
p53	3′-5′	Multiple; proofreading?	Mummenbrauer et al. 1996
APE1/APE2[a]	3′-5′	BER, replication	Wilson 2003; Burkovics et al. 2006
FEN1[a]	5′-3′	Okazaki fragment processing, BER	Harrington and Lieber, 1994
XPG/ERCC5	5′-3′	NER; transcription stabilisation	Habraken et al. 1994a, b
EXO1[a]	5′-3′	Multiple: MMR, DSBR, meiosis	Fiorentini et al. 1997; Lee and Wilson 1999; Tishkoff et al. 1998; Tsubouchi and Ogawa 2000
WRN[b]	3′-5′	Multiple: DNA repair, telomeres	Cooper et al. 2000; Huang et al. 1998, 2000
Dna2	3′-5′	Replication—Okazaki fragment processing	Reviewed in Budd et al. (2009)
MRE11[c]/RAD50/NBS1	3′-5′	DSBR; replication restart	Buis et al. 2008; D'Amours and Jackson 2002
hRAD9	3′-5′	Checkpoint	Bessho and Sancar 2000; Parker et al. 1998
EXDL2	3′-5′[d]	Unknown—ICLR?	Smogorzewska et al. 2010
FAN1	5′-3′	ICLR?	Kratz et al. 2010; MacKay et al. 2010; Smogorzewska et al. 2010
EXOG[a]	5′-3′	Multiple in mitochondria	Cymerman et al. 2008

BER base excision repair, *DSBR* double-strand DNA break repair, *MMR* mismatch repair, *ICLR* interstrand crosslink repair

[a] EXO1 also has (flap) endonucleolytic activity and 3′-5′ polarity, whilst the major activities of FEN1 and APE1 are as Flap endonucleases. EXOG also has endonucleolytic activity

[b] WRN has 3′-5′ exonuclease and a reported 5′-3′ activity in conjunction with Ku70/86

[c] MRE11 contains the exonuclease activity

[d] EXDL2 exonuclease activity is assumed by homology

Arabidopsis and three in each of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*).[1] Within a single nuclease, there may reside multiple specificities: the human APE1 nuclease has both 3′-5′ exonuclease and abasic-site endonuclease activities, while FEN1 is both a structure-specific flap endonuclease and a 5′-3′ exonuclease. Similarly, the RAD2 domain of human EXO1 contains both 5′-3′ exonuclease and flap endonuclease activities (see Table 1 and references therein).

Nucleases may also be partially or fully redundant, depending upon the pathway, and such redundancy may complement functional losses. For example, in the yeast *S. cerevisiae*, 5′ end resection in double-strand DNA break repair can be catalysed by MRE11/Rad50/Xrs2, Exo1 or Rad27 (yeast FEN1) (Moreau et al. 2001). The general trend observed on nuclease mutation, however, is decreased viability and repair as more exonuclease activities are lost, suggesting overlapping but non identical roles.

[1] See http://supfam.cs.bris.ac.uk/SUPERFAMILY/, which also includes detailed phylogeny.

Nucleases involved in DNA replication

Intrinsic proofreading activity of DNA polymerases

DNA replication is a highly accurate and precise process for copying the genome prior to segregation to daughter cells. Accumulation of errors occurring during DNA replication may contribute to both carcinogenesis and cellular ageing. Accuracy in the first instance depends on high fidelity of the replicative DNA polymerases, resulting from a combination

both of a solvent-inaccessible polymerisation active site that allows 10,000-fold discrimination for correct over incorrect Watson–Crick base-pairing of substrate with template, and editing which involves a 40° shift of the new duplex DNA into the 3′-5′ exonuclease active site for proofreading (Franklin et al. 2001). This results in a base misincorporation rate during DNA replication in yeast of $\sim 1 \times 10^{-5}$ for DNA polymerase δ and approximately double that for DNA polymerase ε (Nick McElhinny et al. 2009). Note that human polymerase ε has a 5.5 fold higher fidelity of nucleotide incorporation (Korona et al. 2010) than its orthologue in yeast.

Replication fidelity rates vary slightly depending upon the base incorporated, suggesting that levels of individual nucleotides in the environment can modulate fidelity (Chen et al. 2000), thus replication stress occurs when nucleotide pools are imbalanced, e.g. following treatment with hydroxyurea. Translesion DNA polymerases can take over from faithful DNA polymerases in cases of DNA damage where correct templating is not possible, but these have solvent-accessible active sites and lack proofreading capacity, leading to very high error rates (Kunkel 2009) and accounting for the highly mutagenic effects of lesions that block the replicative polymerases.

Since the high degree of replicative DNA polymerase accuracy requires intrinsic proofreading activity whereby incorrectly incorporated bases are excised by a 3′-5′ exonuclease subunit of the polymerase (Hubscher et al. 2002), it is not surprising that mutation of the proofreading domains of either pol δ or pol ε increases the error rate for base substitution 12–13-fold in yeast (Nick McElhinny et al. 2009) or sevenfold for mutated human pol ε (Korona et al. 2010). Such loss of fidelity can have major biological consequences, as seen by the development of cancer in mice which have lost the proofreading function of polymerase δ in vivo (Goldsby et al. 2001). While there have yet been no studies directly linking *nuclear* DNA polymerase fidelity loss with ageing,[2] it is notable that polymerase ε is required to prevent replicative senescence in budding yeast that lack telomerase activity (Deshpande et al. 2011). Moreover, instability of the nuclear genome can trigger apoptosis, neoplastic change or senescence, leading to organismal ageing.

Extrinsic proofreaders that act in concert with DNA polymerases

Proofreading during DNA synthesis does not necessarily have to proceed via intramolecular activity of intrinsic exonuclease domains of the DNA polymerase, since priming from a mismatch may be orders of magnitude slower than from paired bases (Huang et al. 1992), allowing time for intermolecular intervention. A number of exonucleases may supply extrinsic proofreading activity for DNA polymerases that do not possess their own capacity such as DNA polymerase α, which synthesises initiator DNA (iDNA) at the start of each new Okazaki fragment, and DNA polymerase β, important in repair. Yeast studies with DNA polymerases bearing point mutations in their exonuclease sites provide evidence that the proofreading component of polymerase δ can lend its exonuclease function to polymerase α to correct errors during lagging strand replication. Mutation of pol δ exonuclease prevented correction of the large number of errors generated by a mutant pol α (L868M which in vivo confers a mutator phenotype), while mutation of pol ε exonuclease had no effect (Nick McElhinny et al. 2006, 2009; Pavlov et al. 2006; Perrino and Loeb 1990).

The high error rate of non-proofreading DNA pol α may also be corrected in vivo by the action of ExoN, a 45.3-kDa 3′-5′ exonuclease with the ability to remove 3′ mismatched termini from double-stranded DNA (dsDNA), including the nucleotide analogues 1-beta-D-arabinofuranosylcytosine monophosphate (araC) and 9-beta-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate. ExoN is reported to interact with DNA pol α and may supply it with proofreading capability (Brown et al. 2002; Perrino and Loeb 1990; Skalski et al. 2000). ExoN upregulation by approximately sixfold in araC-resistant leukemia cells suggests that these cancer cells overcome drug treatment through efficient removal of drug-induced mismatches, and hence ExoN is likely to be a clinically relevant target in cancer (Skalski et al. 2000) and possibly age-related accumulation of genetic damage. It is important to note, however, that the genetic locus of ExoN has not yet been specified, so it might prove to be a known

[2] However, loss of fidelity of the mitochondrial replicative DNA polymerase γ is strongly associated with premature ageing phenotypes in mice—see later.

Table 2 Relationship of nuclease superfamilies, families and individual proteins

S F	IPR002297 DNA-directed DNA-polymerase, family A, mitochondria						IPR020045 5′-3′ exonuclease C terminal domain (CTD)		IPR012547 PD-(D/E)xK	
	IPR012337 RNaseH-like		IPR016265 DNA-directed DNA polymerase, family A, mitochondria, subgroup	IPR001604 DNA/RNA_non sp_Endonuclease	IPR020821 Extracellular Endonuclease subunit A	IPR018524 DNA/RNA non-specific endonuclease active site	IPR004808 ExoDNase_II	IPR004808 ExoDNase III	IPR014808 DNA_replication _fac_Dna2	IPR014883 VRR_NUC
F	IPR013520 Exonuclease, RNase T/DNA polymerase III	IPR002562 3′-5′ exonuclease	IPR006133 DNA-directed DNA polymerase, family B, exonuclease domain				IPR006086 RAD2/XPG family IPR000097 AP endonuclease family 1			
P	TREX1	WRN	Pol delta	Pol gamma	EXOG	ENDOG	FEN1	APE1	Dna2	FAN1
	TREX2	EXDL2	Pol epsilon		ENDOG		EXO1			
							XPG/ ERCC5			
							RAD2			

Note that some proteins (e.g. Fen1) constitute their own family or subgroup

SF superfamily, *F* family, *P* protein (see also http://www.ebi.ac.uk/interpro/)

exonuclease, or possibly a novel isoform or duplication of one already described.

Autonomous proofreaders

Autonomous 3′-5′ exonucleases are, as their name suggests, nucleases that are not necessarily intrinsic to DNA polymerases. They may be fairly non-specific and may act with overlapping functionality depending upon the DNA polymerase involved and the type of proofreading required.

TREX1 and TREX2

The mammalian proteins TREX1 and TREX2 (three prime repair exonucleases) are small homodimeric exonucleases that cleave DNA non-processively from the 3′ end (Hoss et al. 1999; Mazur and Perrino 1999, 2001a, b). The TREX proteins make up the bulk of nuclear 3′-5′ exonuclease activity in mammals (Mazur and Perrino 2001a), suggesting that they serve an important role in metazoan nuclear genome maintenance, possibly acting as autonomous proofreaders (note that there are no TREX homologues in yeast). For example, TREX1 can remove mismatched DNA at a strand end (Mazur and Perrino 2001a), a required activity of a proofreader nuclease during DNA replication.

TREX1, whilst predominantly a cytoplasmic protein, is upregulated via the AP-1/Fos transcriptional pathway and relocated to the nucleus under conditions of genotoxic stress such as UV damage (Christmann et al. 2010). This may reflect both a role for TREX1 in DNA repair and in dealing with damage during DNA replication. However, TREX1$^{-/-}$ null mice show no increase in spontaneous mutation (although induced mutation has not been tested) or increase in cancer, but this might merely show that the nuclear function of TREX1 may be complemented by one of the other autonomous proofreaders, perhaps TREX2.

TREX1 in the cytoplasm appears to degrade single DNA strands arising from processing of aberrant replication intermediates that migrate to the endoplasmic reticulum (ER) after S-phase (Yang et al. 2007). Furthermore, it participates in Granzyme-A-mediated cell death in concert with the NM23-H1 nuclease (Chowdhury et al. 2006), resulting in DNA fragmentation characteristic of apoptosis. As loss-of-function

mutations in the TREX1 gene cause the human disease Aicardi–Goutieres syndrome (a neurological syndrome that has symptomatic overlap with systemic lupus erythematosis—Crow et al. 2006; Lindahl et al. 2009), this suggests that non-degradation of ER/cytoplasmic DNA moieties might lead to immune dysfunction based upon an immune response to self-DNA, particularly if the DNA enters the secretory pathway via the ER. Notably, release of DNA from damaged cells is known to trigger inflammatory responses through 'damage-associated molecular patterns' (DAMPS) which are likely to trigger an innate immune response (Zhang et al. 2010), as such factors closely resemble 'pathogen-associated molecular patterns' (PAMPS) to which the innate immune system is tuned. Indeed, mice null for Trex1 only live for one-fifth as long as wild-type mice and develop inflammatory myocarditis (Morita et al. 2004), consistent with autoimmunity and/or premature ageing, since high levels of inflammation are associated with premature ageing (the 'inflamm-aging' hypothesis e.g. Franceschi et al. 2007). Removal of the aberrant cytoplasmic DNA by TREX1 probably mutes DNA damage signalling and checkpoint activation so that the inflammatory response cannot mount.

Defective TREX1 is associated not only with Aicardi–Goutieres syndrome but also with ataxia telangiectasia-like symptoms (Lindahl et al. 2009) and autosomal dominant retinal vasculopathy with cerebral leukodystrophy (Richards et al. 2007), caused by C-terminal truncation and accompanied by loss of perinuclear localisation (see Table 3). This gives rise to the onset of stroke, dementia, loss of visual acuity and other pathologies prematurely in middle age, with death occurring within 10 years of disease onset (Richards et al. 2007). Pathologies of this type become more common in old age; early onset might thus suggest defects in TREX1 or similar exonuclease activities.

Thus TREX1 has both nuclear and cytoplasmic roles, possibly as an autonomous proofreader in replication but also in removing potential inflammatory triggers. Similarly, human TREX2, which is 44% homologous to TREX1, shows punctate nuclear staining with some cytoplasmic localisation. It is cell cycle regulated, being downregulated during G2/M; like TREX1, its levels are highest in S-phase and lowest at mitosis (Chen et al. 2007). HeLa cells in which TREX2 has been knocked out show reduced cellular proliferation (Chen et al. 2007), suggesting an as yet undetermined role in proliferative DNA metabolism for TREX2 but consistent with action as an extrinsic proofreader.

p53

The tumour suppressor protein p53 has been suggested to possess 3′-5′ exonuclease activity (Mummenbrauer et al. 1996), in addition to its better-characterised roles as a transcriptional activator and an inducer of apoptosis (reviewed in Cox and Lane 1995; Green and Kroemer 2009; Vousden and Prives 2009). The 3′-5′ exonuclease activity of p53 is active on both single-stranded DNA (ssDNA) and dsDNA and has a preference for removing mismatches from replicating strand DNA, while paired bases inhibit the exonuclease activity (Huang et al. 1998). p53 may act by enhancing a further nuclease, AEN (apoptosis enhancing nuclease), to result in the DNA fragmentation seen in p53-dependent apoptosis (Kawase et al. 2008). However, p53 is found to co-localise with DNA synthesis during S-phase and might itself provide proofreading functionality for DNA polymerase α, since it enhances the replication fidelity of error-prone pol α but not of high-fidelity proofreading pol ε (Hollstein et al. 1996). Thus a novel way in which p53 might safeguard the genome is by increasing replication fidelity when necessary, for example under conditions of genomic stress following genotoxic damage. It is notable that over half of all human cancers have p53 mutations, frequently in the DNA binding and exonuclease domain of the protein.[3] p53 is also known to be critical in establishing cell senescence, though it is likely that this is primarily through its transcriptional activation of p21 (El-Deiry et al. 1993; Noda et al. 1994) rather than through its nuclease activity.

APE1 and APE2

A novel class of proteins that may also act as autonomous proofreaders are the AP endonucleases. There are four types of AP endonucleases, based upon the sites of incision: class I and class II incise such that 3′-hydroxyl and 5′-phosphate ends arise. Class III and class IV instead generate the reverse—a 3′-phosphate

[3] See http://p53.free.fr.

Table 3 Clinical features associated with exonuclease mutation

Protein	Disease	Notes
Polymerase proofreader	Cancer	Lack of proofreading by replicative or repair polymerases leads to increased DNA mutation and cancer predisposition
TREX1	Aicardi–Goutieres syndrome, lupus, Cree encephalitis	Deleterious DNA mutation leads to immune system dysfunction by disruption of the inflammatory response
p53	Cancer	Most p53 mutations found in cancers are in the DNA-binding core region, which contains the exonuclease activity, suggesting that loss of the proofreading functionality promotes cancer via mutation (cf. polymerase proofreaders)
APE1	Cancer	Deficient BER causes genome instability and predisposition to cancer
FEN1	Cancer, autoimmunity, chronic inflammation, possibly trinucleotide repeat expansion disease	Mutator phenotype from nuclease mutation (compromised OFP and BER) can cause cancer; chronic inflammation resulting from FEN1 dysfunction promotes cancer progression. Trinucleotide repeat expansion on FEN1 mutation in yeast and mouse models; role in repeat expansion less clear in humans (Huntington's disease triplet repeats stable in absence of FEN1 nuclease) though possibly implicated in e.g. Friedreich's ataxia
XPG/ERCC5	Xeroderma pigmentosum (XP), Cockayne syndrome (CS), trichothiodystrophy (TTD), cancer	Dysfunctional NER leading to sensitivity to sunlight (UV) and increased genome instability. Some mutations can cause symptoms of Cockayne syndrome or TTD (premature ageing, neural and physical defects, skin and hair abnormalities). Note that mutation of the related XPF (endonuclease) results in severe progeroid phenotypes
EXO1	Cancer, immune deficiency	Cancer from compromised mismatch repair (HNPCC: human non-polyposis colon cancer). Compromised immunoglobulin production owing to lack of class switching and somatic hypermutation
WRN	Werner syndrome (WS)	Segmental adult-onset progeroid syndrome resulting from loss of the RecQ helicase/exonuclease WRN, which functions in multiple genome stability pathways (notably BER, replication restart and telomere maintenance); causes many symptoms associated with normal ageing to present early (for example cancer, atherosclerosis and heart disease)
DNA2	None described	Defective DNA replication might cause genome instability and a rise in cancer predisposition
MRE11 (as part of MRN)	Nijmegen breakage syndrome (NBS), ataxia telangiectasia-like (AT-L)	A member of the MRN complex (with NBS1 and Rad50) and associates with ATM/ATR. Compromised DSBR and HJ resolution causes microcephaly and other physical defects, immunodeficiency, UV sensitivity and lymphoma predisposition
hRAD9	None described	Loss of the RAD9 DNA damage checkpoint might cause aberrant cellular division/loss of apoptosis, presumably allowing cellular dysfunction and possible cancer predisposition
FAN1	Fanconi's anaemia (FA)	Nuclease associated with FA; symptoms are early cancer (mainly acute myelogenous leukaemia) and bone marrow failure. Also associated with congenital defects, including physical and developmental disabilities, and early death
EXDL2	None described	Putative association with cancer (see FAN1); may have a mitochondrial DNA maintenance function

Table 3 (continued)

Protein	Disease	Notes
POLG	Premature ageing and multiple clinical features (see notes)	Mutation of mitochondrial polymerase gamma proofreading exonuclease leads to accelerated ageing symptoms in mice. The human mitochondrial genome is densely coded meaning that any base substitution errors from loss of proofreading have a high chance of disrupting mitochondrial genes and hence can cause neuromuscular disorders through disruption of ATP production, e.g. Leigh's syndrome, NARP, MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy with ragged red fibres), LHON (Leber's hereditary optic neuropathy)
EXOG/ENDOG	None specifically associated (see notes)	DNA editing and processing defects give rise to mitochondrial DNA instability and may cause mitochondrial diseases as listed above (see POLG)

and a 5′-hydroxyl (Myles and Sancar 1989). Human APE1 belongs to the most common class II. Both APE1 and APE2 (Hadi and Wilson 2000) have been shown to possess specific, non-processive but nevertheless possibly important abilities to cleave 3′-termini from DNA. APE1 removes nucleotides from matched, mismatched and beta-*l*-dioxolane-cytidine nicked DNA (Chou and Cheng 2002, 2003), with a marked preference for 3′-mismatched and nicked substrates. APE2 also has a preference for removal of mismatched bases (Burkovics et al. 2006). It remains to be determined whether and how much these proteins contribute to proofreading during replication or whether they utilise this functionality only within the context of DNA repair.

Replication fidelity is enhanced through nucleases acting in mismatch repair

In addition to proofreading by intrinsic or extrinsic exonucleases, a further mechanism that can increase replication fidelity by up to 1,000-fold involves the mismatch repair (MMR) machinery. Errors in mismatch repair are linked to microsatellite instability and cancer. Eukaryotic MMR requires not only the well-characterised MSH (MutS homologue) proteins which survey the genome for both mismatches and small insertion/deletion loops but also exonucleases such as EXO1 (see Hsieh and Yamane 2008 and references therein).

Eukaryotic EXO1 is a member of the Rad2/FEN1 family of nucleases (Lee and Wilson 1999) (Table 2) and is one of four nucleases that may be involved in MMR, although it is not (as originally reported) an orthologue of the *Escherichia coli* MMR protein EXO1 (Genschel et al. 2002; Tishkoff et al. 1997, 1998). There are some reports that loss of EXO1 gives rise to one form of the MMR-deficient syndrome human nonpolyposis colon cancer, HNPCC (Wu et al. 2001), but a direct association has been questioned (Thompson et al. 2004). Eukaryotic EXO1 is involved in various DNA metabolic pathways other than MMR, including meiosis (Fiorentini et al. 1997) and recombination (Tsubouchi and Ogawa 2000). EXO1 may also play a role in resecting ends at broken replication forks to prevent generation of recombinogenic intermediates.

Human EXO1 has 5′-3′ activity on both dsDNA and ssDNA, although its activity is greater on 3′-overhang and blunt duplex substrates, including those containing gaps or nicks than on 5′ overhang duplex or ssDNA (Lee and Wilson 1999). It can also degrade RNA in vitro. EXO1 is also involved in 3′-nick directed repair, suggesting either that it might have a cryptic 3′-5′ activity or that it is required to activate another as yet uncharacterised 3′-5′ nuclease (Genschel et al. 2002). EXO1 has been shown to resect DNA in vitro, with DNA affinity increased by the RecQ helicase BLM and loading and processivity by the MRN complex and RPA (Nimonkar et al. 2011). In yeast, the flap endonuclease activity of EXO1 has been suggested to be a functional backup for the major Okazaki fragment processing flap endonuclease FEN1 (see "FEN1"), again showing the overlapping redundancies of many

cellular nucleases. Moreover human EXO1 can complement its yeast homologue (Qiu et al. 1999).

Notably, it has recently been shown that a SNP in the EXO1 promoter leading to higher levels of EXO1 expression is enriched in female centenarians (Nebel et al. 2009), suggesting a possible role for EXO1 activity in prevention of premature ageing, possibly by reducing cancer risk. By contrast, *deletion* of the nuclease activity of EXO1 in short-lived late-generation telomerase-deficient mice rescues lifespan (Schaetzlein et al. 2007). This rather unexpected finding may result from repression of the cyclin-kinase inhibitor p21, which leads to the increased lifespan in terc$^{-/-}$ mice, as observed in mice lacking other components of MMR such as PMS2 (Siegl-Cachedenier et al. 2007) and which directly phenocopies p21$^{-/-}$ Terc$^{-/-}$ mice (Choudhury et al. 2007). Thus it is possible that rescue of longevity upon EXO1 loss in telomerase null mice was effected by suppression of the DNA damage response checkpoint—perhaps via lack of EXO1 end-processing and recruitment of downstream response elements—to allow proliferation in cells with uncapped telomeres and other DNA damage. Taken together, these data might suggest that upregulation of EXO1 is only useful for long lifespan in the presence of robust telomeres and DNA proofreading/repair.

Processing DNA intermediates at DNA replication forks

During the maturation step of DNA replication, Okazaki fragments on the lagging strand of the replication fork must be processed to remove both the RNA primers generated by primase and the iDNA synthesised by error-prone DNA pol α, prior to ligation into high molecular weight DNA. Several models of Okazaki fragment processing (OFP) have been proposed (reviewed in Budd et al. 2009), all requiring exonucleases, particularly the flap endonuclease/exonuclease 1, FEN1. The DNA flaps and similar structures arising during OFP are likely also to be formed during DNA lesion processing, and the nucleases important in OFP have also been shown to play roles in DNA repair.

FEN1

FEN1 is a structure-specific endo/exonuclease originally cloned from mouse cells (Harrington and Lieber 1994) and subsequently humans (Hiraoka et al. 1995) and is essential for DNA replication in vitro (Waga et al. 1994). The FEN1 homologue in budding yeast is encoded by the *rad27* gene. It acts during Okazaki fragment processing in DNA replication to remove the terminal deoxyribonucleotide (or ribonucleotide) by endonucleolytic cleavage at the elbow of DNA junctions bearing a 5′ flap generated by incoming DNA pol δ on the lagging strand (Budd et al. 2009). In addition, FEN1 can act exonucleolytically in a 5′-3′ direction on flap substrates.

Recruitment of FEN1 to sites of exonuclease action involves direct binding to the sliding clamp PCNA through a conserved PCNA-interacting motif (PIP, Cox 1997; Warbrick et al. 1997), the interaction positioning FEN1 to act preferentially as an exonuclease rather than an endonuclease (Hosfield et al. 1998a, b). The mode of action has been elucidated by X-ray crystallography, showing that association with PCNA allows flexibility around a hinge region in FEN1 that brings the enzyme's nuclease active site in direct contact with the DNA substrate (Sakurai et al. 2005).

Furthermore, FEN1 has recently been crystallised both alone (Sakurai et al. 2008; Tsutakawa et al. 2011) and in association with the Rad9–Rad1–Hus1 (9-1-1) DNA damage checkpoint complex (Dore et al. 2009). Analysis of the structures of FEN1 (Tsutakawa et al. 2011) and EXO1 (Orans et al. 2011), which were solved at similar times, suggest a unified mechanism for this type of nuclease involving DNA bending and two nucleotide 'fraying' of one strand (Orans et al. 2011).

Mutation of FEN1's nuclease active site (E160D) makes cells more susceptible to chemically induced cancers (Xu et al. 2011). Loss of only one copy of FEN1 increases colon cancer incidence in mice heterozygous for mutation of the Apc tumour suppressor, with Fen1 halpoinsufficiency leading to rapid tumour progression (Kucherlapati et al. 2002). FEN1 also shows gap endonuclease (GEN) activity, which is critical for the resolution of stalled replication forks following DNA damage (Zheng et al. 2005). Notably, these authors showed that a mutation in FEN1 that abolished GEN activity (E178A) removed FEN1's ability to rescue camptothecin and UV sensitivity in a yeast Fen1 mutant.[4] Moreover, transgenic mice

[4] Note that the related human GEN1 (yeast Yen1) appears to act exclusively as an endonuclease (Ip et al. 2008, *Nature* 456:357–361) and so is not discussed in this review.

bearing a point mutation in FEN1 that abolished the majority of 5′ exonuclease and GEN activity developed significant inflammation and autoimmunity, together with high cancer incidence (Zheng et al. 2007). An inability to recruit FEN1 to sites of action through mutation-critical aromatic residues within its PCNA-interacting peptide (F343A F344A) (Warbrick et al. 1997) also results in loss of DNA processing and aneuploid cancers (Zheng et al. 2011).

Mutational studies in yeast have suggested that FEN1 restrains recombination between short DNA sequences (Negritto et al. 2001) such as the flaps arising during strand displacement DNA synthesis on the lagging strand of the replication fork or during DNA repair. These findings are also consistent with the triplet repeat expansion phenotype observed on FEN1/Rad27 mutation (Liu et al. 2004; White et al. 1999) and with its involvement in long patch BER along with its partner PCNA (Frosina et al. 1996). (Note that FEN1 is important for removal of oxidative damage in both the nucleus (Asagoshi et al. 2010) and in mitochondria (Liu et al. 2008)). Mutation of pol ε in a Rad27 null background led to elevated +1 frameshifts on homonucleotide runs (Kirchner et al. 2000) suggesting a role for FEN1 either in mismatch repair or proofreading, in addition to its roles in OFP and BER. Notably, mutation of FEN1 leads to decreased yeast lifespan (Hoopes et al. 2002). Hence loss of function of FEN1 results in a mutator phenotype that predisposes to cancer and signs of premature ageing.

WRN

WRN is a multifunctional replication/repair RecQ-helicase family member (Brosh et al. 2001b) that in deficiency causes the premature ageing Werner's syndrome, an adult-onset progeria phenocopying early many normal ageing diseases such as atherosclerosis, cancer and cataracts. WRN association with FEN1 may be important in Okazaki fragment processing during DNA replication as FEN1's nuclease activity is stimulated by binding to the helicase domain of WRN (Brosh et al. 2002a).

Unlike other RecQ helicases, in addition to helicase activity, WRN has a well-characterised 3′-5′ exonuclease domain (Huang et al. 2000) which is highly conserved and of the DnaQ exonuclease family (Perry et al. 2006) (Table 2). In *Drosophila*, and other invertebrate and plant species, the exonuclease appears as an autonomous protein (Boubriak et al. 2009; Cox et al. 2007; Plchova et al. 2003; Saunders et al. 2008; Sekelsky et al. 1999).

While WRN exonuclease acts on both single-stranded (Machwe et al. 2006) and duplex DNA with overhanging ends, it is also active on substrates that resemble either DNA replication intermediates or structures found during the processing of DNA damage (Brosh et al. 2002b). However, in vitro without other factors, WRN cannot cleave blunt-ended duplex DNA. In DNA replication, WRN is implicated in processing unusual DNA structures that, if left unprocessed, would result in replication fork regression and formation of recombinogenic intermediates including Holliday junctions (HJs). This is supported by the accumulation of stalled replication forks in cells lacking WRN protein (Rodriguez-Lopez et al. 2002; Sidorova et al. 2008), and it is likely that this reflects a need for the nuclease activity of WRN, since many of the defects observed in WS cells, including reduced proliferative capacity, low S-phase fraction and drug sensitivity, can be corrected by ectopic expression of a bacterial Holliday junction resolvase (Rodriguez-Lopez et al. 2007). Recruitment of WRN to stalled replication forks may occur through binding to PCNA via a classical PIP (Lebel et al. 1999; Rodriguez-Lopez et al. 2003).

Thus WRN may be involved in directing the appropriate pathway during recombinational repair, for example in response to collapsed replication forks or other sources of double-strand breaks (Plchova et al. 2003; Rodriguez-Lopez et al. 2002; Saintigny et al. 2002; Swanson et al. 2004). It may, like other RecQs such as yeast Rqh1 (in combination with EXO1 or the cross-over junction nuclease MUS81-EME1) (Doe et al. 2002), direct non-cross over pathways, perhaps by removing a potentially invasive DNA strand arising from replication fork stalling or dissociation of leading and lagging strand polymerases. Indeed, *Rqh1* null yeast are hyperrecombinant, while ectopic expression of human WRN in such cells represses recombination (Yamagata et al. 1998). That this suppression of recombination results from the DNA exonuclease activity of WRN is supported by the finding that fruit flies deficient in the orthologue of human WRN exonuclease, DmWRNexo, show extremely high levels of mitotic recombination (Saunders et al. 2008) and marked developmental abnormalities

(R. Lasala et al., manuscript in preparation). The DNA-end binding Ku70/86 dimer specifically and strongly stimulates WRN's exonuclease activity (Comai and Li 2004; Li and Comai 2002; Li et al. 2004, 2005), changing its specificity to allow degradation of blunt and even 5′-protruding ends (Cooper et al. 2000), suggesting a particular role for WRN during Ku-directed repair such as non-homologous end joining (NHEJ) or in an alternative pathway to degrade free ends at broken replication forks.

WRN exonuclease is implicated in other DNA metabolic pathways in addition to its likely role as an accessory protein at the replication fork. For example, WRN may play an important role at the telomere, including telomeric D-loop resolution (Opresko et al. 2004). It is likely that this requires intramolecular co-operation between the intrinsic helicase and the exonuclease activities of WRN such that the helicase unwinds unusual DNA structures (including G4 quadruplexes which mimic telomeric structures) and is followed by exonuclease activity to process the resulting ends. Indeed, a K577M point mutation within the helicase domain has a dominant negative effect on any subsequent exonuclease activity and prevents lagging strand Okazaki fragment processing at the telomere (Crabbe et al. 2004). (Note that WRN also binds to telomere proteins POT1 and TRF2 (Opresko et al. 2002, 2005)). Interestingly, WRN also interacts physically with and stimulates EXO1 (Aggarwal et al. 2010; Sharma et al. 2003), a nuclease that is implicated both in Okazaki fragment processing and in telomere processing. This interaction may be important for either WRN or EXO1, or both, to process stalled or regressed replication forks and to maintain the T and D loops at telomeres. Loss of telomere integrity drives replicative senescence and ageing, and prolonged replication stress can similarly result in 'deep' senescence and contribute to organismal ageing.

WRN exonuclease can also function as an extrinsic proofreader for DNA polymerase β during base excision repair (BER) (Harrigan et al. 2006). Studies based upon the X-ray crystal structure of human WRN exonuclease domain give a firm assignment of editing functionality (Perry et al. 2006), akin to end-processing activities of other members of the DnaQ family. Moreover, deletion of the exonuclease domain results in cells that are hypersensitive to DNA damage (Kashino et al. 2005). Confusingly, p53, itself a nuclease, strongly inhibits the exonuclease activity of WRN (Brosh et al. 2001a), highlighting the complex synergistic/antagonistic interplay between cellular nucleases. Since loss of WRN causes the segmental progeroid Werner's syndrome (Huang et al. 1998; Yu et al. 1996), this provides a direct evidential link between genome instability and ageing.

Dna2

Dna2 is a combined helicase/nuclease that interacts with FEN1 and is likely to be involved in Okazaki fragment processing, as yeast cells with a mutant allele of *Dna2* (*dna2-1*) can only synthesize short stretches of DNA under restrictive conditions (Budd et al. 2009). It is likely that Dna2 is recruited to process Okazaki fragments under conditions when long flaps arise during pol δ-mediated strand displacement of the primer DNA and iDNA, as these flaps become coated with RPA and resistant to cleavage by FEN1. Indeed, recruitment of Dna2 is likely to occur through direct interaction with RPA. Exonuclease cleavage of the flap DNA by Dna2 eventually results in flaps short enough to be processed by FEN1 (reviewed in Kao and Bambara 2003; MacNeill 2001; Budd et al. 2009).

In addition to a helicase domain in the C-terminus, Dna2 possesses 3′-5′ exonuclease activity with further 5′ flap endo-exonuclease activity (Fortini et al. 2011) present in the amino terminal half of the protein. The gene is itself essential with deletion mutants being inviable, and yeast cells mutant for both Fen1 and Dna2 are inviable (dna2-1 rad27Δ), while Fen1 overexpression can complement Dna2-1 phenotypes (reviewed in Budd and Campbell 2009), although Dna2 stimulates Fen1's activity (Kao et al. 2004). Consistent with a role in preventing ageing, presumably by ensuring genome stability especially of the repetitive ribosomal DNA (rDNA) or at the telomeres, Dna2 mutants in yeast have a short lifespan (Weitao et al. 2003a, b), and similarly, *Caenorhabditis elegans* lacking worm Dna2 show compromised genome maintenance and shortened lifespan (Lee et al. 2011).

The human homologue of Dna2 is also a helicase/nuclease, which does not have an obvious nuclear localisation signal but in deficiency causes nuclear genome instability (Duxin et al. 2009) and co-localises with DNA (nucleoids) in the mitochondrion. It interacts with and stimulates mitochondrial DNA polymerase γ,

and functions with FEN1 in mitochondrial long-patch BER (Copeland and Longley 2008; Zheng et al. 2008). In vitro reconstituted DSB repair complexes show that in the nucleus, end resection proceeds via two pathways (Nimonkar et al. 2011), one of which involves human DNA2 nuclease activity and the RecQ helicase activity of BLM, stimulated by RPA. RPA also ensures that DNA2 exonuclease activity occurs in the correct 5′-3′ polarity. The MRN complex is found to stimulate activity by recruiting BLM to the site of resection (Nimonkar et al. 2011). DNA2 therefore probably suppresses genome instability via both DNA repair (BER, DSB repair) and recombination pathways.

Nucleases in nucleotide excision repair and transcription

Bulky lesions such as UV-induced pyrimidine dimers that prevent replication fork progression or transcription elongation are generally removed through the nucleotide excision repair (NER) pathway, involving many proteins of the Xeroderma pigmentosum (XP) complementation group. Nucleases including XPG[5] are required to make incisions either side of the lesion, hence mutation of the *incision* activity of XPG gives rise to symptoms of XP (Tian et al. 2004), conferring sensitivity to UV damage and consequently great susceptibility to epithelial cancers via defects in nucleotide excision repair (NER) (Table 3). By contrast, total absence of XPG protein causes symptoms of Cockayne's syndrome (Arenas-Sordo Mde et al. 2006), with developmental defects and premature ageing. These phenotypes are thought to arise through loss of XPG's role in stabilising transcription, rather than its activity in DNA repair (Friedberg and Wood 2007). Hence XPG mutation can give rise to various symptoms of both CS and XP including premature ageing (Friedberg and Wood 2007; Wijnhoven et al. 2007). In addition to its incision activity (O'Donovan et al. 1994; Habraken et al. 1994b), XPG belongs to the FEN1/RAD2 family because of its conserved 5′-3′ exonuclease activity (Habraken et al. 1994a, see Table 2). Of note, XPG has recently been shown to interact with the WRN helicase/exonuclease (Trego et al. 2011) via the C-terminal domain of both. XPG co-localises with WRN during S-phase and stimulates its helicase activity (Trego et al. 2011). Whether this association is important in preventing premature ageing is not yet clear.

[5] also known as ERCC5

Exonucleases involved in double-strand DNA break repair

Mre11

The first nuclease recruited in repair of DNA double-stranded breaks is MRE11 (Stracker et al. 2004), a component of the MRN complex (Mre11–Rad50–Nbs1) which plays a key role early in the DNA damage response (DDR) (Berkovich et al. 2007; Lisby et al. 2004; Mirzoeva and Petrini 2001). MRN detects and localises rapidly to DSBs after damage and secures and shields the frayed DNA ends (Williams and Tainer 2005) whilst activating checkpoint signalling via ATM (Lee and Paull 2007; Williams and Tainer 2005). Two Mre11 and two Rad50 molecules form a heterotetramer that interacts with Nbs1 (Xrs2 in yeast) and can bind both ends of a DSB, although Mre11 can itself form homomultimers (reviewed in Budd and Campbell 2009). Yeast and human Mre11 have multiple nuclease activities (Furuse et al. 1998; Moreau et al. 1999; Paull and Gellert 1998) and in addition have helicase-like activities in that they can cause both strand-annealing (de Jager et al. 2001) and strand dissociation.

Mre11 has 3′-5′ dsDNA exonuclease activity in vitro, as well as dsDNA and ssDNA endonuclease activity, but is specific for blunt or 3′-recessed ends, or, very weakly, for hairpin structures (D'Amours and Jackson 2002). Whilst the 3′-5′ exonuclease activity is necessary for DSB repair (Paull and Gellert 1998), experiments on *S. cerevisiae* mutants show that functions of Mre11 are separable and distinct, with the N-terminal nuclease domain required for DSB repair and the C-terminal dsDNA-binding domain needed for meiotic functions such as chromatin modification (Furuse et al. 1998).

Although removal of the nuclease activities of Mre11 in yeast results in only slight radiation sensitivity (Moreau et al. 1999), Mre11 mutation in mammals results in more severe phenotypes. Inherited hypomorphs cause ataxia telangiectasia-like symptoms such as extreme sensitivity to IR and cerebellar

degeneration (Stewart et al. 1999, see Table 3), even though the nuclease-dead Mre11 mutant retains the ability to associate with the other members of the MRN complex and to activate ATM (Buis et al. 2008). This suggests that Mre11 nuclease activity may be more important in mammals than in yeast, and this importance is not based upon the DNA damage checkpoint acting through ATM but occurs via other roles of Mre11. It is possible that Mre11 is involved in preventing replicative senescence caused by telomere attrition, since in yeast it has been implicated in telomere maintenance acting as a block to replicative senescence (Joseph et al. 2010). Additionally, defective DSB repair has been reported in the progeroid Hutchinson Gilford progeria syndrome (HGPS), with a delay in localization of Mre11 to sites of DSBs suggesting that defects in Mre11 targeting may be associated with premature ageing characteristic of this syndrome (Constantinescu et al. 2010). Hence Mre11 nuclease activity may be necessary to prevent premature ageing.

hRAD9

The hRAD9 protein was discovered by homology to the *S. pombe* rad9 protein that is involved in the early DNA damage response checkpoint. Modified hRAD9, along with hRAD1 and hHus1, form the human 9-1-1 complex (Volkmer and Karnitz 1999), a heterotrimeric clamp complex structurally (Xu et al. 2009) and functionally analogous to PCNA that is loaded onto damaged DNA tracts (Burtelow et al. 2000; Carr 2002) by an RF-C like complex and is hyperphosphorylated in a damage-dependent manner, for example by ATM after ionising radiation (IR) (reviewed in Cox and Kearsey 2009). Such phosphorylation is required for IR-induced G1/S checkpoint activation (Chen et al. 2001a) but also after hydroxyurea-induced replication stress or UV-induced DNA damage. hRAD9 has 3′-5′ exonuclease activity (Bessho and Sancar 2000); as hRAD9 quickly localises to double-strand breaks, this suggests that the nuclease activity is important during early DNA processing. The 9-1-1 complex can also bind to and stimulate FEN1 nuclease activity (Wang et al. 2004) and also interacts with and enhances the function of DNA polymerase β, although not pol δ nor pol α (Toueille et al. 2004). hRad9 alone (not as part of the 9-1-1 complex) is also implicated in numerous other pathways; for example in ribonucleotide synthesis via stimulation of the carbamoyl phosphate synthetase activity of CAD (Lindsey-Boltz et al. 2004) and in apoptosis by interaction with members of the Bcl-2 family of proteins (Komatsu et al. 2000). Mouse rad9 nulls are not viable (Hopkins et al. 2004) presumably because Rad9 has so many different roles that there cannot be full complementation of all its activities. The exact role(s) of hRad9 nuclease activity has not yet been elucidated, nor its link with ageing, but in the nematode worm *C. elegans*, the 9-1-1 complex is implicated in telomere maintenance (Meier et al. 2006). Whether this is the case in higher organisms with an overt effect on ageing has yet to be determined.

Interstrand crosslink (ICL) repair

Interstrand crosslinks (ICLs) are chemical links between the bases (or backbones) on opposite strands of duplex DNA. They block progress of the replication fork, causing stalling; ICLs must therefore be removed in order for cells to proliferate and for their long-term survival. Deficiencies in ICL repair give rise to Fanconi's anaemia (FA), a genome instability syndrome characterized by increased sensitivity to agents that promote ICLs in DNA (e.g. mitomycin C), and FA patients show many gross phenotypic abnormalities (Auerbach 1995), with greatly elevated predisposition to cancer (Alter 1996) associated with marked chromosomal instability. The FA protein core complex monoubiquitinates the ID complex comprising FANCI and FANCD2 (Garcia-Higuera et al. 2001), which localises to BRCA-containing nuclear DNA repair foci along with the recombination protein RAD51 (Taniguchi et al. 2002) and promotes removal of the DNA tract containing the crosslink.

FAN1

Short hairpin (shRNAi) screens to identify novel factors that confer resistance to ICL-inducing mitomycin C very recently identified FAN1 (Fanconi-associated nuclease 1) as a novel but important exonuclease involved in the ICL repair pathway (Smogorzewska et al. 2010). Furthermore, FAN1 was independently described as a putative nuclease that interacts with FANCD2. Absence of FAN1 was

found to increase cellular sensitivity to ICL-inducing agents such as mitomycin C and cisplatin (Liu et al. 2010; MacKay et al. 2010).

FAN1 is a protein containing an N-terminal ubiquitin-binding domain and a C-terminal VRR_nuc nuclease domain (ancient lineage, see Table 2) and localises to interstrand crosslinks (ICL) along with monoubiquitinated FANCD2 (Kratz et al. 2010; Liu et al. 2010), a process requiring its ubiquitin-binding domain. Under mitomycin C treatment, FAN1 co-localises with FANCD2 at sites of damaged DNA, but since FAN1 depletion does not affect the ability of FANCD2 to become monoubiquitinated or localise to damage, FAN1 is likely to act downstream of, and may be recruited to sites of damage by, monoubiquitinated FANCD2.

Purified FAN1 is a structure-specific endonuclease capable of cleaving branched structures such as replication fork-like structures, splayed DNA arms and 5′ or 3′ flaps (Liu et al. 2010), but not duplex DNA. Like FEN1, FAN1 is also a 5′-3′ exonuclease as well as a flap endonuclease. Monoubiquitination of FANCD2—and thus the concomitant recruitment of FAN1 to damage foci—is required for the 'unhooking' step in removal of the ICL (Knipscheer et al. 2009). During removal, dsDNA excisions generating double-strand breaks are made either side of the crosslink in order to remove the crosslinked portion of the DNA. At least one of these incisions is catalysed by MUS81-EME1, a heterodimeric endonuclease (Ciccia et al. 2003) that can cleave branched structures and resolve Holliday junctions (Chen et al. 2001b). It is possible that MUS81-EME1 may also make the second incision, though the excision repair factor XPF-ERCC1 is another candidate (Fekairi et al. 2009). Alternatively, the structure arising from the first incision may resemble a 5′ flap, which could act as a substrate for the endonuclease activity of FAN1. While there is no difference in the rate of formation of cisplatin-induced or mitomycin C-induced DSBs in a FAN1-depleted background, their disappearance (removal) is slower than in controls (Kratz et al. 2010; MacKay et al. 2010), suggesting that FAN1 is not required to introduce DSBs, but rather is involved in their repair. Since DSB repair often occurs via homologous recombination (HR), this strongly implies that the FAN1 nuclease is involved in DNA processing during the latter stages of HR, but does not preclude the possibility that FAN1 may act in other as yet undiscovered pathways. The VRR_nuc domain is associated with the PD-(D/E)XK nuclease superfamily (including type III restriction enzymes and the replication/repair helicase/nuclease DNA2). The multiple nucleolytic abilities of FAN1 imply a multifunctional capacity similar to ExoG or Mre11. Whether the primary role of FAN1 is indeed in ICL repair (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Smogorzewska et al. 2010) or lies in other DNA transactions such as removal of flaps at stalled replication forks remains to be determined.

EXDL2

The genetic screen that isolated FAN1 (Smogorzewska et al. 2010) also identified a further putative exonuclease, EXDL2, which has sequence homology to the 3′-5′ exonuclease domain of WRN helicase/exonuclease, a DnaQ family nuclease (see "WRN"). While there is little published information concerning EXDL2, bioinformatics analysis shows that it has the DEDDy motif conserved in WRN and many related exonucleases. Various different isoforms of EXDL2 are reported; there is also a possible association with cancer.[6] RNAi depletion of EXDL2 has no effect on ubiquitination of the Fanconi's ID complex, leading Smogorzewska and colleagues (Smogorzewska et al. 2010) to suggest that EXDL2 may act in an ICL repair pathway either downstream of, or parallel to, the FA repair complex. In *Drosophila*, flies with an insertional mutation in the EXDL2 orthologue, encoded by *CG6744*, show slightly elevated rates of chromosomal recombination (Cox et al. 2007), implicating EXDL2 in nuclear DNA metabolism consistent with a role in repair. Intriguingly, EXDL2 in early *Xenopus* development has been localised by in situ hybridisation to the mitochondrial cloud (Cuykendall and Houston 2010), suggesting that it may also be involved in mitochondrial DNA metabolism, consistent with bioinformatics predictions of mitochondrial localisation of at least some of the isoforms of human EXDL2 (Mason and Cox, unpublished). The similarity between EXDL2 and WRN and its possible involvement in not only nuclear genome stability, perhaps in the ICL repair pathway, but also in mitochondrial DNA (mtDNA)

[6] http://www.genecards.org/cgi-bin/carddisp.pl?gene=EXD2&gc_id=GC14P069658&rf=/home/genecards/current/website/carddisp.pl&asd=17#asd

maintenance make EXDL2 an interesting candidate for further research into possible links with ageing.

Mitochondrial nucleases

Mitochondria have their own highly conserved DNA replication and maintenance apparatus, which includes mitochondrial-specific nucleases. In humans, the circular mitochondrial genome is only 16.569 kb and encodes some components of the electron transport chain together with rRNAs and transfer RNAs (tRNAs) required for mitochondrial mRNA translation (the majority of gene products used in the mitochondria are, however, encoded by nuclear genes and the proteins subsequently imported across the mitochondrial membranes).

The mitochondrial genome has a mode of DNA replication unlike that of the nuclear genome, involving large regions of single-stranded DNA and use of several possible origins that may act bidirectionally or unidirectionally (Falkenberg et al. 2007; Yasukawa et al. 2009) and mediated by the single mitochondrial DNA polymerase γ, POLG. DNA repair pathways within mitochondria include MMR, BER and possibly DSB repair. Interestingly, nuclear repair factors including the nuclease FEN1 are implicated in maintaining mtDNA integrity (Kalifa et al. 2009). Accumulation of mitochondrial genome mutations and deletions has been reported with increasing chronological age, resulting in mitochondria with defects in metabolism, leading to the mitochondrial hypothesis of ageing (Harman 1972; Miquel et al. 1980). Links between telomere attrition resulting in mtDNA damage and loss of mitochondrial function have recently been drawn (Passos et al. 2010; Sahin and Depinho 2010). Thus maintenance of mtDNA integrity may be critical in preventing premature ageing, but until recently, little has been reported on the importance of nucleases in this process.

Proofreading nuclease of mitochondrial DNA POLG

As is the case for the nuclear genome, mitochondrial DNA requires high-fidelity replication, which is conducted by POLG. The importance of exonucleolytic proofreading during mtDNA replication is clear from the phenotype of mice lacking mitochondrial polymerase proofreading through mutation of POLG, which show early-onset of many age-related phenotypes (Trifunovic et al. 2004).[7] Mice carrying high levels of mitochondrial DNA mutation show sarcopenia and mitochondrial dysfunction in skeletal muscle (Hiona et al. 2010), although this can be blunted by endurance exercise (Safdar et al. 2011). They also show an accelerated age-related loss of retinal function (Kong et al. 2011). Interestingly, this high mitochondrial mutation load alters the ability of hematopoietic stem cell lineages to undergo appropriate differentiation, leading to defects such as anaemia and lymphopenia similar to those caused by the premature senescence of this compartment (Norddahl et al. 2011). Hence loss of mtDNA integrity via loss of the proofreading function of polymerase γ is strongly associated with ageing.

EXOG/EndoG

In addition to the intrinsic proofreading nuclease activity of POLG, in *S. cerevisiae* the endo/exonuclease Nucp1 (Dake et al. 1988; Vincent et al. 1988) is the major nuclease in yeast mitochondria and is involved in a number of functions including mitochondrial DNA metabolism, recombination and cell death (Buttner et al. 2007; Zassenhaus and Denniger 1994). Nucp1 can degrade ssRNA and has DNA endonuclease activity (on both ssDNA and dsDNA) and 5′-3′ exonuclease activity (on dsDNA). The mammalian homologue, EndoG, was discovered as a nuclease involved in apoptosis (Li et al. 2001), where it is translocated to the nucleus and degrades chromatin in a caspase-independent manner. Active EndoG outside the mitochondria quickly induces cell death (Schafer et al. 2004). Like its yeast homologue, human EndoG is required for normal cellular proliferation (Huang et al. 2006).

Interestingly, mammalian EndoG acts only as an endonuclease; unlike the multifunctional Nuc1p, it notably lacks the 5′-3′ exonuclease activity used to generate gaps in dsDNA during recombination and repair. Recently, a novel EndoG paralogue named EXOG was discovered in mitochondria, which is thought to have arisen during ancestral gene duplication (Cymerman et al. 2008). Human EXOG homodimer possesses the 5′-3′ exonuclease activity that is

[7] (although the absolute cause of this phenotype has been questioned—see Kraytsberg et al. (2009))

missing in EndoG, as well as its own endonuclease functionality with some preference towards ssDNA, and it can nick supercoiled DNA. Thus EXOG has the activity necessary to generate ssDNA gaps. Together, mammalian EndoG and EXOG recapitulate all the functions of yeast Nuc1p. EndoG has been reported to lie in the mitochondrial inter-membrane space away from the DNA (Ohsato et al. 2002), whilst EXOG is located in the inner membrane where the mitochondrial DNA attaches (Cymerman et al. 2008), suggesting a functional as well as a physical split, with EndoG responsible for the cell death function performed by Nuc1p in yeast and EXOG involved in cellular proliferation. Both exonic (Cymerman et al. 2008) and intronic SNPs have been reported in the ExoG gene; the latter is associated with type 2 diabetes (Moritani et al. 2007), a disease linked with age.

Conclusions

Many different mechanisms have been proposed to account for cellular and organismal ageing, including the adverse impact of oxidative damage on proteins, lipids and DNA, genome instability arising from defects in maintenance mechanisms, mitochondrial dysfunction, defects in autophagy, aberrant metabolic signalling through the IGF1 axis and mTOR, degradation of the stem cell compartment and telomere attrition. That these mechanisms are important in ageing has been demonstrated in several recent experimental models. For example, Jaskelioff and colleagues have shown that short-term telomerase reactivation can restore normal tissue integrity and function even to aged tissues in mice (Jaskelioff et al. 2011), demonstrating the importance of correct telomere maintenance (which may involve not only telomerase but also nucleases including EXO1 and WRN) in preventing tissue homeostasis collapse and ageing. Telomere integrity is also thought to be important in preventing premature ageing of stem cells populations (Sahin and DePinho 2010). Inhibition of the IGF-1/mTOR axis through rapamycin administration significantly increased lifespan in adult mice (Harrison et al. 2009), verifying the importance of IGF1-mTOR in ageing, while nutrient restriction had a similar impact in increasing primate lifespan (Colman et al. 2009; reviewed in Cox (2009); Cox and Mattison 2009).

It would at first sight seem obvious that deficiency of exonucleases, whose normal role is to promote genome stability, would result in premature cell senescence through accumulation of DNA damage. The exonucleases discussed above do show the importance of correct genome maintenance in preventing the onset of premature ageing or age-associated disease such as diabetes and cancer. However, in addition to the direct role in DNA stability, there appears to be much more complex interplay between nucleases and other pathways of ageing, with significant cross-talk between metabolic signalling and DNA damage. Perhaps nowhere is this exemplified more clearly than in the recent description of a severe progeroid phenotype resulting from mutation of the nucleotide excision repair nuclease, XPF, with a patient showing not only signs of both CS and XP (deficient global genomic NER and transcription coupled NER) but also severe effects on liver, blood, muscle and neurological systems (Niedernhofer et al. 2006). Mice engineered to lack ERCC1 similarly showed profound systemic progeroid symptoms and died before sexual maturity. These results suggest that XPF-ERCC1 are involved not only in NER but also in other critical systems, perhaps including interstrand crosslink repair. Perhaps most interestingly, in this mouse model, the IGF1 nutrient response axis was altered, presumably in response to the presence of unrepaired DNA damage (Niedernhofer et al. 2006). Hence ageing may be triggered by initial loss of a single nuclease activity which impacts on multiple downstream regulatory pathways resulting in the loss of tissue homeostasis characteristic of whole organismal ageing.

The very tight association between mitochondrial genome maintenance defects and ageing strongly supports the idea that precise processing of mtDNA is critical to stave off ageing. Nucleases including POLG and EXOG are important to the cell to prevent premature accumulation of mtDNA mutations. Multiple nucleases have evolved to fulfill the functions required for each separate pathway, although there is often redundancy and overlap in functionality, shown by the dearth of human diseases that arise from a nuclease deficiency. However, it is suggestive of the importance of exonucleases that the diseases that do arise from their deficiency are systemic—premature ageing, genome-wide destabilization and general inflammation are all hallmark phenotypes. It is highly probable that a combination of mitochondrial nucleases, together with nucleases acting on the genomic DNA within the

nucleus (particularly the progeroid-associated WRN), play a central role in preventing accumulation of damage that would otherwise give rise to a senescent phenotype. Such nucleases therefore present promising targets for further study and in developing agents either to promote precocious senescence (e.g. in cancer therapy) or anti-ageing therapies based on supporting or augmenting the role of these nucleases.

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