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Review

Human syndromes with genomic instability and multiprotein machines that repair DNA double-strand breaks*

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*This review is dedicated to Liles (Dr. Matilde H. Navarrete), a great woman and scientist that shared with us her enthusiasm with cell beauty and left us on May, 2002 after a long and brave fight against uncontrolled proliferation

Summary. The present report deals with the functional relationships among protein complexes which, when mutated, are responsible for four human syndromes displaying cancer proneness, and whose cells are deficient in DNA double-strand break (DSB) repair. In some of them, the cells are also unable to activate the proper checkpoint, while in the others an unduly override of the checkpoint-induced arrest occurs. As a consequence, all these patients display genome instability. In ataxia-telangiectasia, the mutated protein (ATM) is a kinase, which acts as a transducer of DNA damage signalling. The defective protein in the ataxia-telangiectasia-like disorder is a DNase (the Mre11 nuclease) that *in vivo* produces single-strand tails at both sides of DSBs. Mre11 is always present with the Rad50 ATPase in a protein machine: the nuclease complex. In mammals, this complex also contains nibrin, the protein mutated in the Nijmegen syndrome. Nibrin confers new abilities to the nuclease complex, and can also bind to BRCA1 (one of the two proteins mutated in familial breast cancer). BRCA1 has a central motif that binds with high affinity to cruciform DNA, a structure present in places where the DNA loops are anchored to the chromosomal axis or scaffold. The BRCA1•cruciform DNA complex should be released to allow the nuclease complex to work in DNA recombinational repair of DSBs. BRCA1 also acts as a scaffold for the assembly of ATPases such as Rad51, responsible for the somatic homologous recombination. Loss of the BRCA1 gene prevents cell survival after exposure to cross-linkers. The BRCA1-RING domain is an E3-ubiquitin ligase. It can mono-ubiquitinate the FANCD2 protein, mutated in one of the Fanconi anemia complementation groups, to regulate it. Finally, during DNA replication, the nuclease complex and its activating ATM kinase are integrated in the BRCA1-associated surveillance complex (BASC) that contains, among others, enzymes required for mismatch excision repair. In short, the proteins missing in these syndromes have in common their BRCA1-mediated assembly into multimeric machines responsible for the surveillance of DNA replication, DSB recombinational repair, and the removal of DNA cross-links.

Key words: Ionizing radiation, Double-strand breaks, Multimeric repair machines, Ataxia telangiectasia, Ataxia telangiectasia-like disorder, Nijmegen breakage syndrome, Fanconi anemia, Familial breast and ovary cancer, BRCA1-associated surveillance complex

Introduction

XXI century biologists should deal with the properties of multimeric complexes that constitute protein machines which may display improved or even different capabilities in relation to those shown by any of their components. Changes in a single one of the components alters the activity of the complex as a whole. Furthermore, some of these complexes are part of specific signal transduction pathways that are conserved by evolution as modules containing some of their upstream regulators and downstream effectors (Hartwell et al., 1999).

There are several cancer-prone, autosomal recessive syndromes, that are hypersensitive to clastogenic agents. The phenotype of their cells is characterized by their inability to remove double-strand breaks and by the presence of genome instability. These syndromes are the following: ataxia-telangiectasia (A-T); the ataxia-telangiectasia-like disorder (ATLD); the Nijmegen breakage syndrome; and the Fanconi anemia (FA). The

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Table 1. Some syndromes with genomic instability and cancer proneness.

SYNDROME	SHORTHAND	MISSING PROTEIN	PROTEIN FUNCTION	SENSITIVITY
Ataxia-telangiectasia	AT	ATM	kinase	IR
AT-like disorder	ATLD	Mre11	nuclease	I R
Nijmegen Breakage Syndrome	NBS	Nibrin, NBS1	?	I R
Fanconi anemia	FA	FANCD2	?	cross-linkers + IR

IR: ionizing radiation

Table 2. Clinical and cellular phenotypes in patients with ataxia-telangiectasia (AT), the AT-like disorder (ATLD), the Nijmegen breakage syndrome (NBS) and Fanconi anemia (FA).

	AT	ATLD	NBS	FA
Clinical phenotype				
ataxia	+	+	-	-
oculocutaneous telangiectasia	+	±	-	-
elevated serum alpha-protein	+	-	-	-
radiation hypersensitivity	+	+	+	+
cancer proneness	+	±	+	+
immunodeficiency	+	-	+	?
microcephaly	-	-	+	+
mental retardation	-	-	±	-
premature aging	+	+	?	+
congenital malformations	±	-	+	+
hyperpigmentation spots	±	+	+	+
stunted growth	±	+	+	+
gonadal dysgenesis	+	+	±	+
Cellular phenotype				
chromosomal aberrations*	+	+	+	+
hypersensitivity to radiation	+	+	+	+
hypersensitivity to cross-linkers	-	-	-	+
defective G2 checkpoint	+	±	±	-
defective intra-S checkpoints**	+	+	+	?
defective G1 and spindle checkpoints	+	+	+	?

*: spontaneously occurring in peripheral lymphocytes. Data on AT, NBS and FA from Pincheira et al. (2001; 1998 and 1988, respectively). **: formerly known as radio-resistant DNA synthesis. +: present; -: absent; ±: contradictory data; ?: unknown

cells of this last syndrome are selectively hypersensitive to DNA cross-linkers (Table 1).

The incidence of these four syndromes in the population is quite different. Thus, AT is present in about 1 in 40,000 live births (Gatti, 1998), while there are less than 100 individuals registered in the Nijmegen syndrome record, most of them of Slavic origin. The frequency of the Ataxia-telangiectasia-like disorder remains to be elucidated, as the two first families with ATLD were only distinguished from true AT patients in December 1999 (Stewart et al., 1999). The incidence of Fanconi anemia is the highest of all the four syndromes. Actually, up to 0.5 % of the world population is probably carrying one of the mutated FA genes (Auerbach et al., 1998).

The phenotypes of the patients affected by these four syndromes are pleitropic in nature and many of their diagnostic features develop gradually with age. Ataxia-telangiectasia (AT) diagnosis includes ataxia by progressive loss of Purkinje cells in the cerebellum, with an onset from 1 to 3 years of age; ocular apraxia after 2 years of age while telangiectasia may appear many years after the ataxia. The patients show elevated α-fetoprotein, immunodeficiency, high sensitivity to radiation, and predisposition to cancer, usually lymphoma.

The different clinical features of the four syndromes are displayed in the upper part of Table 2. Proper diagnosis is relevant, as the high radiosensitivity of patients of any of these syndromes discards the use of radiation therapy for them in case they develop a neoplasia.

In spite of the gradual development of symptoms it is still possible to distinguish these syndromes by clinical traits, with the only exception of FA, where clinical phenotypes can be different even in monozygotic twins. In these patients with disturbances in oxygen metabolism and bone marrow aplasia, the exquisite sensitivity of their cells to cross-linkers is the best diagnostic marker (Auerbach et al., 1998).

A noticeable feature of the phenotype of these syndromes is that they are either unable to activate cycle checkpoint (AT) after being challenged their DNA, or they unduly override the checkpoint induced arrest, enduring a process known as checkpoint "adaptation" (Weinert and Hartwell, 1988), commented on later (ATLD, NBS and FA) (see Table 2). Because of these facts, the cells of all these patients defective in double-strand break (DSB) repair display also genomic instability, a landmark of cancer cells (Petrini, 2000).

Molecular motifs and domains in the proteins mutated in the syndromes with inability to repair double-strand breaks

In the present review, the relationships among the proteins mutated in these selected four syndromes are considered. The number of aminoacids of each protein is shown at the right part of the bar representing it (Fig. 1). This figure also shows the main motifs and the known interacting domains of each of these proteins.

ATM (Ataxia-Telangiectasia Mutated) is a member of the Phosphatydil Inositol 3 kinase (PI3K) family of lipid kinases (Savitsky et al., 1995). They have lost their capability to phosphorylate lipid substrates. Instead, they

are S/T-Q directed kinases, i.e. they phosphorylate serine or threonine residues, followed by glutamine at the +1 position. The three main members of this family are the ATM kinase, the ATR (ATM and Rad3-related) kinase, and the DNA-PKcs (DNA-damage-dependent Protein Kinase catalytic subunit). Their targets are proteins mostly involved in the negative control of cycle progression for ensuring the integrity of the genome (Shiloh, 2001).

ATM is a large (3056 aminoacid-long) kinase that displays a recognizable leucine motif common to many DNA-binding proteins in the first molecule half, while its kinase domain is close to its C-terminus. In this motif, the α-helices of two proteins can interact to give rise to a coiled-coil dimer. The motifs labelled as *ATR* and *ATR´* are also found in the human ATR kinase and in other ATM kinase homologues in yeast and *Drosophila* (Jeggo et al., 1998). Though their functions are not yet fully understood, the first but not the last of these ATR motifs (Fig. 1) is also present in members of this PI3K family that still retain the competence to phosphorylate lipids (Jeggo et al., 1998; Abraham, 2001).

Most if not all of the ATM targets are checkpoint proteins, the tumour suppressor protein TP53 (formerly p53) being one prominent member. This TP53 protein, dubbed the guardian of the genome, controls the G0 to G1 transition through the TP53-CDKN1A-Retinoblastoma protein-E2F transcription factor pathway (Lukas et al., 1996). ATM phosphorylates also the BRCA1 checkpoint protein in response to the presence of dsDNA breaks (Cortez et al., 1999).

The ATM kinase phosphorylates also, in response to ionising radiation, seven Ser-Gln/ Thr-Gln motifs of the Chk2 kinase (Matsuoka et al., 2000). The ATM-Chk2 module then stops cycle progression. ATM also phosphorylates the FANCD2 protein in response to DNA breaks (D'Andrea et al., 2001).

Mre11 is a 708-long aminoacid protein that, in vivo, operates as a 5' to 3'exonuclease (DNase) resecting DSB ends (Hoeijmakers, 2001; Moreau et al., 2001). However, in vitro Mre11 is a 3' to 5' exonuclease for single and double-stranded (ss- and ds-) DNA and also a ssDNA-endonuclease that introduces cuts into intact DNA hairpins (De Lange and Petrini, 2000).

The *in vivo* formation of 3´ single strand tails at both sides of a double-strand break by the Mre11 component of the nuclease complex is an early processing step for homologous recombinational repair of the DSBs. The nuclease domain of the Mre11 is located in its amino-terminal region (Fig. 1). In this region, Mre11 contains conserved motifs found in phosphatases and in the *E. coli* dsDNA-exonuclease. These motifs can form a ß-α-ß-α-ß secondary structure.

The Mre11 N-terminus also contains the domain binding to nibrin, the third member of the nuclease complex in vertebrates (Fig. 1). Close to the C-terminal region, the Mre11 nuclease possesses two DNA binding domains. Though the Mre11 DNase has the motif present in ATM substrates (Kim et al., 1999), ATM-dependent phosphorylation of Mre11 has not been yet reported.

As the Mre11 nuclease is always interacting with the Rad50 in the nuclease complex, both in mammals and yeasts (Hofner et al., 2001), Rad50 has also been depicted in Fig. 1. The Rad50 ATPase is a member of the extremely well conserved SMC family (Structural Maintenance of Chromosomes) (Hirano, 1999; Strunnikov and Jessberger, 1999). All the members of this family possess two globular DNA-binding domains, one at each tip, known as the Walker sites (Walker et al., 1982). They are present in multiple ATP-dependent enzymes.

There is something very interesting about the Rad50 ATPase. Each Rad50 molecule is actually half a machine, as the two Walker sites Rad50 has in its tips are only the N- and C-terminus halves of a single ATPase. Thus, the ATPase is only reconstituted when two proper tips interact (Hopfner et al., 2001). The Rad50 ATPase had two large heptad coiled-coil domains in between both Walker sites, where Mre11 and Rad50 interact (Hopfner et al., 2001). A central hinge region separates the two coiled-coil regions (Anderson et al. 2002). Surprisingly, the flexible hinge in the middle of Rad50 only possesses a half of a whole Zn-finger motif (Sharples and Leach, 1995). Thus, only the formation of a Rad50 homodimer will reconstitute the whole Zn-finger motif, making the hinge region competent to bind DNA.

In vivo, the functionality of the Mre11•Rad50 complex is ensured because both components form a heterotetramer. It contains two molecules of each of the members of the complex, forming a single DNA processing head that contains both Walker sites, so that the complex will have ATPase activity and its hinge region will bind DNA (Hopfner et al., 2001). A complex containing SMC5 and SMC6 and a novel non-SMC component is involved in both recombinational DNA repair and in proliferation (Fujioka et al., 2002). Other SMC1 and SMC3 complexes are responsible for the assembly of a mammalian recombination complex that controls meiosis (Jessberger et al., 1996).

The dimeric nuclease complex of *Escherichia coli* is formed by one C and one D polypeptide that possess motifs analogous to those in Rad50 and Mre11, respectively (Connelly et al., 1997). The extraordinary conservation of the nuclease machine suggests its early acquisition in the evolution and the advantage it provides to living organisms.

Other SMC family members form multimeric machines with non-SMC proteins. Cohesins and condensins are the best known of these complexes. These ATPases act as chromosomal chaperones that control the dynamic changes taking place in chromatin throughout the whole cell cycle (Strunnikov and Jessberger, 1999). Thus, SMC1 and SMC3 cohesins help to keep sister chromatids together from the time they are formed up to their segregation in anaphase (Giménez-Abián et al., 2002). On the other hand, the SMC2 and

SMC4 condensins form multimeric complexes that regulate the condensation cycle of different chromatin domains, affecting the expression of the genes contained in them.

Nibrin, or NBS1 (also known as p95, and whose ortholog in yeast is Xsr2) is the protein mutated in the Nijmegen syndrome (Varon et al., 1998). It is a 754 aminoacid-long protein that forms part of the nuclease complex in eukaryotic cells. A glimpse of its role may be acquired by looking at its motifs.

The C-terminus of nibrin interacts with the N-terminus of the Mre11 nuclease (Tauchi et al., 2001). In its N-terminus it has a fork-head motif that binds to phosphopeptides, probably protecting them from phosphatase attack. Next to it, nibrin possesses the BRCT (BReast cancer C-Terminus) motif that allows its interaction with the BRCA1 protein and with any other BRCT-containing protein present in the cell (Fig. 1).

The recently isolated protein FANCD2 is mutated in the D2 complementation group of the Fanconi anemia patients (Timmers et al., 2001). FANCD2 is a 1451 aminoacid-long protein, whose C-terminal region has a DNA-binding domain of the HMG type (High-Mobility Group of chromosomal nonhistone proteins). This motif possesses AT hooks that bind to AT-rich stretches present in the DNA regions known as SAR or MAR (Scaffold or Matrix Attachment Regions). Through them, the DNA loops attach to either the chromosomal scaffold in the mitotic chromosome or to the nuclear matrix in interphase cells (De Belle et al., 1998). This is the region where DNA cruciform structures accumulate. Their binding by HMG-proteins (Bianchi et al., 1989) produces kinking that diminishes the number of cruciform structures present in DNA supercoils (Reeves, 2001; Nakamura et al., 2001).

The FANCD2 protein may be regulated by ubiquitination of its lysine residue located in the 561position of the chain of aminoacids forming it.

The ATM kinase, the nuclease complex and FANCD2 are not only involved in the replacement of DNA segments possessing double-strand breaks, but also in the control of normal cycle progression, probably by ensuring a correct replication process.

Cell response to damage in DNA bases or nucleotides

The cell is subjected to both endogenous and exogenous DNA damage. The cell oxidative metabolism and errors produced during DNA replication are the main sources of endogenous DNA damage. As soon as DNA is damaged, the cell initiates a general DNA damage response. This involves the activation of a specific DNA repair mechanism and of a common checkpoint pathway that transiently prevents the activation of the cycle machinery (Cyclin-Dependent Kinases or CDKs). Thus, as a consequence of the checkpoint operation, the time taken for the cell before an irreversible transition between two subsequent cycle phases is delayed (Zou and Elledge, 2000). This additional time helps the proliferating cell to repair its DNA.

From all the lesions inducible in DNA, some may be directly reversed in minutes. However, the cell repairs its DNA lesions mostly by one of the three mechanisms it is endowed with: base excision repair (BER); nucleotide excision repair (NER); or mismatch repair (MMR). The enzymes involved in them are different. However, in all of them, an endonuclease produces a cut in the intact DNA chain and the damaged base or nucleotide is excised. Then, the synthesis of the correct base or nucleotide takes place by semiconservative replication, using the complementary DNA strand as a template. Finally, the integrity of the repaired strand of the DNA helix is sealed by a DNA ligase, in an ATP-dependent process.

Repair of the endogenous damage produced by cellular metabolism (mostly oxidative damage) and by ionizing radiation in the DNA bases is usually carried out by base excision repair (Friedberg, 2000). On the other hand, nucleotide excision repair (NER) preferentially removes bulky adducts caused by environmental genotoxics and UV-induced pyrimidine that produce helix distortions and interfere with base pairing (Hoeijmakers, 2001). NER is the most versatile of the repair pathways. It removes a 30 nucleotide-long segment of DNA which is comprised of the wrong nucleotide. Humans deficient in NER repair suffer from xeroderma pigmentosum, another rare autosomal recessive disorder. The cells of xeroderma pigmentosum patients are extremely sensitive to ultraviolet. These patients can be subdivided into seven different complementation groups, each of them with defects in different XP proteins. Some are involved in recognising and binding to the DNA damage site (XPC, XPA), others are helicases that unwind locally DNA (XPB and XPD) and others still are DNases (XPG and XPF endonucleases) that carry out the incision step of thephoto-product (Batty and Wood, 2000; Wood et al., 2001).

Mismatch excision repair (MMR) corrects occasional errors produced in DNA during replication or during recombination. Thus, MMR is preferentially active in late S, G2 and prophase of the proliferating cells. Unpaired, mispaired bases and loops repaired by MMR are recognised by the distortion they produce in the DNA double helix. Proteins involved in MMR either recognise mismatches (MSH2, MSH3 y MSH6), or are nucleases homologues of the bacterial MutL endonuclease (MLH1, MLH3 and PMS2) (Jiricny, 2002). These latter ones initiate a cut in the DNA sequences possessing a mismatch. Some of these proteins are mutated in families with Hereditary Nonpolyposis Colorectal Cancer (HNPCC). A few of these patients, however, have mutations in the 5´ to 3´exonuclease EXO1 (Wu et al., 2001) which is also involved in MMR (Shevelev and Hübscher, 2002).

Homologous recombinational repair for the replacement of DSBs

The DSB constitutes the most difficult lesion to repair in a cell and, also, the most dangerous one, as the persistence of a single DSB compromises cell viability in yeast (Frankenberg-Schwager and Frankenberg, 1990). Ionizing radiation produces mostly DNA lesions that are first processed into single-strand breaks, and then into double-strand ones (Benítez-Bribiesca and Sánchez-Suárez, 1999), though up to 10% of the final DSBs can be produced directly (Huang et al., 1992). The repair of DSBs always involves a DNA recombination process.

There are two alternative pathways for recombinational repair of DBSs (Fig. 2). One is the Homologous Recombination (HR) repair route. HR repair tends to be error-free. On the other hand, the alternative route to repair DSBs, the Non-Homologous End-Joining (NHEJ) recombinational repair tends to be error-prone.

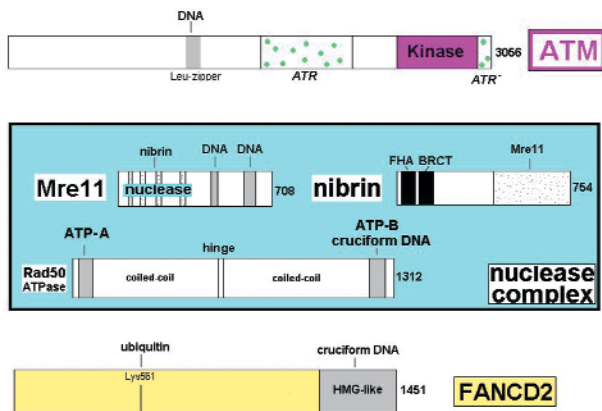

Fig. 1. Schematic representation of the proteins mutated in different syndromes, with some of their recognizable motifs and domains. At the right of each, the number of aminoacids present in the corresponding protein is shown. The ATM kinase (mutated in ataxia-telangiectasia) possesses a DNA-binding domain of the leucine-zipper type, in the first half close to the N-terminus. Its kinase domain is close to the C-terminus. There are two regions (ATR and ATR´) present also in other members of the PI-3K family. However, the ATR´ one is missing in the family members that still retain lipid kinase activity. The nuclease complex is formed by Mre11, the nuclease mutated in the ataxia-telangiectasia-like disorder (ATLD). The 4 vertical segments in the region where Mre11 binds to nibrin correspond to phosphoesterase motifs. Mre 11 has also two DNA-binding domains in its C-teminus. The nibrin (NBS1) protein is mutated in the Nijmegen syndrome. Its C-terminus interacts with the N-terminus of Mre11 in the mammalian nuclease complex. Nibrin also contains a forkhead associated domain (FHA). Finally, nibrin can interact with the BRCA1 protein because both possess BRCT domains. The chromosomal Rad50 ATPase is a permanent member of the nuclease complex. It contains two ATP-binding domains at both termini, the last one binding preferentially to cruciform DNA. The rest of the molecule is formed by two coiled-coil domains, separated by a central hinge region. The FANCD2 protein, mutated in one of the Fanconi anemia proteins, has a lysine residue in its N-terminus half, that can be ubiquitinated. Its carboxyl-terminal region has a domain similar to that present in chromosomal non-histone proteins (High Mobility Group, HMG), with high affinity for cruciform DNA.

The HR process

The process of homologous recombination is schematically depicted in Fig. 2. The nuclease complex (Rad50•Mre11•nibrin), *in vivo*, functions as a 5' to 3' DNase, giving rise to a 3´ single strand DNA which overhangs on both sides of the break. The EXO1

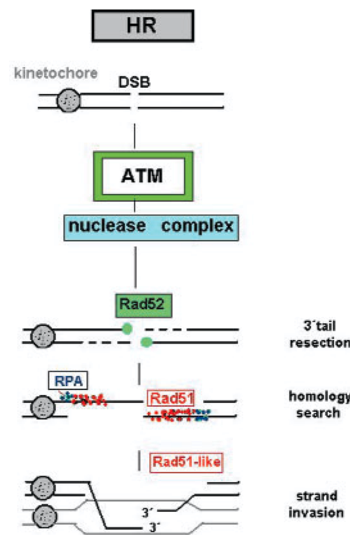

Fig. 2. The Homologous Recombinational (HR) repair of DSBs. A set of chromosomal ATPases that operate in this pathway are depicted: Rad52, Rad51 and Rad51 paralogs (dubbed in this particular figure as Rad51-like). Rad52 binds to the 3´ borders of DSBs. In vivo, the nuclease complex (Rad50•Mre11•nibrin) operates in the 5' to 3' direction to produce ssDNA. RPA (replication protein A) binds earlier than Rad51 to ssDNA. Rad51 and some of its paralogs produce the invasion strand on a homologous chromosome or sister chromatid. The replication (in the 5' to 3' direction) uses the intact copy as a template.

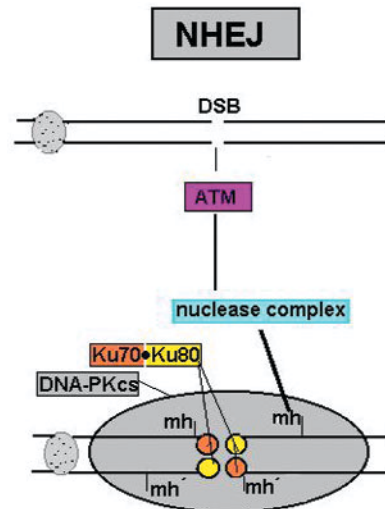

Fig. 3. The non-homologous end-joining (NHEJ) recombinational mechanism for DSB repair. The Ku70•Ku80 dimer binds strongly to the DSB and attracts the DNA-PK catalytic subunit, forming the active DNA-PK trimer. The nuclease complex helps to find microhomologies where to bind the DSB ends. Black intermediate points, as throughout this whole paper, represent formation of a complex between different polypeptides or between different subcomplexes.

nuclease (5' to 3') that works in the maintenance of chromosomal telomeres can also substitute for the in vivo Mre11 exonuclease in the process of homologous recombinational repair (Lewis et al., 2002). Once 3'DNA tails have been produced around the original DSB by the nuclease complex, the single-strand binding protein RPA (a trimeric complex) binds to them (Hendrickson, 1997; Haber, 1998). Rad51 is then targeted at the DNA•RPA complex (Golub et al., 1998) by Rad52 (New et al., 1998). Rad51 displaces the RPA protein, progressively substituting it (Fig. 2) in the maturing repair complex that forms around the Rad51 helical filaments (Lee et al., 2000). HR will take place on them. The yeast Rad51 protein and its bacterial homologue RecA show a preferential binding to GT-rich sequences and, also, an increased invasion of supercoiled DNA by homologous GT-rich regions. Thus, these regions behave as universal pairing sequences in HR (Tracy et al., 1997; Masson et al., 2001).

The Rad51-binding protein BRCA2, mutated in some familial breast cancer (Venkitaraman, 2001), is also required for HR (Xia et al., 2001). First of all, BRCA2 collaborates in the assembly of the Rad51 complex in vivo, after ionizing radiation (Yuan et al., 1999). The BRCA2•Rad51 complex also operates in the strand invasion step (Scully et al., 1997; Chen et al. 1998) of the homologous recombination process (Fig. 2), a step that has been reconstituted in vitro (McIlwraith et al., 2000). After this step, Rad51 starts to look for homology in either a sister chromatid or in the homologous chromosome, probably when still bound to BRCA2. Later on Rad51, or any other DNA-dependent ATPases of the Rad51 family, stimulates the pairing of homologues (Kanaar and Hoeijmakers, 1998), an ATP-dependent process that gives rise to the formation of heteroduplex DNA (Fig. 2).

After DNA replication, always taking place in the 5' to 3' direction, the two Holliday junctions formed between sister chromatids are solved by enzymes (resolvases) that carry out the recombination between transposons integrated in such structures. Nick ligation will finally restore the intact DNA segment that substitutes for the one that initially contained the DSB.

While the overexpression of either Rad51 or Rad52 ATPases increases homologous recombination (Park, 1995; Vispe et al., 1998), it is depressed when both of them are simultaneously overexpressed (Kim et al., 2001).

The multiple roles of Rad52 are not yet well known. In principle, the binding of either Rad52 or of the Ku70·Ku80 dimer seems to select between the homologous or the non-homologous recombinational repair pathways for DSBs, respectively. Rad52 must be an adaptor or even a catalyst for Rad51 effects. Rad52 is a good candidate to protect the 3´ DNA ends from the nuclease attack (Van Dyck et al., 1999) after annealing ssDNA (Van Dyck et al., 2001), as depicted in Fig. 2. Both Rad51 and Rad52 form complexes with the ubiquitin-like protein UBL1 that may modify their activity (Li et al., 2000). Certainly, we need to learn more about the multiple abilities of Rad52 in the context of homologous recombination.

The proteins involved in HR

Rad51, the recombinase protein, is the main player of the homologous recombinational repair pathway. Actually, Rad51displays the highest (69%) homology between yeast and humans amongst all the proteins involved in the HR repair process (Shinohara and Ogawa, 1995). Rad51 is a DNA-dependent ATPase, homologue of the *E. coli* RecA protein, that forms heptameric long filaments along ssDNA. Rad51 is a member of the Rad52 epistasis group of DNA-dependent ATPases, Rad50 (the component of the nuclease complex) being another member of this group. Other members are Rad51 paralogs, i.e., proteins encoded by genes derived from a common ancestor in man, but which keep a modest 20% homology with Rad51. These paralogs are XRCC3 (one of the human X ray-Repair Cross-Complementation genes), Rad 51B, C and D, Rad54L and B (Wood et al., 2001). As members of the same epistasis group any of the genes encoding these proteins can suppress the expression of any other of them.

The filamentous Rad51C paralog binds to circular ssDNA (Kurumizaka et al., 2001; Masson et al., 2001), where the recombinase behaves as the catalytic subunit. Rad52 substitutes for the XRCC3 paralog of Rad51 in the process of homologous recombinational repair (Fujimori et al., 2001). Rad 51 may, in turn, interact with and/or substitute for Rad52 (McIlwraith et al., 2000). The nuclease complex is probably involved in the repair of capped DSBs to ensure that illegitimate chromosomal rearrangements do not take place in them (Lobachev et al., 2002). Thus, it is also possible that the heptameric ring formed by Rad52 around DNA (Stasiak et al., 2000) would also be present in the 3' DNA overhang throughout most of the HR process.

Non-homologous end-joining recombination for repair of DSBs

The alternative non-homologous end-joining mechanism for DNA recombinational repair does not require any homologous chromosome or sister chromatid to use as a template. Thus, NHEJ is the right mechanism for the replacement of double-strand breaks in the still unreplicated sequences of the somatic cells, either proliferating in G1 or in the unreplicated sequences of a nucleus replicating during S phase, and also in the cells which are quiescent in G0. This non-homologous end-joining mechanism (NHEJ) for recombinational repair of DSBs is depicted in Fig. 3.

The NHEJ pathway involves proteins such as those forming the Ku70 •Ku80 dimer that possess basic motifs acting as nuclear localization signals (Bertinato et al., 2000). The Ku70•Ku80 complex binds, on one side, to

DNA breaks and, on the other, to the catalytic subunit of the DNA-damage dependent Protein Kinase (DNA-PKcs), giving rise to the active DNA-PK machine (Lee and Kim, 2002). DNA-PKcs is a 4127 aminoacid-long member of the PI-3 kinase family whose activity is increased by dephosphorylation, brought about by a PP2A-like enzyme (Douglas et al., 2001).

The nuclease complex is also required for this recombinational repair (Huang and Dynan, 2002). The NHEJ mechanism uses microhomologies or sequences, which are usually no longer than 5 bp (mh and mh' in Fig. 2), to bind one end of the double-strand breaks to them. The nuclease complex seems to be responsible for the selection of these microhomologies (Paull and Gellert, 2000). Because of the direct binding of the DNA end to a close sequence with some homology, the NHEJ recombinational repair is error-prone.

Moreover, at least in S. cerevisiae, the nuclease complex stimulates the activity of the XRCC4•ligase IV

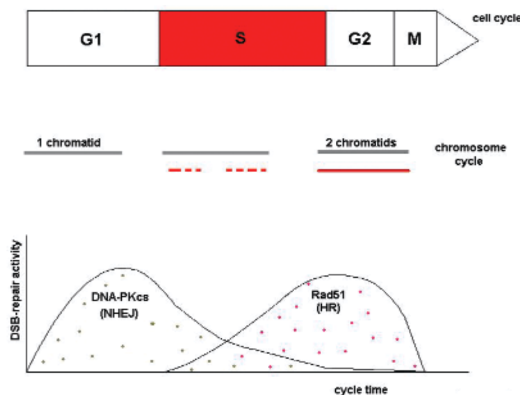

Fig. 4. The HR pathway to repair DSBs is always chosen when an intact sister chromosome is available in the cell. The upper bar represents the different phases of the cell cycle, while the mid lines represent the chromosomal cycle, with 1- and 2 chromatid chromosomes in G1 and G2, respectively. The lower part represents the NHEJ (DNA-PKcs-dependent) and HR (RAD51-dependent) pathways (modified from Hendrickson, 1997).

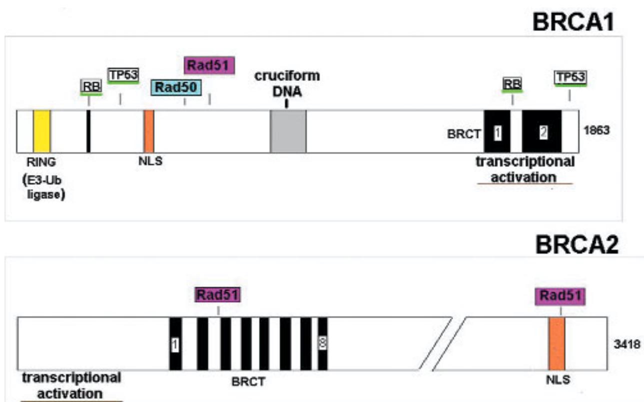

Fig. 5. Recognizable motifs and domains present in the tumour suppressor proteins BRCA1 and BRCA2. Figures at the right of both bars represent number of aminoacids. The two motifs (1, 2) at the carboxyl terminus of BRCA1 are the BRCT ones. One of them is present in members of the BRCT family of proteins. BRCA1 has a RING domain at its N-teminal region, with E3-ubiquitin ligase activity. The central DNA-binding domain has a high binding affinity for cruciform DNA. NLS represents the Nuclear Localization Signal. Binding domains to the checkpoint proteins RB (retinoblastoma) and TP53, as well as to Rad50 (member of the nuclease complex Fig. 1) and Rad51 (in Fig. 2) are also shown.

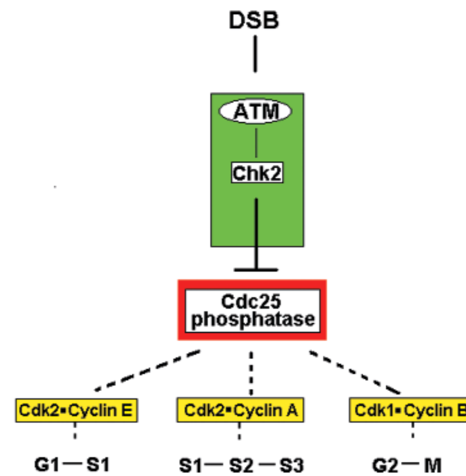

Fig. 6. The transcription-independent ATM-Chk2-Cdc25 checkpoint pathway that is specific for the response to ionizing radiation. The line ending in an inverted T-shape means that the function of the next protein if blocked. In this way, activation of all other downstream processes will also be blocked, as represented by discontinuous lines. A central point between two polypeptides, as in CDK1•Cyclin B, means that both polypeptides form a functional complex or protein machine. At the bottom of Figs. 6, 7 and 8, G1, G2 and M correspond to the pre-replicative, post-replicative and mitotic phases of the cell cycle. S1, S2 and S3 are three sequential thirds of the whole S period.

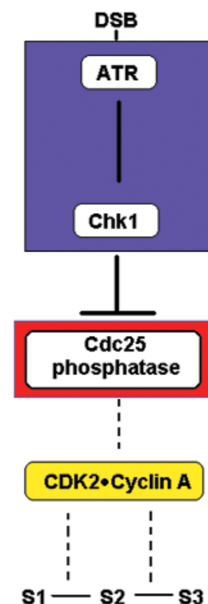

Fig. 7. The ATM-Chk1-Cdc25 phosphatase checkpoint pathway. Symbols as in Fig. 6.

end-joining machine that operates downstream in the NHEJ pathway (Paull, 2001). Mutations in the ligase IV component of this complex also increase radiosensitivity and genome instability (Gatti, 2001).

While other proteins involved in the NHEJ pathway still remain unknown, Rad52 may be one of them, as Rad52$^{-/-}$ mutants have 100-fold less NHEJ than their wild type counterparts, in yeast (Kramer et al., 1994).

Error-proneness of the NHEJ mechanism is probably tolerated in cells with small effective genome. Thus, the fraction of the genome encoding proteins is estimated to represent around a modest 3% in higher eukaryotes (Ji, 1999).

NHEJ is also used in vertebrates for the formation of antibodies, operating in the Variable-Diversity-Joining process (V(D)J) (Grawunder and Harfst, 2001). It should be noticed that in such a programmed activity, NHEJ tends to be error-free.

The choice of one of the two recombinational repair pathways

Paradigmatic homologous recombination takes place during meiosis, between the homologous chromosomes that are in the bivalent after the long meiotic prophase (Troelstra and Jaspers, 1994). The crossing over that takes place between chromatids from parental and maternal chromosomes results in recombinant chromosomes, different from the paternal and maternal chromosomes they derived from. Thus crossing over is a mechanism for changing gene linkage in the chromosomes, increasing gene heterogeneity in a population.

On the other hand, in the somatic cells, homologous recombination can use either the undamaged copy present in its homologous chromosome or in its sister chromatid as a template to recreate the damaged one (Lee et al., 1997). Recombination between sister chromatids is between 2 or 3 orders of magnitude more frequent than between homologues (Johnson and Jasin, 2001). As shown in the bottom part of Fig. 4, homologous recombination (Rad51-dependent) between sisters can only take place when the damaged DNA has been previously replicated, i.e from early S to midmitosis (Hendrickson, 1997). Moreover, in this cycle window, the two identical sister copies are in close position. Incidentally, HR repair between sister chromatids is also the most accurate one, as the sequences that replace the damaged ones are identical to them.

The BRCT proteins

The BRCT (BRCA1 protein Carboxyl Terminus) proteins possess at least one of the two globular domains found in the carboxyl terminus of BRCA1, one of the two proteins mutated in familial breast and ovarian cancer. BRCT motifs are mostly found in proteins related to DNA repair and checkpoint pathways activated by DNA damage (Bork et al., 1997). They are found, for instance, in nibrin, in the tumour suppressor protein TP53 and in some proteins mutated in Fanconi anemia (Deng and Brodie, 2000). The BRCT domain allows the protein to bind DNA and to form homodimers, as well as heterodimers with other BRCT proteins (Huyton et al., 2000).

Since nibrin, the component of the mammalian nuclease complex, is one of these proteins that can interact with the BRCA1 and BRCA2 proteins and because both BRCA1 and BRCA2 interact with the Rad51-family of chromosomal DNA-ATPases involved in homologous recombination (Fig. 2), the motifs and domains present in both BRCA 1 and 2 proteins are also shown in Fig. 5.

BRCA1 is a 1863 aminoacid-long protein with two of the BRCT motifs that characterize the BRCT proteins in its C-terminus. BRCA2 have eight such motifs, but positioned in the middle part of its 3418 aminoacid-long protein instead. Moreover, the first BRCT motif in BRCA1 recruits one cofactor (COBRA1) whose binding induces large-scale chromatin decondensation (Ye et al., 2001). This chromatin remodelling effect, independent of histone hyperacetylation, might be a crucial step in the access of a DNA lesion to the repair complex.

BRCA1 may modify in another way location, activity, and degradation of a protein. Thus, BRCA1 possesses in its N-terminus a RING domain (Fan et al., 2001). It, as any RING domain, possesses E3 ubiquitin-ligase activity. This activity is greatly enhanced by association to BARD1 (BRCA1-Associated RING Domain), another RING protein (Kerr and Ashword, 2001; Chen et al., 2002; Irminger-Finger and Leung, 2002).

Coming back to BRCA1 features (Fig. 5), it has two domains interacting with the checkpoint or tumour suppressor protein TP53 and two others interacting with the RB (RetinoBlastoma) protein, the TP53 downstream target (López-Sáez et al., 1998). The presence of these interacting domains supports the BRCA1 involvement in the G0 to G1 transition. It also supports its possible role in the transcription-dependent activation of checkpoint pathways that prevent cycle progression, specifically in the ATM-TP53-CDKN1A pathway that controls both G1 and G2 progression and that will later be commented on (Fig. 8).

The binding of the direct CDK-inhibitor CDKN1A (formerly p21) to the CDK2•Cyclin E dimer inactivates this protein machine. As a consequence, the RB•E2F transcription factor complex remains unphosphorylated and the G1 to S transition remains blocked (López-Sáez et al., 1998). Both the G0 to G1 and the G1 to S transitions are usually licensed in a permanent way in immortalized cell lines and in tumours. It is obvious that an unimpeded continuous proliferation is better achieved when the cells automatically initiate a new cell cycle and also trigger nuclear replication with no checkpoint restrictions.

BRCA1 also possesses two contiguous domains that

interact with the Rad50 chromosomal ATPase (the component of the nuclease complex, Fig. 1) and with the Rad51 ATPase, the recombinase enzyme that controls homologous recombination (Fig. 2).

The BRCA1 protein is phosphorylated by the ATM kinase in response to ionizing radiation in its 1387 serine residue, as well as in the 1423 and 1524 ones, two residues also being phosphorylated after UV radiation. Finally, the Chk2 kinase involved in the ATM-Chk2-Cdc25 checkpoint phosphorylates the BRCA1 serine residues in positions 988 and 1497. The multiple BRCA1-activating inputs support the extreme BRCA1 sensitivity to the damage induced for ionizing radiation in the DNA of human cells.

Cruciform DNA and its binding proteins

In the middle of the BRCA1 protein, there is a domain that strongly binds to cruciform DNA (Fig. 5). But there are also binding domains with such a preference in the Rad50-ATPase integrated in the nuclease complex (Fig. 1), as well as in the FANCD2 protein (Fig. 1). Protein binding to cruciform DNA takes place in a sequence-independent manner and, when the BRCA1•cruciform DNA interactions are observed, multiple DNA branches irradiate from each of these sites (Paull et al., 2001). As earlier commented, the binding of BRCA1 to DNA inhibits the activity of the nuclease complex. Therefore, BRCA1 should be displaced from the cruciform DNA to allow the free access of the nuclease complex (Paull et al., 2001).

Cruciform DNA is preferentially located in the chromosome at the base of the DNA loops, in the SAR o MAR regions (Scaffold or Matrix Attachment regions) (Earnshaw and Laemmli, 1984). In the mitotic chromosomes, these regions are at the interface between the DNA forming the axial component of the chromosome, i.e., the chromosome scaffold, and also the DNA loops anchored to such scaffold. The spreading of histone-depleted chromosomes provides a good vision of the sites of the chromosomal scaffold that should possess a bunch of hairpin structures to which loops are anchored at a 90° angle (Mullinger and Johnson, 1979, 1980). The DNA scaffold regions operate as boundaries for the genes found in different contiguous loops (Gerasimova and Corces, 1998). In interphase, the scaffold region (or SAR region) that becomes a part of the nuclear matrix (the MAR region) would support the assembly of replication and recombination protein machines on it. In interphase, when the chromosomal scaffold becomes a part of the nuclear matrix, these machines become immobilized in the nucleus (Cook, 1991).

The processing of a single damaged base in the interphase chromatin into a large missing chromatid segment seems to involve transduction of some signal up to the basis of the so-called Revell loop (Bryant, 1998). These signals would induce the chromosome rearrangements, probably involving the hyperphosphorylation of large chromatid segments (Rogakou et al., 1999). The so-called Revell loop would be transiently closed in a place where sister chromatid exchanges take place. This loop may be similar to a conventional loop anchored to either the mitotic chromosomal scaffold (Earnshaw and Laemmli, 1984) or to the interphasic nuclear matrix.

BRCA2 in HR repair

BRCA2 is mostly involved in the homologous recombinational repair of DNA double-strand breaks (Venkitaraman, 2001). It shares some common features with BRCA1, but BRCA2 also displays some unique functions (Bertwistle and Ashworth, 1998). The BRCA2 protein, mutated in other familial breast and ovarian cancer patients, possesses, apart from its eight central BRCT domains, two domains which interact with the recombinase (Rad51), one of them overlapping its BRCT domains while the other is close to the nuclear localization signal, in its C-terminus. The BRCA2 protein seems to be an indispensable component of the Rad51-dependent HR pathway, though all its possible roles have not yet been fully resolved. Thus, the lack of the BRCA2 gene highly increases the sensitivity of the cell to DNA cross-linker agents (Kraakman-van der Zwet et al., 2002). Some relationship may, then, exist between BRCA2 and the proteins mutated in Fanconi anemia cells. This needs further investigation.

The sensors of DNA damage

At present, no univocal relationship has been established between a specific DNA lesion and a sensor (Friedberg, 2000). The ATM and ATR kinases should be part of a damage sensor complex that can directly bind to DNA ends (Durocher and Jackson, 2001), as the trimeric DNA-PK complex that binds after ionizing radiation, and also after UV (Lee and Kim, 2002).

The presence of functionally redundant mechanisms for dealing with a cellular process is the rule. This is specially so when applied to sensors of DNA damage. Most of the 15 members of the BASC complex that surveys DNA replication in the S phase of the cell cycle can bind to DNA (Wang et al., 2000), as commented below (Fig. 10).

As early as 1988, Weinert and Hartwell defined that the Rad9 protein involved in detecting DNA breaks was responsible for activating a checkpoint pathway that induced a G2 arrest that was proportional to the amount of DNA damage. Thus, Rad9 recruits ATM to DNA breaks and also acts as a scaffold to accumulate Chk2 kinase molecules, facilitating their ATM-dependent activation (Gilbert et al., 2001).

Ataxia-telangiectasia: the ATM kinase that activates checkpoint pathways

Both ATM/ATR kinases are proximal transducers of

antimitogenic signals produced by the presence of DNA damage (Abraham, 2001). The ATM kinase is activated by ionizing radiation throughout the whole cell cycle, i.e. independently from the cycle phase when the DNA damage is produced and detected (Pandita et al., 2000).

Both the ATM and the ATR kinases phosphorylate serine or threonine followed by glutamine (the S/T-Q motif) in multiple target proteins. Nibrin, the component of the mammalian nuclease complex that operates in recombinational repair of double-strand breaks (Fig. 2), is one of their targets. Surprisingly, the nuclease complex is an upstream requirement for the activation of checkpoint pathways in the presence of double-strand breaks (Grenon et al., 2001).

Because the ATM and ATR kinases are involved in the general response to double-strand breaks, inducing their repair, both kinases are tumor suppressor proteins. Both kinases also play an important role in the activation of checkpoint pathways that prevent irreversible cycle transitions when DSBs are present (Fig. 6). The antimitogenic signal transduced by the checkpoint mechanism activated by these kinases in the presence of DSBs may either be transcription-dependent or transcription-independent. There are two transcription-independent checkpoint pathways. They are responsible for a transient stop in cycle progression. Such a block operates shortly after the presence of DNA damage has been detected in the cells. On the other hand, the transcription-dependent pathway activated by DNA damage always represents a tardy response.

The ATM-Chk2-Cdc25 checkpoint pathway

In response to ionizing radiation, the early activation of checkpoints to stop cycle progression usually takes place by the transcription-independent pathway depicted in Fig. 6 (Matsuoka et al., 1998). Essentially, the pathway involves the upstream ATM kinase and its effector kinase Chk2. Chk2 is often named according to its homologues in *Saccharomyces cerevisiae* (Rad53) or in *Schizosaccharomyces pombe* (Cds1).

The Chk2 kinase phosphorylates the Cdc25 phosphatase to inactivate it. This phosphatase is a component of the enzymatic route that controls cell progression throughout different CDKs (cyclin-dependent kinases), though it controls preferentially the G_2 DNA damage checkpoint (Passalaris et al., 1999; O'Connell et al., 2000). The Chk2 kinase possesses FHA domains, such as those also found in nibrin, that reinforce phosphorylation by preventing the dephosphorylation of aminoacids in a protein (Durocher et al., 2000).

Only when the Cdc25 phosphatase is active, i.e when it is not blocked by the Chk2 kinase, does it remove the inhibitory phosphates present in the threonine14-tyrosine15 residues of the catalytic subunit of the already intranuclear CDK-cyclin complex. Then, the CDK located downstream of the Cdc25 becomes active (López-Sáez et al., 1998).

Fig. 6 also displays the different CDKs under Cdc25 regulation in the interphase of the proliferating cells. They comprise the Cyclin E•CDK2 for the G1 to S transition; the Cyclin A•CDK2 for the subsequent replication checkpoints, and the Cyclin B•CDK1 for entry into mitosis (Bulavin et al., 2002).

Around half of all human tumours that are missing a functional TP53 are recalcitrant to conventional antitumor therapy. As they can still maintain in their G2 the ATM-Chk2-Cdc25 module, it is the most feasible target for therapy in these patients (Yao et al., 1996).

The ATR-Chk1-Cdc25 checkpoint pathway

The ATR kinase transduces DNA damage signals produced by ultraviolet radiation in any phase of the cell cycle or by those produced by stalled replication forks during S phase, when replication takes place. ATR initiates the ATR-Chk1kinase-Cdc25 phosphatase module that interrupts CDK activation and, subsequently, prevents cycle progression (Fig. 7). The Chk1 kinase phosphorylates serine 216 of the Cdc25 phosphatase. The presence of phosphoserine transforms this position into a binding site for a 14-3-3 protein (Rad24). The CDC25•14-3-3 dimer is then exported out of the nucleus because of the nuclear exclusion signal which the 14-3-3 protein possesses (López-Girona et al. 1999). Separation of enzyme and its target in different cycle compartments reinforces the Cdc25 inability to activate nuclear CDKs.

Though the ATM-Chk2-Cdc25 is the preferential pathway used by the cell to delay G2 progression in response to ionizing radiation, a BRCA1-dependent-Chk1 pathway is also present in G2 (Yarden et al., 2002). Moreover, in spite of the apparent differences between both checkpoint pathways, they complement each other, especially when the canonical one has become out of order (Boddy et al., 1998). Thus, the ATM-Chk2-Cdc25 checkpoint pathway activated by DNA damage is inactive in the ATM-missing ataxia-telangiectasia patients (Chen et al., 1999). In response to ionizing radiation, the A-T cells activate, after some delay, a G2 checkpoint. The caffeine sensitivity of this surrogate pathway indicates that the ATR-Chk1-Cdc25 module could be responsible for it (Pincheira et al. 2001).

Transcription-dependent cycle blocks induced in the presence of DNA damage

There is a transcription-dependent mechanism that constitutes a late response to the presence of DSBs in the DNA. The transcription-dependent pathway is the ATM-TP53-CDKN1A pathway (Fig. 8). In response to γ-ray irradiation, the ATM kinase phosphorylates both the serine 15 of the tumor suppressor protein TP53 (Abraham, 2001), and its binding protein 53BP1 (53 Binding Protein 1) (Anderson et al., 2001).

The TP53 tumor suppressor protein is a transcription

factor that induces the transcription of the gene encoding CDKN1, the direct inhibitor of CDKs, formerly known as p21 (Schmidt-Ullrich et al., 2000). The CDKN1A binds directly to the Cdk4/6•cyclin D that controls the onset of proliferation (or G0 to G1 transition). It also binds to the Cdk2•cyclin E and to the Cdk1•Cyclin B dimers that control G1 to S and G2 to mitosis transitions, respectively (Bunz et al., 1998).

The transcription-dependent checkpoints also suppress the expression of genes such as the CDK-regulatory subunits, the cyclins. This response takes some hours to become established in a cell (Bulavin et al., 2002). Thus, it may be responsible for its exit from cell cycle, towards G0 and G0,2 quiescent states (2n2C and 2n4C DNA contents, respectively).

Ataxia-telangiectasia-like disorder and the Nijmegen syndrome: Mre11 and nibrin in the mammalian nuclease complex

The proteins mutated in both the ATLD (the Mre11 nuclease) and the Nijmegen syndrome (nibrin or NBS1) form, together with the Rad50-ATPase (Fig. 1), the nuclease complex. Nibrin is an acquisition of higher eukaryotes, while the other two proteins or analogues are also found in lower eukaryotes and bacteria, respectively. The operation of the nuclease complex as a DNase is crucial for both pathways of recombinational repair: HR and NHEJ (De Jager et al., 2001).

The incorporation of nibrin to the mammalian nuclease complex provides it with four new competences: 1) licenses the ATP-dependent activity of Rad50; 2) reverses to 5' to 3' the Mre11 endonuclease activity in the ds to ssDNA transitions, producing 3´ overhangs; 3) partially unwinds DNA duplexes; and 4) cleaves fully paired hairpins that sustain cruciform DNA structures (Paull and Gellert, 1999). The nibrin domains responsible for the acquisition of the new competences of the nuclease complex are differently distributed (Desai-Mehta et al., 2001).

The ATM kinase phosphorylates nibrin in four of its serines (those in positions 278, 343, 397 and 615) in response to DNA damage (Wu et al., 2000; Yuan et al.,

Fig. 8. The transcription-dependent checkpoint pathway ATM kinase – the tumour suppressor protein TP53 – the direct CDK inhibitor CDKN1A (formerly p21), whose binding to the different cyclin•CDK dimers inactivates them.

Fig. 9. The Fanconi anemia proteins corresponding to different complementation groups (A to G). Five of these proteins form a complex, downstream of the FANCB protein and upstream of the FANCD2 one. This latter one binds to BRCA1 and co-localizes with it in nuclear foci after DNA damage.

Fig. 10. The early repair foci formed after ionizing radiation contains the ATM kinase, BRCA1 and BRCA2. They also contain the nuclease complex, the DNA helicase mutated in the Bloom´s syndrome (BLM), and the ssDNA single binding protein RPA. Moreover, the Rad9•Hus1•Rad1 sliding clamp is loaded around the DSB by the Rad17 clamp loader.

Fig. 11. Main components of the replication complex (BASC). BASC typically contains the BRCA1 protein and the ATR kinase. It also has the nuclease complex and the BLM helicase also found in the repair complex. It contains the clamp PCNA (the processivity factor for DNA polymerases δ and ε), as well as the clamp loader RFC (replication factor C). Enzymes of the post-replicative mismatch repair system (MSH2, MSH6, MLH1, MLH3 and PMS2) are also present in it.

2000; Shiloh et al. 2001). The activated nibrin can in turn interact with BRCT proteins. Because of this interaction, the whole nuclease complex can be transported into the nucleus, as both BRCA1 and 2 proteins have nucler localization signals (Fig. 6). Though the nuclease complex is also effectively transported to nucleus in lower eukaryotes in the absence of nibrin, the underlying mechanism is unknown. Nibrin, in the mammalian cells, either potentiates the transport capability of the dimeric nuclease complex or substitutes for it.

The multiple relationships between nibrin and ATM can be illustrated by an example: ATM phosphorylates nibrin in the presence of DNA damage to initiate DSB repair (Gatei et al., 2000). In turn, nibrin is required for activation of the ATM-Chk2 checkpoint (Brown et al., 1999; Chaturvedi et al., 1999) to delay cycle progression in response to ionizing radiation (Buscemi et al., 2001).

Lastly, the nuclease complex has other important roles in addition to DSB recombinational repair. Thus, it is involved in the maintenance of telomeres (Zhu et al. 2000; Ranganathan et al., 2001), and also in the production of the DSBs required for meiotic (homologous) recombination (Haber, 1998).

Fanconi anemia: the FANCD2•BRCA1 complex

FANCD2 is the most evolutively conserved of the eight proteins mutated in the different Fanconi anemia patients. The mutated FANCD2 is only found in a small fraction of them. However, all patients share the same clinical phenotype, suggesting that they may function either forming complexes or being part of a sequence in the same pathway. The relationships between the FANC proteins correspond to both situations (Fig. 9). Thus, five of them (A, C, E, F and G) are assembled into a complex, downstream of the FANCB protein, while the FANCD2 will be the effector of such a complex (Grompe and D'Andrea, 2001). On the other hand, the still uncloned FANCD1 might operate downstream of FANCD2 or be unrelated to this pathway (Siddique et al., 2001).

FANCD2 is subjected to regulation by both ATM and BRCA1. Thus, it is phosphorylated by ATM in response to ionizing radiation (D'Andrea et al., 2001), and phosphorylated FANCD2 forms a heterodimer with BRCA1, in which FANCD2 is ubiquitinated. This FANCD2•BRCA1 complex accumulates in repair foci (Joenje and Arwert, 2001).

Ubiquitination tags proteins for their degradation in the proteasome (Hershko and Ciechanover, 1998; Sudakin et al., 2001). Mono-ubiquitination may also result in protein inactivation, as when the MDM2 oncogene, another RING protein, mono-ubiquitinates the tumor suppressor protein TP53, allowing the operation of TP53 nuclear exclusion signals (Boyd et al., 2000; Geyer et al., 2000). In this way, the TP53 transcription factor is separated from its nuclear DNA target.

However, monoubiquitination may activate a protein, instead. This is the case for histone H1 in *Drosophila* (Pham and Sauer, 2000), histone H2B in yeast (Robzyk et al., 2000), and some transcription factors (Hicke, 2001). Thus BRCA1 ubiquitinates mammalian histone H2AX (Chen et al., 2002) that results in its immediate (~2 min) recruitment into repair foci (Paull et al., 2000). This is probably the case for FANCD2 that can then displace to cross-linked DNA sites (Kerr and Ashworth, 2001; Moynahan et al., 2001). This would explain why mutations in the BRCA1-RING domain predispose to cancer (Ruffner et al., 2001). Cancellation of the ubiquitin-dependent degradation of RNA polymerase II (Woudstra et al., 2002) may also cooperate in this effect, by preventing the inhibition of transcription as it would facilitate DNA repair (except for the transcription-coupled one).

The development of bone marrow aplasia, typical from Fanconi anemia, in Nijmegen´s syndrome patients also suggests a possible connection between both syndromes (Resnick et al., 2002).

The DSB repair complex

In response to DSBs produced after ionizing radiation, a repair complex is formed. It shares some of the BASC components, such as BRCA1 itself, the nuclease complex, the RPA trimer and also the BLM helicase (right part of Fig. 7). Co-localization experiments prove the involvement of the histone H2AX in the recruitment of the nuclease complex to the nuclear foci (Paull et al., 2000) where the ubiquitinated FANCD2•BRCA1 complex also co-localizes (García-Higuera et al., 2001).

The repair complex contains the ATM kinase instead of the ATR one. It often contains the ring clamp that forms around a DNA break, i.e. the Rad9•Hus1•Rad1 complex. This complex forms a PCNA-like ring structure that works as a sliding clamp and a processivity factor for the DNA polymerases that are around a DNA lesion and are responsible for its repair. The Rad9•Hus1•Rad1trimer is loaded by the Rad17 protein around damaged DNA (Venclovas and Thelen, 2000), in a way that is similar to the loading of the PCNA trimer by the RFC (Replication Factor C) around replicating DNA. Both clamp loaders (Rad17 and RFC) are pentameric complexes with ATPase activity (Burtelow et al., 2001). They contain four small proteins with Walker A and B sites similar to those in the Rad50 chromosomal ATPase of the nuclease complex (Fig. 1).

The repair complex also possesses the BRCA2 protein. BRCA2 seems to be indispensable for Rad51 formation of the DNA heteroduplex formed among the 3' single strand overhangs of the chromatid possessing the DSB and both chains of the intact sister chromatid (Fig. 10).

One of the mismatch repair proteins, MSH2, which also recognizes Holliday junctions in DNA (Alani et al., 1997) such as those produced during HR (Fig. 2), may also be in it (Fig. 2). However, its presence has only

been documented in a replication surveillance complex (Fig. 11).

The BASC complex

The BASC (BRCA1-Associated genome Surveillance Complex) is a complex larger than 2 MDa that integrates over 15 subunits (Fig. 11). It recognizes DNA damage in replicating or in freshly replicated DNA, i.e. in the proliferating cells in S and G2 cycle phases (Wang et al., 2000). Some of their subunits are small complexes themselves. The BRCA1 checkpoint protein is the member that gives name to this supercomplex. BRCA1 seems to act as a scaffold for both replication and repair proteins (Futaki and Liu, 2001).

Apart from containing the nuclease recombination complex, the BASC complex also houses the ATM protein kinase, the trimeric PCNA clamp and its loader, the pentameric replication factor C (RFC) (Fig. 7).

Additional components of the BASC supercomplex are the mitsmatch repair proteins MSH2, MSH6, MLH1 and the recently found MLH3. They are mutated in hereditary nonpolyposis colorectal cancer (Boland, 1998). Mismatch repair is the primary short-term process that corrects helix distorsions that are the consequence of base-base and insertion/deletion mispairs produced by errors during DNA replication. Recently, it has been proved that the nuclease Mre11 also participates in this process (Giannini et al. 2002).

The mismatch repair pathway of DNA repair should take place in the freshly replicated sister chromatids, from mid S up to G2 completion (Fig. 3). Mispairs that are not repaired by this process should be preferentially repaired by homologous recombination after the processing nucleases have produced DSBs.

The accumulation of repair proteins in nuclear foci formed in response to DNA damage

Co-immunodetection of the various repair proteins in the cell is a useful tool to understand time and topological differences in the response to DNA damage. Co-immunodetection of the three proteins of the mammalian nuclease complex in nuclear foci achieves its maximum after ionizing radiation (Zhong et al., 1999), supporting the close and cooperative association of multiple nuclease complex molecules in DNA repair. However, nuclease complex foci are also present, though to a lesser extent, during replication.

Two facts are noticeable in the formation of nuclear foci: they do not form after UV irradiation (Petrini, 1999; Mirtzoeva and Petrini, 2001) and they are heterogeneous in composition. Thus, the foci formed in response to ionizing radiation contain either the nuclease complex or the Rad51-ATPase responsible for HR, but not both (Maser et al., 1997). Finally, Rad52, the heptameric protein that forms a ring around the DSBs, only accumulates in foci formed in cells that are in the process of replication (Lisby et al., 2001).

Truncation experiments proved that the forkhead-associated domain of nibrin is a strict requirement for the formation of the foci containing the nuclease complex (Tauchi et al., 2001). However, it is unknown whether these foci may represent the sites at which DNA repair has initiated and whether they remain even if the repair has not been completed (Petrini, 1999).

In the replicating nuclei, IR induces the exit of BRCA1 from the BASC while PCNA (the processivity factor for DNA polymerases δ and ε) remains in them. On the other hand, irradiation of the G1 cells should only be repaired by NHEJ (Fig. 4). In these irradiated cells in G1, BRCA1 decreases in nuclear foci, while their content in nuclease complex remains high (Wang et al., 2000). It seems that PCNA interacts with the Ku70•Ku80•DNA-PKcs heterotrimers so that PCNA may have some role in the NHEJ mechanism (Balajee and Geard, 2001).

The BLM helicase (mutated in the Bloom's syndrome) is a homologue of the bacterial RecQ helicases (Ellis et al., 1995). This syndrome is characterized by its high rate of sister chromatid exchanges and its hypersensitivity to alkyl purines and, even more so, to ultraviolet. The BLM helicase is only incorporated in the nuclear foci containing the nuclease complex from mid S up to G2 completion (Bischof et al., 2001), i.e., when sister chromatids are present in the proliferating cell. During replication, BLM seems to be required for the positioning of the nuclease complex at any stalled replication fork (Franchitto and Picchierri, 2002).

Lastly, while Rad51 and BRCA2 colocalize during HR repair in nuclear foci that do not contain the nuclease complex, Rad51 and BRCA1 colocalize in them during both HR and NHEJ recombinational repair (Xia et al., 2001).

Topological approaches provide information on the dynamic changes taking place in the different DNA repair machines to be assembled in eukaryotic cells.

Cell cycle checkpoints and checkpoint adaptation

The proliferating cells are always under the constraint of the time mechanism provided by the cycle of activation and desactivation of the cyclin-dependent kinases (CDKs). The CDKs are the crucial elements of the machinery that make the cell advance throughout each of the different phases as well as throughout the transitions between subsequent cycle phases.

In the proliferating cells, in response to ionizing radiation, the general damage response activates simultaneously the most adequate repair pathway, and also a cycle-specific subpathway or checkpoint. The checkpoint pathways transiently stop the CDK-based time mechanism, by interfering with CDK activation (Figs. 6-8). In this way, the checkpoint provides additional time to deal with the processing of DNA damage. When the damage is finally reversed, the

checkpoint pathway switches off and the cell continues advancing as a consequence of its cycle programme (Hartwell and Weinert, 1989).

Saturation of DNA repair takes place more usually in the proliferating than in the quiescent cells, because of the above commented time constraints that the former are submitted to. The checkpoint mechanisms caducate with time (Hartwell and Weinert, 1989; Paulovich et al., 1997). Hence, the proliferating cells, temporarily blocked in a cycle phase, override unduly the checkpoint block after a time, entering into the subsequent cycle phase without being prepared for it. This is the process known as checkpoint adaptation, that involves the achievement of tolerance to the presence of unrepaired damage. The cell adapted to the presence of some DNA damage behaves gains a chance to repair itself later on, while the unadapted starts at once a programme for cell death.

Induction of a lengthened time in the cycle phase previous to the checkpoint-surveyed transition supresses genome instability that is always secondary to checkpoint adaptation. Thus, the use of an antimicrotubular agent to stop mitosis is able to prevent the appearance of genome instability (Fasullo et al., 1998). However, adaptation to checkpoints is not a passive process, but a programmed activity instead, as a specific pattern of expression is needed for the adaptation to take place (Bennet et al., 2001, Lee et al., 2001).

Genome instability: the persistence of unrepaired DSBs

Eukaryotic cells can support the presence of some persistent DSBs. In McClintock's words, "the genome may reorganize itself when faced with a difficulty for which it is unprepared" (McClintock, 1984). Genome instability is the basis of novel experiments in evolution taking place in a stressed genome when a DSB is introduced into a G1 nucleus. The "natural experiments in evolution" taking place in an individual cell may lead from small deletions to rearrangements of its whole genome, mediated by activation of potentially mobile transposons. This is the genomic instability observed in immortalized cell lines.

In the proliferating cells, the developing of genome instability is the consequence of the undue override of a mitotic checkpoint block. Thus, DNA breaks entering into a G1 nucleus should induce ectopic fusion between sequences that can be far apart, by NHEJ repair recombination, either in the same chromosomes or in two different ones. The recombinant chromosome formed will possess two centromeres. Migration of each of these centromeres to different spindle poles will result in the stretching of the chromosomal segment between both centromeres, after formation of a chromosomal bridge in ana-telophase. Bridges will be solved by a random ectopic cut, often accomplished by the growing cytokinetic plate. This new chromosomal break will again initiate a new breakage-fusion-bridge cycle, as summarized by McClintock (1984). The continuing breakage-fusion-bridge cycle will ensure genome instability for following generations.

Induction of apoptosis in the presence of DNA damage

During the transient cycle block a checkpoint induces, the cell may still follow a different developmental fate. It may start its regulated suicide by inducing a programme of gene expression named apoptosis (Rudin and Thompson, 1998). This induction apparently relies on the simultaneous and contradictory presence of both antimitogenic and mitogenic signals in the cell. For this induction to occur, both the ATM kinase (Friedberg, 2000) and also the cycle checkpoint protein TP53 (formerly known as p53) must be active. Apoptosis is characterized, at the cell level, by condensation and fragmentation of chromatin and nucleus. Apoptosis is a strictly regulated mechanism for the removal of damaged, mutated, aged or unnecesary cells. As a consequence, decreased apoptosis correlates with an over-increased proliferation. When it occurs in cells with genomic instability, the diminution of apoptosis favours cell transformation.

Conclusion

The study of the proteins responsible for the human syndromes here considered underscores intermediate steps in the DSB removal by recombination through the formation of protein heterocomplexes. The presence of breaks in the mitotic chromosomes of all these patients proves that the cell has unduly overriden the G2 damage checkpoint. Thus, their cells are "adapted" to the presence of persistent DNA damage (Hartwell and Kastan, 1994).

The intimate relationships between DNA replication, recombination, transcription and repair are obvious today (Flores-Rozas and Kolodner, 2000). On one side, the presence of a common structural core in different proteins involved in replication, repair and meiotic recombination provides an explanation for their alternative positioning on DNA (Egelman, 2000). On the other side, the interchange among the different proteins in the complexes provides the mechanistic basis for such a replacement and sustains the multifunctionality of the subsequent DNA repair-related heterocomplexes.

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