Molecular Radiation Biology

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Learning Objectives

- To gain familiarity with biomolecules that undergo radiolysis after ionizing radiation (IR) and to learn about some of the damaged products and the expected biological consequences
- To understand how IR influences various DNA repair mechanisms, cell cycle phases, and cell death mechanisms as well as associated signaling cascades that are involved
- To get knowledge on higher order chromatin organization and its connection to DNA damage repair
- To be able to distinguish between cell survival and cell viability and understand different *in vitro* and *in vivo* assays used to evaluate clonogenic capacity

- To understand chromosomal aberrations including chromosomal translocations in different cell cycle phases, formation of micronuclei, radiation-induced foci, and their dependence on the type of the incidental radiation as well as to acknowledge the health risks of such cellular damages
- To get familiar with mechanisms of oxidative stress, telomeres/senescence, and immunity in the context of cancer biology and/or radiation response
- To get acquainted with the types and underlying mechanisms of cellular hyper-radiosensitivity
- To describe how radiation resistance can be induced by external factors such as hypoxia and previous low-dose exposure or as part of the tumor cell evolution
- To get knowledge on the role of epigenetic factors, e.g., various types of RNAs, extracellular vesicles, as well as DNA methylation; histone modification; and gene expression in the cellular radiation response
- To define signatures of radiation response comprised of changes at gene transcription level and their biological consequences
- To become acquainted with CRISPR-CAS9 genome editing system and its application in molecular biology science as well as in DNA DSB repair analyses

3.1 Radiolysis Products with Carbohydrates, Proteins, and Lipids

As described in Chap. 2, ionizing radiation (IR) can interact with matter directly, via molecule ionization, or indirectly, via the radiolysis of water. The result of this interaction is highly reactive ionized molecules that undergo a rapid cascade of chemical reactions, which leads to the breaking of chemical bonds. The radiolytic damage of biomolecules, such as carbohydrates, lipids, and proteins, is described as an indirect effect following water radiolysis and depends on biomolecule concentration in the irradiated medium. The products of water radiolysis—radicals—are often found in clusters and react with the biomolecules present within cells before they have a chance to diffuse and form a homogeneous distribution of products. To date, the studies on radiation-induced damage of these biomolecules are mainly based on the radical analysis of model molecules or on the molecular analysis of cellular mixtures after irradiation. Figure 3.1 shows an overview of the radiolysis products described in this chapter. The description of radiolysis products of the different biomolecules clearly demonstrates possible interactions and reactions between radicals and subcellular targets [1].

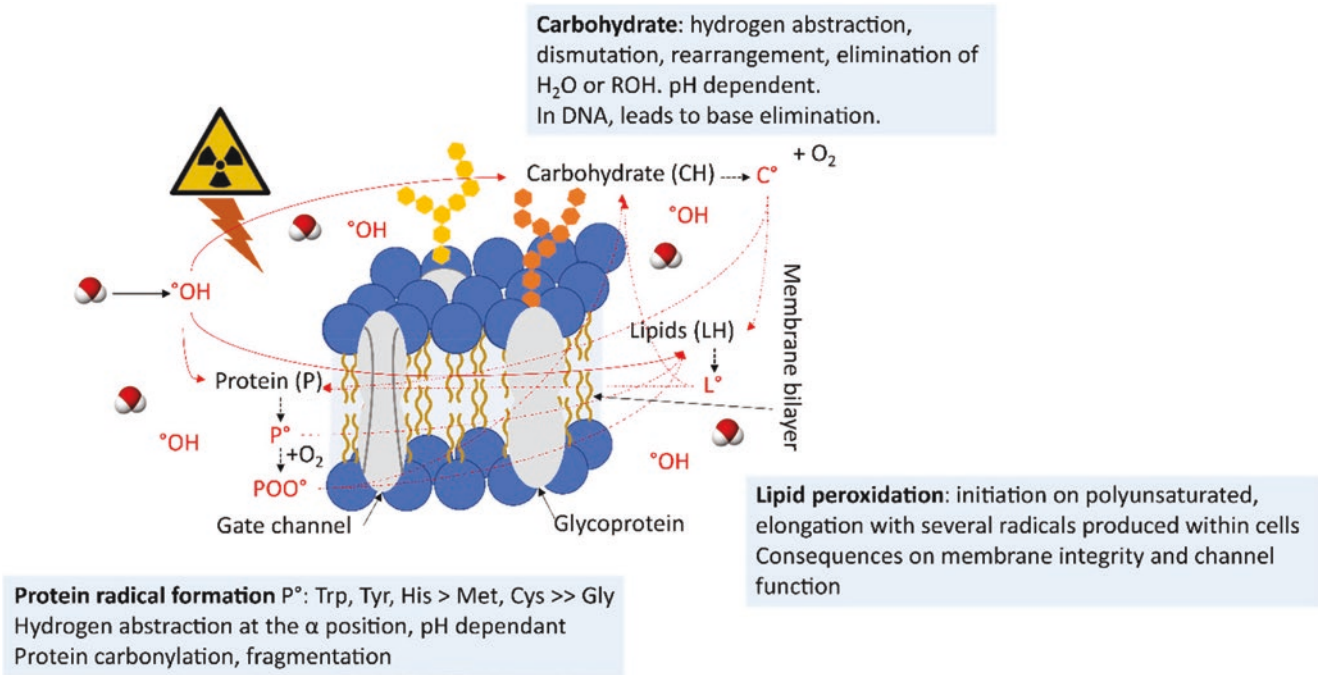

Fig. 3.1 Summary of the radicals produced with proteins, lipids, and carbohydrates following external IR exposure. Cellular exposure to IR leads to dissociation of biological macromolecules. Radiolysis of carbohydrates, proteins, and lipids is explained in their respective blue boxes. *PO* protein radicals, *CO* carbohydrate radicals, *LO* lipid radicals, *OOH* hydroxyl radicals, *POOO* protein peroxyl radicals, *Trp* tryptophan, *Tyr* tyrosine, *His* histidine, *Met* methionine, *Cys* cysteine, *Gly* glycine, *ROH* alcohol—an analog of water where R is alkyl group, O is oxygen atom, and H is hydrogen atom

3.1.1 Carbohydrates

Carbohydrates are hydrated organic molecules consisting of carbon (C), hydrogen (H), and oxygen (O), characterized by the formula $C_x(H_2O)_y$, where x and y denote the numbers of carbon or water in the molecule. Chemically, most carbohydrates are polyhydroxy aldehydes, ketones, alcohols, and acids, which can polymerize, form connected chains of molecules, and, therefore, become more complex [2]. In biological media, such as cells, some carbohydrates are a major energy source for all non-photosynthetic organisms (e.g., glycogen), and others have vital structural functions (e.g., chitin, cellulose) or are essential components of RNA, DNA, and biochemical cofactor synthesis (e.g., adenosine mono/di/triphosphate).

Investigations of ionization damage to carbohydrates were done mainly in the fields of food and DNA [3]. Food irradiation can be used to extend shelf life (0.5–3.0 kGy), to inhibit sprouting (0.03–0.12 kGy), for insect disinfestation (0.2–0.8 kGy) and parasite disinfestation (0.1–3.0 kGy), and to eliminate pathogenic bacteria that do not form spores (1.5–7.0 kGy). In this context, it is important to know the chemical transformations occurring at a molecular level, including carbohydrates, that might have an adverse impact on the nutritional, sensory, or functional state of food [4]. In DNA, the sugar moiety plays an important role in the radiation-induced strand breaking process, even if not all the carbohydrate alterations are implied [3].

Model molecules of carbohydrates, such as ethylene glycol, glycerol, and glucose, were used to understand radiation products yielded from carbohydrates. Furthermore, they were used to study the formation of radicals via electron spin resonance (ESR) and electron paramagnetic resonance (EPR) or molecular products via high-performance liquid chromatography-mass spectrometry (HPLC-MS_2) [4].

The radiolysis of carbohydrates in aqueous system is pH dependent and occurs mainly by an indirect interaction of hydroxyl radical (°OH) with C–H bonds producing carbohydrate radicals. In contrast, carbohydrates react slowly with superoxide radicals (coming from solvated electrons) and scarcely with °H radicals [3, 4]. The carbohydrate radicals readily react with molecular oxygen or experience dismutation, dimerization, and elimination of alcohol or water (the most ubiquitous). Thus, radiolysis of carbohydrate inside the DNA molecule can lead to a degradation of the sugar structure and a loss of the base.

3.1.2 Lipids

Lipids are small organic molecules, representing 21% of the eukaryotic cell content. Biochemically, they originate entirely or in part from carbanion-based condensations of thioesters, forming fatty acids, which are components of triacylglycerols (TAGs), phospholipids, and sphingolipids, or by carbocation-based condensation of isoprene units, forming isoprenol derivatives including sterols [2]. Lipids perform many essential functions in the cell including signaling and energy storage (due to their highly reduced state) and are the hydrophobic units of bilayers that form cellular and organellar membranes, which contribute to their function and topology.

In aqueous biological media, during IR, lipids (mostly polyunsaturated acids) are likely to undergo lipid peroxidation. This is initiated by some water radiolysis species and presence of endogenous transition metals [5] and propagates the chain reaction and produces several other organic reactive radicals. These primary and secondary radicals, being able to penetrate the membrane interior, may react either with the lipid matrix or with integral membrane proteins.

This radio-induced lipid peroxidation can thus contribute to the loss of cellular function through the inactivation of membrane enzymes and even of cytoplasmic (i.e., water soluble) proteins. Moreover, consequences include also perturbation of membrane function itself (thinning, change of structure or charge distribution, polarity) and consequently some carrier ion complexes and ion channels: efficiency can increase due to accumulation of polar oxidation products, but also be inhibited due to depolarization following conductance leakage [6].

3.1.3 Proteins

Proteins are biomolecules made of many linear chains of amino acid residues arranged in a three-dimensional structure, with various binding types (covalent or weak electrostatic bonds). Proteins constitute about 74% of the eukaryotic cell organic content. Amino acids, peptides, and proteins undergo a variety of reactions with radio-induced radicals which in most cases are pH dependent. These reactions involve mostly hydrogen abstraction at the α position of the amino acid, electron transfer, addition, fragmentation and rearrangement, dimerization, disproportionation, and substitution [7]. Many studies showed that the most reactive amino acids are the aromatic (Trp, Tyr, His) and sulfur-containing (Met, Cys) amino acids, whereas the least reactive is glycine (Gly) [7, 8]. Once generated, the formed protein radicals can interact with oxygen, yielding a peroxyl radical, and with other biological components for instance yielding other reactive radicals or initiating lipid peroxidation.

Some of the most commonly measured oxidative protein modifications are protein carbonyl groups originating from the oxidation of the amino acid residues or their side chains [9]. This leads to the formation of carbonyl derivatives, protein backbone cleavage, or beta scission of side-chain alk-

oxyl radicals of aliphatic residues (e.g., Ala, Val). In addition, oxidation of the sulfur of cysteine residues can lead to disulfur bond rearrangement.

Studies performed in biological media, e.g., cells, tend to show that in case of hydroxyl radicals coming from external irradiation, damage to DNA and lipids is a secondary process and proteins are more likely the initial targets, due to their relative amount and reactivity [7, 8] (Box 3.1).

Box 3.1 In a Nutshell: Radiolysis Products with Carbohydrates, Proteins and Lipids

- Radiolysis of carbohydrates and proteins occurs mostly via OH, begins with an abstraction of one hydrogen atom, and is pH dependent.
- Radiolysis of the carbohydrates within DNA may result in the loss of the base and thus DNA damage.
- Lipids are likely to undergo peroxidation following IR processes, initiating a chain reaction leading to the production of organic reactive radicals.
- Lipid peroxidation may lead to the loss of cellular functions including those associated with membranes.
- In proteins, the most reactive amino acids are the aromatic (Trp, Tyr, His) and sulfur-containing (Met, Cys) ones, whereas the least reactive is glycine (Gly).
- Protein radicals may react with oxygen-yielding peroxyl radicals or with other biological compounds such as lipids, leading to lipid peroxidation or formation of other reactive radicals.
- Some of the most measured oxidative protein modifications are protein carbonyl groups.
- In cells, proteins are the initial targets, due to their relative amount and reactivity.

3.2 Types of Radiation-Induced Lesions in DNA

In contrast to the above-described effects of IR in carbohydrates, lipids, and proteins, DNA radiolytic lesions occur both directly and indirectly, with the proportion being dependent on radiation type (α, β, γ, heavier ions). Deoxyribonucleic acid (DNA) molecules are, unlike other biomolecules within a cell, unique, and if they get damaged and stay unrepaired, this may lead to serious and often lethal consequences.

Due to the importance of DNA, cells have a complex DNA damage response system, consisting of several interrelated signaling pathways, which can recognize the damage and initiate its repair. DNA can be damaged by different mutagens, such as oxidizing agents and alkylating agents, as well as by IR or UV light. However, the type of DNA damage depends on the type of mutagen, as well as the type, dose, and energy of radiation.

3.2.1 DNA Structure

DNA is a large molecule composed of two polynucleotide chains that coil around each other to constitute a double-stranded helix structure. DNA molecules carry the genetic information for most biological processes. The two antiparallel DNA strands are connected by hydrogen bonds, and the backbone of each strand is composed of nucleotides. Each nucleotide consists of an alternating sugar (2-deoxyribose), a phosphate group, and one of the four nitrogen-containing nucleobases [adenine (A), cytosine (C), guanine (G), or thymine (T)]. The structure of the bases is shown in Fig. 3.2. Two of the bases, thymine and cytosine, are single-ring groups (pyrimidines), whereas two other bases, adenine and guanine, are double-ring groups (purines).

On one strand, nucleotides are joined to another by covalent bonds between the sugar of one nucleotide and the phosphate group of the next one (phosphodiester bond). The

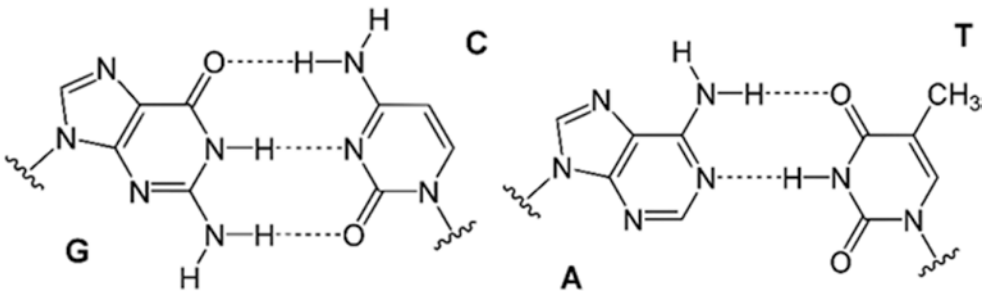

Fig. 3.2 The four DNA bases with respective hydrogen bonds (dashed lines). *G* guanine, *C* cytosine, *A* adenine, *T* thymine

bases on the opposite strands are complementary, adenine pairs with thymine and guanine pairs with cytosine through hydrogen bonds [10].

3.2.2 Damage of Sugar and Bases

A base lesion is defined as a modification (oxidation, alkylation, and deamination) of the chemical structure of one of the four DNA bases. Modification can occur through the loss of an electron, called oxidation, the transfer of an alkyl group, called alkylation, or the removal of an amino group, called deamination. After the break of the N-glycosidic bond between the DNA base and the 2-deoxyribose, a base can get lost and an abasic site can be created [11]. A representation of base lesion and abasic site is shown in Fig. 3.3. Sugar and base damages are quite easy for the cell to repair, as will be shown in Sect. 3.4.

Most of the sugar and base modifications are due to the hydroxyl radical (OH°). This radical reacts with the bases by addition to double bonds and by abstraction of hydrogen from the methyl group of thymine or from any C–H bond, but more likely from the C4 and C5 positions of the deoxyribose [12]. Pyrimidine base modifications are more readily formed after radiation compared with purines. The main radiation-induced base degradation products can be found in the work of Cadet and Wagner [13].

3.2.3 DNA Cross-Links

A DNA–DNA intrastrand cross-link (intra CL) is formed when chemical bonds are created between two DNA bases of the same DNA strand, while a DNA–DNA interstrand cross-link (inter CL) is created when the chemical bonds are between bases of opposing strands. A chemical cross-link can also be generated with another endo- or exogenous molecule such as surrounding proteins to produce a DNA-protein cross-link (DPC). A DPC is formed as a covalent linkage between the protein and DNA after radiation-induced generation of DNA base radicals and amino acid radicals, mostly via hydroxyl radicals, which interact with each other [12]. A representation of the cross-links is given in Fig. 3.4.

They are problematic since replication and transcription mechanisms require a separation of the DNA strands. The most frequent cross-links observed are between tyrosine and thymine, tyrosine and cytosine, or lysine and thymine.

3.2.4 Single-Strand Breaks

Single-strand breaks (SSBs) result from endogenous processes and exposure to exogenous agents such as radiation and chemicals. A representation of this process is given in Fig. 3.5. More frequently, IR creates free highly reactive radicals, especially hydroxyl radicals (OH°), which may react with nearby DNA and produce an SSB. The repair of SSB is rather simple, as it will be discussed in Sect. 3.4, and thus most of the time, an SSB does not cause any serious problems to the cell. The quantity of SSBs increases linearly with the IR dose applied, and their formation decreases when the linear energy transfer (LET) increases [14].

3.2.5 Double-Strand Breaks

Double-strand breaks (DSBs) are produced when two SSBs on the two opposite DNA strands appear in close vicinity (one or two helix turns, thus about 15–20 DNA base pairs apart) [11]. Since DSBs are considered as the most important

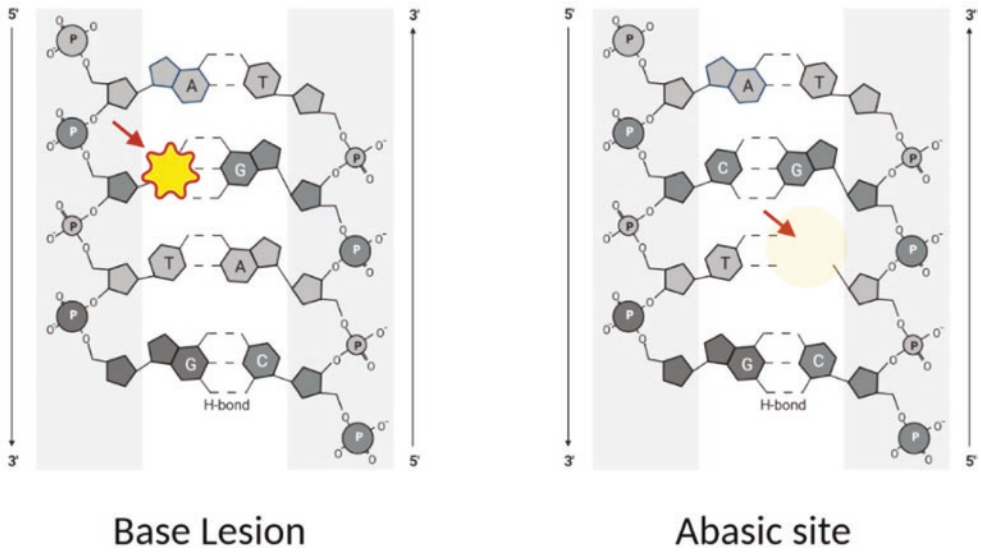

Fig. 3.3 Examples of DNA base damages. In base lesions, the chemical structure of any DNA base is modified (highlighted with yellow and red), whereas in abasic sites, the N-glycosidic bond between the DNA base and the 2-deoxyribose is broken (as shown with red arrow). *G* guanine, *C* cytosine, *A* adenine, *T* thymine, *H-bond* hydrogen bond, *P* phosphate

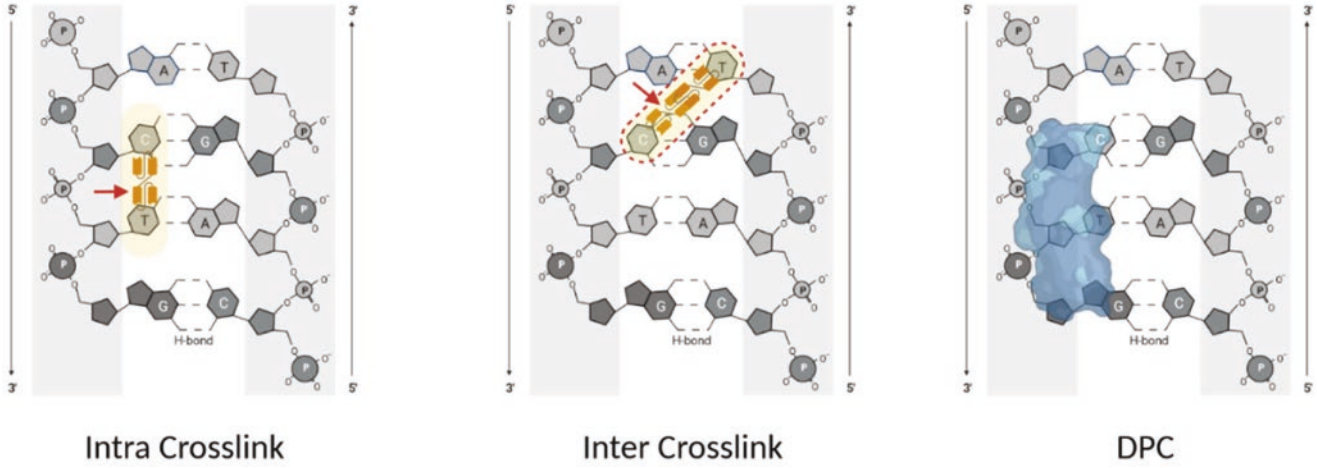

Fig. 3.4 Examples of DNA cross-links. Chemical bonds (yellow) are created between two DNA bases within the same DNA strand (intra cross-link) or opposite strands of double-stranded DNA (inter cross-link). Proteins (blue) can become cross-linked to DNA to form DNA-protein cross-link (DPC). *G* guanine, *C* cytosine, *A* adenine, *T* thymine, *H-bond* hydrogen bond, *P* phosphate

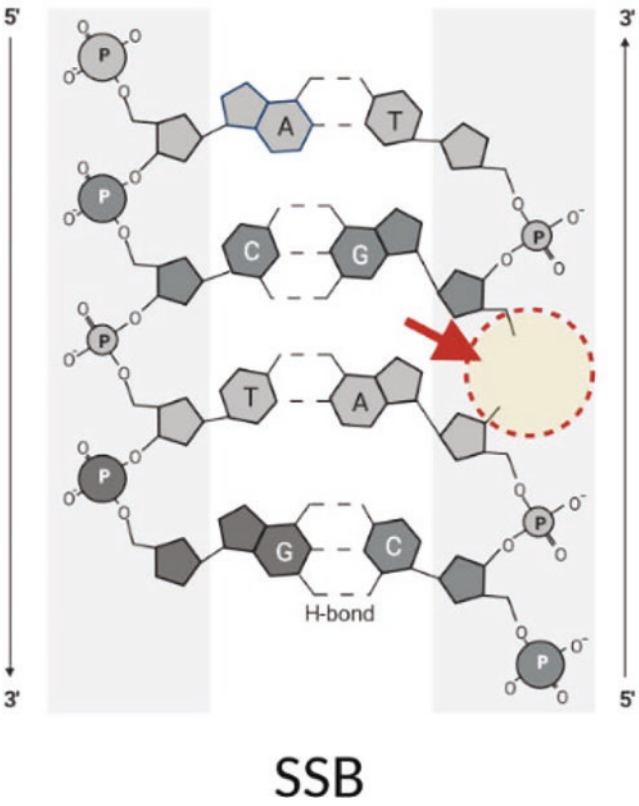

Fig. 3.5 Single-strand breaks (SSB): an illustration of a single-strand break in DNA. *G* guanine, *C* cytosine, *A* adenine, *T* thymine, *H-bond* hydrogen bond, *P* phosphate

cause of cell death after IR, understanding their mechanisms of formation is essential. Radiation-induced DSBs increase linearly with radiation doses up to several hundred Gray (Gy) and have been detected at as low as 1 mGy [15]. As explained in Chap. 2, low linear energy transfer (LET) IR consists of electrons and photons that liberate secondary electrons and produce reactive oxygen species (ROS). However, even if they can create closely spaced lesions, the collision between particles and atoms in tissues is infrequent, thus leading to less, randomly distributed DSBs. On the contrary, the damages induced by high-LET particles are distributed along the particle tracks, which exhibit higher rates of collision and lead to nonrandom DSB distributions. Furthermore, there is a complexity of the nature of the DSBs formed according to the dose and the type of radiations, which influence the DNA damage response (DDR) and its efficacy. One can talk about "clean DSBs," produced by hydrolysis of the phosphodiester bonds, which are easier to repair compared to "dirty DSBs," which contain residual modified sugar residues produced by reaction of the 2-deoxyribose with hydroxyl radicals [11] (see Fig. 3.6). "Dirty" DSBs are more frequently created by high-LET heavy ions or α particles.

Induction of DSB lesions by radiation is reviewed by Sage and Shikazono [16]. The ROS produced by the water radiolysis mediated by irradiation induces oxidized bases and loss of bases. Both lesions are repaired by base excision repair (BER, see Sect. 3.4), which can lead to DSB formation. Usually, DNA gaps of 1 or 2 nucleotides are filled by DNA polymerase and sealed by DNA ligase IIIα. During this process, SSBs can be generated in both DNA strands, and when they are close enough lead to a DSB. Moreover, the repair of a cluster lesion, e.g., an SSB opposite to an oxidative DNA lesion, could also result in the formation of a DSB as a result of irradiation. Additionally, through replication, if a damage is complex, e.g., effect on DNA secondary structures, formation of abasic sites, cross-links, and effect on DNA-binding proteins, the replication fork can stall and a DSB might occur. Moreover, conformational variables of the chromatin, which is a dynamic entity, and nuclear factors might affect DSB formation caused by radiation-induced

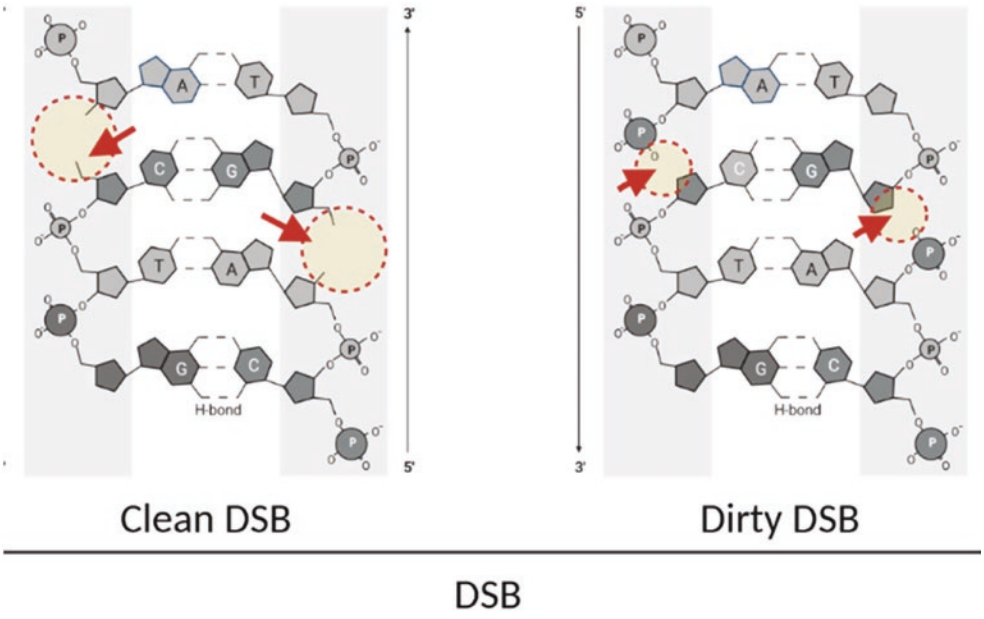

Fig. 3.6 Double-strand breaks (DSB): an illustration depicting different types of double-strand breaks in DNA. *G* guanine, *C* cytosine, *A* adenine, *T* thymine, *H-bond* hydrogen bond, *P* phosphate

radicals across the genome and according to the different points of the cell cycle.

3.2.6 Complex DNA Damage

Complex DNA damages, described as clustered DNA damages, are also named "locally multiple damaged sites" (LMDSs). LMDSs consist of closely spaced DNA lesions within a short DNA segment and are responsible for an increased cellular lethality since they are more difficult to repair. Two or more DNA lesions of the same or different type may be induced by IR within one or two helical turns of the DNA molecule, on the opposite strand. This clustered bistranded damage can be SSBs, DSBs, oxidized bases, and abasic sites. For example, at a dose of 1 Gy of IR, all this damage can be generated isolated or up to 10 bp apart [17]. Furthermore, the number of lesions per cluster depends on the radiation type and dose [18]. Experimental and theoretical studies have evidenced an increased complexity of the DNA damage induced by high-LET IR due to clustered ionizations, making complex DNA damage the signature of high-LET IR. Indeed, such lesions are considered the most important ones in terms of biological effects since they are the most challenging for the DNA repair machinery.

3.2.7 Overview of Ionizing Radiation-Induced DNA Damage

Not all cellular DNA damage is caused by exogenous factors; it can also be the result of cell metabolism as well as other normal cell processes. An overview of the average yield of DNA damage by endogenous factors per day and by low- and high-LET IR by 1 Gy is given in Table 3.1. One can see that even though the number of particles in the nucleus for high-LET radiation is much lower compared to low-LET radiation, the number of ionizations is the same. The dose deposition profile of high-LET IR induces more localized, complex, and clustered damages, which are more difficult to repair.

Table 3.1 Comparison of DNA damage for endogenous factors and low- or high-LET radiations

	Endogenous/cell/day	Low-LET IR/Gy	High-LET IR/Gy
Tracks in nucleus	–	1000	A few <1
Ionizations in nucleus	–	100,000	100,000
Ionizations in DNA	–	1500	1500
Base damage	16,000	10,000	10,000
DNA single-strand breaks	10,000–55,000	700–1000	300–600
DNA double-strand breaks	8	40	>40
Cross-link DNA/DNA	8	30	–
Cross-link DNA/protein	A few	150	–
Locally multiple damaged sites	A few	Increased with LET	

The number of tracks in the cell nucleus as well as the number of induced damages for high-LET IR depends on the particle type and energy; therefore, the given values represent only an estimate

3.2.8 UV Radiation-Induced DNA Damage

Ultraviolet (UV) light (100–400 nm) is a natural genotoxic agent able to induce deleterious effects affecting biological processes and structures, but also DNA structure, leading to a genomic instability [19]. DNA damage induced by UV is

mainly pyrimidine dimers, oxidized bases, as well as SSBs and DSBs. Nucleotides absorb UV radiations, which raise the DNA base to a highly reactive singlet or triplet state, leading therefore to photochemical reactions. The chemical nature and the amount of DNA damage strongly depend on the wavelength of the incident photons. Three main types of DNA lesions are formed involving two successive pyrimidine bases (CC, TT, TC, and CT) and leading to a DNA double-helix distortion: cyclobutane pyrimidine dimers (CPDs), pyrimidine 6-4 pyrimidone photoproducts (6-4PPs), and their Dewar isomers. The most energetic part of the solar spectrum corresponding to UVB (290–320 nm) leads to the formation of CPDs and 6-4PPs, whereas less energetic but 20 times more intense UVA (320–400 nm) also induces the formation of CPDs associated with a wide variety of lesions such as single-strand breaks and oxidized bases. Furthermore, in addition of the direct photolesions induced, some indirect DNA damage can occur through the production of ROS, especially hydroxyl radicals (OH°) and RNS. ROS can induce the oxidation of pyrimidine and purine bases, and also the deoxyribose backbone of DNA, such as the induction of the most frequent, i.e., the 8-hydroxyguanine (8-oxo-G) and in a smaller extent SSBs and DSBs. Moreover, the ROS induced by UV can lead to the alkylation of bases and to cross-linking of DNA–DNA or DNA-protein. CPDs and 6-4PPs are mostly formed between TT and TC, and in less proportion for CT and CC sequences. Additionally, the chromatin structure, as well as the composition of the neighboring nucleotide sequence of pyrimidine dimers, also influences the formation of UV-induced DNA damage. More recently, some studies discussed the influence of the epigenetic markers (DNA methylation, histone posttranslational modifications) in the induction of UV-induced lesions at a particular locus. Indeed, the methylation of DNA at C5 of cytosine (5-mC) was associated with an increase by 80% of the CPD yield and a decrease by 3 of the 6-4PP [20] (Box 3.2).

Box 3.2 In a Nutshell: Types of Radiation-Induced Lesions in DNA

- Deoxyribonucleic acid (DNA) is a large molecule composed of two polynucleotide chains that coil around each other to constitute a double-stranded helix structure.
- IR can cause DNA base or sugar damage, single- or double-strand breaks, DNA interstrand, intrastrand, or protein cross-links.
- DSBs are considered to be one of the most serious DNA lesions.
- High-LET IR induces more localized, complex, as well as clustered damage, which has the most serious potential biological consequences.

3.3 Types of DNA Repair Pathways

As described above, various types of DNA lesions occur through endogenous and exogeneous factors frequently in a human cell. Depending on the complexity, these lesions challenge cellular genomic integrity. At the time of cell division, many cellular processes are coordinated to ensure the maintenance of the stable genome and ascertain the preservation of the nuclear material [21]. These processes are known as the DNA damage response (DDR). The types of DNA damage and their primary repair pathway are listed in Table 3.2. The DDR signaling capacity can, if not sufficient, cause problems for the cell to maintain genome stable, which may result in a mutation. This may, as a last consequence, trigger transformation into a tumor or cancer cell. As DNA damage occurs physically, it can be repaired; however, when the mutation is established, the alterations that took place in the base sequence cannot be repaired. Accordingly, it is essential for normal cells to maintain DDR function to avoid such process.

3.3.1 Base Excision Repair

Base excision repair (BER) is the most common and important DNA repair process involved in removing minor DNA base defects. Many BER genes are extremely maintained from bacteria to humans demonstrating that BER is a fundamental repair process [22]. BER is a well-studied pathway for damage repair caused by respiration, spontaneous hydrolysis, and alkylation events, such as single-nucleotide bases (small, non-helix-distorting base lesions), that occur hundreds of times every day in each cell [23]. Thus, the BER system is critical to eliminate damaged bases that could otherwise produce mispair mutations or DNA replication breakdowns. In BER, SSBs are formed and repaired in an organized chain of events involving multiple proteins. Within BER, two pathways are simultaneously active: short patch repair (SP-BER), which is used to eliminate a broken base which has a non-bulky character, and long patch repair (LP-BER), which can replace the area in which the damaged DNA base is found. A schematical view of SP- and LP-BER can be found in Fig. 3.7.

In BER, specialized proteins called glycosylases recognize and remove the majority of the damaged DNA bases. There are multiple glycosylases, each of which is unique to a certain form of base damage. All these enzymes have, as their primary function, to cut out the base which got damaged yet without impacting the DNA backbone, causing further damage in an abasic place in the DNA (either apurinic or apyrimidinic site) [25]. Although each DNA glycosylase is specialized to a certain substrate and works in a distinct manner, they all have a single principal way of action: first, tak-

Table 3.2 DNA damage repair mechanisms

DNA repair mechanism	DNA damaging/genotoxic agents	DNA lesion feature	DNA damage example	DNA repair features
Base excision repair (BER)	Reactive oxygen species, X-rays, alkylating agents	Oxidative lesion	Oxidation (8-oxo-G) uracil, single-strand break	Removal of base by N-glycosylase abasic sugar removal, replacement
Nucleotide excision repair (NER)	UV lights and polycyclic aromatic hydrocarbons	Helix-distortion lesion	Bulky adducts, intrastrand cross-link	Removal of DNA fragment and replacement
Mismatch repair (MMR)	Replication	Replication error	A–G mismatch, T–C mismatch, insertion, deletion	Removal of strand by exonuclease, digestion, and replacement
Double-strand break repair (DSBR)	X-rays, ionizing radiations, reactive oxygen species, anti-tumor agents	Double-strand DNA breaks	Double-strand break, interstrand cross-link	Unwinding, alignment, ligation

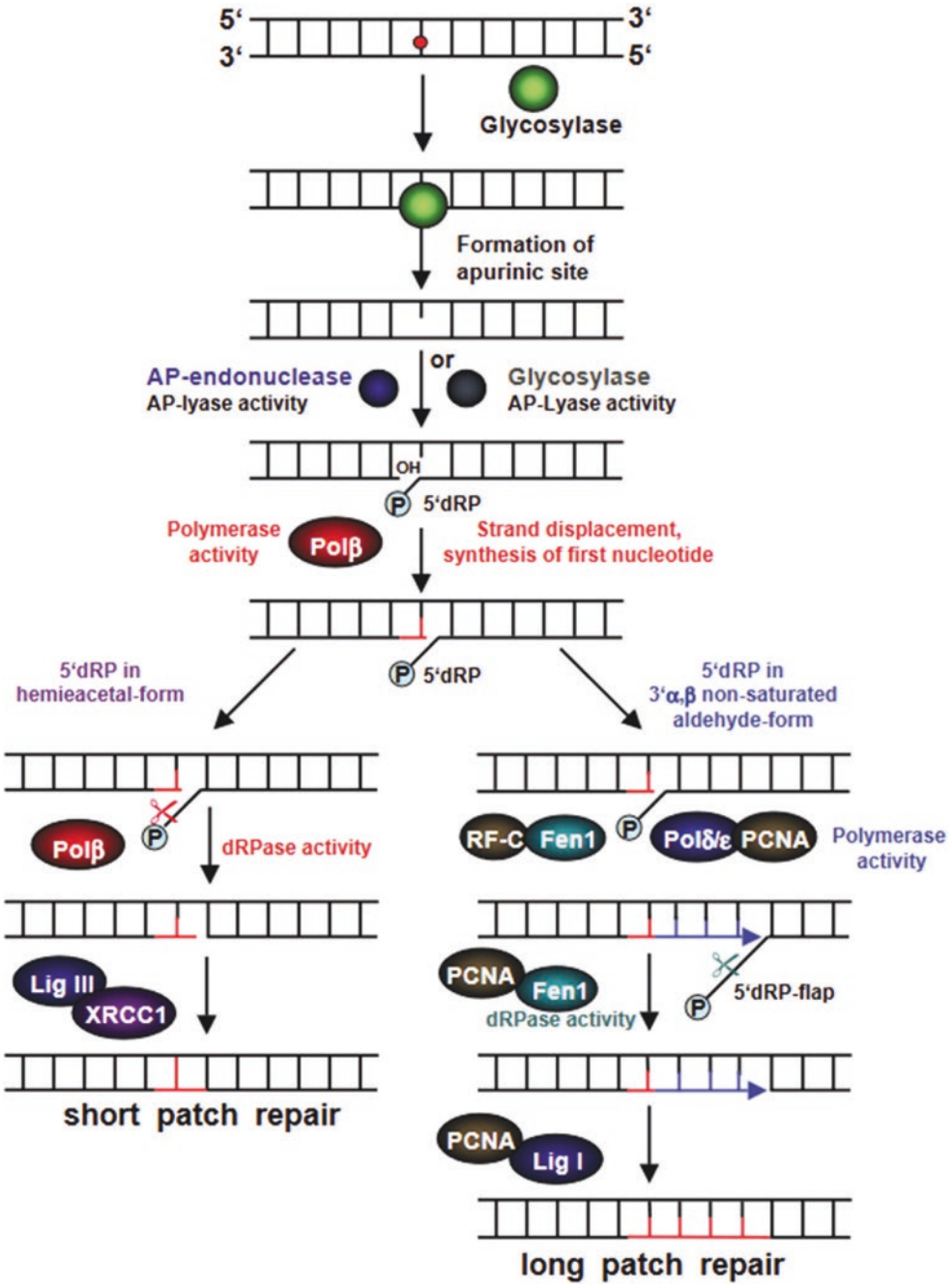

Fig. 3.7 Short and long patch base excision repair: recognition of the DNA lesion occurs by a specific DNA glycosylase which removes the damaged base by hydrolyzing the N-glycosidic bond. The remaining AP site is processed by APE. Depending on the cleavability of the resulting 5′dRP by Polβ, repair is performed via the short or long patch BER pathway. Reproduced with permission from [24]. *AP-endonuclease* apurinic/apyrimidinic endonuclease, *AP-lyase* apurinic/apyrimidinic lyase, *OH* hydroxide, *P* phosphate, *5’dRP* 5′ deoxyribose phosphate, *Lig III* ligase III, *XRCC1* X-ray repair cross-complementing 1, *RF-C* replication factor C, *Fen1* flap structure-specific endonuclease 1, *PCNA* proliferating cell nuclear antigen, *Lig I* ligase I

ing the damaged base outside the DNA helix, thus assisting the detection of bases with minute alterations, and, second, triggering the cutting of an N-glycosidic bond, which in turn enables the formation of an abasic site [22]. Humans have 11 DNA glycosylases, which are classified as monofunctional (removing a base which results in formation of an AP site), bifunctional (removing a base and cutting the DNA backbone close to the damaged base), or Nei-like (which removes the base but also cuts each side of it).

Once the monofunctional DNA glycosylase has created the AP site, another repair enzyme, AP endonuclease 1 (APE1), incises and hydrolyzes the AP site, removing the base followed by the sugar residue, cutting the DNA backbone, and as a result an SSB is formed. APE1 also operates on bifunctional glycosylase products, creating a one-nucleotide gap product after hydrolysis. Polynucleotide kinase phosphatase (PNKP), whose product is suitable for DNA polymerase action, is required for the repair of oxidized DNA bases. When there is a gap or SSB is formed, poly(ADP-ribose) polymerase 1 is activated (PARP1) [23]. In this way, the integrity of the break can be maintained. PARP1 also orchestrates, via its poly(ADP-ribosyl)ation activity, a cascade of proteins binding to the SSBs with the main aim to detect and promote its further repair.

The most common polymerase used in BER is DNA polymerase (Pol), which fills the gap with the proper nucleotide and catalyzes a lyase reaction. SP-BER is linked by the DNA ligase III-XRCC1-mediated mechanism to complete the process [25]. In contrast to SP-BER, LP-BER occurs when a lesion is resistant to Pol cleavage, and polymerases such as PCNA, flap endonuclease 1 (FEN1), and PARP are recruited. While displacing the broken strand, the polymerase synthesizes DNA and inserts a repair patch consisting of 2–12 of the correct nucleotides into the gap. The repair synthesis is carried out by the T complex of the replication factor C (RFC)/proliferating cell nuclear antigen (PCNA)/DNA polymerase δ/ε. Here, the lap endonuclease 1 (FEN1) acts by taking out the flap structure that is overhanging the damaged base site, and the nick that is formed is ligated by DNA ligase I [14]. SP-BER and LP-BER primarily differ in how many of the DNA bases are cut out during the repair (see Fig. 3.8). SP-BER only replaces the bases which are damaged, whereas LP-BER cuts out and replaces up to ten nucleotides.

IR-induced base damage is effectively repaired by BER. BER deficiencies can result in a higher mutation rate but seldom cause cellular radiosensitivity [26]. The X-ray cross-complementing factor 1 (XRCC1) gene mutation, which causes a 1.7-fold increase in radiation sensitivity, is an exception. The radiation sensitivity of XRCC1-deficient cells, on the other hand, could be due to XRCC1's involvement in other repair processes, such as SSB repair. Reduced repair and radiosensitization can be caused by mutations, deletions, or inhibition of either of these genes.

In both BER and SSB repair, DNA polymerase beta (pol) is a key enzyme. Under some situations, cells lacking pol or expressing a dominant negative construct to pol, which inhibits its function, have been demonstrated to be more vulnerable to ionizing radiation in vitro [27]. Small-molecule medicines that block PARP1 have also been produced. The PARP inhibitors are a medication that targets BER and SSB repair and are now being tested in clinical trials for cancer treatment, as described in Chap. 6 (Box 3.3).

Box 3.3 In a Nutshell: Base Excision Repair

- BER is a specific repair mechanism that is used to handle DNA base damage.
- BER removes single-nucleotide base lesions (small, non-helix-distorting base lesions) from the genome.
- SP-BER and LP-BER are two complementary BER systems essential for removing base damage and fixing SSB in DNA, minimizing mutagenesis but differing in what base damages they can handle.
- BER inhibitors have showed potential as radio/chemosensitizers in a variety of malignancies, or they can create synthetic deadly alliances with common cancer mutations.

3.3.2 Nucleotide Excision Repair

From unicellular bacteria to complex humans and plants, nucleotide excision repair (NER) works in a similar way. In humans, NER is known for its one-of-a-kind repair process to remove photolesions caused by UV radiation. However, there is one circumstance in which NER genes can influence the IR response. More DNA cross-links are formed when cells are irradiated under hypoxia than when irradiated under normoxic circumstances. Excision activity of two NER genes, DNA excision repair protein (ERCC1) and DNA repair endonuclease (XPF), is required for such cross-links, among other things. Defects in either of these genes may cause hypoxic cells to become more radiosensitive. As a result, the status of the NER pathway is relevant to radiotherapy in combination with specific chemotherapeutic drugs, as well as hypoxic tumors treated only with radiotherapy [28].

The principle of NER is shown in Fig. 3.8. The lesion-recognizing NER factors look for unpaired single-stranded DNA on the other side of the damaged strand [22]. The oligonucleotide that contains the lesion is eliminated, and to restore the DNA to its original form, a repair patch is created using

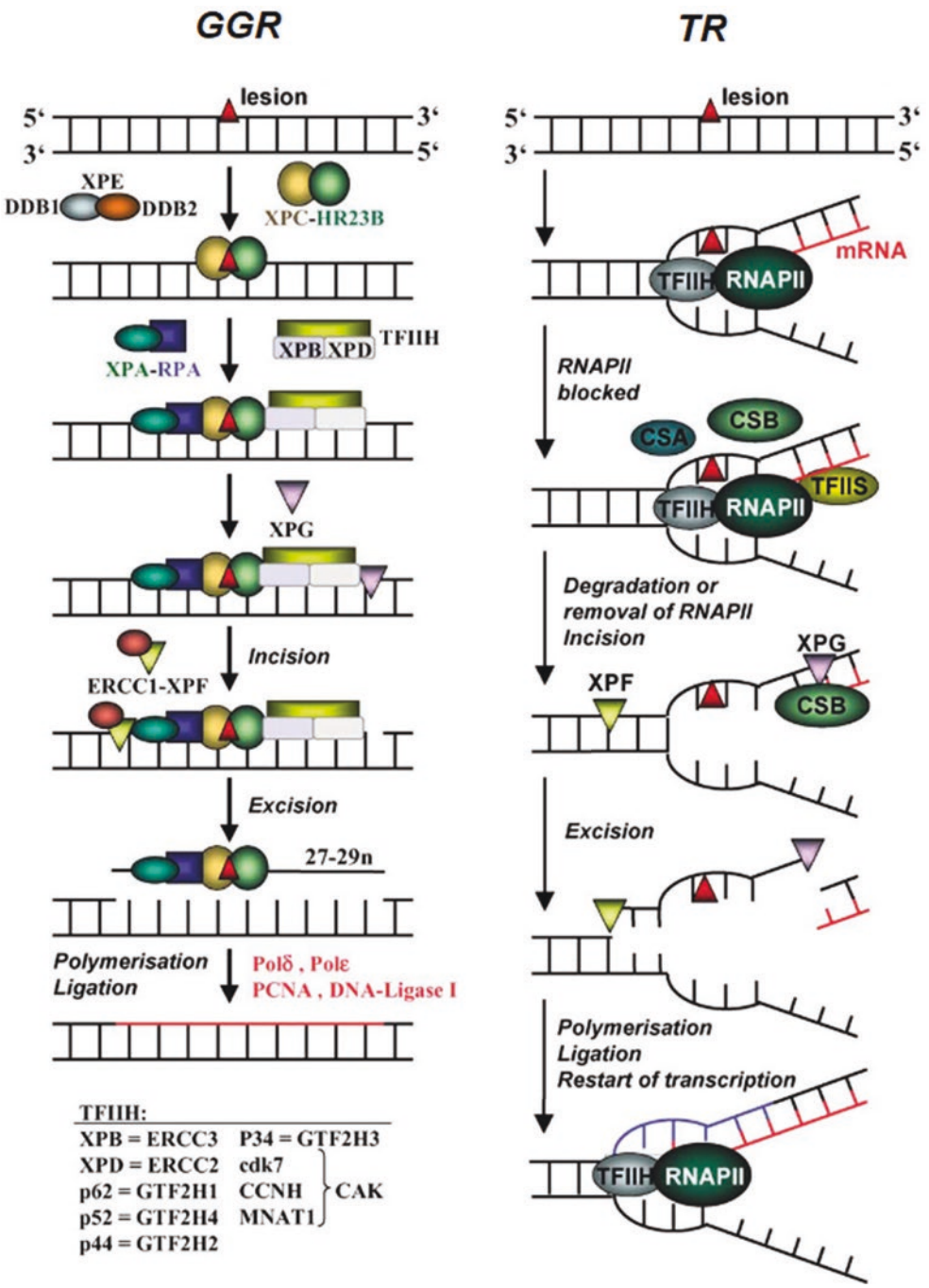

Fig. 3.8 Nucleotide excision repair (NER) pathway: during global genomic repair (GGR), recognition of the DNA lesion occurs by XPC–HR23B, RPA–XPA, or DDB1–DDB2. DNA unwinding is performed by the transcription factor TFIIH and excision of the lesion by XPG and XPF–ERCC1. Finally, resynthesis occurs by Polδ or Polε and ligation by DNA ligase I. During transcription-coupled repair (TCR), the induction of the lesion results in blockage of RNAPII. This leads to assembly of CSA, CSB, and/or TFIIS at the site of the lesion, by which RNAPII is removed from the DNA or displaced from the lesion, making it accessible to the exonucleases XPF–Ercc1 and XPG cleaving the lesion-containing DNA strand. Resynthesis again occurs by Polδ or Polε and ligation by DNA ligase I. 23B: Reproduced with permission from Christmann et al. [24]. *DDB1* DNA damage-binding protein 1, *DDB2* DNA damage-binding protein 2, *RPA* replication protein A, *TFIIH* transcription factor IIH, *ERCC1* excision repair cross-complementing group 1 protein, *Polyδ/ε* DNA polymerase delta/epsilon, *PCNA* proliferating cell nuclear antigen, *Lig1* DNA ligase 1, *RNAPII* RNA polymerase II, *CSA and CSB* Cockayne syndrome factors A and B, *TFIIS* transcription initiation factor IIS, *HR23B* homologous recombinational repair group 23B

the opposite undamaged complementary strand as a template. With varied degrees of success, NER eliminates lesions from the entire genome and can be separated into two paths [24]:

1. Global Genome Repair (GGR or GG-NER): GG-NER is a genome-wide process, i.e., lesions can be eliminated from DNA that encodes, or not, for genes.
2. Transcription-Coupled Repair (TCR or TC-NER): TC-NER exclusively eliminates lesions in the DNA strands of genes that are actively transcribed. If a DNA strand that is actively transcribed is broken, the RNA polymerase could inhibit DNA repair by blocking access to damage sites. TC-NER has evolved to overcome RNA polymerase's barrier by essentially eliminating it from the damage site, allowing repair proteins access.

In the early damage recognition phase, the two NER sub-pathways vary. In GGR, the NER proteins are recruited by the stalled RNA polymerase in collaboration with Cockayne syndrome protein B and A (CSB and CSA). In TCR, the NER proteins are engaged by the stalled RNA polymerase in collaboration with CSB and CSA [14].

Mutations in the NER genes do not cause IR sensitivity. However, defective NER increases sensitivity to UV-induced DNA damage and anticancer drugs that create bulky adducts, such as alkylating agents. Human DNA repair deficiency such as xeroderma pigmentosum, in which individuals are hypersensitive to UV radiation, is caused by germline mutations in the NER genes [14] (Box 3.4).

Box 3.4 In a Nutshell: Nucleotide Excision Repair

- Nucleotide excision repair (NER) is a technique for removing bulky adducts from DNA, chiefly those caused by UV.
- Defects in certain NER proteins may result in enhanced radiosensitivity of hypoxic cells.
- Large DNA lesions like thymine dimers and cisplatin adducts are repaired using a DNA repair pathway.
- The two types of NER pathways are global genome repair (GGR or GG-NER) and transcription-coupled repair (TCR) (TCR or TC-NER).

3.3.3 Mismatch Repair

The mismatch repair (MMR) system has a role after the cellul replication process, where sometimes incorrect bases pair with each other (which is called a mismatch). Therefore, MMR aids in keeping DNA homeostasis and plays a major role in evolutionary genomic stability [29]. Its basic purpose is to rectify the small insertion-deletion loops (indels) and the base-base mispairs that are spontaneously generated at the time of DNA replication. These mis-incorporated bases have escaped the proofreading action of replication polymerase. Usually, the polymerase that carries out the DNA synthesis process is not completely error-free. The DNA polymerase on average makes one mistake for every 10^5 nucleotides [29], which implies that ~100,000 errors arise through each S phase of the cell. Even though the DNA polymerase is there to ascertain that such mistakes do not occur, a few mutations can go unnoticed by it and hence the MMR-associated genes act as the second line of defense. However, if the cell is deficient in the MMR process, these errors remain uncorrected. Therefore, the mutational rate and sequence length modification in the microsatellites, which is a known trait of tumor cells, increase. The relevance of MMR in radiation-induced damage and cellular radiosensitivity is a matter of controversy. The mismatch repair (MMR) pathway was first discovered in *E. coli* cells [30]. Researchers have explored and understood that the MMR pathways and its associated proteins are evolutionarily conserved in almost all organisms including humans [31]. MMR works by inserting or deleting the mispaired bases by recognizing the mispaired lesion; excision, i.e., removal of the erroneous strand; and DNA resynthesis and gap repair by filling it with the correct resynthesized DNA.

The parent strand, which includes a palindrome DNA sequence "GATC" and adenine, is methylated by the enzyme deoxy-adenine-methylase. However, after replication when there are two new incorrect strands, methylation in the newly formed daughter strand is not seen [32] (Fig. 3.9). Such alterations are recognized and repaired by the methyl mismatch repair. The specific region of mispairing is recognized by the Mut S protein, which is coupled by the MutL. The activity of MutS is stimulated by the heterodimer MSH2–MSH6, along with MutSα. The MutSα recognizes small IDLS comprising 1–2 nucleotides, whereas the MSH2–MSH6 identifies longer insertion-deletion loop-type mismatches. After the binding of MutS to the DNA, it is followed by the ATP-dependent prerequisite of MutL homolog (MSH) complex. The parent strand is recognized by the MutL, which brings the misrepaired region nearer and leads to a loop formation around the area. Another protein, MutH, an endonuclease enzyme, performs the activity of cleaving. Next, UVr-D, a helicase, releases the cut strand leading to the formation of a gap where the new error-free or accurate nucleotide sequence is included by the polymerase 1 and joined by ligase. Cells that are deficient in the MMR proteins exhibit a high frequency of mutations and also irreversible microsatellite instability. Accordingly, individuals with germline mutations in MMR genes are more susceptible to various types of cancers [33] (Box 3.5).

Box 3.5 In a Nutshell: Mismatch Repair

- MMR targets DNA mismatches that arise mainly during replication, as well as repairing mismatches that occur in DNA following treatment with alkylating agents.
- The MMR pathway detects and repairs erroneous insertions, deletions, and base substitutions that have not been detected by the proofreading function of DNA polymerase during DNA replication, thus maintaining the genome stability.
- It works by recognition of mispair, excision of the affected strand, and filling of the gap.

3.3.4 Double-Strand Break Repair

Double-strand breaks (DSBs) are the most lethal kind of DNA damage because even one uncorrected DSB can result in loss of genetic information and finally lead to cell death. Moreover, such unrepaired or misrepaired DSBs can lead to

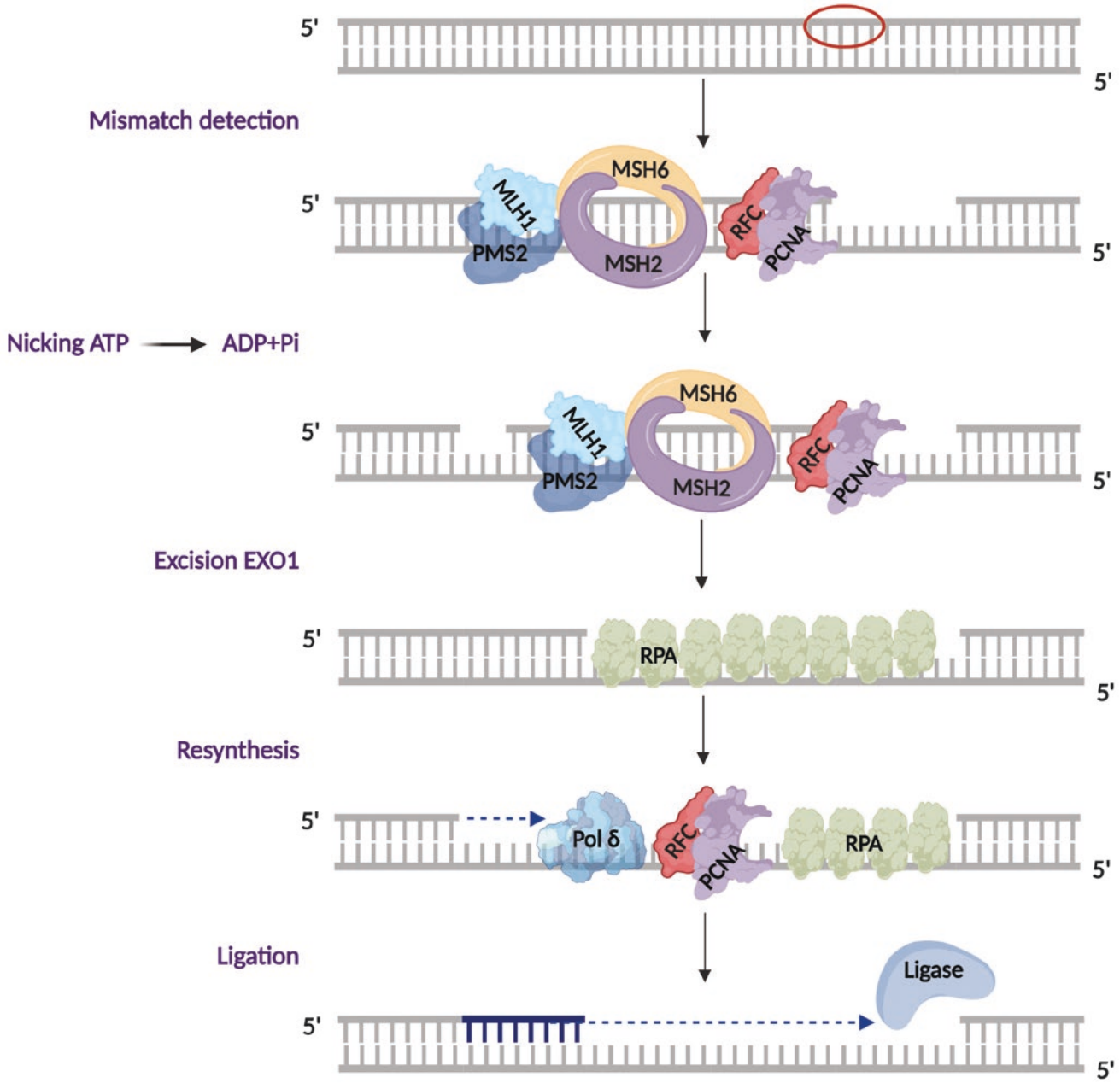

Fig. 3.9 Overview of eukaryotic mismatch repair system. In the human cell, the predominantly found MutSα (MSH2–MSH6) or the MutSβ recognizes the DNA mismatch repair and initiates its repair. Some of the crucial molecules which participate in the repair are the MutLα (MLH1-PMS2), the proliferating cell nuclear antigen (PCNA), and the replication factor (RCF). EXO1 catalyzes the repair, and ligase finally ligates the repaired DNA

augmented genomic instability and eventually tumorigenesis [21]. Accordingly, for a cell to pursue its genetic information, a functional DSB repair system is of major importance. As a result, cells have evolved a dedicated response to identify and mend DSBs. For repair of DNA DSBs, two principal pathways are used, namely homologous recombination (HR) and Non-homologous end joining (NHEJ).

These pathways differ with respect to the use of homologous template DNA as well as in DNA repair fidelity. HR utilizes undamaged sister chromatid as its template to repair the damage, and therefore it is error-free. However, NHEJ works by eliminating the damaged DNA followed by direct ligation and hence is error-prone. As HR needs an undamaged template, it only operates in late S and G2, in contrast to NHEJ, which has the capacity for DSB repair regardless of the cell's position in the cell cycle phase [33].

3.3.4.1 Homologous Recombination (HR)

The homologous recombination (HR) molecular pathway is associated with a large number of cellular processes, from

imparting genetic diversity to DNA repair or replication. HR is evolutionarily conserved from bacteria to mammalian cells. This pathway is essential for fixing DNA damages with high accuracy by using the genomic code of the chromosomal copy which was not damaged [34]. HR works by precisely repairing the DSB, shielding cells from any chromosomal abnormalities such as those observed in many cancers. Throughout the process of DNA replication, HR-associated proteins endorse the faithfulness and restoring of distressed DNA replication forks. This adds sturdiness, serving the replication machinery to circumvent under replication and succeeding segregation tribulations of the chromosome. Inherent HR insufficiency in cells can persuade instability in the genome and further lead to cancer. Conversely, discrepancy in the HR pathway also sensitizes tumors not only to DNA damage treatment but also to other potential DNA repair inhibitors for remedial repair pathways.

For the commencement of the HR pathway, the break site 5′–3′ end resection is a requirement, which not only exposes the single-stranded DNA (ssDNA) overhangs but also averts the NHEJ pathway to repair the DNA breaks (Fig. 3.10) [36]. The repair proteins MRE11 (meiotic recombination 11), RAD50 (RAD50 double-strand break repair protein), and NBS1 (nibrin) form the MRN complex, and together with the ataxia-telangiectasia mutated (ATM) kinase, they are the first to recognize the DNA damage. By attaching to the DNA ends, the MRN complex instigates the process of DNA end resection. Next C-terminal binding protein 1 interacting protein (CtIP) is employed so as to produce the overhangs at the 3′ end of the single-stranded DNA [36]. The preference of the choice of repair pathway is governed by the p53-binding protein 1 (53BP1) and breast cancer-associated protein 1 (BRCA1) contrasting activity in addition to the MRE11 resection activity. Whenever a DNA break is identified, both BRCA1 and 53BP1 compete to govern the commitment of the cell to undergo NHEJ or HR, respectively. By hindering the DNA end resection and concurrently securing two double-stranded DNA (dsDNA) ends, facilitating their successive ligation, 53BP1 supports the NHEJ pathway [37]. The mechanism by which BRCA1 suppresses 53BP1 still remains uncertain. Ubiquitination of CtIP occurs when BRCA1 interacts with BRCA1-associated RING domain protein 1 (BARD1). This subsequently enhances the affinity of CtIP for DNA and as a consequence promotes resection [37]. At this time, the DNA ends are protected and prevented from resection by replication timing regulatory factor 1 (RIF1), which is a 53BP1-interacting partner and a Shieldin complex. The increased HR activity can be attributed to either the loss of 53BP1 or the Shieldin complex that weakens the NHEJ pathway. Blocking wide-ranging end resection is central, meant for preventing the hyper-recombination by HR and stopping the loss of genetic material. Some other lethal repairing pathways like break-induced replication (BIR) or single-strand annealing (SSA) can lead to wide-ranging resection whose outcome is loss of heterozygosity [35].

A full functional HR pathway can be utilized after the DNA end resection. A detailed review of this process can be found in the work of Ranjha et al. [38]. The canonical HR pathway not only restores a direct DSB, but also repairs damage created by stalled or collapsed replication forks [21]. As soon as an extensive resection is executed by the action of several nucleases, cells are obligated to follow a homology-governed mode of repair. The DSB goes through a nuclease-driven progression known as DNA end resection in order to produce 3′-end ssDNA segments all through HR. This is crucial for the searching and strand invasion that occurs later during the recombination process. Along with the CtIP nuclease, DNA end resection is instigated by the MRE11 nuclease within the MRN complex. MRN/CtIP in combination with Bloom syndrome protein (BLM) or exonuclease 1 (EXO1) and DNA replication helicase/nuclease (DNA2) arbitrates the short- as well as long-term resections. During this resection, the 3′ ends of ssDNA get exposed that are rapidly covered by replication protein A (RPA) complex. The ssDNA region covered by RPA further recruits and stimulates the ataxia-telangiectasia and Rad3-related (ATR) kinase. This in turn triggers the checkpoint kinase 1 (Chk1) kinase. The RPA coating not only ascertains the nondegradation of ssDNA overhangs but also avoids the formation of secondary structures. To form the presynaptic filament, RAD51 dislocates RPA, which is then involved in the action of several RAD51 mediator proteins. To construct a displacement loop (D-loop), the RAD51 nucleoprotein filament explores a homologous sequence to occupy and dislocate one strand of the homologous template. This structure aids in the formation of a heteroduplex by pairing the broken strand with the displaced strand, and DNA synthesis at the break site repairs for any missing nucleotides. The outcome of the second end capture leads to the configuration of a double-Holliday junction (dHJ). The resolution of such an intermediate occurs either by a resolution mechanism or by a dissolution, which makes it susceptible to crossover (CO) or noncrossover (NCO). On the other hand, at the time of synthesis-dependent strand annealing (SDSA), no more than one-end invasion takes place, therefore leading to the formation of a single-Holliday junction. This transitional structure is suspended into an NCO. The HR repair pathway is known to also involve chromatin modifiers, remodelers, and even integration of histone variant so as to deal with the obstructions that the nucleosomes produce to the resection machinery. HR is active during the late S phase and the G2 phase and therefore is able to utilize the sister chromatid as a guiding template to repair the DSBs. Hence, this pathway is error-free [38].

3.3.4.2 Non-homologous End Joining

The Nonhomologous end joining pathway (NHEJ) pathway (Fig. 3.11) has long been demonstrated to be central in

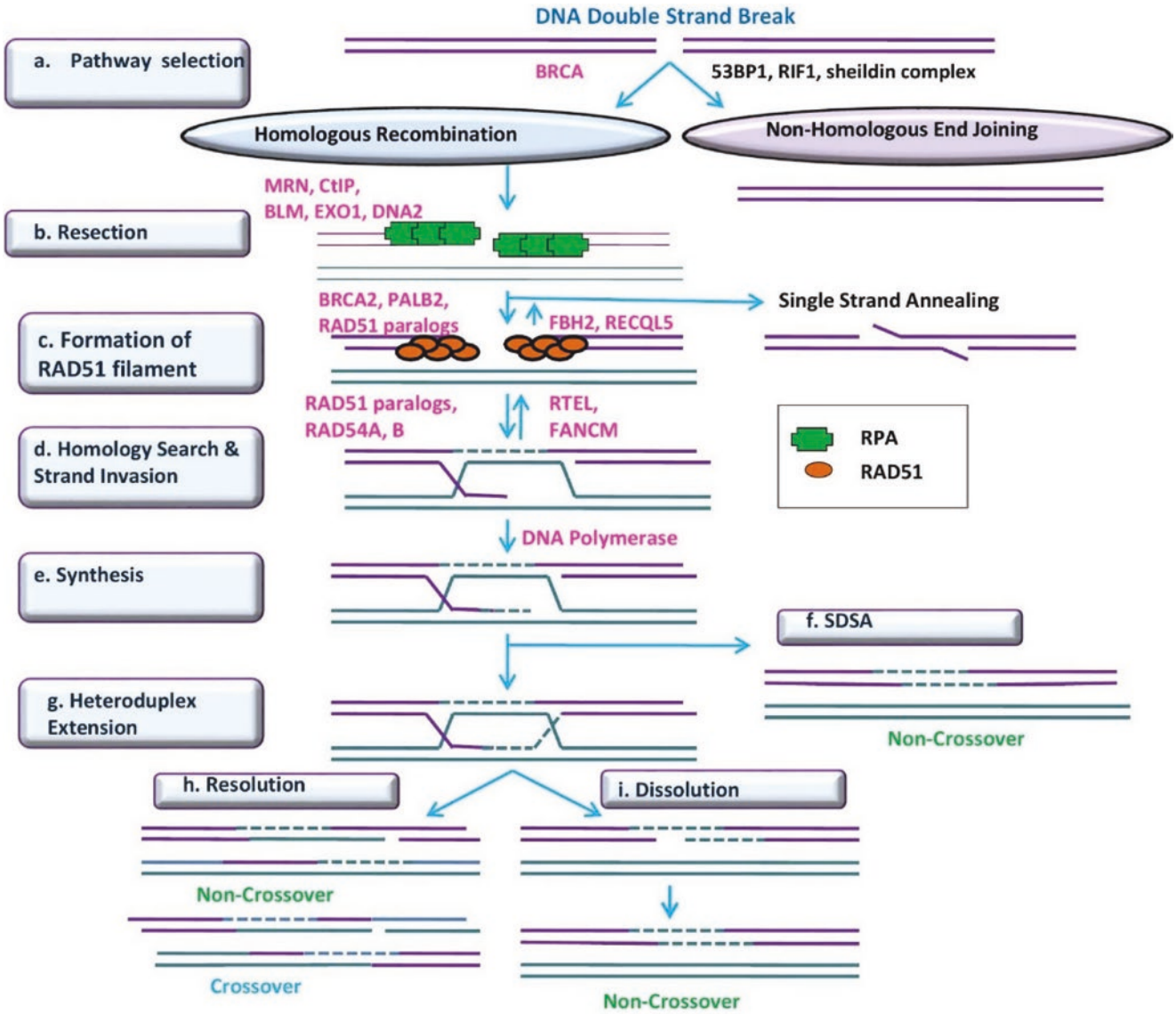

Fig. 3.10 Overview of homologous recombination (HR) pathways in double-strand break repair. When cells suffer a DSB (purple lines), they can repair them either by HR, with the help of a template that is homologous (turquoise lines), or by the NHEJ pathway. (**a**) BRCA1 promotes the HR pathways, whereas the Shieldin complex, RIF1, and 53BP1 promote the NHEJ pathway. (**b**) The resection process is performed by the MRN complex along with CtIP, EXO1, BLM, and DNA2 that form the 3′ ssDNA overhangs. These overhangs are then coated with the RPA (green boxes), which is later shifted by the RAD51 (brown circles). On the other hand, single-strand annealing occurs in case of the RAD-independent repair process, where annealing of the complementary DNA sequences takes place followed by overhangs cleaved by the flap endonuclease and finally the ends of the DNA are ligated. (**c**) Positive regulators of RAD51 such as RAD51 paralogs, BRCA2, and PALB2 aid in the formation of the RAD51 filament, whereas RECQL5 and FBH2 negatively regulate RAD51. (**d**) The RAD51 paralogs and RAD54A-B support the RAD51-mediated homology searching and strand invasion. At the same time, FANCM and RTEL negatively govern the RAD51-mediated D loops. (**e**) The homologous template in the form of sister chromatid or a homologous chromosome is used by the DNA polymerases to copy the missing sequence. (**f**) The DNA is resolved into a noncrossover product when SDSA dislodges the D loop. (**g**) In case there is an extension of the heteroduplex and development of Holliday junction created by the second-end capture, the intermediate states can be resolved by either resolution or dissolution. (**h**) The outcome of resolution is both the crossover and noncrossover products. (**i**) The outcome of dissolution is a noncrossover product. Adapted with permission (CCBY) from Sullivan and Bernstein [35]. Abbreviations: *DSB* double-strand DNA break, *HR* homologous recombination, *NHEJ* Non-homologous end joining, *BRCA1* breast cancer gene 1, *RIF1* Rap1-interacting factor 1, *53BP1* p53-binding protein 1, *MRN* MRE11–RAD51–NBS1 complex, *CtIP* CtBP-interacting protein, *EXO1* exonuclease 1, *BLM* Bloom's syndrome helicase, *RecQ* helicase-like gene, *DNA2* DNA replication helicase/nuclease 2, *ssDNA* single-stranded DNA, *RPA* replication protein A, *RAD51* RAD51 recombinase, *PALB2* partner and localizer of BRCA2, *RECQL5* RecQ-like helicase 5, *FBH2* also GNA11, G protein subunit alpha 11, *FANCM* FA complementation group M, *RTEL* regulator of telomere elongation helicase 1, *SDSA* synthesis-dependent strand annealing

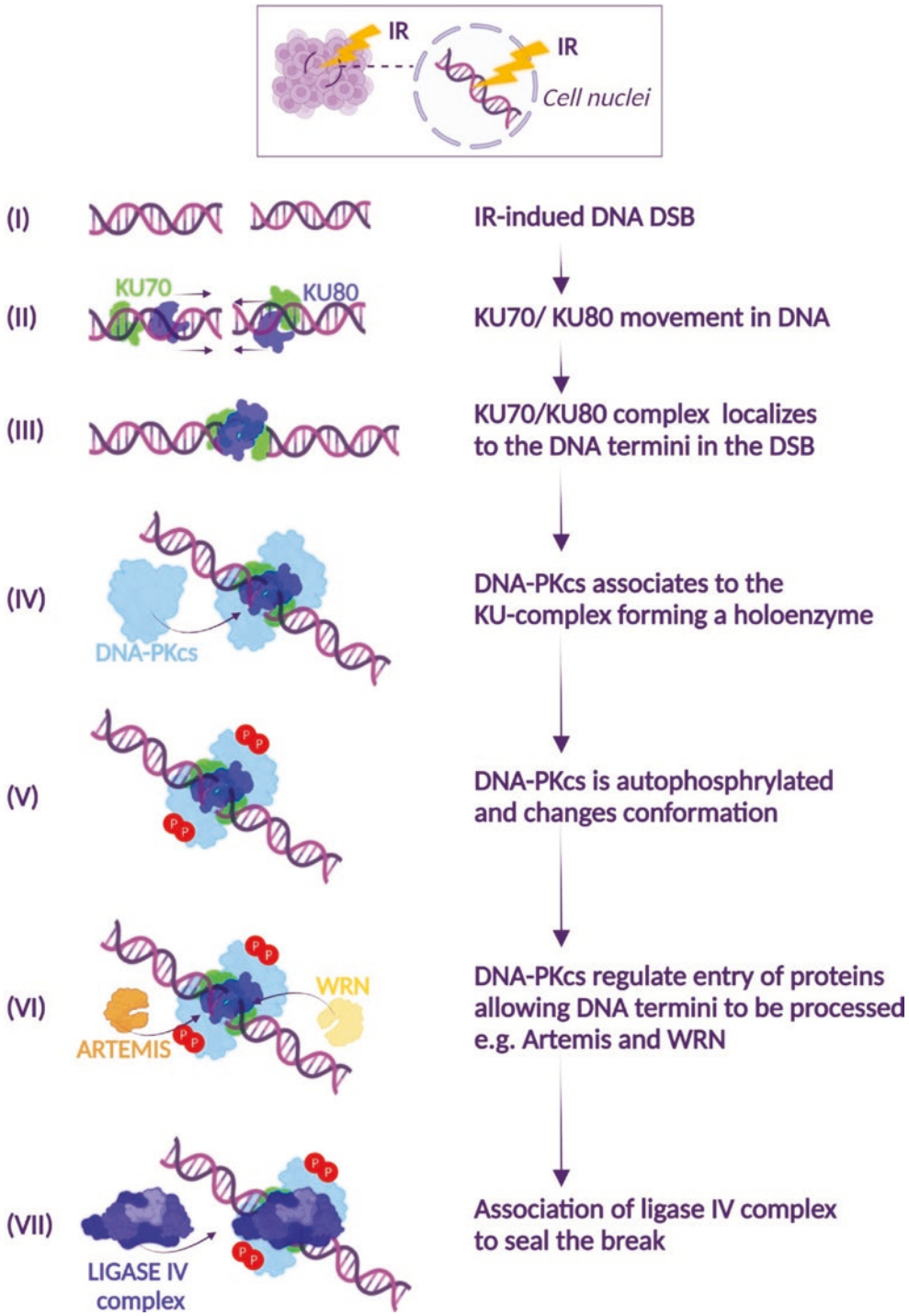

Fig. 3.11 Schematic of the principal steps of NHEJ. (I) IR triggers the formation of DNA DSB in the cell nucleus. (II) To act on these, the NHEJ pathway commences with the movement of Ku (Ku70/Ku80) proteins towards the loose ends in the DNA DSB. (III) Ku70/Ku80 forms a complex embracing the ends protecting DNA integrity. DNA DSBs with noncomplex termini can be ligated directly after this step as end processing is not required. (IV) When the ends in the DSB require end trimming, the DNA-PKcs is recruited onto DNA via association to the Ku70/Ku80 complex forming a platform for subsequent steps. (V) Once associated to Ku proteins and DNA, DNA-PKcs undergoes autophosphorylation which changes its conformation. (VI) In this way, DNA-PKcs is active as a kinase and regulates the association of multiple DNA end-trimming proteins (e.g., Artemis, WRN, Polμ/λ, PNK), which restores the nucleotides at the termini allowing ligation to take place. (VII) The ligation step is controlled by the DNA ligase IV complexes, which apart from ligase IV also include XRCC4, XLF, and PAXX. At the end of the trimming and ligation step, some bases may be lost causing loss of genomic information which may cause mutations. Abbreviations: *DNA DSB* DNA double-strand break, *NHEJ* Non-homologous end joining, *Ku* dimeric Ku70/Ku80 protein complex, *DNA-PKcs* DNA-dependent protein kinase catalytic subunit, *WRN* protein deleted in Werner syndrome, *Polμ/λ* DNA polymerase μ/λ, *PNK* polynucleotide kinase, *XRCC4* X-ray repair cross-complementing protein 4, *XLF* XRCC4-like factor, *PAXX* paralog of XRCC4 and XLF

repairing DNA DSBs, and cells deficient in some of these signaling components are known to be very IR sensitive [39]. Moreover, NHEJ has a critical role in V(D)J-recombination when B and T lymphocytes are developed in the immune system. This is also illustrated by severe combined immunodeficiency (SCID) patients who, due to lack or alteration in some of the NHEJ components including the catalytical subunit of DNA-PK (DNA-PKcs) as well as others, have T and B lymphocytes that do not have proper function [39]. Importantly, cells from such patients also display high IR sensitivity.

The NHEJ process starts at the DNA end termini, also known as the break synapsis, where a heteromeric complex of the Ku proteins, Ku70/Ku80, forms a ringlike structure around the DNA. The Ku70/Ku80 complex then moves towards the break to bring the free DNA ends together and protect them from nuclease digestion (Fig. 3.11). This is critical for NHEJ function and for IR sensitivity as cells deficient in either Ku subunits have impaired NHEJ and also are IR sensitive [41].

The end structures within the DNA DSB which are sensed and protected by the Ku protein complexes are 3′ or 5′ overhangs, blunt ends, closed hairpin, and complex structures including those found in IR-induced DSBs [41]. The current understanding is that the Ku complex heterodimer slides along the DNA strand and multiple subunits align onto DNA to form a protein scaffold. The end structure in the DSB, i.e., the blunt ends, 3′ or 5′ overhangs, thereafter dictates what route the NHEJ takes as some proteins are required for certain end termini to be processed prior to ligation while others are not [41, 42]. For example, when the end termini have some regions with certain nucleotides that overlap, the ends are ligated by the DNA ligase IV and X-ray repair cross-complementing 4 (XRCC4) complex alone. However, in the majority of the cases, the DNA protein kinase catalytic subunit (DNA-PKcs) orchestrates the reactions forming a holocomplex with the Ku proteins on the DNA [42] (Fig. 3.11).

DNA-PKcs is a kinase with the capacity to phosphorylate proteins on serine or threonine resides. It belongs to a protein family also named the PIK kinases to which also ATM and ATR belong. DNA-PKcs requires DNA binding for its kinase activity to control the end-processing activity within NHEJ as well as inactivation of its own function [42]. Thus, when the Ku complex binds DNA-PKcs, it causes autophosphorylation of multiple residues in the kinase domain and thereafter DNA-PKcs can phosphorylate its downstream substrates.

Multiple studies in rodent and human cells using various genetic approaches have shown that a defective DNA-PKcs activity impairs the repair of some but not all IR-induced DNA DSBs, but nevertheless causes increased radiation sensitivity [39]. To further study the function of DNA-PKcs for repair of IR or chemotherapy-induced DNA damage, inhibitors towards the kinase pocket have been developed, some of which have also been demonstrated to function as IR sensitizers of tumor cells and in tumor-bearing mice (reviewed in the work of Myers et al. [43]). All in all, it is clear that DNA-PKcs orchestrates the NHEJ pathway, but despite decades of research, the understanding of the entire molecular mechanisms is still not complete.

The end processing of the nucleotides is required as a DNA DSB seldom has the 3′OH and 5′P termini that are required for ligation. Therefore, the ends in the DNA DSB need to be processed by exonucleases such as Artemis, which has intrinsic 5′ exonuclease function and 5′ exonuclease acquired once in complex with DNA-PKcs [44]. The critical role for Artemis in the NHEJ processing has been shown as cells deficient in Artemis are sensitive to IR. However, Artemis is only required for repair of a subset of ~10–20% of the DNA DSBs, while the others are rejoined efficiently in the absence of Artemis. Therefore, it has been suggested that Artemis is responsible for repair of DNA DSBs that display slow repair kinetics. Apart from Artemis, there are also other proteins involved in the end-processing activity including Werner syndrome ATP-dependent helicase (WRN). It exhibits helicase and exonuclease function and suppresses 5′ end resection as well as HR by blocking MRE11 and CtIP association. Other examples are the polynucleotide phosphatase/kinase (PNKP) and tyrosyl-DNA phosphodiesterase 1 (TDP1) that modify the phosphorylation of the nucleotides and trim the ends to a state allowing ligation to take place. As some nucleotides may be lost in the end termini, the DNA polymerase μ and DNA polymerase λ are also part of the end-trimming activity in NHEJ.

Ligation of broken ends by NHEJ is carried out in a protein complex, which bridges around the DNA end in the DSB. The complex contains, among other proteins, XRCC4, DNA ligase IV, and XRCC4-like factor (*XLF*). Out of all the proteins involved in NHEJ, DNA ligase IV stands out when it comes to repair of DNA DSBs because mice, in which this gene is disrupted, experience lethality as embryos and dissection of such embryos have revealed extensive apoptosis, in particular in the nervous system [45]. Both ligase IV and XLF mutations, that impair their function, are reported in humans in different tumor types, e.g., leukemias and lymphomas, with the patients showing various degrees of deficiency in B and T lymphocyte function [46] (Box 3.6).

Box 3.6 In a Nutshell: Non-homologous End-Joining

- The NHEJ pathway plays a crucial role in the repair of DNA DSBs generated endogenously and by IR.
- NHEJ has less fidelity in repair than HR and may therefore in certain circumstances cause mutations.
- NHEJ deficiency results in increased radiation sensitivity.
- Some of the NHEJ pathway components, e.g., DNA ligase IV, are essential for NHEJ repair, while others are required for efficient repair of certain subsets of DNA DSBs.
- NHEJ components, e.g., DNA-PKcs, offer a target that can be used for radiation sensitization purposes in various tumor types.

3.3.4.3 Alternative DSB Repair Pathways

Cells fundamentally utilize two conventional mechanisms to repair their DSBs, i.e., the HR and the NHEJ pathways. However, in recent times, a third pathway is discovered which is known as the alternative NHEJ (alt-NHEJ or aNHEJ), microhomology-mediated end joining (MMEJ), and B (backup)-NHEJ. This is an extremely error-prone pathway that operates in NHEJ-proficient as well as -deficient cells. Unlike HR, this pathway does not require any long homologous DNA templates and is therefore called as "alternative end-joining" pathways. This mechanism typically but not always depends on the microhomologies that exist at or near the DNA DSB ends, which implicates that it might not be completely divergent from the mechanism of HR. The junctions of this repair pathway demonstrated overlapping microhomologies of 3–16 nucleotides as well as nucleotide deletions. Earlier, it was known that the NHEJ pathway could recover short microhomologous region of up to five nucleotides in mammalian cells. However, the alt-NHEJ can operate even in the NHEJ-deficient cells [47]. It is a unique pathway that is seen to be ongoing throughout the cell cycle but found to be augmented in the G2 phase when compared to the G1 phase. Although it is arguable if there are other alt-NHEJ overlapping pathways, there is evidence of a microhomology-mediated end joining (MMEJ) that involves the arrangement of microhomologous series on the inner side of the broken ends prior to fusion and is linked with deletion adjoining the original DSB. This is also an error-prone pathway leading to chromosomal translocations.

One of the characteristics of alt-NHEJ is the excessive deletions and frequent microhomologies at the junction, while such microhomologies are not always present. The exclusivity of alt-NHEJ products implicates the usage of end resection-promoting enzymes, their association of proteins that get benefitted from the microhomologies that can support the intermediates to stabilize, nucleases competent of eliminating the noncompatible 5′ and 3′ overhangs, and finally ligation. The MRE11 complex and CtIP in end resection are known to facilitate the alt-NHEJ, and DNA ligase III emerges to uphold the ligation step.

It is observed that the microhomology-mediated DNA repair proceedings take place via RAD52-dependent single-strand annealing (SSA)-type machinery where the minimum SSA-dependent DSB repair lies between 5 and 29 base pairs of homology. In this mechanism, it is mandatory to have direct repeats on both the sides of the DNA break. Since SSA does not involve any strand invasion events, it is independent of RAD51. As MMEJ depends on the already existing microhomologies around the break, its probable mode of action is associated with SSA. Finally, for the sealing event, MMEJ depends on ligase III [47].

3.4 Importance of Chromatin Architecture (at Nano- and Microscale) in DNA Damage and Repair

3.4.1 Multifaceted Importance of Chromatin Architecture in DNA Damage Induction and Repair

Although repair processes have been intensively investigated for decades, many principal questions concerning the mechanisms of radiation DNA damage induction and repair remain open [reviewed in the work of Falk and Hausmann [48]]. Chromatin in the cell nucleus is arranged into numerous hierarchical levels (Fig. 3.12) from micrometer to nanometer, which leads to the formation of a three-dimensional (3D) architectural chromatin network.

This network is dynamic and influenced by the cellular status and ongoing processes in the cell nucleus. Chromatin architecture is precisely regulated by physical and biochemical regulation systems and, in turn, regulates global and local genome functions. Local chromatin arrangement thus both reflects and determines the functions of the particular genetic locus, such as its transcriptional activity. Importantly in the context of radiobiology, nonrandom chromatin architecture seems to co-determine the response of cells to irradiation in numerous ways: First, in a tight interplay with physical characteristics of the radiation, functional chromatin structure states increase or decrease DNA susceptibility to DNA damage induction. Second, the chromatin architecture acts as an additional level of DSB repair regulation, cooperating with "standard" biochemical genetic and epigenetic regulation systems. Chromatin architecture may regulate DSB repair at individual DSB sites and also globally, via tuning the transcription intensity of genes involved in DNA repair and other processes related to the complex response of cells to radiation DNA damage (e.g., cell cycle progression or apoptosis). Theoretically, chromatin architecture might collect and unify

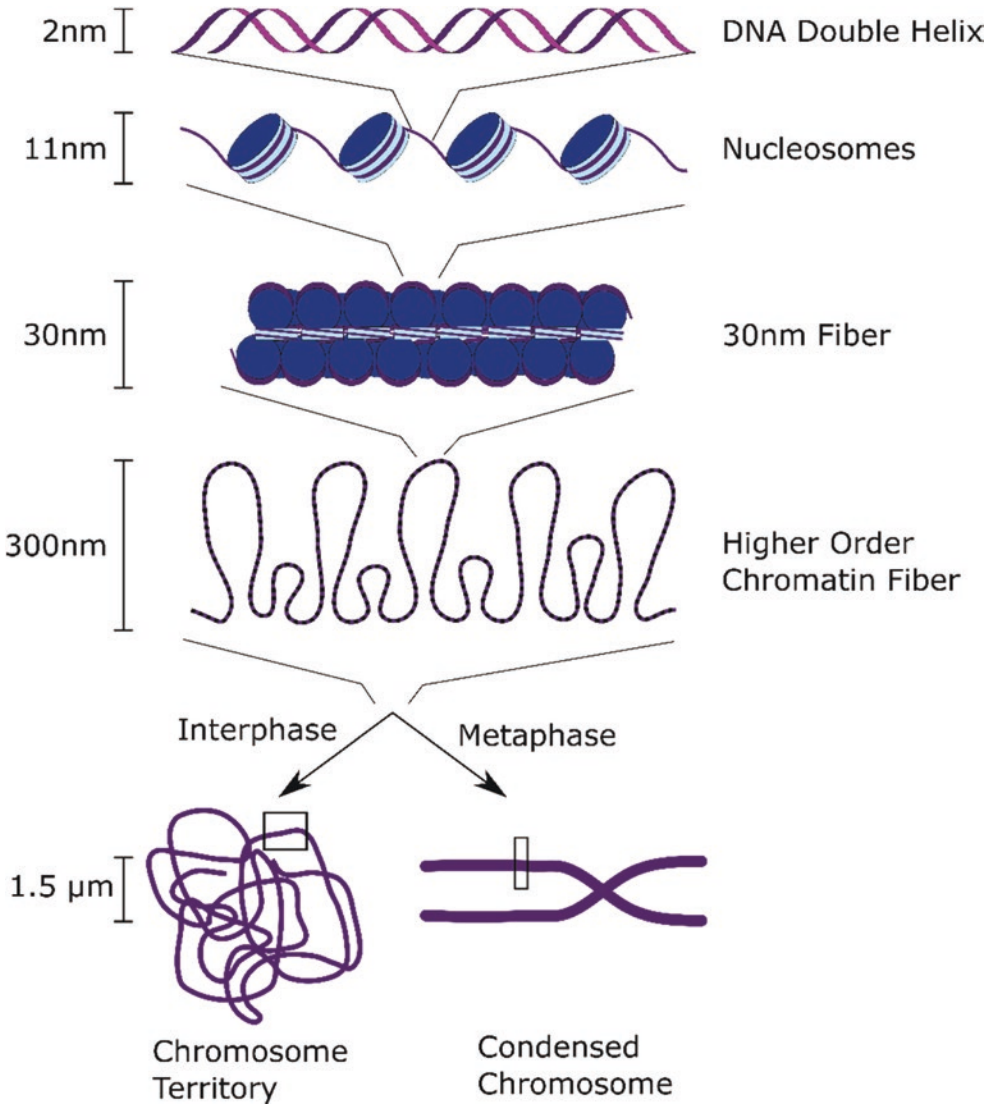

Fig. 3.12 Structure of DNA organization. The DNA forms a double-helix structure, which is wrapped around histones forming so-called nucleosomes. The nucleosomes form complex fibers of 30 nm size, which themselves form the higher order chromatin fibers, which are in the range of 300 nm. In the interphase, these fibers build the chromatin territories, where territories from different chromosomes can overlap, forming so-called networks. In the metaphase, the higher order chromatin fibers are condensed to form chromosomes. (Adapted with permission (CCBY) from Liu et al. [40])

signals of other different signaling networks (biochemical, epigenetic) and transfer these heterogeneous signals into single integrated output signal represented by a specific architectural status of the chromatin network that can be easily interpreted by the cell. Chromatin architecture might thus impersonate a "roofing" regulatory system based on simple physical laws, which allows for a sufficiently fast decision-making process for the optimal repair mechanism at each individual DNA damage site.

Different types (low LET vs. high LET) of IR interact with chromatin in specific ways. Therefore, the relationship between the radiation quality, architecture of structurally and functionally distinct chromatin domains, and DSB induction, repair, and misrepair play a role in the cellular radiation response. Genetically active, decondensed euchromatin and mostly inactive, condensed heterochromatin are the two traditionally recognized structurally and functionally distinct chromatin domains, which affect radiation response. However, it should be noted that radiation response differences may be even more prominent for other chromatin architectural and functional counterparts [49], such as RIDGE (regions of increased gene expression) and anti-RIDGE domains [50], which have even more precisely defined function and more homogenous architecture as compared to euchromatin and heterochromatin (Box 3.7).

Box 3.7 In a Nutshell: Importance of Chromatin Architecture

- DNA is organized in structural units ranging from micrometers to nanometers, forming 3D chromatin architecture.
- Chromatin architecture is a key factor determining local damage induction by radiation.
- Chromatin architecture operates with genetic and epigenetic regulatory factors orchestrating DNA damage response.

3.4.2 DNA Damage and Repair in the Context of Chromatin Architecture at the Microscale

DNA damage and repair processes can be related to specific cell states and chromatin architectures. The spatiotemporal sequence of repair protein binding to DSB and surrounding phosphorylated and thus activated H2AX histone (called γH2AX) sites can be analyzed using microscopy (Fig. 3.13). The analysis of the formation and subsequent dissociation of repair complexes, and the structure of these complexes, brought deep insights into the mechanisms of the two main DSB repair pathways in human cells, nonhomologous end-joining (NHEJ) and homologous recombination (HR)—as discussed above.

The most obvious architectonical chromatin types are condensed (hetero)chromatin with only a low number of active genes and decondensed (eu)chromatin, which is generally considered as genetically (transcriptionally) active. It has been shown that condensed chromatin protects DNA from free radicals generated by ionizing radiation [51], but, at the same time, it is this condensed architecture and a high content of repetitive sequences that complicate and slow down the repair of DSBs located in heterochromatic domains. The protective function against free radicals of the heterochromatic status does not seem to simply result from high condensation of heterochromatin domains but rather from a high amount of proteins that specifically bind to heterochromatin and interact with radiation-induced free radicals before they can damage DNA [51]. However, if a DSB occurs in heterochromatin, its condensed architecture must decondense first in order to allow the formation of huge repair complexes and continuation of repair processes [52]. Moreover, numerous studies indicate that the slower repair of heterochromatic DSBs not only reflects this necessity for the decondensation of a damaged chromatin domain but also points to a slower repair mechanism, specifically homologous recombination (HR) [48]. HR in heterochromatin could be superior over NHEJ for numerous structural reasons and therefore preferred by the architecture of heterochromatin domain; however, at the same time, repetitive sequences present in heterochromatin are a clear contraindication for this repair mechanism. This paradox can be again explained and overcome by the already described heterochromatin decondensation at the beginning of repair. The RAD51 recombinase, which is responsible for complementary DNA strand search and exchange, can bind to heterochromatic DSB sites only upon heterochromatin decondensation and protrusion of a DSB to the domain surface, which ensures spatial separation of the damaged DNA ends from repeats remaining embedded within the heterochromatin domain. HR is thus evidently regulated by chromatin architecture changes, which also ensure the fidelity of this repair mechanism [48]. It remains unknown whether NHEJ or other repair pathways are also associated with some specific chromatin architecture requirements and rearrangements, similar to HR. However, some recent studies suggest that epigenetic and structural regulations are involved in repair pathway selection at individual DSB sites, as it is discussed later. The key properties of hetero- and euchromatin as mentioned here are summarized in Table 3.3.

A serious consequence of irradiation is the formation of chromosomal aberrations, and the chromatin architecture significantly participates in this process. The severity and complexity of the genetic damage are related to the complexity of the underlying DNA damage. The connection between dam-

Table 3.3 Properties of hetero- and euchromatin

Heterochromatin	Euchromatin
Condensed DNA	Decondensed DNA
Low amount of active genes	Transcriptionally active
Protection of DNA from radicals through condensed structure and high amount of radical catching proteins clustering around DNA	No radical protection
	No decondensation necessary and therefore fast repair
Slow repair due to necessary decondensation	No preference of repair mechanisms defined by chromatin architecture
Homologous recombination superior to nonhomologous end joining	

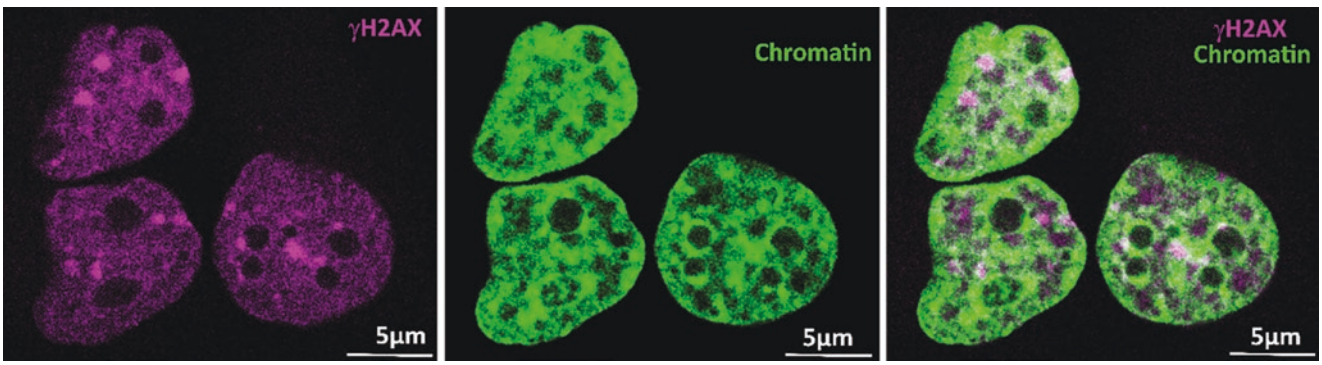

Fig. 3.13 Localization of DNA damage on chromatin: radiation damage induced by high-LET alpha particle radiation microscopically visualized by γH2AX as a biomarker for double-strand breaks (left, magenta), chromatin labeling (middle, green), and merge of the two (right)

age complexity and radiation type was discussed in Sect. 3.2. An additional factor defining the complexity is the chromatin state, and radiation interacts with this. These interactions can be illustrated on the example of chromosomal translocation formation upon irradiation of euchromatin and heterochromatin with low-LET and high-LET radiation, respectively. The type of radiation, chromatin architecture, and consequently initiated DSB repair processes participate in a specific way in free DNA-end misrejoining (review [53, 54]).

The probability of a chromosomal translocation formation between two specific genetic loci, i.e., the linking of the ends of different chromosomes after induction of DSB in both chromosomes at the same time, depends on spatial (3D) separation of these loci in the cell nucleus. Chromatin is non-randomly organized in the cell nucleus, though on the probabilistic basis, this means that chromosomal translocations between some genetic locus pairs appear more frequently than translocations between other pairs. This expectation was confirmed by experiments with interphase cells exposed to neutrons or high-LET particles where translocations appeared most frequently between the neighboring chromosomal territories or even genetic loci statistically located in close proximity [55]. Overall, there are two hypotheses used to explain the processes related to repair of DSB in the context of chromatin organization:

1. Position-first hypothesis: It considers DSBs as immobile structures and emphasizes the role of (preset) chromatin architecture in determining the probability of a chromatin exchange between two specific genetic loci.
2. Breakage-first hypothesis: It considers DSBs as mobile and gives the chromatin architecture a subsidiary role.

Both hypotheses explain different phenomena occurring. While the position-first hypothesis works well in explaining the enhanced probability of translocations to be formed by neighboring chromosomes, it does not allow chromatin exchanges between spatially more distant genetic loci, though such translocations were experimentally observed. Furthermore, although complex chromosomal translocations are only occasional events upon cell exposure to photonic (low-LET) radiation, they do occur. As DSBs are dispersed through the cell nucleus and thus spatially separated in cells irradiated with low-LET radiation, formation of complex translocation between three or more DSBs can hardly be explained without involving DSB movement. Both observations can be explained by the breakage-first hypothesis. However, the idea of highly mobile chromatin at DSB sites in cells exposed to low-LET radiation, where chromatin is not locally fragmented as in cells exposed to high-LET particle radiation, has not been generally confirmed. The explanation of this paradox came with the spatiotemporal tracking of individual radiation-induced protein accumulations (foci) [52], showing the majority of "immobile" DSBs accompanied with a small proportion of highly mobile DSB lesions or by subdiffusive nature of DSB loci [56]. The increased mobility correlated with DSB localization in heterochromatin and can thus be attributed to chromatin decondensation at the beginning of heterochromatin repair process, leading to the protrusion of DSBs onto the surface of heterochromatin domains. Numerous DSBs thus accumulate in nuclear subcompartments of a limited volume, which increases the probability of their mutual interactions and consequently chromatin exchanges even among multiple DSBs.

After irradiation with high-LET particles, on the other hand, locally concentrated energy deposition causes serious chromatin fragmentation and mobilization within cell nucleus micro-volumes along the particle tracks. This situation allows mutual contacts of many short chromatin fragments from one or several neighboring chromosomes and thus easy formation of complex chromatin translocations, irrespectively of the original chromatin architecture and chromatin architecture changes during repair. Chromosomal translocations in cells exposed to high-LET radiation thus occur due to physical rather than biological (repair) processes. We have already mentioned that heterochromatin architecture protects DNA from low-LET radiation as heterochromatin-binding proteins prevent DNA interaction with free radicals, mostly mediating harmful effects of low-LET radiation. With high-LET radiation, however, most damage to DNA is caused by the direct effect of radiation particles or emitted secondary electrons. In this case, heterochromatin represents a more dangerous chromatin architecture, as particles cannot be stopped by any chromatin architecture and heterochromatin provides more DNA targets per a volume unit compared to euchromatin. Hence, in cells exposed to high-LET radiation, translocations in heterochromatin tend to be more complex than in euchromatin (Box 3.8).

Box 3.8 In a Nutshell: DNA Damage and Repair in the Context of Chromatin Architecture

- Hetero- and euchromatin form different chromatin architectural regions within a cell nucleus resulting in different consequences of radiation damage induction.
- Chromosomal aberrations after low-LET radiation can be explained through the "position-first hypothesis" in combination with chromatin decondensation in heterochromatic regions.
- Chromosomal aberrations after high-LET radiation occur due to physical fragmentation of DNA rather due to biological processes.
- Heterochromatin protects DNA from indirect damage (mainly induced by low-LET radiation) but is more sensitive to direct damage (mainly induced by high-LET radiation).

3.4.3 DNA Damage and Repair Processes at the Nanoscale

Using a variety of tools of super-resolution microscopy and image data computing has revealed that γH2AX foci in cell nuclei exposed to low-LET X-rays are subdivided into several equally sized, functionally relevant clusters. The number of clusters increased with the radiation dose according to the well-known linear-quadratic dependence and decreased at later time periods postirradiation. Calculations of the persistence of homology revealed a highly similar topology of γH2AX and other repair protein clusters, especially when these clusters were closely associated with heterochromatin regions. During the repair period, size and topology of these clusters seem to be maintained as long as they are attached to chromatin at actively repairing DSB sites. These findings suggest a functional relevance of the focus/cluster topology [57].

For instance, while the γH2AX clusters had a typical diameter of about 400 nm–600 m, the MRE11 clusters were smaller (about 200 nm) and usually completely embedded within γH2AX clusters [58]. The sizes of clusters were independent of repair time and cell type. On the other hand, the topological similarity of clusters followed the dynamics of the repair protein interaction with chromatin; that is, binding to damage sites was accompanied by ordering while detachments caused the relaxation of topological arrangements. In contrast, γH2AX and MRE11 clusters spontaneously occurring in the nonirradiated cells (e.g., due to replication defects) did not show this topological similarity.

Recent studies discovered spatial distribution changes of tri-methylated H3K9 histone (H3K9me3), ALU repeat sequences (ALU), or long interspersed nuclear element (LINE)-like L1 sequences, indicating chromatin reorganization or movement and DNA strand relaxation after radiation exposure, followed by recovery during repair [59]. Altogether, described results suggest a functional relevance of chromatin and repair focus nano-architecture in DSB repair process and their regulation (Box 3.9).

Box 3.9 In a Nutshell: DNA Damage and Repair Processes on the Nanoscale

- DNA repair locations marked by γH2AX and 53BP1 are subdivided into functional clusters at the nanoscale, in a manner which is cell type and radiation type specific.
- Other repair protein clusters are smaller and are embedded in the γH2AX and 53BP1 clusters.
- After damage induction, chromatin is reorganized accompanied by DNA movement.
- Chromatin reorganization is recovered during DNA repair.

3.5 Consequences of DNA Damage Misrepair or Unrepair

Lack of repair (unrepair) and misrepair of DNA damage can lead to increased chromosome breaks or rearrangements and mutations usually referred to as a status of genomic or genetic instability (GI). GI is usually associated with loss of cell cycle control, senescence, and cell death and in humans with pathological disorders including premature aging and predisposition to various types of cancer and inherited diseases [60]. On the other hand, GI is also fundamental for evolution and induction of genetic diversity. It is known that genomic integrity is carefully supervised by specific surveillance mechanisms like DNA damage checkpoint, DNA repair, or mitotic checkpoint. A deficiency in the regulation of any of these mechanisms often leads to GI, which can predispose a cell to malignant transformation [61].

3.5.1 DNA Lesions and Repair

In huge DNA molecules in the cell, nucleus genes are present. These genes are responsible for the development and function of the cell and the whole organism, because they code proteins. Due to this fact, unrepaired or misrepaired DNA lesions, which can lead to gene mutations, can promote changes in the structure of the encoded protein or lead to the decrease or complete loss of its expression. The types of DNA lesions occurring were already discussed in Sect. 3.3. Based on the current experimental and theoretical evidence, the most repair-resistant lesions are not the single ones but a combination of them in a short DNA segment of 10–20 bp called clustered damage. Clustered DNA lesions are considered the signature of ionizing radiations especially for particle radiation [45]. Various studies suggest that the probability for a break or other DNA lesion to be incorrectly processed and amended is fairly low when damage is spatially separated but increases drastically when multiple breaks and/or non-break lesions coincide. For an analytical description of DNA repair pathways, the reader can refer to Sect. 3.4. As was already mentioned in Sect. 3.3, the DNA molecule consists of nucleotides (deoxyribose + phosphate group + base), which can be for simplicity named based on the four bases [adenine (A), cytosine (C), guanine (G), thymine (T)]. Thus, the DNA alphabet is a very easy one; it only consists of four letters. These four letters are then combined to give rise to groups of three, which define the amino acids that are then the new alphabet for the translation to proteins. For more details on DNA-to-RNA transcription and RNA-to-protein translation, see for example [62]. Even if the cells have a very sophisticated DNA damage response and repair system, it may happen that not all the damage is removed. A mutation is when a permanent change in the DNA sequence occurs.

Mutations can be divided into somatic or germline mutation in terms of what kind of cell is affected. A germline mutation occurs in a sperm or in an egg and can be passed to offspring. Somatic mutations occur in cells of the body and cannot be passed to next generations. Mutations can also be grouped as point or chromosomal mutations. Point mutations are when a single nucleotide is replaced with another single nucleotide, or deleted, or inserted in a place that it should not be. Point mutations do not always have significant consequences on the encoded protein. For example, as is shown in Table 3.4, the mutation can be silent. This means that even if there is a change in the original DNA sequence, the final product of the transcription will be the same, because there are several combinations of the DNA alphabet that lead to the same amino acid. In other cases, the mutation can lead to the change of the final amino acid (missense mutation) or to the creation of a stop codon (nonsense mutation), which then affects the final protein.

Table 3.4 Point mutations and their consequences

		Point mutations		
	No mutation	Silent	Missense	Nonsense
DNA	TTC	TTT	TCC	ATC
mRNA	AAG	AAA	AGG	UAG
Amino acid	Lysine (Lys)	Lysine (Lys)	Arginine (Arg)	Stop

3.5.2 Mitotic Cell Death, Senescence, Cytoplasmic DNA

Mitotic cell death, also called mitotic catastrophe (MC), is the process when a cell dies during or right after mitosis [63]. It can be triggered by DNA damage and its mis- and unrepair and therefore through radiation. MC can be both a caspase-dependent, regulated and caspase-independent, unregulated pathway of cell death. Some characteristic morphologies can be found in Fig. 3.14a.

Senescence in biology refers to a process by which a cell ages and permanently and irreversibly stops dividing but does not die [63]. The number of senescent cells increases with age, but senescence also plays an important role during development as well as during wound healing and can be triggered by radiation. In culture, senescent cells exhibit a different morphology compared to non-senescent cells, called "fried egg" appearance (see Fig. 3.14b). It was shown that among other features, the radiation dose plays a major role in the induction of either senescence or apoptosis and necrosis. In some cell lines, senescence is the major response to low doses of radiation, whereas higher doses lead to apoptosis or necrosis. In IR-treated tissue, enhanced senescence may lead to pathogenic onsets, such as loss of organ function.

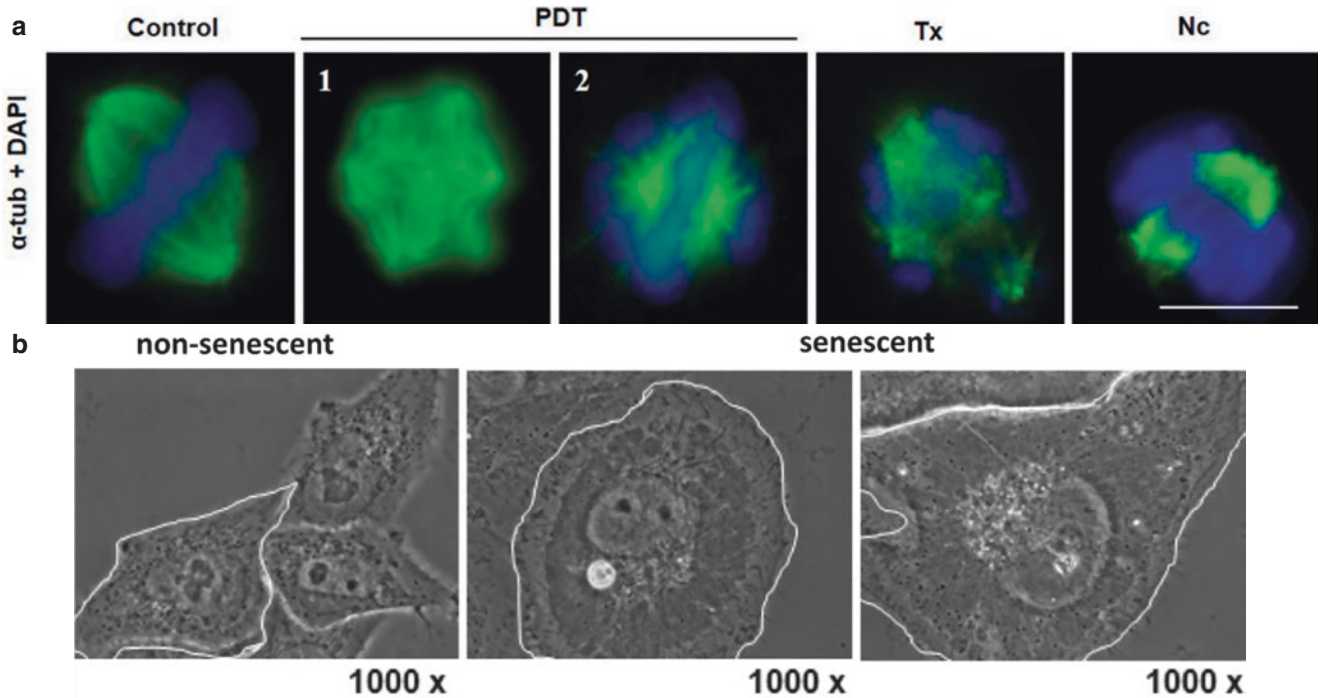

Fig. 3.14 Morphologies of mitotic catastrophe (**a**) and senescence (**b**). (**a**) Fluorescence image of cancer cells undergoing mitosis. The DNA is labeled with DAPI and mitotic spindles using α-tubulin staining. The cells exhibiting mitotic catastrophe are treated with photodynamic therapy (PDT), Taxol (Tx), or nocodazole (Nc). The control shows normal mitotic spindles. The treated cells show various types of altered spindles and mitosis. Scale bar: 10 μm. Reproduced with permission (CCBY) from Mascaraque et al. [64]. (**b**) Phase-contrast images of Chang cells. Senescence was induced using 1 mM of deferoxamine. (Reproduced with permission (CCBY) from Kwon et al. [65])

Recent advances in the field indicate that a further consequence of DNA damage misrepair or unrepair can be the release of cytoplasmic DNA that can also trigger immune responses. In general, it is widely accepted that immune signaling can be activated by the presence of DNA in unusual locations, such as the cytoplasm or the endosomes, as DNA is normally located in the nucleus of eukaryotic cells. Emerging evidence indicates a cross talk between DNA repair machinery and the immune system, and more specifically it has been discovered that DDR factors like DNA repair proteins can enhance innate immune signaling [66]. Defects in DDR and proper processing of DNA damage can therefore trigger a multitude of cellular phenotypes, including autoinflammatory disease, cellular senescence, and cancer. Genotoxic agents such as radiations or high oxidative stress can act as the primary instigators for immune signaling activation through the release of a wide range of biological and chemical factors often referred to as "danger signals" or damage-associated molecular patterns (DAMPs) [67] (Box 3.10).

Box 3.10 In a Nutshell: Consequences of DNA Damage Misrepair and Unrepair

- Genomic instability (GI) collectively refers to a status of increased DNA changes, chromosomal rearrangements, and enhanced tendency for genetic alterations occurring during cell division.
- Unrepaired or misrepaired DNA lesions can lead to chromosomal mutations, which can lead to cell death or loss of genetic material, thus promoting GI.
- Mitotic cell death is the process of a cell dying in relation to mitosis and can be triggered by radiation-induced damages.
- Senescence is the status of irreversible cell cycle arrest, which occurs naturally during aging but can be triggered by radiation, which can lead to pathological onsets.
- Cytoplasmic DNA and DNA repair defects can trigger immune response.

3.6 Cytogenetics and DNA Damage Measurements for Assessment of Radiation Effects

Cytogenetic techniques can be used to analyze chromosomal aberrations in metaphase and morphological abnormalities of DNA content in interphase nuclei. The applicability of these aberrations in the fields of biological dosimetry, clinical cytogenetics, and environmental monitoring is based on a large number of radiobiological and DNA-repair theories.

3.6.1 Micronuclei and Other Nuclear Anomalies

As described before, when cells are exposed to a variety of genotoxic agents (chemical/physical/radiation/DNA-damaging agents), they cause defects in DNA, chromosomes, and other cellular components. Radiation induces extensive DNA damage such as DSBs that, if misrepaired or unrepaired, ordinarily result in asymmetrical chromosome rearrangements and exchanges, which may lead to formation of small chromatinic bodies also known as micronuclei (MN) (see Fig. 3.15). MN are tiny extranuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that were not incorporated into the nucleus after cell division and are surrounded by a membrane. As a variety of genotoxic agents may damage DNA and the mitotic machinery by multiple mechanisms, leading to MN formation, MN are not IR specific.

It is now well established that MN are formed from acentric chromatid fragments caused by misrepaired or unrepaired DNA breaks or lagging acentric chromosomes due to mitotic spindle failure at an anaphase. Additionally, the formation of DNA DSBs and MN is sometimes the result of simultaneous excision repair of damages (e.g., 8-oxo-deoxyguanosine) and inappropriate bases' (e.g., uracil) incorporation in proximity on opposite complementary DNA strands.

A whole chromosome lagging behind (chromosome mal-segregation) during anaphase also results in MN formation. Mal-segregation usually happens due to absence or inappropriate attachment of spindle microtubules to chromosome kinetochore. However, the potential mechanisms behind the formation of MN are hypomethylation repeat sequences in centromeric and pericentromeric DNA, defects in kinetochore proteins or assembly, dysfunctional spindle, defective anaphase checkpoint genes, and malfunctioning in cell cycle control system. Sometimes, mis-segregation events occur when the centromeres of the dicentric chromosomes are pulled towards opposite poles of cells with sufficient forces to detach the chromosome from spindle during anaphase, thus resulting in micronucleus formation from whole chromosome loss.

Furthermore, multiple extrachromosomal acentric double minutes (DMs), cytogenetic hallmarks of genomic amplification, can aggregate after DNA damage and generate cytoplasmic MN that are subsequently eliminated from the cell.

Other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) (see Fig. 3.15) are sensi-

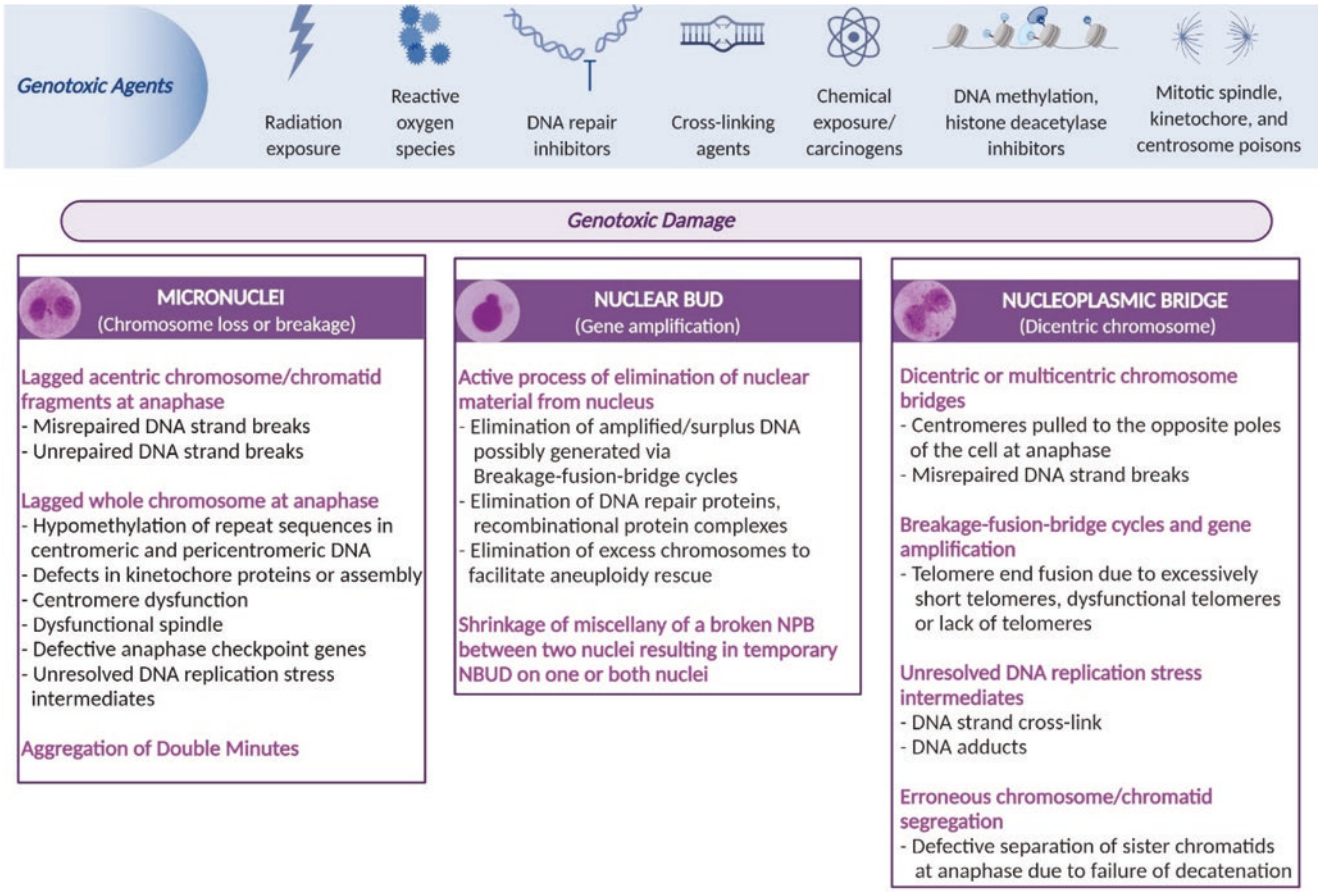

Fig. 3.15 Mechanisms by which genotoxic agents cause micronuclei and other nuclear anomalies. Micronuclei (MN) can originate from lagging acentric chromosomes or chromatid fragments or whole chromosomes at anaphase in mitosis. Nuclear bud (NBUD) formation represents the process of extrusion of the amplified/surplus DNA, DNA repair-recombinational protein complexes, and possibly excess chromosomes from aneuploidic cells. Nucleoplasmic bridges (NPBs) originate from dicentric chromosomes. This arises because the centromeres of dicentric chromosomes are often pulled in opposite directions and defective separation of sister chromatids occurs during anaphase leading to bridge formation, which can be observed as an NPB in telophase

tive and reliable biomarkers for early genotoxic instability and chromosomal breakages and rearrangements. NPBs originate as an aftereffect of misrepair of DNA strand breaks or failure of complete chromatid separation to opposite poles of the cell during anaphase. It can also originate from telomere end-to-end fusion mechanism, a fundamental indication of and a marker for loss of telomere function, which is caused by (a) excessively short telomeres, (b) dysfunctional telomeres due to loss of telomere-binding proteins without telomere erosion, (c) inappropriate assembly of telomere-capping protein structure, (d) defects in recombinational repair proteins, or (e) lack of telomeres. Another distinctive nuclear anomaly, NBUDs, is one of the precursors of MN and is associated with chromosomal instability events. Most NBUDs originate from interstitial or terminal acentric fragments and represent the expulsion of undesirable amplified extrachromosomal DNA content, which localizes to specific sites at the periphery of the nucleus and is eventually eliminated via nuclear budding during the S phase of cell cycle. It is also plausible that NBUDs might occur after elimination of DNA repair-protein complexes in the cytoplasm (Box 3.11).

Box 3.11 In a Nutshell: Micronuclei and Other Nuclear Anomalies

- Micronuclei are small extranuclear bodies surrounded by a membrane that contain damaged chromosome fragments or even whole chromosomes. The genetic information encoded in the MN DNA will get lost and lead to large genomic consequences.
- Chromosome segregation errors and/or fragment loss at anaphase ("inter-cell bridges") and exclusion of acentric fragments from daughter nuclei lead to formation of MN in the cytoplasm.

- Micronuclei occur outside the main cellular nucleus and are prone to rupturing, which leads to changes in DNA that can drive cancer development.
- Extensive DNA damage may cause dicentric/concatenated ring chromosomes and acentric chromatid/chromosome fragments, which can result in the formation of a nucleoplasmic bridge (NPB) at anaphase and micronuclei, respectively.
- Nuclear buds (NBUDs) are the result of elimination of amplified extrachromosomal DNA, which adheres to the nucleus by a thin nucleoplasmic connection, and are observed as double minute-type micronucleus bodies.

Micronucleus assays are frequently used to assess genotoxicity and cytotoxicity of different chemical and physical factors, including IR-induced DNA damage. The cytokinesis-block micronucleus assay can measure MN, NPBs, and NBUDs. A diverse range of reliable micronucleus tests (Fig. 3.16) are executed with different cell types, eventually reflecting chromosomal aberrations, ongoing DNA injury, initial stage in the development of genomic instability, and tumorigenesis. In the widely used cytokinesis-blocked MN assay, MN are scored in once-divided binucleated cells, where cytokinesis is blocked with addition of cytochalasin B, an inhibitor of microfilament ring assembly necessary for the completion of cytokinesis. In order to get statistically solid results, a huge amount of cells need to be scored. Therefore, automatic analysis of MN boosts the reliability of

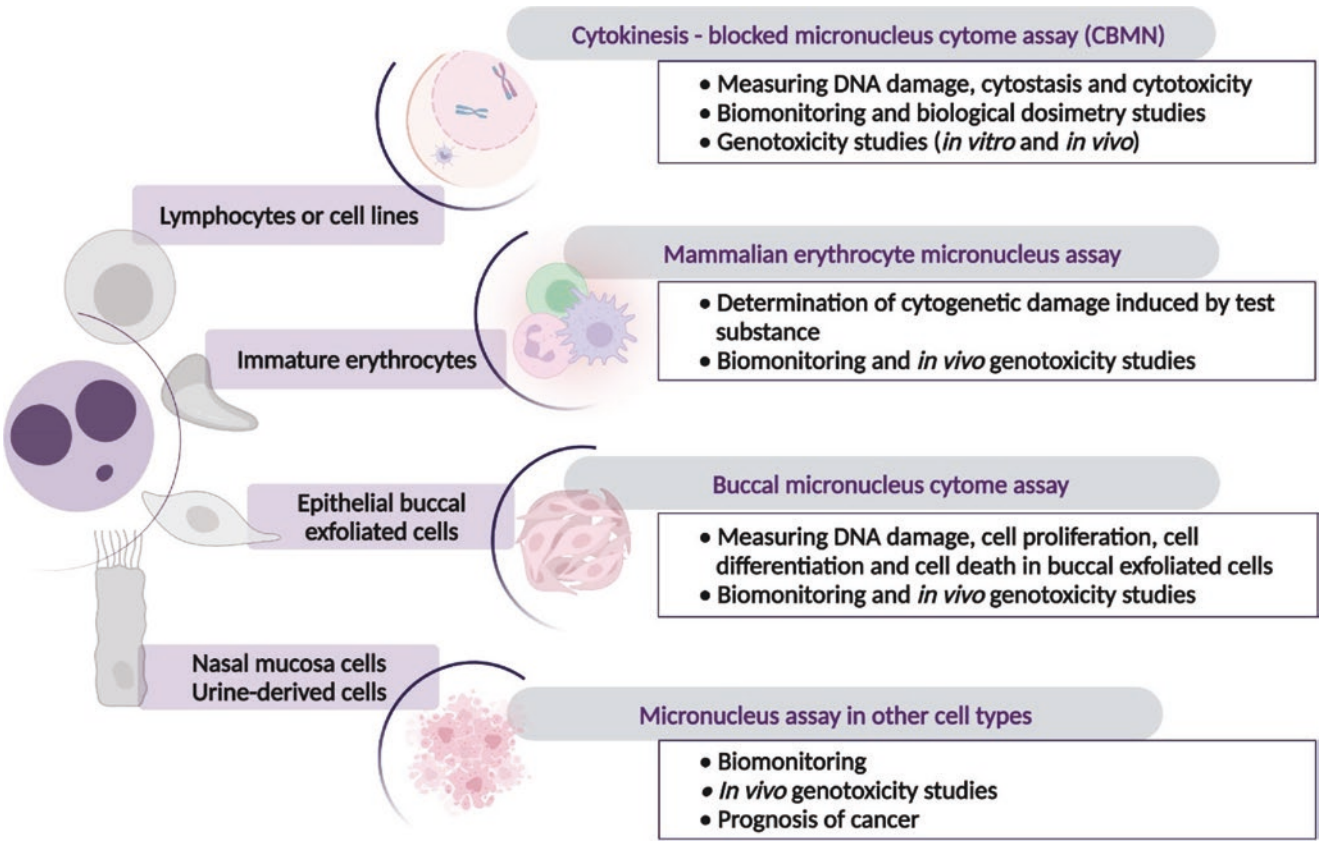

Fig. 3.16 Depending on the cell type, different micronucleus assays can be employed to assess and determine the genotoxicity and cytotoxicity of different chemical and physical factors. Applications of each assay are outlined in their respective boxes. The most popular CBMN assay can be applied to cultured human lymphocytes or cell lines to measure MN and other chromosomal instability biomarkers such as NPBs and NBUD. The mammalian erythrocyte micronucleus assay is performed on immature erythrocytes from bone marrow to determine cytogenetic damage after radiation exposure. The buccal micronucleus cytome assay is done in rapidly dividing buccal epithelial exfoliated cells (oral cavity) to analyze MN and other cytogenetic biomarkers (source of DNA damage, cytotoxicity, etc.). Occasionally, MN assay is performed on nasal mucosa cells or urine-derived cells for detection of chromosomal damage caused by environmental and lifestyle factors, occupational exposures, prognosis of cancer, and certain diseases. Although the objective and method of performance are similar to CBMN or bone marrow MN assays, these tests have not gained much popularity so far

the assays. Concomitantly, it increases the statistical validity after analyzing a large number of cells in one go. Additionally, the existing automatic/semiautomatic micronucleus scoring by microscopic systems, by flow cytometry and imaging flow cytometry, gives high accuracy and sensitivity and leads to rapid analysis (Box 3.12).

Box 3.12 In a Nutshell: The Use of Micronucleus Assay

- Micronucleus assays are used to assess genotoxicity and cytotoxicity of radiation.
- Depending on cell type, different MN assays are used.
- Automated analysis of MN boosts the reliability and statistical validity.

3.6.2 Chromosomal Aberrations

Chromosomal mutations, also called chromosomal aberrations (CA), are observed at the first mitosis after irradiation and are those that incorporate chromosomal changes, such as deletions, inversions, insertions, substitutions, duplications, or translocations of parts of chromosomes. For better understanding, some types of mutations are shown in Fig. 3.17.

The mutations shown can also lead to other aberrations. Three which should be mentioned are dicentric and ring chromosomes as well as acentric fragments as shown in Fig. 3.18. A dicentric chromosome is created when two chromosomes with two centromeres are fused. In metaphase, they are visible as one chromosome with two centromeres. This aberration will most likely die during mitosis. Acentric fragments are either fragments of a single chromosome or fused parts of different chromosomes containing no centromere. A ring chromosome is a chromosome which has two breaks on the opposing ends and is fused to form a ring. Both aberrations cannot be pulled into a daughter cell and most likely will, together with the encoded genetic information, be lost during mitosis [68]. According to the severity of the chromosomal aberration, the cell will more likely die; in some cases, it can get transformed to a cancer cell or, in case of germ line cell or a cell in early embryogenesis, several genetic disorders can occur [69]. For a more detailed view on this, refer to Chaps. 2 and 7.

The frequency of radiation-induced CAs rises with increasing radiation dose to the cells. Different types of CAs depend on the phase of cell cycle at which the nucleus is exposed to irradiation. Chromosome-type aberrations (Table 3.5) occur when pre-synthetic phase (G_1) is exposed to irradiation, while chromatid-type aberrations (Table 3.6) appear if irradiation occurs during post-synthetic phase (G_2). In chromosome-type aberrations, more than one break is unable to rejoin at the correct ends that often results in abnormal chromosomes. There is much hidden damage present, some of which is transmitted to future cell generations. In

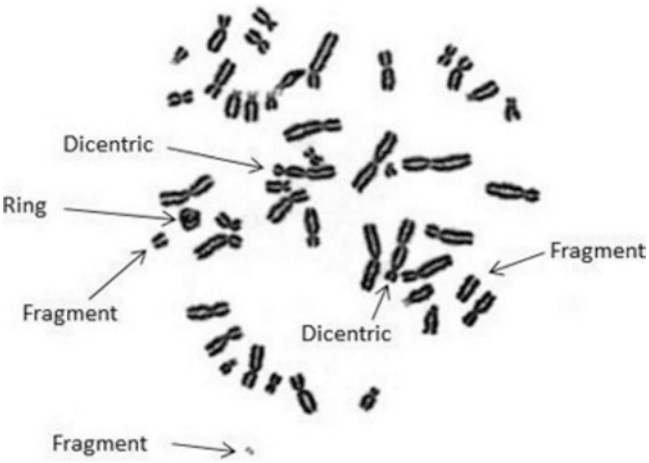

Fig. 3.18 Human metaphase cell irradiated with 5 Gy gamma rays. Two dicentric chromosomes, three acentric fragments, and a ring chromosome could be found. From https://www.qst.go.jp/site/nirs-english/1369.html (accessed 05/2022)

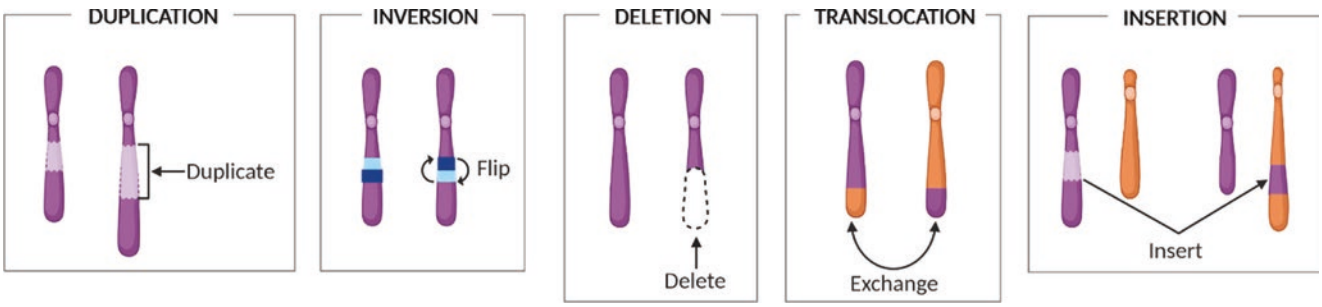

Fig. 3.17 Types of chromosomal mutations. Nonlethal aberrations are observed at the first mitosis after irradiation. Duplication: one or more copies of a DNA segment/a region of a chromosome are formed. Inversion: A segment of a chromosome breaks off and reinserts in reverse orientation within the same chromosome. Deletion: A part of a chromosome/one or more nucleotides from a segment of DNA are missing or deleted. Translocation: It involves two chromosomes in which a piece of one chromosome breaks off and rejoins to another chromosome. Insertion: A segment of one chromosome is removed and inserted to another chromosome or the same chromosome

Table 3.5 Chromosome-type aberrations

Dicentrics	When G1 phase is exposed to irradiation, it causes chromatid breaks in two different chromosomes, which rejoin during S phase and can be seen as dicentric at M phase. Two centromeres in one chromosome appear in dicentrics via breakage-fusion-bridge cycle. These are relatively easy to detect and the main aberration used for biodosimetry
Chromosomal gap	Random achromatic lesions can occur at both the chromatids of a metaphase chromosome, which can be visible as non-stained/lightly stained thinner region. The width of this region is less than the width of chromatid arm
Acentric chromosomal fragments	When single or double breaks occur in the same chromosome arm, either at the end of a chromosome or between centromere and telomere region, it will produce terminal or interstitial acentric fragments, respectively. These acentric chromosomal fragments (without centromere) are lost during anaphase. These are generally associated with dicentric chromosomes
Ring chromosome (centric ring/ acentric ring)	Usually, they result from two terminal breaks in both chromosome arms (chromatids), followed by fusion of the broken ends together to form a circular (centric ring) chromosome, leading to the loss of genetic material. Alternatively, the subtelomeric sequences or telomere-telomere fusion with no deletion also results in complete acentric ring chromosomes
Terminal and interstitial deletion (excess acentrics)	A terminal deletion is the loss of the end of a chromosome (telomere), leaving longer acentric fragment than the width of the chromatid. Interstitial deletion occurs when two breaks are induced in interstitial region and the terminal part rejoins the main body of the chromosome, generating double minutes as acentric fragments
Reciprocal translocation	Reciprocal (complete or two-way) translocations involve non-acrocentric chromosomes, and it occurs when two different (nonhomologous) chromosomes have exchanged segments with each other
Marker chromosome	Marker chromosomes are often referred to as mysterious supernumerary piece of chromosomal material. In addition to normal chromosomes, these are small additional structurally abnormal metacentric/centric chromosome fragments whose genetic origin is unknown; however, it can be determined by FISH analysis using specific probes

Table 3.6 Chromatid-type aberrations

Chromatid gaps (achromatic lesions)	Chromatid gap is a non-staining or very lightly stained region (achromatic lesion) of a single chromatid in which there is a minimal misalignment of the chromatid. The width of this region is less than the width of chromatid arm
Isochromatid deletions	The double breaks (often called isochromatid breaks) at the same position on both chromatids are an apparent exception to the definition of chromatid aberrations. They may be induced upon irradiation in the S and G_2 phases of the cell cycle *Isochromatid deletions with complete and incomplete sister union (SU)*: The side-by-side ends of isochromatid breaks usually undergo a cross union to produce U-shaped fragments *Isochromatid deletion without unions (NU: nonunions)*: Occasionally, the sister union does not occur and such sister nonunions may be in either the proximal (centric) or the distal (acentric) fragments. They are cited as NUp (nonunion proximal) and NUd (nonunion distal), respectively
Terminal and interstitial deletion	Loss of terminal end of one of the chromatids of a chromosome
Symmetric interchanges	Symmetrical chromatid exchanges are equivalents of chromosome-type reciprocal translocation. Exchanges that yield a balanced interchange of genetic material between two identical sister chromatids (i.e., SCE) with no loss of genetic material and no mechanical problems at mitosis
Asymmetric interchanges	Inter-arm interchanges and asymmetrical chromatid exchanges are equivalents of chromosome-type dicentrics. The segments of chromatids are differently joined up, yielding an acentric and dicentric chromatid
Intra-chromatid exchanges/ intra-arm interchanges	Chromatid exchanges may occur between non-sister chromatids of paired homologous chromosomes or between sister chromatids of a homologous chromosome. These exchanges may result in symmetrical or asymmetrical interchanged forms such as intra-chromatid exchange with centric ring, inter-chromatid exchange with dicentric, pericentric inversion, and duplication/deletion
Triradials	A three-armed configuration occurs when there is an interaction between one chromosome with an isochromatid deletion and a second having a chromatid deletion

contrast, radiation can induce chromatid aberrations during late S and G2 phases, when sister chromatids are being duplicated and the DNA DSBs may result in chromatid breaks (deletions), interchanges, or triradials. Mostly, sister chromatids or non-sister chromatids of homologous chromosomes are affected by all the breaks and rejoins. The chromosomal aberrations serve as a biological dosimeter—an indicator of radiation exposure. Furthermore, radiation-induced CAs delineate an early marker of late effects, including cell killing and transformation.

A series of methods and techniques (Fig. 3.19) have been developing to assess stable or unstable type of CAs in order to evaluate the potential of a test compound (chemical/mutagen/radiation exposure). Human peripheral blood lymphocytes offer unique possibilities to study somatic cell division (in vitro) and thus have been utilized for detection of CAs (Box 3.13).

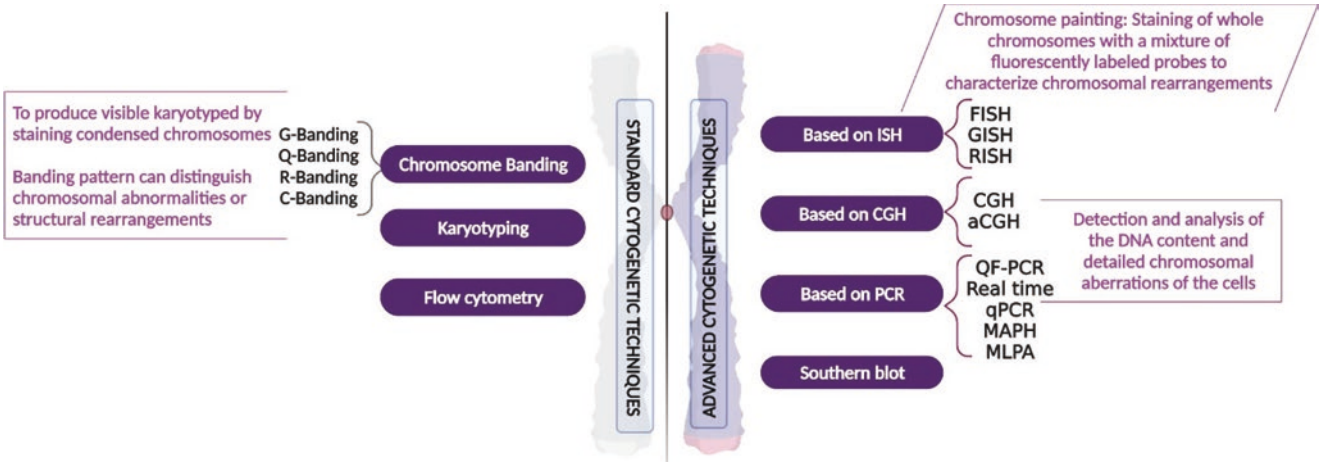

Fig. 3.19 Techniques to assess constitutional or acquired chromosomal abnormalities using standard banding techniques (left) or advanced molecular cytogenetic techniques (right). Standard cytogenetic techniques are traditionally performed by karyotyping of stained metaphase chromosomes or by flow cytometry. Chromosome banding is used to produce alternating light and dark regions, also referred to as "cytogenetic bands," along a chromosome with the use of special stains (abbreviations are listed below). Chromosome banding patterns are essential in pairing and ordering all the chromosomes, known as karyotyping. Flow cytometry-based procedures have been developed to assess numerical (ploidy) and structural (telomere length) chromosomal aberrations in mitotic cells largely based on DNA content. To overcome the limitations of the banding analysis, advanced cytogenetic techniques are introduced. In techniques based on ISH, fluorescently labeled "painting" probes are used to localize nucleic acid sequences. FISH identifies chromosomal rearrangements and mapping-specific genes on individual mitotic chromosomes. GISH determines the origin of genomes or chromatins in hybrids. RISH reveals cellular patterns of mRNA expression in cells. CGH-based techniques provide an overview of chromosome ploidy level (gain and loss) throughout the whole genome. CGH with the use of microarrays—aCGH—detects aneuploidies, deletions, duplications, and amplifications based on DNA content. Southern blotting and PCR-based molecular cytogenetic techniques have good potential to detect chromosomal abnormalities from trace amounts of specific regions of DNA/RNA. *G-banding* Giemsa banding, *Q-banding* quinacrine fluorescence banding, *R-banding* reverse banding, *C-banding* centromere banding, *ISH* in situ hybridization, *FISH* fluorescence in situ hybridization, *GISH* genomic in situ hybridization, *RISH* RNA in situ hybridization, *CGH* comparative genomic hybridization, *aCGH* array comparative genomic hybridization, *QF-PCR* quantitative fluorescence polymerase chain reaction, *qPCR* quantitative polymerase chain reaction, *MAPH* multiplex amplifiable probe hybridization, *MLPA* multiplex ligation-dependent probe amplification

Box 3.13 In a Nutshell: Chromosome-Type and Chromatid-Type Abberations

- Radiation-induced breakage and improper rejoining in pre-replication (G_1) chromosomes may lead to *chromosome-type* aberrations.
- Radiation-induced breakage and inappropriate rejoining in post-replication (late S or G_2) chromosomes may lead to *chromatid-type* aberrations.
- Since the radiation-induced aberrations in G_0 lymphocytes are of the chromosome type, all paired acentric fragments are to be classified as chromosome-type terminal deletions and not isochromatid deletions.
- Unstable aberrations like dicentrics, rings, and anaphase bridges are lethal to cells and not passed on to the progeny. Small deletions and stable symmetric translocations are nonlethal and are passed on to the progeny; thus, they may have genetic consequences.

From the mentioned chromosomal mutations, the translocations are especially dangerous as, in contrast to many other types of chromosomal aberrations, they can be tolerated by the cells. They usually neither cause loss of genetic material nor mitotic cell death and are thus transmitted to the next cell generations. At the same time, translocations are highly oncogenic or affect cell physiology in other ways. Translocations may be simple; reciprocal; i.e., if chromatin fragments are exchanged between two chromosomes; or even complex [70]. Translocations mostly arise due to erroneous DNA end joining by classical NHEJ or mutagenic alternative repair pathways. Although homologous recombination is generally considered a highly precise repair mechanism, recombination between repetitive sequences especially in heterochromatin may also lead to chromatin exchanges [48]. In addition, HR can trigger chromosomal translocations when its intermediates are resolved by crossover between allelic or nonhomologous chromosomes [70]. Although translocations are not associated with extensive losses of the genetic material, they can generate fusion genes (and proteins) with aberrant, often oncogenic, functions. An

example could be the reciprocal translocation t(9;22)(q34;q11) between genes BCR and ABL [71], which is responsible for the development of the well-known chronic myeloid leukemia (for terminology and categorization of translocation types, the reader is referred to specialized books on medical genetics or cytogenetics, e.g., Griffiths et al. [70]). In addition to formation of fusion genes, translocations may activate proto-oncogenes by repositioning them along or between the DNA molecules into a close proximity of a strong promoter of some other gene. If the reading frame of the translocated gene is shifted, its function may be lost. However, the gene activity can be changed also epigenetically, if a gene is moved into an incorrect chromatin environment. This is often a cause of the tumor suppressor silencing, after a tumor suppressor is translocated close to a genetically inactive heterochromatin domain. In the context of radiobiology, it is important to emphasize that cell exposures to different radiation types lead to different types of translocations. Cells irradiated with photonic radiation with low LET mostly contain interchromosomal translocations where one chromatin fragment is translocated to another chromosome or two fragments are reciprocally exchanged between two chromosomes. These lesions are usually simple, but the proportion of complex translocations increases with the radiation dose. Cells exposed to a particle high-LET radiation, on the other hand, mostly suffer from complex chromosomal translocations arising as the consequence of extensive chromatin fragmentation by highly localized energy deposition along the particle tracks [72]. For the same reason, high-LET radiation preferential generates intrachromosomal translocations affecting a single chromosome at multiple sites. To explain this phenomenon, it should be emphasized that chromosomes in the interphase cells occur in the form of chromosomal territories with only a limited extent of mutual intermingling along their borders, as explained in Sect. 3.5. Hence, the areas of chromosome territory borders where translocations between the neighboring chromosomes can be formed represent only a small proportion of the nuclear volume along the radiation particle track [53, 54]. With increasing doses and more particles transversing a single nucleus, however, extensive rearrangements of the genome affecting high numbers of chromosomes can be detected (Box 3.14).

Box 3.14 In a Nutshell: Chromosomal Translocations

- Chromosomal translocations are the consequence of illegitimate rejoining of DNA double-strand breaks generated by radiation.
- Chromosomal translocations pose a risk of formation of a fusion gene/protein with oncogenic functions; even single translocation may be a sufficient genetic defect to initiate leukemia.
- While low-LET radiation generates mostly simple translocations, exposure to high-LET radiation leads to complex genotype rearrangements.
- Due to the character of energy deposition, low-LET radiation produces predominantly interchromosomal translocations; higher occurrence of intrachromosomal translocations is then a sign of a high-LET exposure.

3.6.3 Premature Chromosome Condensation

Chromosome condensation, the landmark event at the onset of prophase, is the dramatic reorganization of the isolated patches of long thin chromatin strands at the nuclear periphery into compact short chromosomes that can be visualized at metaphase during mitosis or meiosis in eukaryotic cells. Maturation-promoting factor (also called mitosis-promoting factor or M phase-promoting factor, abbreviated MPF), the $p34^{cdc2}$/cyclin B complex, serves as a master cell cycle regulator for the M-phase transition and chromatin condensation by phosphorylated condensins (Fig. 3.20). MPF activity mainly depends on the cellular concentration of cyclin B, which usually oscillates through cell cycle. During cell division, chromatin condenses and individualizes to discrete chromosomes, which are further segregated by mitotic spindle fibers. Once divided, chromatin decondenses to re-establish its interphase structure component facilitating DNA replication and protein-making processes.

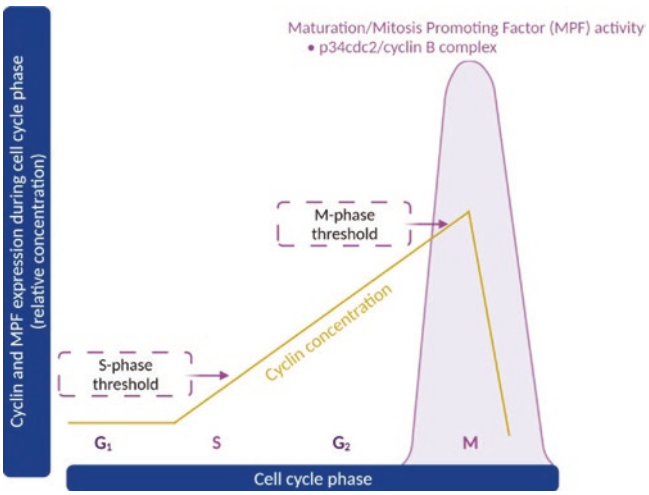

Fig. 3.20 The presence and action of MPF protein in the cell control premature chromosome condensation induction. Cyclin B oscillates through the cell cycle being undetectable during interphase, very low in G1, gradually increasing from S, reaching maximum in G2, and decreasing abruptly at G2/M transition. This corresponds to the MPF activity during cell cycle. *MPF* maturation/mitosis-promoting factor

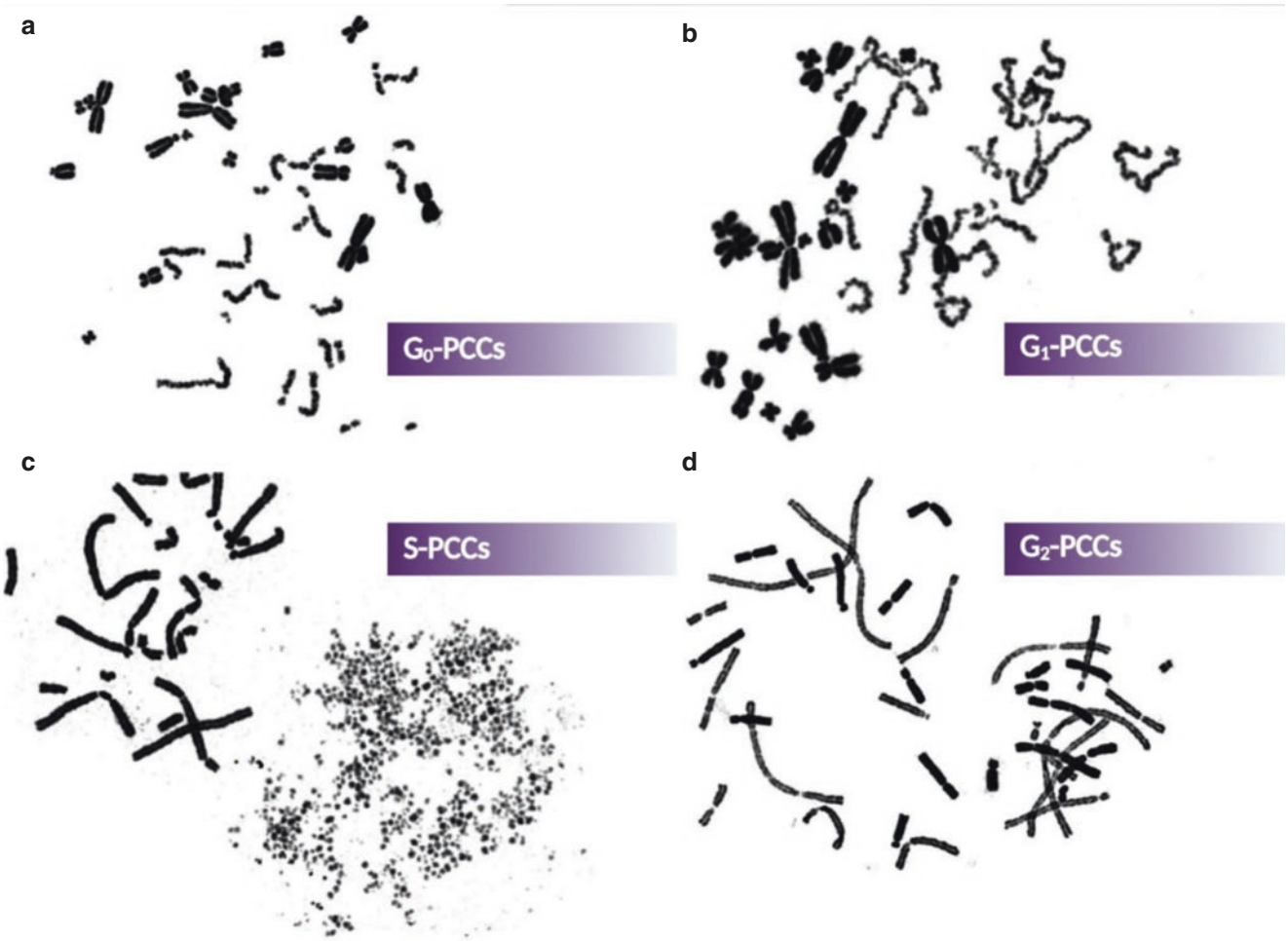

Fig. 3.21 Premature chromosome condensations (PCCs) at various stages of the cell cycle: darkly stained metaphase chromosomes belong to mitotic CHO cells, whereas the lighter stained to the interphase CHO cells. (**a**) G0-PCCs, (**b**) G1-PCCs, (**c**) S-PCCs (reproduced with permission (CCBY) from Pantelias et al. [73]), (**d**) G2-PCCs. *CHO* Chinese hamster ovary

Chromosome condensation may also occur prematurely in interphase test cells when they are fused to mitotic cells or chemically using specific phosphatase inhibitors. The most common approach is the use of Chinese hamster ovary (CHO) cells as mitotic inducer cells. Following cell fusion, the MPF present in a mitotic cell interacts with the interphase nucleus causing dissolution of its nuclear membrane and premature chromosome condensation of interphase chromosomes. This phenomenon is known as *premature chromosome condensation (PCC)*. The morphology of prematurely condensed chromosomes (PCCs) depends on the stage of the interphase cell in the cell cycle (i.e., G_0, G_1, S, and G_2) (Fig. 3.21). PCCs in G_0-phase cells exhibit single chromatids, highly condensed and distinct. During the G_1 phase, G_1-PCCs are despiralized single chromatid chromosomes, while chromosomes condensed during the S phase (S-PCCs) have a "pulverized" appearance because of less condensed chromatin at the sites of replication [73]. Condensation during the G_2 phase (G_2-PCCs) yields distinct elongated double-chromatid chromosomes. Consequently, cell fusion-mediated or chemical induction of PCCs has been proven a powerful cytogenetic tool in radiobiology to study the conversion of radiation-induced DNA lesions into chromosomal aberrations at various cell cycle stages since it enables visualization and quantification of radiation-induced numerical and structural chromosomal alterations directly in interphase cells.

PCC can be induced either by fusion of human lymphocytes with mitotic cells (fusion-mediated PCC) or with the use of specific chemicals (chemical-induced PCC).

In the case of fusion-mediated PCC, the condensation was at first achieved with the use of fusogenic viruses (such as Sendai virus or its equivalent). However, an important disadvantage of this method is that the fusion efficiency depends on various notable factors [74]. These difficulties were overcome by using cell-fusing chemical agents (e.g., polyethylene glycol—PEG). PEG overcomes these difficulties and can be widely used for radiation cytogenetic studies.

Chemical-induced PCC exploits specific inhibitors for serine/threonine protein phosphatase, which can activate endogenous intracellular MPF, which is much simpler and easier than fusion-induced PCC. Chemicals that can be used for the achievement of drug-induced PCC are okadaic acid, calyculin A, 2-aminopurine, staurosporine, wortmannin, and sodium vanadate. A limitation of this method is that no PCC can be induced in G_0 resting-phase cells (Box 3.15).

Box 3.15 In a Nutshell: Premature Chromosome Condensation

- The appearance of a prematurely condensed interphase chromosome depends on the stage of cell cycle.
- PCC can be done in two main ways either by the fusion of human lymphocytes with mitotic cells (fusion-mediated PCC) or by the use of chemicals (chemical-induced PCC).
- G1-PCC displays very long single chromatids; PCC in an early, middle, and late S-phase cell shows crushed and pulverized appearance of both single and sister chromatids; G2-PCC demonstrates still long separated sister chromatids with no clearly visible centromere.
- The dephosphorylated active form of MPF, a p34cdc2/cyclin B complex, promotes chromosome condensation in meiotic and mitotic cells.
- Upon inhibition of protein phosphatase enzymes, cdc25 and cyclin B/cdc2 complex is activated which promotes condensation of chromosomes prematurely.

Because of its unique properties, PCC is used for visualizing and scoring chromosomal damage induced by radiation or other clastogenic agents, measuring the induction yield and repair kinetics of chromosome damage in cells at various cell cycle stages immediately after irradiation. It can also be used for the study of condensation dynamics and conformational changes that occur during the cell cycle. The data obtained using the PCC assay can correlate radiation-induced DNA damage and CAs observable at metaphase [75].

Mitotic cell fusion-induced PCC in human lymphocytes (G_0-PCC) allows early detection of cytogenetic damage in interphase, the stage of human lymphocytes in peripheral blood, and is the most suitable technique especially for biodosimetry applications in radiation emergency accidents as well as for triage biodosimetry [76]. A later ring PCC (rPCC) assay is an alternative biodosimetry method to the "gold standard" cytogenetic approach (dicentric analysis in metaphase) for high-dose exposure to radiation and can be applied in a simulated mass casualty accident either after chemical induction of PCC [77] or by means of cell fusion providing a much faster assessment of dose [78].

3.6.4 Chromothripsis-Like Alterations

During the last decade, it has been reported that high-LET radiation induces chromothripsis-like complex chromosomal alterations, resembling the phenomenon of chromothripsis appearing in tumors [79]. The term chromothripsis arises from the Greek dialect (chromo for chromosome and thripsis for shattering into pieces), and it was initially described in 2011 by Stephens et al. [80]. Rather than a progressive accumulation of sequential alterations induced in the genome, chromothripsis is a process where chromosome segments undergo tremendous but localized shattering and random rearrangements in a single catastrophic event. Inaccurate rejoining of the induced chromosome fragments results in a new genomic arrangement and the formation of complex chromosomal aberrations that may trigger carcinogenesis (Fig. 3.22).

The mechanisms responsible for chromothripsis are still under debate. However, studies have shown several situations that could be catastrophic for the cell and result in chromothripsis. One possible mechanism proposed is that DNA damage such as DSBs and chromosomal aberrations may cause aberrant mitosis and formation of MN including one or more chromosomes that may undergo localized shattering and chromothripsis. Chromosome shuttering and chromothripsis may emerge in MN when the main nucleus enters mitosis while DNA is still being replicated within micronuclei. Additionally, PCC induces a mechanical stress in the asynchronous micronucleated cells leading to chromosome shattering [73]. Random genomic rearrangements in micronuclei can then be integrated into the cell's genome, triggering amplification of oncogenes and cancer development [81]. Other additional mechanisms have also been proposed, such as dicentric chromosome formation, telomere erosion, and abortive apoptosis [82].

Regarding radiation-induced chromothripsis-like chromosomal alterations, it was tested recently whether clustered DNA lesions and chromatin decompaction induced by high-LET irradiation can subsequently evolve in localized chromosome shattering in chromosome domains along the particle tracks. This is a critical risk for chromothripsis to occur, and the results obtained provided experimental evidence that high-LET particle radiation is effective in inducing chromothripsis-like aberrations, which can be used as a fingerprint of high-LET exposure [83]. These discoveries are valuable in the fields of radiation oncology and space radiation protection, since chromothripsis-like aberrations can be responsible for adverse effects and increase the hazard for secondary induced cancer.

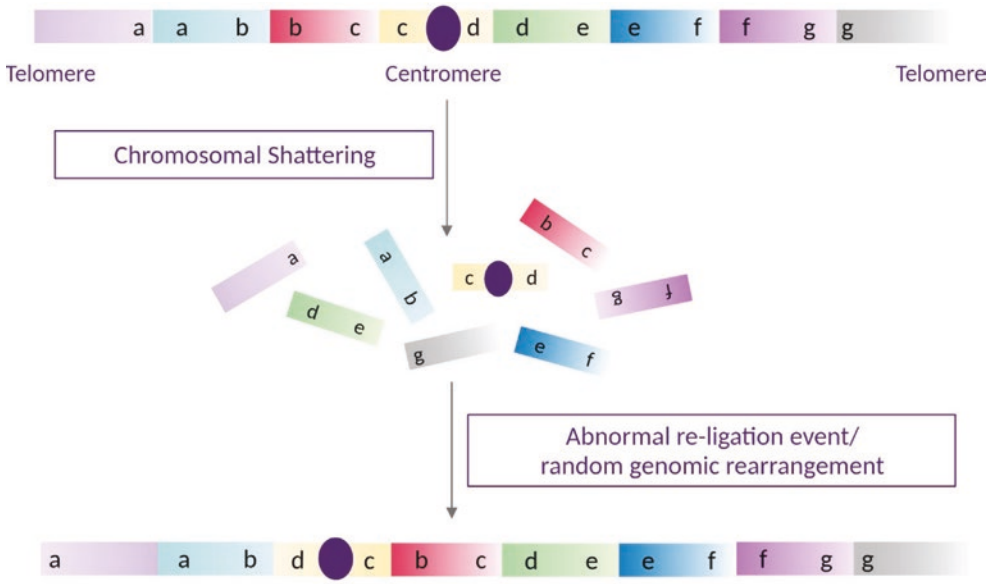

Fig. 3.22 Schematic illustration of chromothripsis. It is a phenomenon where one single catastrophic event leads to a massive and localized shattering of one or few chromosomes. Shattered chromosome fragments are not properly rejoined resulting in a new genome configuration and a large number of complicated chromosomal aberrations

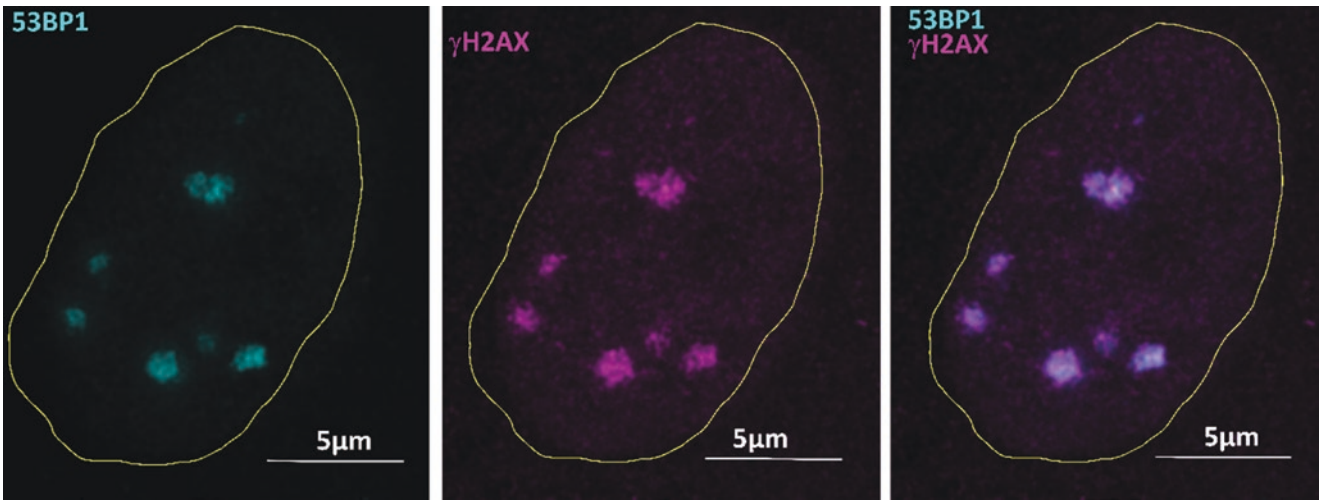

Fig. 3.23 Radiation-induced DNA damage foci. 53BP1 (left, cyan) and γH2AX (middle, magenta) foci in HeLa cells irradiated with 1.2 Gy alpha particles and spatially fixed at 60 min postirradiation. Colocalization of γH2AX and 53BP1 foci is shown (right). Yellow line indicates the cell nucleus

3.6.5 Ionizing Radiation-Induced Foci (IRIF)

This chapter is dedicated to the importance of ionizing radiation-induced foci (IRIF) (Fig. 3.23) in DNA damage measurements. Traditional biomarkers of radiation exposures are chromosomal aberrations and micronuclei. In contrast to quantification of these biomarkers, which emerge due to repair errors in some cells only, IRIF of certain proteins and posttranslational modifications are formed in all cells on all DSB damages, almost immediately after irradiation. Hence, these IRIF can be considered specific biomarkers of DSB lesions [84]. This allows easier and faster victim triage. Moreover, naturally occurring amplification of the DSB damage signal, associated with extensive focal accumulation of γH2AX and numerous repair proteins at DSB sites (for detailed description on DNA repair, see Sect. 3.4), offers the unprecedented sensitivity of radiation dose estimation via the pure counting of IRIF on immunofluorescence microscopy images [84]. The radiation dose absorbed by the cells can be estimated by simple counting of such IRIF or, more automatically, by measuring the integrated intensity of the IRIF signal for high numbers of individual cells by flow cytometry [85]. Under the optimal conditions, especially the time range around 30 min after irradiation, the reported minimal detectable values lie in the range of mGy [86].

Furthermore, DNA damage induction and repair processes can be studied in individual cells using the IRIF assay. In practice, this is important in situations where individual

cells can be differentially affected by irradiation, such as in the cases of a partial-body exposure. The ability to study individual cells is critically important also for radiobiological research as individual cells, even if irradiated homogeneously, appear in different phases of the cell cycle, belong to specific (cancer) cell clones, may be to a various extent affected by the bystander effect, etc.

On the other hand, the biochemical nature of IRIF means that their formation potentially depends on various factors, which may introduce some variability to DSB quantification. It remains a subject of discussion whether all DSBs necessarily require IRIF formation for successful repair. Additionally, some foci may persist at DSB sites even after the break rejoining. A real obstacle could follow from the fact that IRIF occur, to some extent, in nonirradiated cells. However, recent results have proved that the spontaneously forming foci differ in size and topology from the radiation-induced ones. So, staining patterns corresponding, for instance, to replication-stressed or apoptotic cells can be distinguished from IRIF related to DNA repair [87]. Importantly, this phenomenon is more prominent only in cancer cells, which are not relevant for biodosimetry. In any case, "the second γH2AX assay intercomparison exercise" carried out in the framework of the European biodosimetry network (RENEB) confirmed a high fidelity of irradiated victims' triage (dose categorization, rather than dosimetry) based on IRIF detection of the postradiation modification of histone variant H2AX, called γH2AX [84].

γH2AX is formed by the phosphorylation of histone H2AX at ser139 [57]. This process is mediated by ATM, ATR, and DNA-PK kinases, appears in minutes after DNA breakage, and spreads over ~2 Mbps of DSB-surrounding chromatin. Due to this extent of chromatin modification, γH2AX can be microscopically visualized as compact IRIF at DSB sites of 400–600 nm size as described in Sect. 3.5.

The number of γH2AX foci at a particular time postirradiation corresponds to a dynamic equilibrium between the IRIF formation and disassembly as shown in Fig. 3.24. This is the reason why the maximum γH2AX numbers per cell are detected with a short delay after irradiation and the numbers of counted γH2AX are slightly lower compared to physically detected DNA breaks (PFGE, comet assay) [49].

For most cell types, the peak number of γH2AX is detected in the time window between 30 min and 1 h postirradiation on average, and some shift to later postirradiation times may appear in cancer cells as they often suffer from DSB repair defects. If the integrated γH2AX signal is measured by flow cytometry, the maximal values are measured later than with focus counting, at about 1 h postirradiation, as the size of γH2AX foci grows longer than their number [49]. After reaching the peak value, the number of γH2AX foci rapidly reduces (Fig. 3.24) and, at 24 h postirradiation, only few DSBs that are repaired only with difficulty persist in cells irradiated with medium doses (in order of Gy) of low-LET radiation. However, a substantial proportion of DSBs may still be detected at this late period of time or even after several days postirradiation in

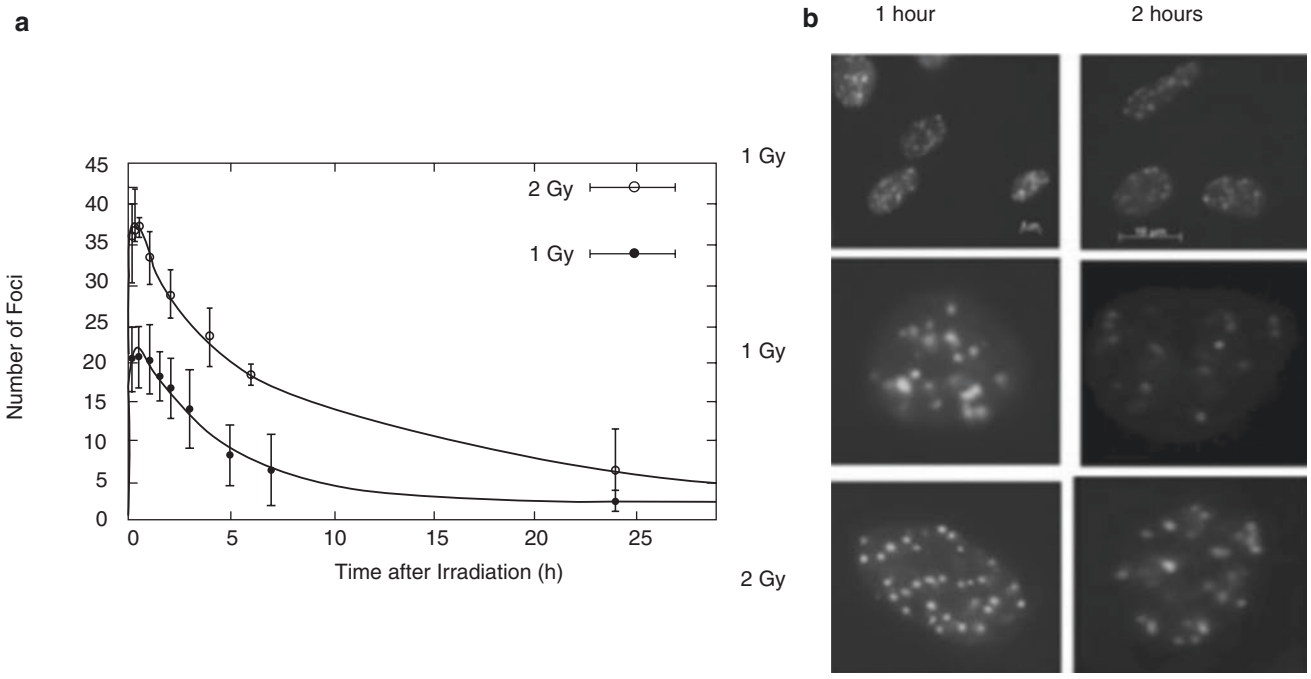

Fig. 3.24 DNA repair kinetics. (**a**) Formation and disassembly of γH2AX foci in human cancer cells irradiated with 1 Gy or 2 Gy X-rays. (**b**) Representative microscopic images for γH2AX foci 1 h and 2 h after X-ray irradiation. (Reproduced with permission (CCBY) from Mariotti et al. [88])

cells exposed to high-LET radiation or high doses of low-LET radiation. From the perspective of biodosimetry, this means that the highest precision of the absorbed dose estimation can be achieved in a few-hour window immediately after irradiation. This requirement can be fulfilled during planned medical care, where, in addition, the monitoring of γH2AX foci formation and disassembly (DSB repair kinetics) may be used to identify patients hypersensitive to radiotherapy or radioresistant tumors. However, in the case of unpredicted accidents with mass screening, IRIF-based biodosimetry must rely on the persistent foci due to the necessary reaction time. This requires suitable mathematical models for the absorbed dose estimation and restricts the method applicability to the acute photon dose range of ~0.5 to ~8.5 Gy and days after exposure (i.e., 1 day after 1 Gy and 14 days after 8.5 Gy) [89]. For military countermeasures, it should also be kept in mind that some chemical warfare agents, such as mustard gas, also generate γH2AX foci. Furthermore, background levels may vary due to non-irradiation-induced IRIF, which are also counted and vary individually, so this assay is best suited for triage rather for accurate dosimetry.

In addition to the analysis of γH2AX IRIF numbers, the spatial distribution of γH2AX foci can be determined by microscopy. This is an important advantage of microscopy over flow cytometry as low-LET and high-LET exposures can be distinguished according to nuclear topology of γH2AX foci [90] as described in Sect. 3.5. On the other hand, flow cytometry offers more room for automation than microscopy and can analyze much higher cell numbers, making it the more suitable method for routine biodosimetry in most circumstances (Box 3.16).

Box 3.16 In a Nutshell: γH2AX as Radiation Damage Marker

- γH2AX IRIF form as the histone H2AX is phosphorylated after DSB induction.
- γH2AX IRIF formation starts a few minutes after irradiation and peaks at 30 min–1 h postirradiation.
- Especially after high-dose irradiation or irradiation with high-LET particles, persistent γH2AX IRIF are left after repair.
- γH2AX IRIF can be used for triage-level biodosimetry by counting foci either in the first hours or persistent foci in microscopic images.

γH2AX attracts numerous proteins with specific signaling and/or repair functions to DSB sites. These proteins, in turn, form IRIF with protein-specific time occurrence and extent of colocalization with γH2AX. Hence, IRIF formed by numerous repair proteins can be used to quantify DSBs and estimate the absorbed dose in the same way as it was described above for γH2AX. Alternatively, repair protein and γH2AX foci can be detected simultaneously to enhance the fidelity of DSB evaluation. Furthermore, the protein composition and structure of IRIF protein complexes (e.g., their specific persistent homology at the nanoscale), and differences of these parameters in specific chromatin domains and after exposure to different types of ionizing radiation, help to understand the mechanisms of DNA repair.

Some proteins like 53BP1 form IRIF morphologically comparable to γH2AX foci. Others, which are required in only a few copies (Ku70 and Ku80), are too tiny and can be visualized only with electron microscopy or super-resolution optical microscopy [91]. Other proteins [such as MRE11, NBS1, or ATM (Fig. 3.25)] create small, but large enough, IRIF to be recognized by standard immunofluorescence microscopy. However, these proteins are, in addition to their IRIF location, also dispersed over the cell nucleus. As IRIF and free aggregates of these proteins may be similar in size, and cannot be discriminated by antibody staining, it is often difficult to reliably distinguish these IRIF from the background [52]. Depending on the function of a particular protein in the repair process, IRIF appear immediately (e.g., MRE11, NBS1, 53BP1) or only later after irradiation (BRCA1, BRCA2, RAD51, etc.). This timing may correspond with repair pathway specificity of a given protein. Some proteins, such as 53BP1 [57], are involved in the regulation of both major DSB repair pathways (NHEJ and HR), while other proteins are selective either for NHEJ or for HR (BRCA1, BRCA2, RAD51).

IRIF of repair pathway nonselective proteins, such as 53BP1, occur in all cells and colocalize with most γH2AX foci [57]. 53BP1 is thus a good DSB marker for biodosimetry, in addition to γH2AX. Moreover, 53BP1 foci have similar size and shape as γH2AX foci so that 53BP1 and γH2AX foci extensively colocalize (Fig. 3.23). This fact improves DSB detection in cells where both types of foci are labeled simultaneously. Co-labeling of γH2AX and 53BP1 foci may be especially useful when cells were exposed to low radiation doses generating only few DSBs or if cancer cells with a strong background signal are analyzed. A significant improvement of DSB number estimation due to γH2AX and 53BP1 co-detection is experienced also in cells exposed to high-LET radiation, where DSBs are extensively clustered and can be thus discriminated only with limitation. However, super-resolution microscopy methods, such as single-molecule localization microscopy (SMLM) or STED microscopy, are necessary for more precise analysis of IRIF foci or even their internal composition and arrangement [48, 57].

It should be noted that not all γH2AX foci necessarily colocalize with 53BP1 (or other repair proteins) at early time periods postirradiation. This includes also the period of 30 min postirradiation when the maximum γH2AX focus

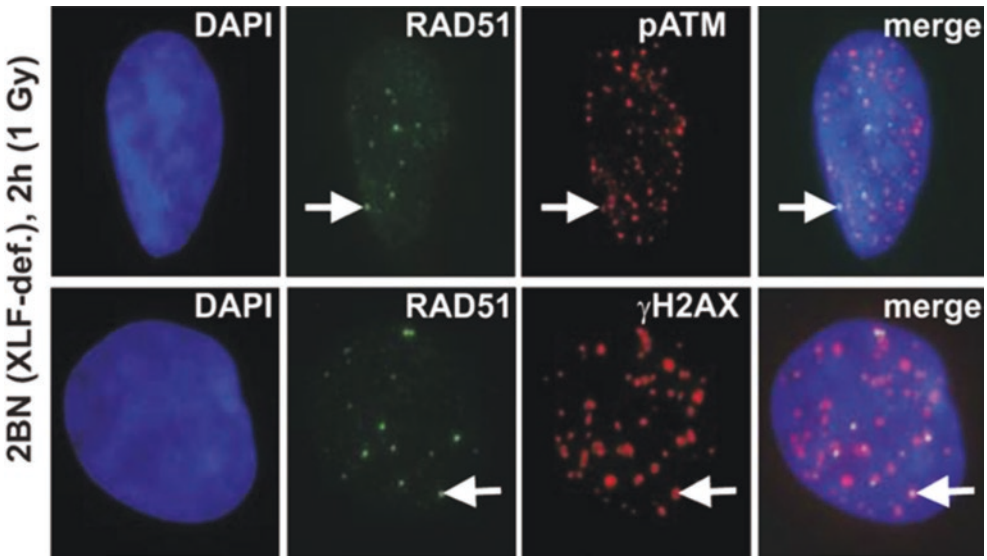

Fig. 3.25 DNA repair protein markers forming small foci. 2BN hTert (XLF-deficient) human fibroblasts were analyzed 2 h post-IR with 1 Gy. Cells were stained against DAPI, pATM, and RAD51, or DAPI, γH2AX, and RAD51. RAD51 is present in a subset of pATM and γH2AX foci. Reproduced with permission (CCBY) from Geuting et al. [92]. *DAPI* 4′,6-diamidino-2-phenylindole used for staining nuclei, *XLF* XRCC4-like factor

Table 3.7 DNA repair proteins and occurrence

Protein/IRIF	Occurrence
γH2AX	All DSB
53BP1	All DSB
NBS1	Part of MRN complex
MRE11	Part of MRN complex
Ku70/80	All DSB
RAD51	Predominantly HR
Brca1	Transition between NHEJ and HR
Brca2	Predominantly HR

numbers are detected. On the other hand, at late time periods after irradiation, 53BP1 foci may persist in cells without being accompanied by γH2AX. These non-colocalizing 53BP1 foci probably label and protect incompletely repaired chromatin [93].

Moreover, as IRIF form also at sites of single-stranded DNA breaks (SSB) or oxidative base damages, co-labeling of γH2AX with suitable markers of these lesions (e.g., XRCC1 or OGG1) [94] can provide information on the complexity of individual DNA damage sites. This information may be correlated to various factors, such as the LET of the incidental radiation or chromatin density and genetic activity at DSB sites [16]. Table 3.7 shows a summary of the IRIF markers mentioned in this section and their occurrence (Box 3.17).

Box 3.17 In a Nutshell: Ionizing Radiation Induced Foci

- Repair protein IRIF, depending on the protein's role throughout repair, can also be used for biodosimetry.
- Repair protein IRIF can be used to understand repair mechanisms and pathways of individual DSB sites.
- IRIF can be used to understand the effect of radiation of different LET.

3.7 Oxidative Stress: Redox Control and Mitochondrial DNA Damage

3.7.1 Oxidative Stress and Consequences for Cell Macromolecules

Exposure to IR induces oxidative damage to cellular molecules such as proteins, lipids, and DNA as a result of oxidative stress (OS), a consequence of the indirect effects of IR (see Chap. 2 and Sect. 3.2), as shown in Fig. 3.26. OS refers to a state of imbalance between oxidants and antioxidants, in favor of oxidants, due to either antioxidant depletion or oxidant accumulation. Oxidants include reactive oxygen (ROS) and nitrogen (RNS) species that comprise free radicals, which are characterized by oneself or more unpaired electrons in the outer shell, and non-radical reactive species. A list of radicals and non-radicals can be found in Table 3.8. Some of these species, e.g., superoxide and hydroxyl radical, are short-lived due to their high reactivity towards other molecules, while others, like hydrogen peroxide, are more stable. Among the ROS, the hydroxyl radical is particularly toxic and involved in the mediation of IR-induced lesions to cell biomolecules. By analogy to OS, nitrosative stress is mentioned when referring to RNS.

Oxidants are produced from exogenous, such as air pollutants, xenobiotics, and IR, and endogenous sources as normal cellular metabolism by-products. Examples are the mitochondrial electron transport chain (ETC), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and peroxidases. Low to moderate ROS levels are crucial in physiological function of cell to avoid oxidative stress involved in aging and several neurodegenerative diseases, diabetes, cancer, atherosclerosis, etc. ROS are also signaling molecules involved in the IR non-targeted effects (see Chap. 2).

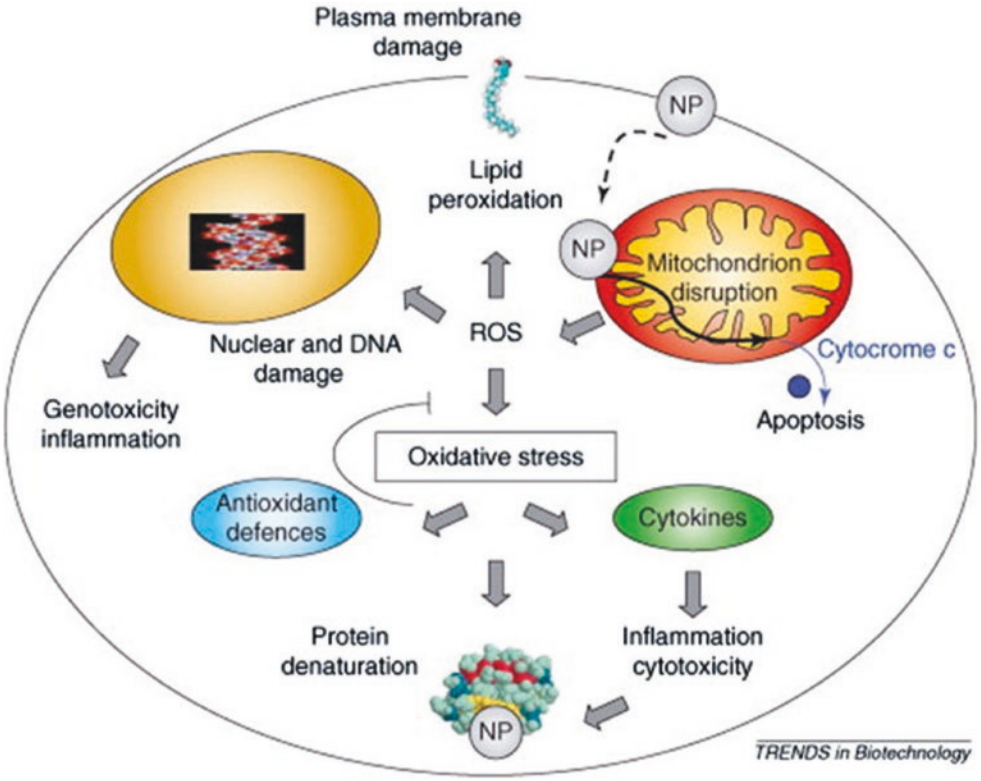

Fig. 3.26 Possible ROS-mediated oxidative stress. Upon exposure to IR, oxidative stress can induce collateral damage, such as lipid peroxidation, protein denaturation, nuclear and DNA damage, mitochondrial damage, and apoptotic death by releasing cytochrome c. Oxidative stress owing to excess ROS generation induces overexpression of antioxidant enzymes in an attempt to control ROS levels. At high levels of oxidative stress, antioxidant defenses are overwhelmed, which leads to inflammatory and cytotoxic responses. (Reproduced with permission from Sanvicens and Marco [95]). *NP* nanoparticles, *ROS* reactive oxygen species

Table 3.8 List of free radicals and non-radicals

Free radicals	Non-radicals
Reactive oxygen species (ROS)	
Superoxide $^{\circ}O_2^-$	Hydrogen peroxide H_2O_2
Hydroxyl °OH	Singlet oxygen $^1O_2$
Peroxyl °ROO	Ozone O_3
Lipid peroxyl LO°O	Hypochlorous acid HOCl
	Lipid peroxide LOOH
Reactive nitrogen species (RNS)	
Nitric oxide °NO	Nitrous acid HNO_2
Nitrogen dioxide $^{\circ}NO_2$	Peroxynitrite $ONOO^-$
	Dinitrogen trioxide N_2O_3

OS occurs in pathologic conditions, when the cellular antioxidant defenses are overwhelmed by free radicals and oxidants. Their great oxidative ability leads to oxidative damages to cellular biomolecules (DNA, proteins, and lipids) resulting in multiple damage affecting cell membrane, cellular signaling, and genome integrity. The accepted radiation biology paradigm considered DNA for a long time as the critical IR target and the primary cause for the harmful effects of IR, due to its content of genetic information, with nucleic acid damage being extensively characterized, without consideration that damaged lipids and proteins may also have detrimental effects on cellular function.

Further targets of radiation-generated ROS are lipids, major constituents of the cell membrane, because of their molecular structure containing abundant reactive double bonds [96]. Upon ROS reaction with polyunsaturated fatty acids (PUFA), chain reactions occur, leading to lipid peroxidation (LP) and generation of toxic decomposition products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), and isoprostanes (IsoPs), which are quantifiable markers of LP reactions. Biological LP consequences include changes in the permeability and fluidity of the membrane lipid bilayer, ion gradient disruption across membrane, and alterations in membrane-associated protein activity [96].

Potential oxidative damage to proteins is multiple, cysteine, methionine, and tyrosine residues. Chemical modifications include oxidation, carbonylation, and nitration and lead to posttranslational modifications inducing conformational changes affecting protein structure and function, i.e., loss of enzyme activity.

While the physical and chemical reactions initiated by radiation occur in less than a millisecond, the resulting biological effects may take hours, days, months, or years to be expressed and may differ among individuals due to varying intrinsic radiosensitivity. In particular, since the oxidative damage extent depends on the antioxidant availability, increased expression of antioxidant defense systems has been linked to decreased radiosensitivity [97].

OS also has a central role within the inflammatory process. ROS such as superoxide can rapidly combine with NO to form other RNS, such as peroxynitrite, and is 3–4 times faster than the dismutation of superoxide by the SOD. The RNS, in turn, induces nitrosative stress, which

adds to the pro-inflammatory burden of ROS. Injured cells release chemoattractant molecules, and NO increases vascular permeability and vasodilation that trigger local inflammation. Neutrophils are the first inflammatory cells to arrive at the site of injury, and the increased expression of intercellular adhesion molecule 1 (ICAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) on disrupted endothelial surfaces contributes to neutrophil extravasation. When leukocytes come into contact with collagen fragments and fibronectin, they release pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), IL-1, and IL-6 that increase ROS production and lead to even greater local inflammation that can perpetuate inducing chronic radiation injury, which in some cases develop into fibrosis [98] (Box 3.18).

Box 3.18 In a Nutshell: Oxidative Stress

- Oxidative stress is characterized by an imbalance between prooxidant molecules and antioxidants.
- Oxidative stress participates in the oxidative damage of cellular components.
- Antioxidants play a key role in stopping the oxidative chain reactions by scavenging the free radical intermediates.
- Excessive generation of ROS, that provokes mitochondrial DNA mutations, impairs the mitochondrial respiratory chain and modifies membrane permeability and mitochondria-associated defense systems.
- Several biomarkers of oxidative stress exist and comprise direct ROS measurement, indirect measure of oxidative stress by quantifying oxidation products, and measure of antioxidant defenses.

3.7.2 Redox Control: Antioxidant Defenses

In order to cope with ROS and RNS, living organisms have evolved essential antioxidant defense mechanisms (Fig. 3.27). These are classified as enzymatic and nonenzymatic systems or as high-molecular-weight and low-molecular-weight compounds. The first line of antioxidant defenses includes the highly abundant glutathione (GSH), catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD). GSH acts directly as an oxidant scavenger or indirectly as a cofactor of several enzymes such as the GPx. SOD exists in three isoforms using different metals as cofactors: SOD1, which is predominantly cytoplasmic; SOD2, which is mitochondrial; and SOD3, which is extracellular. SOD1 and SOD3 contain copper (Cu) and zinc (Zn), whereas SOD2 has manganese (Mn) in its active site. They catalyze the dismutation of $^{\circ}O_2^-$ to H_2O_2 afterwards converted to water by catalase, GPx, or peroxiredoxin (Prx). GPx transforms reduced GSH to its oxidized form (GSSG). GSH pool regenerates by de novo synthesis and glutathione reductase using NADPH as a reducing equivalent. GPx is also involved in hydroperoxide detoxification. Prx is involved in hydroperoxides and peroxynitrite detoxification, using thioredoxin (Trx) as a source of reducing equivalents. The most reactive and highly toxic $^{\circ}OH$ is produced from H_2O_2 in the presence of reduced transition metal, a reaction known as the Fenton reaction. Apart from GSH, nonenzymatic antioxidants include endogenous compounds which are produced in organism (uric acid, lipoic acid, L-arginine …) and exogenous compounds which are supplemented through the diet, i.e., carotenoids, ascorbic acid (vitamin C), vitamin E and derivatives (tocopherols and tocotrienols), polyphenols (curcumin, resveratrol, quercetin …), and others.

Glutathione is the major low-molecular-weight thiol in mammals. It plays a key role in cell resistance against oxidative and nitrosative damage by providing reducing equivalents to enzymes involved in the metabolism of ROS, by eliminating potentially toxic oxidation products, and by reducing oxidized or nitrosated protein thiols. In its reduced form (GSH), glutathione is the principal intracellular antioxidant. The conversion of the oxidized form (GSSG) into GSH is done by glutathione reductase (GR) in the presence of NADPH, which is generated by glucose-6-phosphate dehydrogenase in the pentose phosphate pathway (Fig. 3.27). Hence, any damages to these enzymes can compromise GSH functions. The processes of glutathione synthesis, transport, utilization, and metabolism are tightly controlled to maintain intracellular glutathione homeostasis and redox balance. Glutathione is exclusively synthesized in the cytosol and about 85% of it remains there, mainly in the reduced form. The ratio of GSH:GSSG in the cytosol is conservatively estimated at about 10,000:1–50,000:1, and the concentration of the cytosolic GSH is as high as 10 mM, while GSSG in the cytosol is as low as nanomolar concentration [99]. Directly and indirectly, GSH effectively scavenges free radicals and other reactive species (e.g., hydroxyl radical, lipid peroxyl radical, peroxynitrite, and H_2O_2) through enzymatic reactions, such as those catalyzed by GPxs, glutathione-S-transferases (GST), formaldehyde dehydrogenase, maleylacetoacetate isomerase, and glyoxalase I (Fig. 3.27). GSH also helps to recover other important antioxidants as vitamin C.

OS was shown to promote the activation of redox-sensitive transcription factors such as the nuclear factor erythroid 2-related factor 2 (NRF2) and the nuclear factor kappa B (NF-κB). The NRF2 transcription factor plays a central role in the maintenance of cellular redox homeostasis via the coordinated transcriptional upregulation of numerous antioxidant proteins (Fig. 3.28). These include more than 500

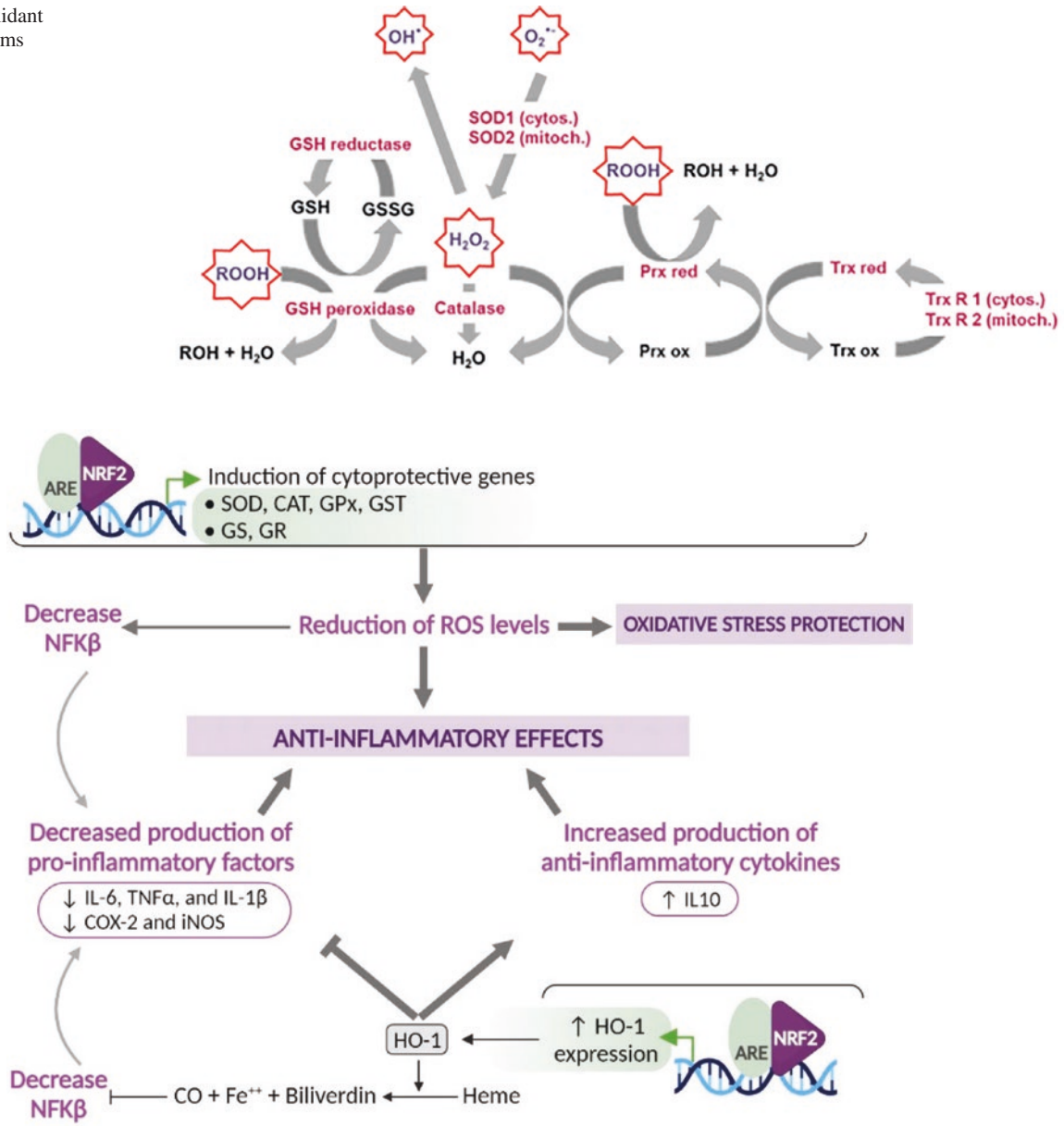

Fig. 3.27 Antioxidant defense mechanisms

Fig. 3.28 NRF2 protection against oxidative stress and excessive inflammatory responses involved in IR injury. NRF2 induces antioxidant response genes, like SOD, CAT, GPX, and GST that enhance ROS elimination. In addition, expression of enzymes such as GR and GS increases GSH cellular content and antioxidant capacity of the cell. Reduction in ROS levels decreases the expression of NFKβ, the main contributor to the inflammatory response. Moreover, NRF2 enhances the expression of HO-1 and its activity in the production of CO that reduces NFKβ activity, pro-inflammatory cytokine secretion (IL-6, TNFα, and IL-1β), and pro-inflammatory enzyme activity (COX-2 and iNOS). *ARE* antioxidant-responsive element, *NRF2* NF-E2-related factor 2, *SOD* superoxide dismutase, *CAT* catalase, *GPx* glutathione peroxidase, *GST* glutathione S-transferase, *GS* glutathione synthetase, *GR* glutathione reductase, *GSH* glutathione, *ROS* reactive oxygen species, *NFKβ* nuclear factor kappa β, *IL-6 and 10* interleukin 6 and 10, *IL-1β* interleukin 1 beta, *TNFα* tumor necrosis factor alpha, *COX-2* cyclooxygenase 2, *iNOS* inducible nitric oxide synthase, *HO-1* heme oxygenase 1

genes that are crucial to metabolize electrophilic attack and protect against OS and inflammatory damage. Kelch-like ECH-associated protein 1 (KEAP1) is a key cytoplasmic repressor of NRF2. KEAP1 interaction with NRF2 leads to NRF2 proteasomal degradation. In the presence of OS or inducers, key "sensor" cysteine thiol groups on KEAP1 are modified, disrupting the degradation process and allowing NRF2 to directly translocate into the nucleus. NRF2 then upregulates the expression of enzymes involved in the synthesis and recycling of GSH, such as the catalytic and modulator subunits of glutamate–cysteine ligase (GCLC and GCLM), GR, GPx, SOD, and several GST. Moreover, several proteins within the redoxin family, such as Trx, TrxRs, Prxs, and sulfiredoxins, are also upregulated by NRF2 [100]

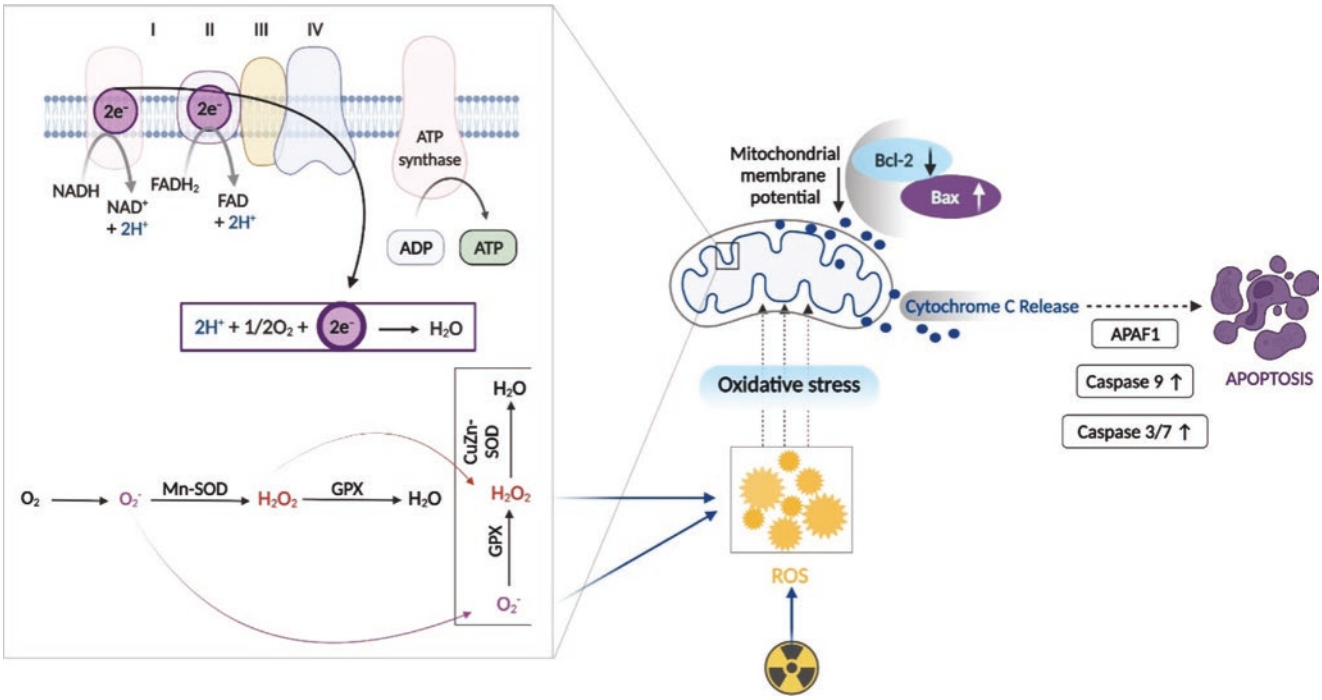

Fig. 3.29 Mitochondria as the key player in radiation-induced oxidative stress-mediated apoptosis. Various stimuli like radiation or improper functioning of the oxidative phosphorylation induce oxidative stress via ROS production. This causes the mitochondria to dysfunction and subsequently leads to cell death by apoptosis. *NAD+* nicotinamide adenine dinucleotide, *NADH* nicotinamide adenine dinucleotide hydrogen, *H+* hydrogen, *FAD* flavin adenine dinucleotide, *FADH2* flavin adenine dinucleotide hydrogen, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate, *Mn-SOD* manganese superoxide dismutase, *GPx* glutathione peroxidase, *H2O2* hydrogen peroxide, *CuZn-SOD* copper zinc superoxide dismutase, *ROS* reactive oxygen species, *Bcl-2* B-cell lymphoma 2, *Bax* Bcl2-associated X, *APAF1* apoptotic protease-activating factor 1

as shown in Fig. 3.29. NRF2 stimulates the mitochondrial biogenesis program through activation of nuclear respiratory factor 1 and indirectly prevents/attenuates inflammation, because NRF2 activation results in the expression of previously mentioned antioxidant enzymes, which detoxify ROS, and in turn this reduces the expression of NLRP3 inflammasome and NFKβ (the main regulator of pro-inflammatory response). Moreover, NRF2 upregulates heme oxygenase activity (HO-1) and increases CO production, which in turn reduces NFKβ activity. In response to this, pro-inflammatory cytokine (IL6 and TNFa) production is reduced, and at the same time the production of anti-inflammatory cytokines (such as IL10) increases. As a consequence of these changes, NRF2 facilitates cells to survive oxidative stress and the inflammatory response that aggravates their cytotoxic effects (Fig. 3.29).

3.7.3 The Role of Mitochondria in Oxidative Stress

Mts are double-membrane multifunctional organelles associated with biosynthesis, metabolism, cell survival, signaling of ROS, etc. In the late 1960s, it was found that radiation could significantly modify the structural form of mts and also the mitochondrial DNA. Human mtDNA is a 16,569 base pair (bp) double-strand circular DNA molecule containing 37 genes, encoding 13 polypeptides for the mt electron transport chain, 2 ribosomal RNA, and 22 transfer RNA for mt protein synthesis. Somatic cells have an average of 100–500 mts with 1–15 mtDNA molecules per mitochondrion.

Although nuclear DNA (nDNA) is the main IR target, mts are constantly removing excess ROS created during energy production and mtDNA is much more vulnerable to IR effects than nDNA. mtDNA is generally repaired less efficiently than nDNA [101], although it uses the same repair mechanisms such as BER, MMR, and HR but not NER and classical NHEJ. Furthermore, the histones for better exposure protection are lacking. Together, this leads to a mutation rate which is 10–1000 times higher than nDNA [102]. Both direct IR exposure and irradiated cell-conditioned medium induce mtDNA damage and alter directed protein synthesis. As a consequence, IR exposure can cause the loss of mt membrane potential, leading to mt undergoing either fission, division of one mitochondrion, or fusion, combination of several mitochondria, autophagy (mitophagy), apoptosis,

modification in the mtDNA copy number per cell (mtDNAcn), and cause DNA damage and mutations, like point mutations or deletions. A common deletion mutation of 4977 base pair deletion in mtDNA genes coding for subunits of the mitochondrial ATPase, NADH dehydrogenase complex I, and cytochrome c oxidase is known as a marker for oxidative damage [101].

Changes in mtDNAcn or mutations in mtDNA both caused by high intra-mtROS control mt-dependent methylation potential of nDNA by decreasing methyltransferase activity and thus causing global DNA hypomethylation or changes in the expression of specific genes [103]. Global DNA methylation levels depend on human mtDNA variants and are also tissue specific and, therefore, may be connected with the differences in susceptibility to the pathogenic processes resulting from IR exposure and OS in different tissues [103].

OS also appears to target the mitochondrial DNA polymerase-γ activity required for replication and repair of mtDNA, thereby reducing the overall repair capacity. Therefore, subsequent to radiation exposure, mtDNA might be damaged, with an ensuing decrease in respiratory chain activity and decrease of mitochondrial function, giving rise to an increased ROS production. Moreover, mutations in mtDNA could lead to an increase in accessibility of reduced components of the ETCs to O_2, which may result in an increase in prooxidant formation. The functional disablement can be weighed by the limitations of the complexes I and III of the mitochondria, reduction of succinate-induced respiratory competence, augmented ROS levels, and increased mitochondrial protein oxidation. The net consequence is persistent metabolic OS that continues to cause de novo oxidative damage to critical biological structures. Such mitochondrial dysfunction can lead to stress signals, which lead to reduced electron transport chain (ETC), and oxidative phosphorylation can cause imbalance in the mitochondrial ROS production, decrease in the mitochondrial membrane potential, and lesser cellular ATP or energy. Although mts are the main producer of ROS, mts themselves can be susceptible to the pathological outcomes once targeted by ROS. By triggering the mitochondrial stress and downstream signaling, the increased levels of free radicals linked to the mtDNA oxidative damage lead to apoptosis.

One of the crucial steps in the process of apoptosis is the permeability transition pore opening (mPTP), followed by drop in the mitochondrial membrane potential. Opening of the pore increases the permeability of the mitochondrial membrane to molecules, leading to mitochondrial swelling and necrosis. NO produced at the basal level (e.g., 5 μM) could S-nitrosylate cyclophilin D (CypD), a critical mPTP regulatory component. This prevents the association of CypD with mPTP that is required for opening the pore and confers a protection to the cell under a stress. On the other hand, NO produced at a high concentration (e.g., 500 μM) could produce peroxynitrite in the presence of large amounts of ROS. Peroxynitrite could oxidize mPTP leading to its opening, which would lead to the opening of mPTP, loss of ATP production, and necrosis. The damaged mitochondria generated excessive ROS like hydrogen peroxide and superoxide anion, which provokes the mitochondrion-driven ROS propagation. ROS themselves accelerate the production of mitochondrial ROS. This process is also called as ROS-instigated ROS release (RIRR) by initiating as inter-mitochondria signaling network [104] (Fig. 3.28). Oxidative insult by radiation to the mt alters the mitochondrial membrane potential and causes the leakage of cytochrome c from the inner membrane compartment, which elicits a sequence of signal transduction progression, the outcome of which is apoptotic cell death. Once the mitochondria are severely stressed, the pro-apoptotic factors like Bax create pores on the mitochondrial membrane, which lets the release of cytochrome c in the cell cytoplasm. It interacts with Apaf-1 to form a complex called apoptosome (Apaf-1, cytochrome c, and ATP). Caspase-9 then gets activated and commences the action of other caspases like caspase-3, -6, and -7. These lead to DNA fragmentation and cell degradation, thereby pushing the cells towards apoptosis. This kind of cell death is known as mitochondrial mediated cell death or intrinsic pathway of apoptosis (Fig. 3.29). However, in this case, apoptosis plays a role in abashing cells that induce excessive ROS.

3.7.4 Oxidative Stress Biomarkers

Biomarkers of OS can be classified as molecules that are modified by interactions with ROS or molecules of the antioxidant system that change in response to increased OS. ROS levels can also be monitored using fluorescent probes of commercial kits, which specifically detect intracellular ROS such as H_2O_2, NO, or $^{\circ}O_2^-$. However, assays that monitor ROS levels are unlikely to be useful for biomonitoring purposes due to the short half-life of ROS and the fact that the response is not specific to radiation exposure.

3.7.4.1 Antioxidant Defenses

S-Glutathionylation is the posttranslational modification of protein cysteine residues by the addition of glutathione. This modification can prevent proteolysis caused by the excessive oxidation of protein cysteine residues under oxidative or nitrosative stress conditions. Measuring S-glutathionylation of the proteins as biomarkers (Fig. 3.30) is hampered by difficulty in accessing the tissue in which these modifications occur. Nevertheless, S-glutathionylation of hemoglobin has been proposed as a biomarker of OS strengthened by finding that it occurs in the circulating erythrocytes in parallel with S-glutathionylation of molecules in the vasculature or myocardium [105].

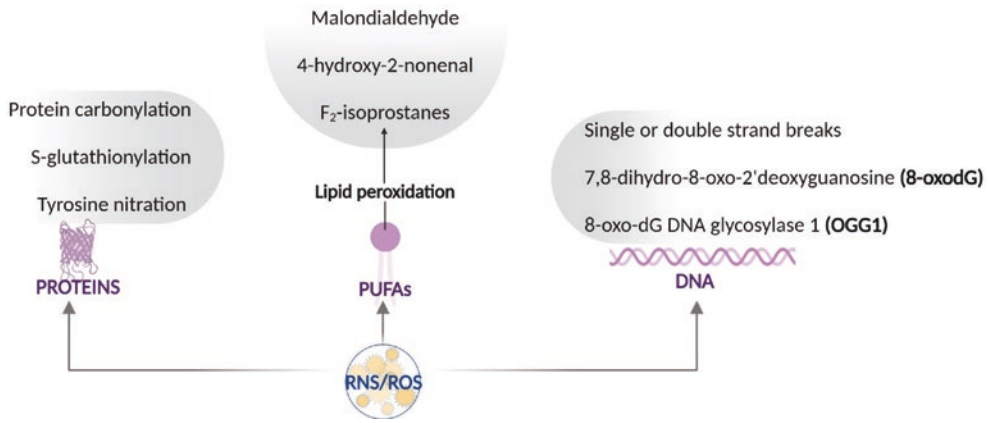

Fig. 3.30 Main oxidative products of DNA, lipids, and proteins. Oxidative products (listed in gray boxes) are formed depending on the free radicals (RNS/ROS) and the biomolecule target (amino acids, proteins, phospholipids, nucleic acids). These products can be used as oxidative stress biomarkers. *RNS* reactive nitrogen species, *ROS* reactive oxygen species

The participation of GSH in antioxidant reactions, either chemically or enzymatically via GPx, results in its own oxidation to GSSG. Decrease in intracellular GSH/GSSG ratio is one of the most used biomarkers of OS. In these conditions, GSSG is preferentially secreted out of the cell, and therefore, blood levels of GSH and GSSG may reflect changes in glutathione status in other less accessible tissues. 6 h after a single dose of irradiation (equivalent to 5 Gy), GSH/GSSG ratio decreases in blood. The decrease in GSH/GSSG is mainly due to an increase in the concentration of GSSG, because GSH levels do not change significantly [106].

3.7.4.2 Total Antioxidant Capacity (TAC) and Other Antioxidant Biomarkers

Antioxidants protect the body from the harmful effects of free radical damage. Thus, measurement of antioxidant levels in target tissues or biofluids has been widely used to assess the extent of oxidant exposure and, in turn, OS. TAC is the measure of the free radical amount scavenged by a test solution, being used to evaluate the antioxidant capacity of biological samples (tissues or biofluids). The TAC system involves enzymes (SOD, CAT, GPxs, and other enzymes), endogenous antioxidants, and dietary antioxidants (mentioned before), which are generally decreased when OS increases. TAC can be easily measured in cells, tissue lysates, and biological fluids by commercial colorimetric kits and represents a global approach (integrated parameter considered as the cumulative effect of all antioxidants of the biological sample) if no specific antioxidant molecule is to be investigated. One of the critical points is that the results obtained with different methods are not always comparable, depending on the different technologies used for their assessment. Moreover, as mentioned by Dr. Sies (who coined the concept of oxidative stress): "neither the term 'total' nor the term 'capacity' are applicable to the in vivo assays using an arbitrarily selected oxidant generator assaying a sample removed from its biological context, which is characterized by enzymatic maintenance of steady state" [107]. For that reason, we agree with him "that investigators should measure individually parameters associated with oxidative stress (GSH, urate, ascorbate, tocopherol, etc.) and antioxidant enzymes activities (in tissues samples and lymphocytes (in the case of blood samples) if their want to have an idea of the exposure of the entire organism to oxidative stress" [108].

3.7.4.3 Oxidation Products of DNA, Lipids, and Proteins

The "comet assay" and newer techniques [e.g., gas chromatography, high-pressure liquid chromatography (HPLC), immunoassays] can distinguish gross DNA damage produced by IR and damage from oxidation (for a detailed description, see Chap. 7). For low doses of radiation, the total number of induced DNA alterations is probably small when compared with the total number of equivalent alterations from endogenous sources. At DNA level, guanine is the most susceptible base to OS, and its oxidation at the C8 of the imidazole ring of deoxyguanosine generates 7,8-dihydro-8-oxo-2′deoxyguanosine (8-oxodG), which is the most predominant and stable DNA oxidative lesion in the genome (Fig. 3.30). A failure to repair oxidized bases creates a risk of mutation during DNA replication. For example, 8-oxodG mispairs with deoxyadenosine (dA) rather than deoxycytosine (dC) resulting in a C-A point mutation, thus increasing the risk of carcinogenesis. Besides the impact of confounding factors like age, sex, and smoking habits, with the help of correction factors, 8-oxodG levels are good and sensitive biological indicators of OS, which can be quantified in serum or urine samples, using HPLC coupled with mass spectrometry [109]. 8-OxodG can be removed by NER or BER with the action of 8-oxodG DNA glycosylase 1 (OGG1), a base excision DNA repair enzyme that cleaves the N-glycosidic bond between the base and the deoxyribose, generating an apurinic/apyrimidinic site (AP) and triggering the BER mechanism. DNA strand breaks and AP sites are effective substrates to activate DNA damage sensor PARP1. Overactivation of PARP1 is associated with apoptosis-inducing factor (AIF)-mediated and caspase-independent cell death. OGG1 seems to guard genome integrity

through lesion repair or cell death depending on the magnitude of guanine oxidation. OGG1 may also be measured as an OS marker.

As previously mentioned, lipid peroxidation products include MDA, 4-HNE, or IsoPs and can be used as oxidative stress biomarkers (Fig. 3.30). The latter are prostaglandin-like molecules formed by the nonenzymatic peroxidation of arachidonic acid (AA). MDA may be formed as a result of enzymatic and free radical peroxidation of PUFAs containing at least three double bonds and is also formed during prostaglandin synthesis. MDA can also react with DNA bases to form deoxyguanosine, deoxyadenosine, and deoxycytidine adducts, and these DNA-MDA adducts have mutagenic effects. Phospholipids containing linoleic acid and AA are considered the main source for 4-HNE production. Many different analytical methods are available for the measurement of MDA, 4-HNE, or IsoPs in biological samples and are reviewed by Tsikas [96].

It has been estimated that proteins scavenge a majority (50–75%) of generated reactive species. To function as biomarkers, protein oxidation products must be stable, accumulate in detectable concentrations, and correlate with OS exposition. Protein carbonylation is an irreversible protein modification, associated with alterations in functional and structural integrity of proteins, contributing to cellular dysfunction and tissue damages. Due to relatively early formation during OS, higher stability in comparison to other oxidation products, and simple analysis methods, protein carbonyls are one of the most OS biomarkers. Protein carbonyls can be easily quantified in plasma, serum, tissue samples, and also saliva by enzyme-linked immunosorbent assay (ELISA) [110].

The reaction between °NO and $°O_2^-$ forms peroxynitrite, which can nitrate tyrosine residues in proteins. This process is in competition with the enzymatic dismutation of $°O_2^-$ and the diffusion of °NO across cells and tissues. Peroxynitrite-mediated damage has been implicated in a wide range of disease pathologies, and 3-nitrotyrosine (3-NT) and nitrated proteins have been established as a footprint of nitro/oxidative biomarker of progression and severity in conditions. The measurement of 3-NT can be performed in plasma, serum, as well as tissue samples by special mass spectrometry. Commercially available ELISAs are usually used in clinical studies due to standardization and easy sample preparation. In turn, several limitations have been highlighted in the literature, such as low sensitivity and minor specificity. This and other protein oxidation biomarkers in human diseases are extensively reviewed by Kehm et al. [110]. The advancement of proteomics will allow us to assess changes in proteins (including the assessment of carbonylated, S-glutathionylation, S-nitrated, and/or N-nitrated derivatives) that serve as biomarkers of exposure to IR. An overview of the oxidation products of DNA, lipids, and proteins formed can be found in Fig. 3.30.

3.8 Cell Cycle Effects

The cell cycle is a fundamental process through which the cell grows and accurately duplicates the genetic material before it divides to give rise to two daughter cells. The cell cycle is divided into two phases: interphase in which the cell spends most of its time, followed by mitosis during which the cell divides into two daughter cells. The interphase has three distinct phases. The first phase is the G1 phase in which the cell grows and prepares itself for DNA synthesis. Second is the S phase, when the cell actually duplicate its DNA. The third phase is the G2 phase, where it prepares itself for mitosis. The duration of G1 varies considerably from cell to cell, while S, G2, and mitosis show less variation. Quiescence is a reversible state of a cell in which it does not divide but retains the ability to reenter cell cycle. This state is also called G0 phase.

The transition from one cell cycle phase to another is controlled by a variety of proteins, cyclins, and cyclin-dependent kinases. If the system identifies any inaccuracies, the transition from one phase to the next will be delayed and the cells arrested in the so-called cell cycle checkpoints [111]. Cells, which enter mitosis with unrepaired DNA damage, will most likely fail to divide properly resulting in cell death. In order to provide time for DNA damage repair or, if repair is not the best solution for inducing cell death, e.g. apoptosis, before DNA synthesis (S phase) and in particular mitosis is initiated, radiation induces arrest in checkpoints at the end of the G1 and G2 phases. Since the process that kills the cells after radiation damage is related to cell division, cells in G0 or cells which are differentiated or in senescence and have lost the ability to proliferate are very resistant to radiation [112].

3.8.1 Cycle-Dependent Kinases and Cyclins

Cell division is a highly regulated progression allowing cells to divide and to generate daughter cells. The regulation is necessary for the recognition and restoration of genetic injury along with the prevention of uninhibited cell division. It is regulated by cyclins and cyclin-dependent kinases (CDKs). CDKs are serine or threonine kinases, which unite with a separate subunit of functional cyclins, which presents domains essential for enzymatic activity. CDKs are known to have a crucial function not only in cell division but also in amending the transcription responses. Hence, the deregulation of CDKs is a characteristic of cancers and utilized for anticancer therapy purposes. On the other hand, cyclins establish the activity of CDKs as their levels keep changing during the cell cycle. Depending on their participation and function during the cell cycle, cyclins are divided into four

categories: G1 cyclins, i.e., D cyclins; G1/S cyclin, i.e., cyclin E; S-phase cyclins, i.e., cyclins E and A; and M-phase cyclins, i.e., B cyclins. Researchers have discovered around 20 CDK-associated proteins, which makes the cell cycle a complex process that involves the combination of CDKs (Cdk1, Cdk2, Cdk3, Cdk5, Cdk4, Cdk6, Cdk7, Cdk8, etc.) and cyclins (A1, A2, B1, B2, B3, C, D1, D2, D3, E1, E2, F, etc.) in distinct phases of the cell cycle endowing extra governance to the cell cycle apparatus (Table 3.9 and Fig. 3.31). Cyclins impart the specificity for substrates and normal cell cycle regulation, which includes the subunit binding, localization, activation/deactivation, etc. to the Cdk/cyclin complexes [113].

Table 3.9 Cyclins, CDKs, and their function throughout cell cycle

Cell cycle phase	Cyclins	CDKs	Functions
G1	Cyclin D	CDK 4, CDK6	Can act in response to external cues, e.g., growth factors and/or mitogens
G1/S	Cyclins E	CDK2	Control the centrosome duplication
S	Cyclins A and E	CDK2	The main targets are helicases and polymerases
M	Cyclins B	CDK1	Control G2/M checkpoint. The cyclins are produced in S phase but are inactive until the synthesis is entirely completed. Phosphorylate several downstream targets

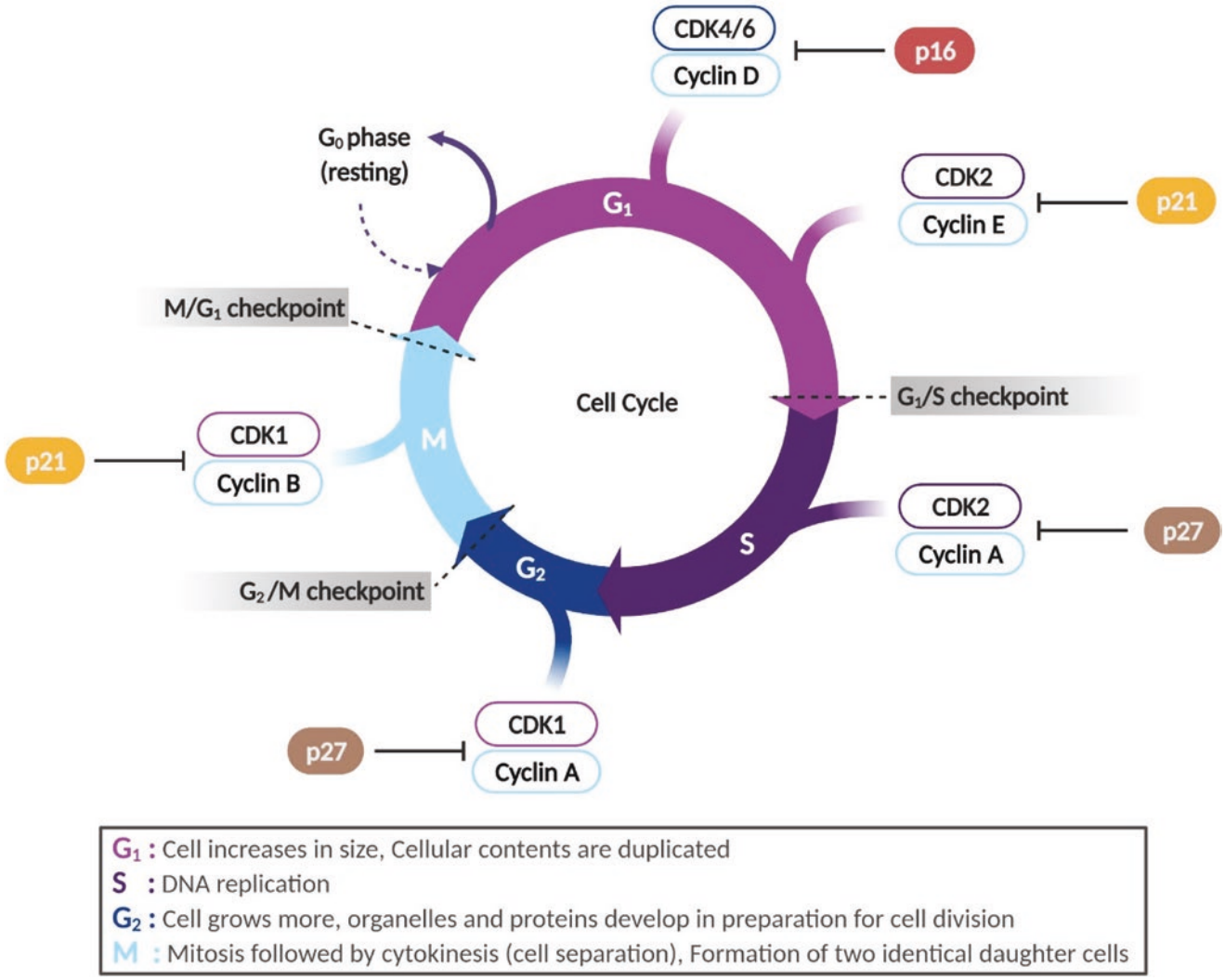

Fig. 3.31 Overview of cell cycle: functions of different phases, cyclins and CDKS, and CDIs

3.8.2 Activation of CDKs by Binding to Cyclins

CDKs have a very limited activity without the presence of a cyclin. To be an active kinase, it should be bound to its cyclin partner and its activity can be further altered by phosphorylation and association of additional proteins like p27. Every CDK/cyclin complex possesses a distinct function that is restricted to a specific cell cycle phase (Table 3.9). Cdk4 and/or Cdk6 are activated by D-type cyclins in the beginning of the G1 phase, and it commences phosphorylation of the retinoblastoma protein (Rb) family (Rb, p107, and p130). This releases the E2F transcription factor and causes the activation and transcription of the E2F-responsive genes that are necessary for the cell cycle progression. The cyclin A and E types are the early E2F-responsive genes. During the later G1 phase, cyclin E binds to Cdk2 to activate it and executes the phosphorylation of Rb (pocket proteins), provoking the further activation of the E2F intervened transcription. This assists in the crossing over of the cell cycle checkpoints at the periphery of the G_1/S phase, and to S-phase commencement. Cdk2 unites with cyclin A and aids the progression of the S phase. During the inception of the S phase, A-type cyclins are synthesized, which phosphorylates proteins associated with DNA replication. Going further, at the time of G_2/M transition, the activity of Cdk1/cyclin A is necessary for the induction of the prophase. Lastly, Cdk1/cyclin B complexes dynamically contribute to the completion of the mitosis process. Cdk1 activity fluctuates throughout cell cycle succession and is proficient of governing varied cell cycle adaptations (G_1/S, S, and G_2/M phases) by connecting with diverse cell cycle phase-associated specific cyclins, and several processes like action of CDK-activating kinase (CAK) and inhibitory phosphorylation on CDK. Regulating the cyclin levels and action of CDK inhibitors during the cell cycle assures that CDKs are active in the precise stage of the cell cycle. Cells exploit many processes such as transcriptional control of cyclin genes and breakdown of cyclins; the transcriptional control of the cyclin subunits is one way that ensures appropriate temporal expression of the cyclins and degradation of cyclins, to confine cyclins to the proper cell cycle phase and to keep them at the accurate concentration [114]. Ubiquitin-mediated protein degradation is one of the most crucial regulatory controls that confine the cyclins to the proper cell cycle phase. However, SCF (Skp1, Cullin, and F-box proteins) and APC/C (anaphase-promoting complex or cydosome) are two ubiquitin proteins involved in the degradation of cyclins. During the G1-S-phase transition, SCF controls degrading G1 cyclins (cyclin D), while APC/C degrades the cyclins of the S phase and mitosis, thus advancing the exit from mitosis. To control the CDK activity, the regulation of cyclin levels is not the only mechanism. Other mechanisms like activation and inhibition of phosphorylation actions on the CDK subunit and existence of inhibitors are critical in controlling cyclin-CDK activity [114].

3.8.3 Inhibitors of Cyclin-Dependent Kinases

CDK inhibitors are a family of proteins that can bind directly to the cyclin-CDK complex and hinder its activity. In the transition of the G1-S phases, these proteins play a very crucial role. CKIs implicated in controlling the S phase and mitotic CKIs are indispensable to avoid early commencement of the S- and M-phase CDKs. However, in human cancers, genes coding these CKIs are often mutated leading to aberrant cell cycle regulation. During normal or extreme conditions (DNA damage, telomere dysfunction, and stress), the functions and activities of the CDK/cyclin complexes are governed and controlled by two families of CKIs. The INK4 family comprises the p16INK4a, p15INK4b, p18INK4c, and p19INK4d which can specifically bind to Cdk4 and Cdk6 and hinder the activity of the D-type cyclin. The other Cip/Kip family (p21Cip1/Waf1/Sdi1, p27Kip1, p57Kip2) obstructs Cdk2/cyclin E, Cdk2/cyclin A, Cdk1/cyclin A, as well as Cdk1/cyclin B activity. The p21 protein hinders the formation of cyclin/CDK protein complexes that are required for the progression from the G1 phase to the S phase of the cell cycle (Box 3.19).

Box 3.19 In a Nutshell: Cell Cycle and Radiation Response

- Irradiated cells display a complex set of responses that can include either progression or arrest of the cell cycle.
- Every phase of the cell cycle has a very specific set of cyclins and cyclin-dependent kinases to perform functions associated with that particular cell cycle phase.

3.8.4 Cell Cycle Phase and Radiosensitivity

To study the variation of radiosensitivity with position in the cell cycle, it is necessary to synchronize the cells to get a population of cells that are all in the same cell cycle phase.

For cells in culture, there are three main techniques.

1. In fluorescence-activated cell sorting (FACS), a flow cytometer is used to sort cells based on fluorescence from a DNA-binding dye, such as Hoechst 33342, which can be used for live cells.
2. Chemically induced cell cycle arrest collects over time all the cells at a cell cycle checkpoint. When the drug is

removed, the cells will go through the cell cycle synchronously for some time before they become more and more asynchronous. The most used drug is hydroxyl urea, which arrests cells at the border between G1 and S. The advantage of this method is that it can also be used in vivo.

3. Mitotic selection was introduced by Terasima and Tolmach [115] and is the most used synchronization method in cell culture in vitro. As cells enter mitosis, they round up and become less attached to the flask bottom. By then shaking the flask, the mitotic cells will detach and can be collected with the medium. The cells can then be irradiated at different time points as they go through cell cycle.

The first age-response curve by Terasima and Tolmach [115] using 3 Gy irradiation of HeLa cells is shown in Fig. 3.32, left panel, together with a curve showing the fraction of labeled cells after pulsed incorporation of [3H]-thymidine during S phase. Cell survival was measured as the ability to form a macroscopic colony. The data indicate four times higher survival if the dose is delivered during early G1 compared to at the start of S phase. Furthermore, there is an increase in cell survival with age during S phase; that is, the radioresistance increases as more and more of the DNA is synthesized. HeLa cells are HPV infected and do not have functional p53, which normally would give the cells time for repair before entering S phase. The cells irradiated early in G1 will have time for repair, which is reflected in a high survival, while the cells in late G1 are more sensitive, because they may enter S phase with unrepaired DNA damages. Cell lines with short G1 are sensitive throughout G1. Terasima and Tolmach also irradiated the synchronized cells with various radiation doses and thereby recorded complete dose-response curves for HeLa cells irradiated in different phases of the cell cycle (see Fig. 3.32, right panel). These curves confirmed the variation in radiosensitivity through the cell cycle, as was demonstrated by the age-response curves. In addition, they also showed that cells irradiated while in mitosis are far more radiosensitive than cells irradiated in any part of interphase.

Measurements of the radiosensitivity of cells in G2 are technically difficult, and it has become customary to suppose that cells are radiosensitive if irradiated in G2. However, the radiosensitivity of cells in G2 has been shown to be dose dependent to a quite different degree than in any other phase of the cell cycle. Cells are hyper-radiosensitive for small radiation doses because the mechanism for early radiation-induced G2 arrest by ATM is not activated by radiation doses in the range below about 0.3–0.5 Gy (Box 3.20).

Box 3.20 In a Nutshell: Radioresistance and Radiosensitivity and Cell Cycle

- Cells increase radioresistance throughout S phase.
- Cell radiosensitivity is highest during mitosis.

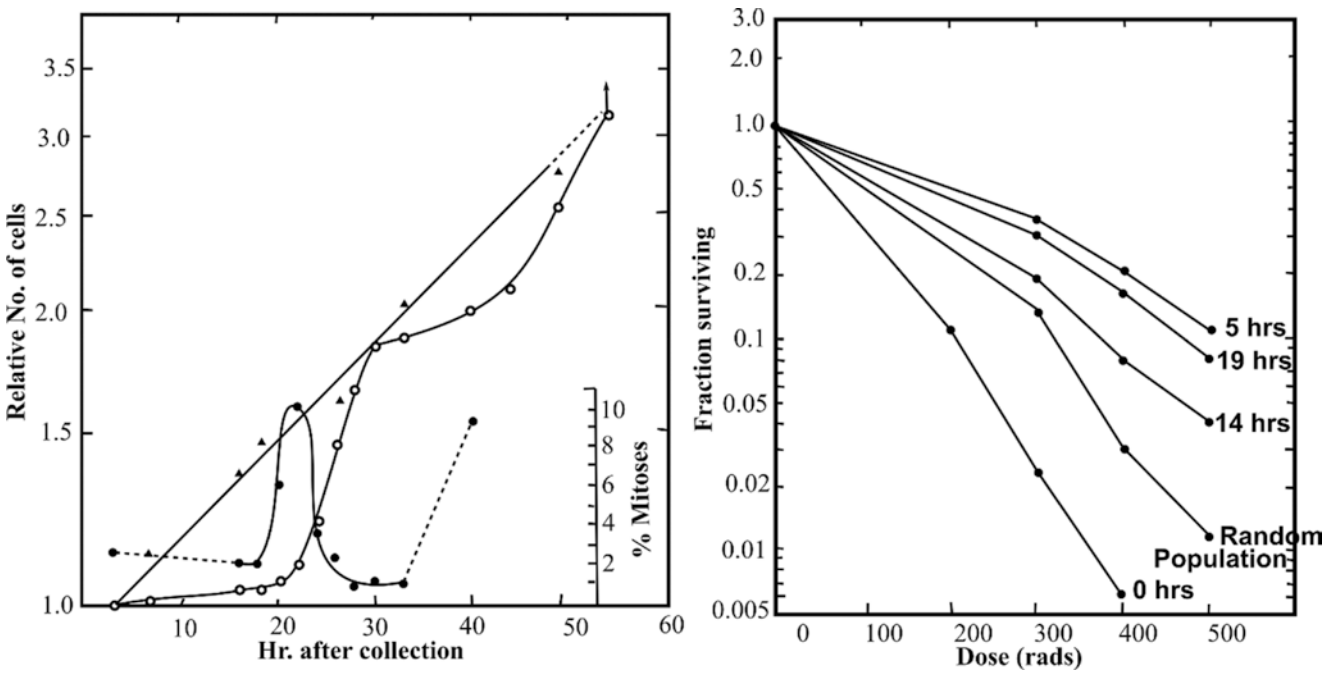

Fig. 3.32 Age-response of cells after radiation. Left: Age-response curves for HeLa-S3 cells (open circles: synchronized cells, triangles: asynchronous cells) irradiated with 3 Gy X-rays (= 300 rad) at different time points after selection in mitosis and the fraction of cells with incorporated [3H]-thymidine in DNA after a 20-min pulse (black circles, right *y*-axis). Right: Dose-response curves for HeLa-S3 cells synchronized by mitotic selection and X-irradiated at different times after selection. 0 h: mitosis, 5 h: early G1 phase, 14 h: S phase, 19 h: late S/G2 phase. [Reproduced with permission from Terasima and Tolmach [115]]

3.9 Telomeres and Senescence

3.9.1 Telomeres and Their Role

Telomeres are nucleoprotein structures located at the end of each linear chromosome in the cell nucleus. They are composed by tandem repeats of the G-rich hexanucleotide TTAGGG and are typically 10–15 kb long [116]. These structures are organized into heterochromatin domains, and they play a significant role in maintaining genome stability. There are at least two very important functions of telomeres in eukaryotes. The first one is the protection of the linear DNA molecules from the DNA repair mechanisms, which may recognize these sites as double-strand breaks. Secondly, they define the maximum number of cell cycles that a cell may undergo [116]. At each cell cycle division, telomeres shorten by 50–200 bp due to the DNA end-replication problem [117]. This problem results from the inefficient copying of the last base pairs of the linear DNA molecule by DNA polymerase. After several cell divisions, the length of telomeres reaches a critical threshold, which means that the cell can no longer divide. The cell has then reached its Hayflick limit, and it proceeds to senescence. Telomere shortening is thus a very-well-known hallmark of cellular senescence and aging. A good example of the telomere shortening is the deficiency of the adaptive immune system in older individuals caused by T cells reaching their Hayflick limit [118].

The telomere attrition can be opposed by an RNA-dependent DNA polymerase known as telomerase. This enzyme can elongate the telomeres by adding 5′-TTAGGG-3′ repeats to the chromosomes 3′ terminal ends. Telomerase is connected with cells' immortality; thus, it is present in germline and malignant cells. There is only little or no telomerase in most somatic cells [118]. This information is summarized in Fig. 3.33. An inverse correlation between the telomere length and the radiation-induced cytogenetic damage was found for lymphocytes, fibroblasts, epithelial cells, and many cancer cell lines. It was shown that telomere shortening leads to chromosome fusion, chromosome bridges, or higher frequencies of micronuclei. Thus, telomere shortening is closely linked to the cell radiosensitivity. Therefore, targeting the telomeres could be a very good radiosensitizing method in our fight with cancer during radiotherapy [116].

3.9.2 Senescence and Its Role

As described in Sect. 3.7, cellular senescence is a cell state triggered by extrinsic (cellular stressors) and intrinsic (physiological processes) factors. It is characterized by a prolonged and generally irreversible cell cycle arrest, associated with secretory features, macromolecular damage, and altered metabolism, with its function to remove potentially harmful cells from the proliferative pool [120]. Senescent cells are

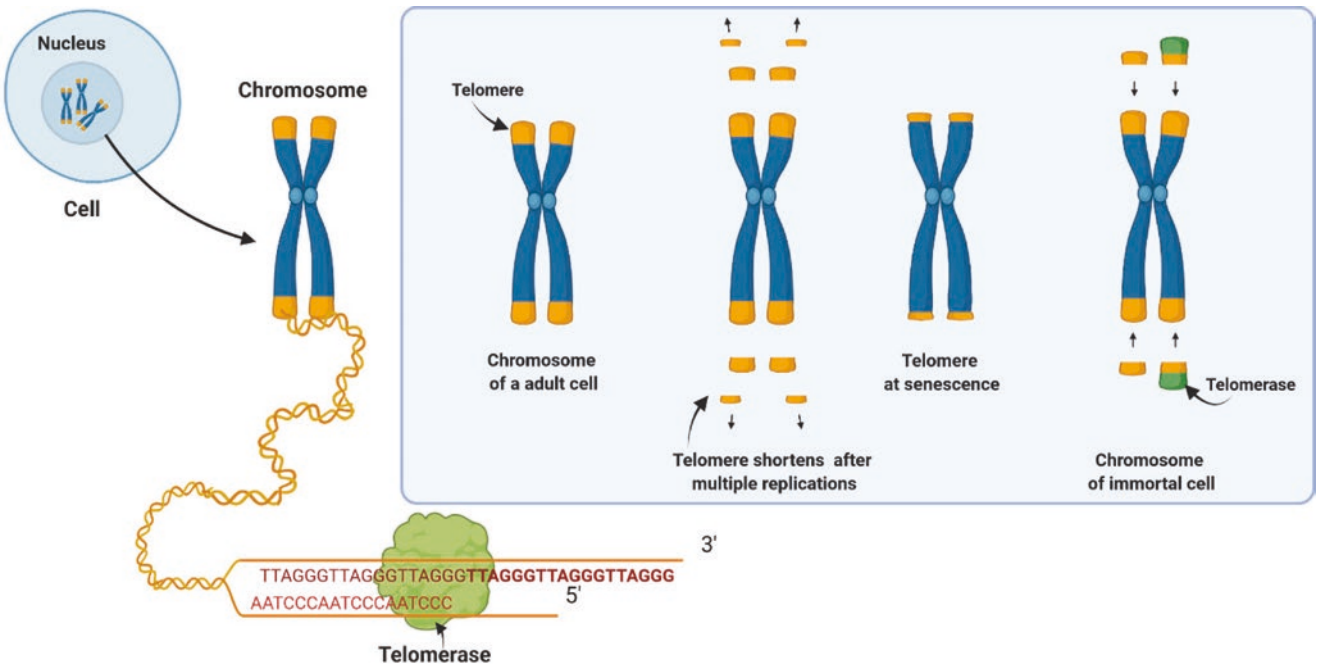

Fig. 3.33 Telomeres, their shortening, the senescence state, and immortal cells. An adult cell chromosome with telomeres and the enzyme telomerase, which plays a crucial role in telomere end lengthening (left). Telomere characteristics in an adult cell's chromosome, after multiple replications, at cell senescence, and when the cell is immortal (left to right, blue box). (Adapted with permission from Aunan et al. [119])

detected at any life stage from embryogenesis (contributes to tissue development) to adulthood (to prevent the proliferation of damaged cells). Yet, senescent cells can also potentiate various aspects of tumorigenesis, including proliferation, metastasis, and immunosuppression by secreting a collection of pro-inflammatory factors collectively termed as senescence-associated secretory phenotype (SASP) [121]. It is important to clarify that senescence is a distinct form of cell cycle arrest and distinct from quiescence, where cells can reenter the cell cycle when favorable growth conditions are restored; terminal differentiation, where cells exhibit functional and morphological changes resulting in loss of original cellular identity; and cell death, where cells are being eliminated and are thus nonfunctional. The existence of multiple senescence programs and the nonspecificity of current senescence markers make it difficult to fully unveil the complex mechanism behind senescence (current understanding presented in Fig. 3.34). It is therefore recommended to apply a multi-marker approach when investigating cellular senescence [120]. Yet, it is currently accepted that two main signaling pathways initiate and maintain the cell cycle arrest: p53–p21–retinoblastoma protein (RB) and p16INK4A–RB. As a consequence, depending on the senescence program, senescent cells express a multitude of hallmarks such as morphological alterations, senescence-associated beta-galactosidase (SA-β-gal), and SASP among others [122].

- Senescence in developmental processes, i.e., in embryogenesis and organogenesis, is induced by paracrine signaling and is mediated by the expression of the cell cycle inhibitor p21. Although SA-β-gal is highly expressed, developmental senescence is not associated with DNA damage, does not secrete the typical range of SASP cytokines, and is independent of p53 and p16INK4a. Senescence in wound healing prevents excessive fibrosis by secreting PDGFA-enriched SASP to stimulate appropriate skin repair. Senescence causes, or at least contributes to, organismal aging through the shortening of telomeres followed by the induction of p16INK4a and resulting in an accumulation of senescent cells over time. Studies by Baker et al. [123], first in BubR1-mutant mice (Cdkn2ap16 knockout mice) and then later in naturally aged mice, demonstrated that in the absence of p16INK4a, it is possible to inhibit the production of senescent cells and improve health span [123]. Also, SASP triggers multiple intercellular communication paths that also promote aging. Finally, the elimination of senescent cells improved several age-associated conditions. Senescence in cancer has shown a dual role as tumor suppressor and tumor promoter. Senescence is a key mechanism of tumor suppression via the inhibition of proliferation of cancer cells or by stimulating immune surveillance. Yet, cells induced to senescence by oncogenes or chemotherapy exhibit stem-

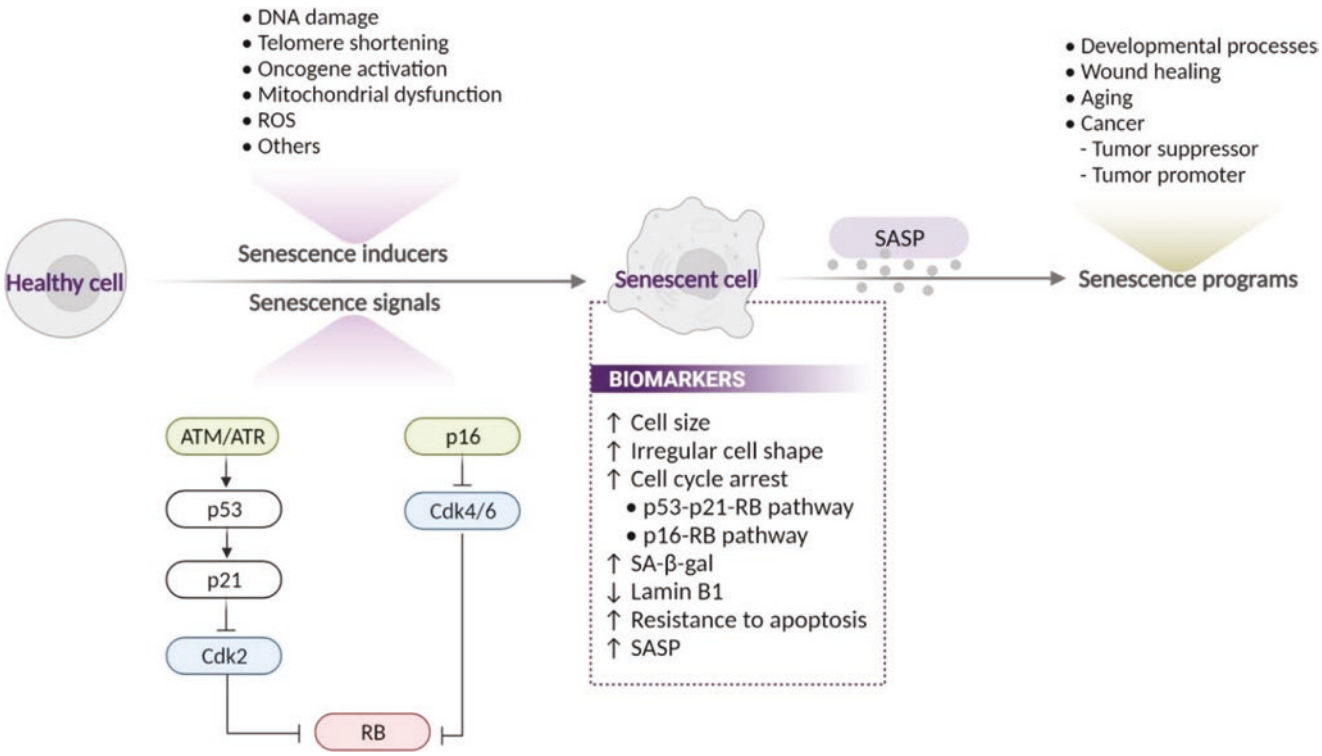

Fig. 3.34 Overview of cellular senescence processes. *ROS* reactive oxygen species, *ATM* ataxia-telangiectasia mutated, *ATR* ATM and Rad3-related protein, *Cdk2/4/6* cyclin-dependent kinase 2/4/6, *RB* retinoblastoma tumor suppressor gene, *SASP* senescence-associated secretory phenotype, *SA-β-gal* senescence-associated beta-galactosidase

like properties that promote cancer. Several stressors can induce cellular senescence and radiation in one of them. Thus, IR may cause cell cycle arrest resulting in a prematurely induced senescence phenotype (including SA-β-gal, p16INK4a, p21, and SASP), which is p53 dependent [121]. Unfortunately, the accumulation of these senescent cells can have a negative impact by promoting tumorigenesis. Thus, eliminating senescent cells from tumors and surrounding healthy tissues may be a successful and beneficial adjuvant strategy (Box 3.21).

Box 3.21 In a Nutshell: Telomeres and Senescence

- Telomeres are part the ending parts of chromosomes, which protect the genome integrity
- Telomeres shorten in each cell division by 50–200 bp due to the DNA end-replication problem.
- Telomere shortening is closely linked to cellular radiosensitivity.
- After several cell divisions, the length of telomeres reaches a critical threshold, the Hayflick limit, and the cell proceeds to senescence.
- Senescence is sometimes addressed as a type of cell death. A cell in senescence cannot proliferate anymore, it lives only metabolically.
- Cellular senescence is characterized by a prolonged and generally irreversible cell cycle arrest, and it functions as a process to remove potentially harmful cells from the proliferative cell pool.
- Senescence is a key mechanism of tumor suppression via the inhibition of proliferation of cancer cells or by stimulating immune surveillance in cancers treated with radiotherapy.

3.10 Cell Death Mechanisms

In response to IR, multiple, molecularly distinct forms of cell death may be initiated. Although the decision points of their initiation are not completely clear, it is known that the level of the DNA damage but also the individual signaling status of different cell death pathways in different cell types, e.g., hematological vs. epithelial cells, influence the decision regarding the cell death route.

The cellular factors that influence include cell type, position in cell cycle when irradiated, DNA repair capacity, as well as functionality of TP53 and similar DNA-damaging sensors [124]. The dose and radiation quality also contribute to the cellular IR response to cell death, and in the tissue, the oxygen levels may impact the cell death route taken [124]. In this section, an overview of four cell death mechanisms are given: (I) mitotic cell death/mitotic catastrophe, (II) apoptosis, (III) necrosis, and (IV) autophagy (Fig. 3.35), some of which are also interconnected in the cell. Furthermore, the underlying molecular mechanisms and importance of these forms of cell death following IR are also described alongside methods of assessment.

3.10.1 Mitotic Cell Death/Mitotic Catastrophe

Mitotic catastrophe (MC) is an important type of IR-induced cell death mechanism, which is triggered when cells enter into the mitotic phase without appropriately completing the S and G2 cell cycle phases [125]. Hence, MC controls cells that are often incapable of successfully completing mitosis. MC works by activating mitotic arrest, and later it may lead to a controlled or a regulated cell death mechanism or senescence. Therefore, MC is a controled cell death that usually follows the intrinsic apoptotic pathway route [124] (Fig. 3.37). MC is also promoted when the proteins that regulate the G2 phase like the $p21^{CDKN1A}$, checkpoint kinases 1 and 2 (CHK1/2), ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia, and Rad3-related protein (ATR) are inhibited. MC basically commences with the irregular condensation of the chromatin around the nucleoli, which looks similar to early chromosome condensation. Cells may die in the same cell cycle or in the successive cell cycle progression or division after IR. The anomalous mitosis in such cases leads to unusual segregation of the chromosomes and cell division. As a consequence, this causes formation of giant cells which exhibit the uncharacteristic nuclear morphology and numerous micronuclei and nuclei. Also, it is noteworthy that MC induced by IR is accompanied with excess duplication of chromosomes and hyper-amplification, which results in a mitosis that is multipolar and later development of micronuclei. DNA damage and flaws in the DNA repair processes lead to centrosome hyper-amplification. Cyclin-dependent kinase 2 (CDK2) and cyclin A or E initiate the amplification of the centrosomes at the boundary of G1/S phase. This is often observed in cells that lack a functional TP53; however, in cells with a functional TP53 and $p21^{CDKN1A}$, which is known as an inhibitor of CDK2, cellular senescence is promoted.

The outcome of MC in the form of cell death can be elicited in the mitotic phase or in the successive interphase. Some cells activate apoptotic pathways in the metaphase that results in delayed apoptosis, i.e., it can take up to 6 days after IR. Cells that get away with the mitotic arrest of the mitotic

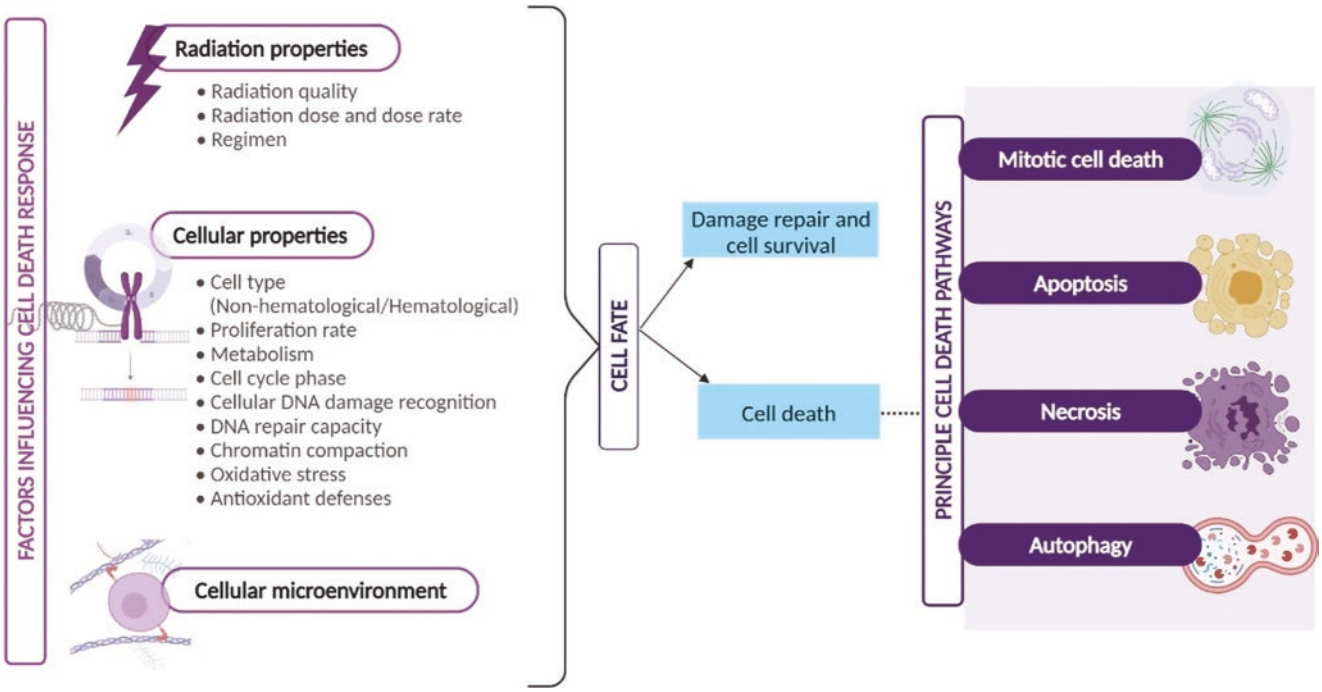

Fig. 3.35 Overview of cell death and cell death-protective mechanisms in response to radiation. Radiation-induced cell death is influenced by different factors, such as radiation factors, cell intrinsic factors, and cellular microenvironment factors (left). Cell death pathways are listed to the right. The mechanisms and importance of these principal cell death forms are described in detail in the text

cell death are frequently observed to have an unsuccessful cytokinesis consequentially exhibiting tetraploid anomalous nuclei developing into giant cells. Giant cells that possess a functional TP53 will eventually undergo apoptosis following the mitochondrial pathway of apoptosis in the subsequent G1 phase. However, cells with mutant TP53 or deficient TP53 function go on with a few number of cell cycles and attain a growing amount of chromosomal anomalies before they finally succumb to either delayed apoptosis or necrotic form of cell death [125]. As the cells that undergo MC are usually the ones who have lost the potential to carry out any further replication, MC is frequently referred to as a genuine type of cell death. One of the most common properties exhibited by cancer cells is that of defects in cell cycle checkpoints. This lets the cells enduring IR-induced damage to hastily inscribe in the mitotic process even with the misrepaired DNA that eventually leads to MC. More than a few cell division attempts can take place before adequate genetic injuries mount up to activate mitotic death, emphasizing why solid tumors frequently display deferred reactions to IR [124]. MC is triggered after IR exhibits diverse mechanisms of action (Table 3.10) [126].

Table 3.10 Examples of IR-induced MC in different tumor cell lines

Inducer of MC	Cell line	Features/signaling components of MC
Ionizing radiation	HeLa (cervical adenocarcinoma)	Increased levels of cyclin B
	U2OS (osteosarcoma)	Checkpoint adaptation
	HT0180 (fibrosarcoma)	Micronucleation
	MOLT4 (leukemia)	Checkpoint adaptation

3.10.1.1 Mode of Action of Mitotic Catastrophe

During MC, the mitotic damage is recognized and guides the cell into one of the three potential antiproliferative fates (Fig. 3.36). In one of them, when cyclin B levels are elevated, the malfunctioning mitotic cells recruit the cell death machinery and die during mitosis. Another cell death pathway that cells can take is by mitotic slippage. Here, cells go out from mitosis and cell death is triggered in the next G1 cell cycle transition. Lastly, cells with a MC character can also undergo senescence after exiting mitosis.

MC may not at all time be accompanied by mitotic arrest. Nevertheless, the mechanism of action that dictates cell fate of subsequent MC continues to remain unclear [127]. When

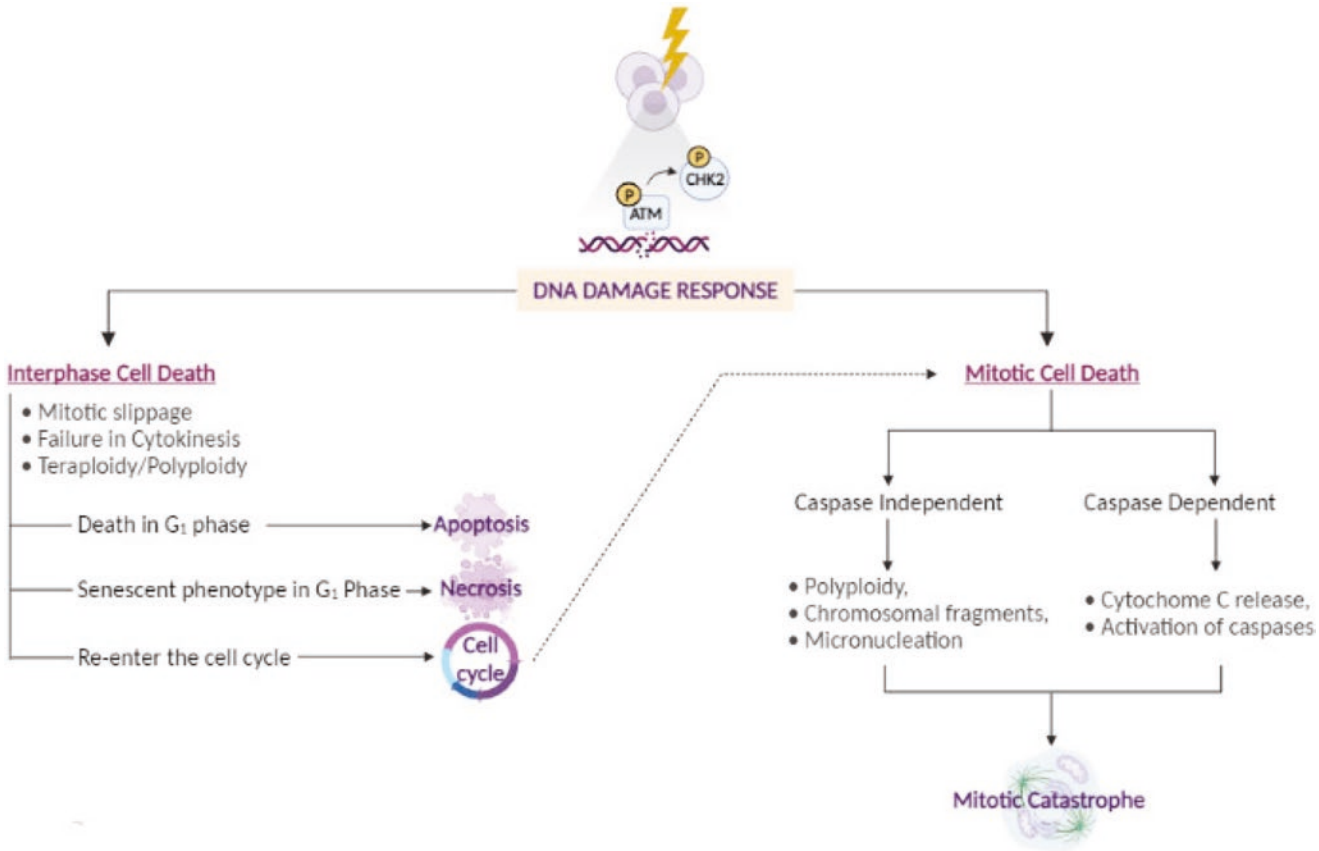

Fig. 3.36 Cell death pathways operative in mitotic catastrophe. Different signaling events triggered in response to a nonfunctional mitosis are shown. Upon DNA damage, cells which lack functional p53 can go out from mitosis without commencing cytokines or initiate cell death even in mitosis. Apoptosis and necrosis signaling in the context of mitotic catastrophe are depicted

mitotic arrest is extended, the amount of cyclin B is decreased albeit the spindle assembly checkpoint (SAC) is functional. As a result, if cyclin B levels drop below the verge that determines mitotic exit, slippage occurs (Box 3.22).

Box 3.22 In a Nutshell: Ionizing Radiation Induced Cell Death

- IR-induced cell death depends on radiation quality, dose as well as cell type, cell cycle position, and functionality in DNA damage signaling.
- Mitotic catastrophe is one of the principal forms of IR-induced cell death that results from early/untimely entry into mitosis, even before the fulfillment of S and G2 phases of the cell cycle.
- The characteristic features of IR-induced mitotic catastrophe are altered nuclear morphology, micronucleation, and formation of multinucleated cells.

3.10.2 Apoptosis

Apoptosis (originally from Greek language translated "falling off") is also known as "cellular suicide." It is a cell death process which may be executed under normal physiology, e.g., organism development, but also in the context of disease. Apoptosis is a highly controlled pathway with distinct molecular features. Thus, some of the rapidly proliferating cells undergo apoptosis, which is an essential part of neurogenesis and tissue development in humans as well as in other mammalians. During apoptosis, cells are disposed in a complex but well-ordered fashion which involves energy-requiring molecularly defined effector mechanisms [128]. To simplify, apoptosis allows the cells to self-destruct with limited tissue damage when they are exposed to different triggers/signals which can be endogenous, e.g., formed DNA damages, telomere shortening, or encountered from the outside of the cell, e.g., cytotoxic or DNA-damaging agents, IR exposure, loss of growth factors, cytokine or glucocorticoid hormone level alterations, or hypoxia [128].

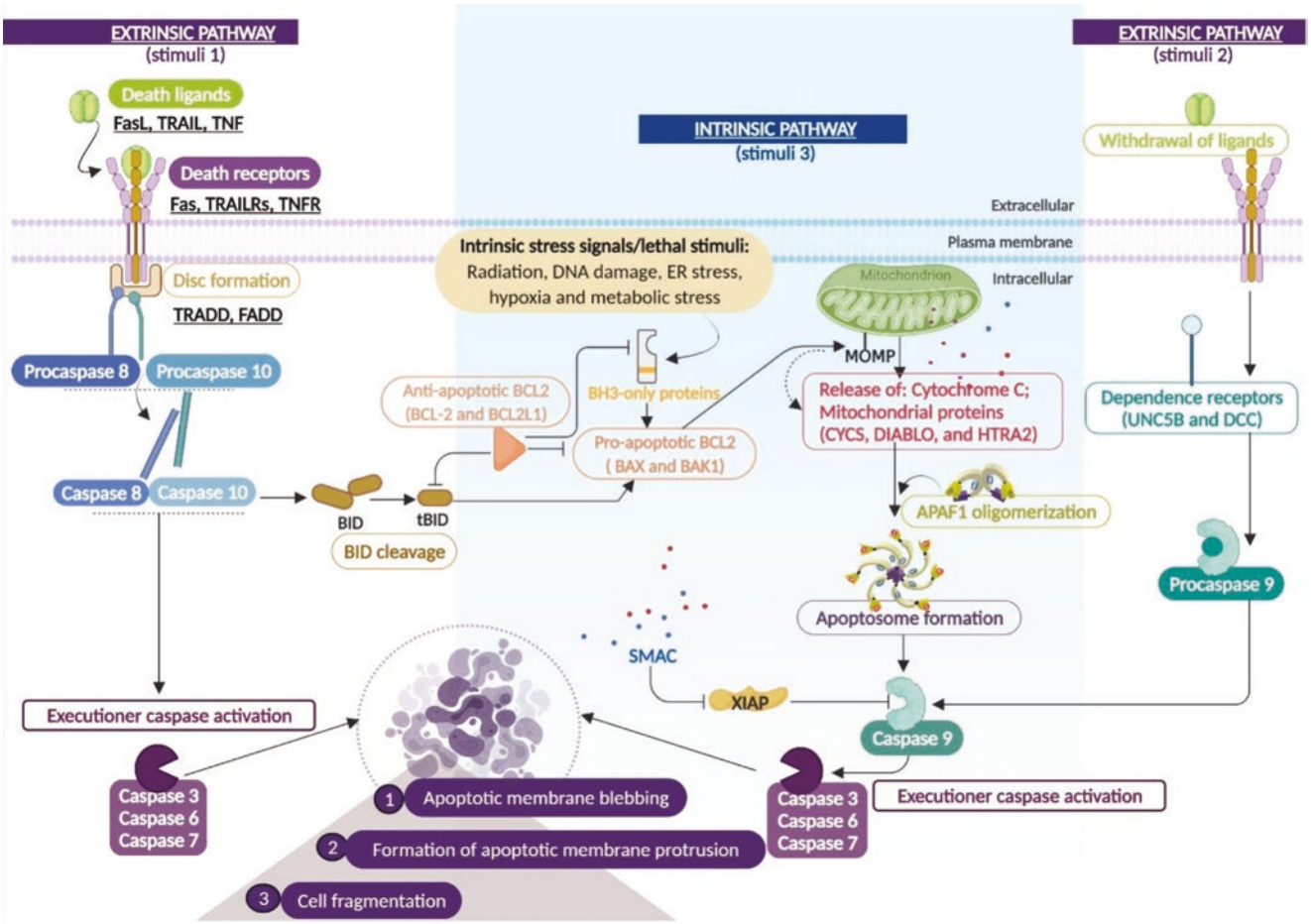

Fig. 3.37 The intrinsic and extrinsic route to apoptosis. Intrinsic stress signals (e.g., DNA damage, hypoxia, metabolic stress) or lethal stimuli (e.g., IR exposure) can induce intrinsic mitochondrial apoptosis (middle). Cleaved or truncated Bid (tBid) can also connect the extrinsic pathway to the intrinsic route. In the extrinsic pathway, ligands for death receptors (left) can trigger caspase activation, but the pathway can also be activated when some dependence receptors are inactivated (right). Abbreviations: *FasL* Fas ligand, *TRAIL* TNF-related apoptosis-inducing ligand, *TNF* tumor necrosis factor, *Fas* Fas cell surface death receptor, *TRAILR* TNF-related apoptosis-inducing ligand receptor, *TNFR* tumor necrosis factor receptor, *TRADD* TNFR1-associated death domain protein, *FADD* Fas-associated protein with death domain, *caspase* cysteine-aspartic proteases, *BID* BH3-interacting domain death agonist, *tBID* truncated BID, *Bcl-2* B-cell lymphoma 2 (an apoptotic inhibitor), *BCL2L1* Bcl-2-like 1, *MOMP* mitochondrial outer membrane permeabilization, *BH3* Bcl-2 homology 3, *DIABLO* direct inhibitor of apoptosis-binding protein with low pI, *APAF-1* apoptotic peptidase-activating factor 1, *Bax* Bcl2-associated X (an apoptotic regulator), *Bak* Bcl-2 homologous antagonist/killer, *XIAP* X-linked inhibitor of apoptosis protein, *SMAC* second mitochondria-derived activator of caspase, *UNC5B* Unc-5 netrin receptor B

Apoptosis results in the production of apoptotic bodies, which are cell fragments, e.g., collapsed cytoskeleton, disassembled nuclear envelope, and fragments of nuclear DNA. An apoptotic cell is also marked by certain "find-me" and "eat-me" signals at the cell surface, which allow the dying cell to be recognized and rapidly engulfed by different macrophage subtypes in the near or distant tissue, thereby avoiding inflammation. A well-recognized potential "eat-me" signal is the expression of phosphatidylserine (PS) on the outer side of plasma membrane, which in turn is being used for assessing early apoptotic cells [129].

In the 1990s, studies which resulted in authors being awarded a Nobel Prize revealed that core machinery components of some apoptotic pathways are highly conserved from nematodes to humans [130]. Subsequently, research on the molecular mechanisms regulating apoptosis has established two major routes of this cell death type, namely intrinsic and extrinsic apoptosis, respectively (Fig. 3.37).

3.10.2.1 Intrinsic Pathway to Apoptotic Execution

Multiple perturbations may trigger intrinsic apoptotic cell death, e.g., growth factor withdrawal, cytokine alterations, endoplasmic reticulum stress, replication stress, formation of reactive oxygen species (ROS), microtubular alterations or mitotic defects, and IR-induced DNA damage. In the context

of DNA damage, mitochondrial release of apoptogenic proteins is central. This commences in part via mitochondrial outer membrane permeabilization (MOMP) that allows cytochrome C and other proteins to be released to cytosol. Once there, cytochrome forms the apoptosome complex together with apoptotic peptidase-activating factor 1 (APAF-1), where pro-caspase-9 is cleaved to active caspase-9 (CASP9). Subsequently, CASP9 cleaves the effector caspases (e.g., caspase-3, caspase-6, and caspase-7), which then causes degradation of cell signaling and structural proteins resulting in an apoptotic morphology. The BCL-2 proteins are regulators of MOMP. These can either promote, e.g., BCL-2-associated X apoptosis regulator (BAX) or BCL-2 antagonist/killer (BAK) or block MOMP, e.g., BCL-2 or BCL-XL members [131]. Another set of BCL-2 members, which only have a BH3 domain, can also promote MOMP, but they act via alleviation of BCL-2 or BCL-XL function or via promotion of BAX/BAK activity. Examples thereof are BCL2-associated agonist of cell death (BAD), BH3-interacting domain death agonist (BID), BCL2-interacting mediator of cell death (BIM), NOXA, and TP53-upregulated modulator of apoptosis (PUMA).

In DNA damage-induced apoptosis, TP53 and BAX/BAK proteins are important. The BCL-2 family members also sense other cellular clues to elicit intrinsic apoptosis including alterations in growth factor receptor/PI3K signaling or microtubule disruption, both of which may have impact in the context of IR-induced cell death. In addition, the mitogen-activated protein kinases 8 and 9 (MAPK8 and MAPK9), more commonly referred to as c-jun N-terminal kinase 1/2 (JNK1/JNK2), are known to regulate the BCL-2 rheostat by phosphorylation of BCL-2 and BAD, via induction of NOXA and PUMA by TP53 transcriptional regulation as well as by association of BIM to microtubuli [132].

3.10.2.2 Extrinsic Pathway to Apoptotic Execution

The extrinsic pathway starts by the activation of membrane receptors, so-called death receptors (DRs), e.g., FAS/CD95 cell surface death receptor and TNF receptor superfamily member 1A (TNFRSF1A)/TNFR1, and is driven by initiator caspases, e.g., caspase-8 (CASP8) and caspase-10 (CASP10). The extrinsic pathway is also used by various immune cells to trigger apoptotic cell death in tumor cells including TRAIL [133]. In addition, the inflammatory cytokine TNF-α produced by activated macrophages, which binds to the TNFR1 and TNFR2 receptors in most human cells, can elicit apoptotic response. Moreover, cytotoxic lymphocytes carry the FasL, which binds and activates the FAS receptor on the surface of the target cell that is followed by death-inducing signaling complex (DISC) formation. Subsequently, adapter proteins bind to the intracellular region of aggregated DISC complex, causing the accumulation of procaspase-8 molecules, which via proteolytic cleavage initiate a proteolytic cascade leading to effector caspase activation. There is also an amplification step where further release of mitochondria-localized pro-apoptotic factors takes place to amplify the initial CASP-3 activation (Box 3.23).

Box 3.23 In a Nutshell: Apoptosis

- Apoptosis is a distinctive and highly controlled form of *programmed cell death*, which requires energy to hit the *self-destruct* button of an affected cell.
- Apoptosis which can be triggered in response to endogenous or exogenous signals is a chain of sequential morphological events during which the early apoptotic cell shrinks and chromatin is irreversibly condensed and cleaved culminating into formation of apoptotic bodies.
- In the mitochondria-mediated or intrinsic route to caspase activation, induction of mitochondrial outer membrane permeabilization (MOMP) is a central event that sets free pro-apoptotic factors such as cytochrome c.
- The BCL-2 proteins can positively and negatively control MOMP.
- The extrinsic pathway is mediated by a death ligand/signal binding to a membrane death receptor and downstream activation of CASP8.

3.10.2.3 Activation of Apoptosis by Ionizing Radiation

IR-induced DNA damages, e.g., unrepaired DNA SSBs or DSBs, primarily trigger apoptosis via the intrinsic pathway [134]; however, at certain IR doses and in certain cell types, the extrinsic apoptotic pathway may also be executed. IR can also initiate mitochondria-mediated signaling in response to ceramide production/formation at the plasma membrane. Moreover, IR can trigger the production of O_2^- and ROS (like H_2O_2 or OH^- radicals), which via release of Ca^{2+} and cytochrome c from mitochondria can cause apoptosis [135].

One important signaling regulator of apoptosis in response to IR is TP53 [136] (Fig. 3.38). Thus, TP53 is phosphorylated in response to DDR signaling, accumulates in the nucleus, and binds to promoters of target genes, e.g., BAX, PUMA, NOXA, p53AIP1, and APAF-1. This results in an alteration in their transcription and hence expression levels, which is followed by mitochondria-mediated apoptosis.

The extrinsic pathway may also play a role in IR-induced apoptosis in which TP53 may upregulate the expression of the FAS receptor and its ligands, which subsequently causes downstream transactivation of initiator CASP8 and apoptosis.

IR may moreover activate the ceramide pathway at the plasma membrane, wherein formation of ROS inflicts lipid

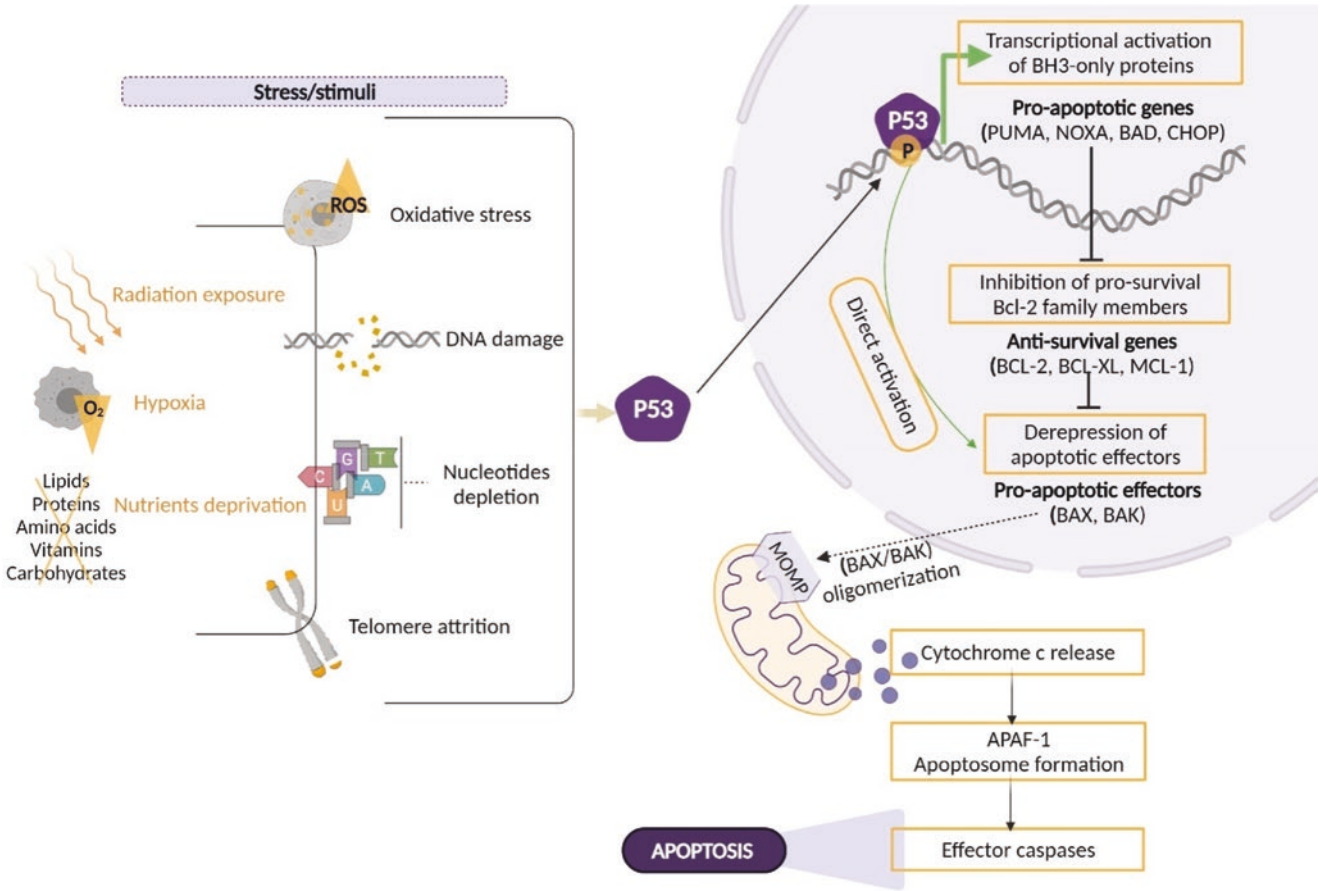

Fig. 3.38 TP53-mediated intrinsic route to apoptosis. The mechanisms of TP53-induced apoptosis through the Bcl-2-regulated pathways in cells undergoing stress are shown. DNA damage triggers stress signaling, which in turn causes stabilization of the TP53 protein in the nucleus. Subsequently, TP53 as a nuclear transcription factor increases the expression of BH3-only proteins such as PUMA and NOXA and downregulation of BCL-2 or BCL-XL expression. The BH3-only proteins bind and inhibit the anti-apoptotic or pro-survival BCL-2 family proteins, so as to unleash the cell death effectors (BAX/BAK) which are often held as hallmarks of apoptosis in affected cells. Oligomerization of BAX/BAK causes MOMP, with subsequent release of cytochrome c, formation of the apoptosome complex, and activation of CASP9 and subsequently effector caspases, which causes apoptotic features of the dying cells. Abbreviations: *ROS* reactive oxygen species, *MOMP* mitochondrial outer membrane permeabilization, *BH3* Bcl-2 homology 3, *PUMA* p53 upregulated modulator of apoptosis, *BAD* Bcl-2-associated agonist of cell death, *CHOP* CCAAT/enhancer-binding protein homologous protein, *Bcl-2* B-cell lymphoma 2 (an apoptotic inhibitor), *Bcl-xL* B-cell lymphoma-extra-large, *Bax* Bcl2-associated X (an apoptotic regulator), *Bak* Bcl2 antagonist killer 1, *APAF-1* apoptotic peptidase-activating factor 1, *caspase* cascade of aspartate-specific cysteine proteases

oxidative damage in the membrane (Fig. 3.39). Subsequently, acid sphingomyelinase is activated, and second messenger ceramide is released as a result of sphingomyelin hydrolysis. IR-induced DNA damage may also trigger mitochondrial ceramide synthase resulting in the accumulation of ceramide which subsequently can induce apoptosis [137].

Ceramide may also activate the RAC1/mitogen-activated protein kinase kinase kinase-1 (MAP3K1) pathway by which MAPK8 and the effector CASP-1, -3, and -6 are induced and which also stimulate the DR pathway. MAPK8/JNK1 is known to be triggered in response to IR as well as other apoptotic stimuli, and depending on the duration of activity, it may induce apoptotic signaling. In summary, the rate of apoptotic events after IR may be executed via different routes and is influenced by cell type, cell cycle phase, dosage number, as well as radiation quality (Box 3.24).

Box 3.24 In a Nutshell: Ionizing Radiation Induced Apoptosis

- IR-induced apoptosis can be executed through intrinsic, extrinsic, or membrane stress (ceramide) pathways.
- IR may trigger apoptosis via mitochondria where TP53 regulation of the BCL-2 family proteins is of major importance.

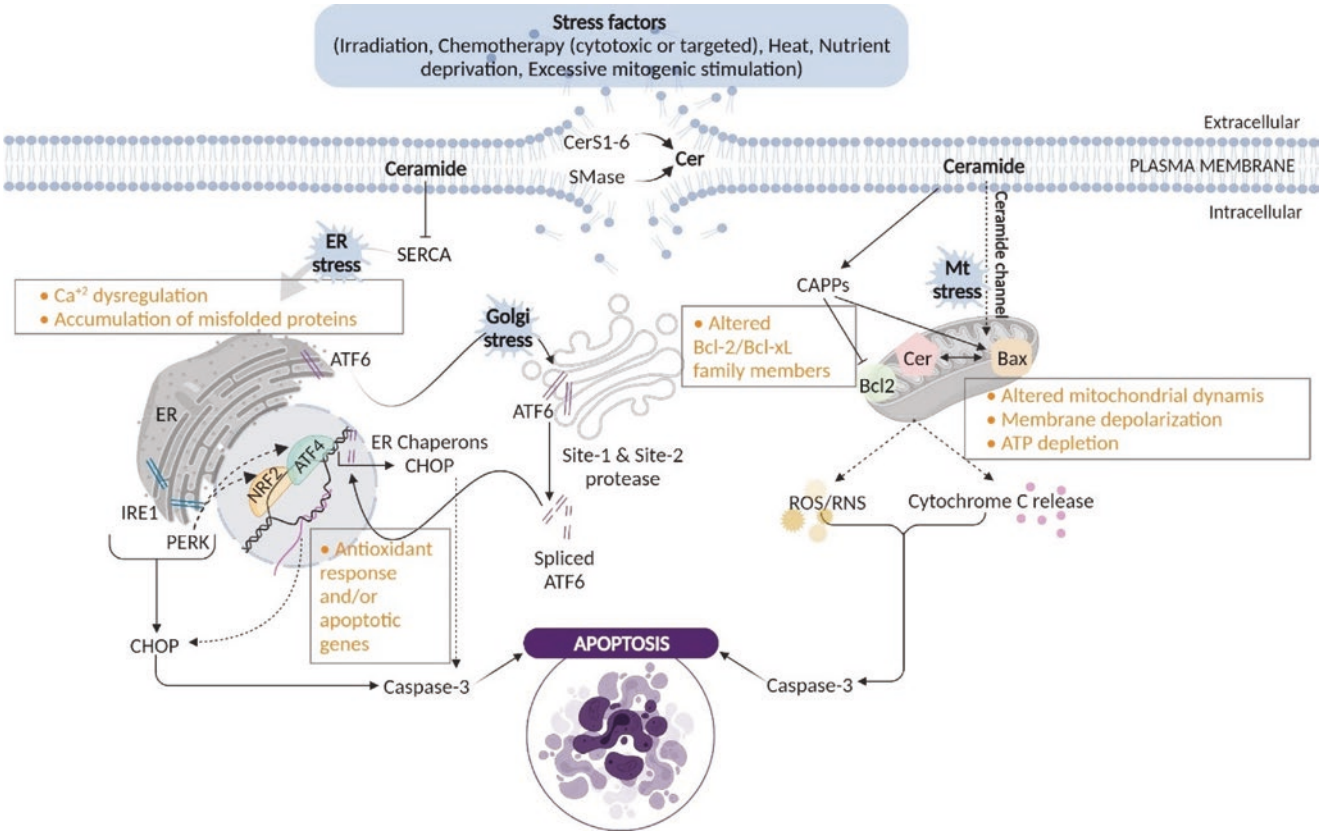

Fig. 3.39 Overview of ceramide signaling and connection to the apoptotic machinery. IR-induced lipid oxidative damage causes sphingomyelinase activation at the plasma membrane, followed by hydrolysis of sphingomyelin and release of ceramide. High dose of IR-induced DNA DSBs can also trigger the mitochondrial ceramide synthase for *de novo* synthesis of ceramide. Inhibition of SERCA and calcium depletion in ER promote ER stress. Expression of downstream pro-apoptotic factor, e.g., CHOP, increases. The UPR activator proteins, ATF6, IRE1, and PERK, alter ER stress. The PERK pathway via ATF4-dependent NRF2 expression triggers the CHOP-mediated apoptotic pathway. CHOP can also be induced by spliced ATF-6 (in Golgi), which regulates the Bcl-2 protein family. CAPPs can alter the BCL-2 protein family, which determines the commitment of cells to apoptosis. Abbreviations: *Cer* ceramide, *CerS1–6* a family of six ceramide synthases, *SMase* sphingomyelinase, *SERCA* sarco-endoplasmic reticulum calcium transport ATPase, *ER* endoplasmic reticulum, *ATF6* activating transcription factor 6, *IRE1* inositol-requiring enzyme 1, *PERK* protein kinase R-like ER kinase, *NRF2* nuclear factor erythroid 2-related factor-2, *ATF4* activating transcription factor 4, *CHOP* CCAAT/enhancer-binding protein homologous protein, *Mt* mitochondria, *CAPPs* ceramide-activated protein phosphatase, *Bcl-2* B-cell lymphoma 2 (an apoptotic inhibitor), *Bcl-xL* B-cell lymphoma-extra-large, *Bax* Bcl-2-associated X (an apoptotic regulator), *RNS* reactive nitrogen species, *ATP* adenosine triphosphate

3.10.2.4 Methods to Detect Apoptotic Cell Death

The apoptotic cell features, i.e., cell morphology, and the activation of different apoptotic signaling routes giving rise to distinguishable phenotypes have been extensively studied with multiple methods at hand. The detection of apoptosis includes methods (Fig. 3.40) related to membrane alterations, e.g., PS exposure monitored by annexin V association [129]; DNA fragmentation assessment; cytotoxicity and cell proliferation assays; analyses of mitochondrial effects, i.e., cell permeabilization; loss of mitochondrial potential; BCL-2 family protein complex formation; association of the apoptosome or DISC complex in cytosol; and pro-caspase cleavage later via different antibody-based, enzymatic assays or by flow cytometry [138]. Moreover, less frequently used technologies such as light-scattering flow cytometry and time-lapse microscopy perfusion platform can be performed to avoid underestimating the extent and timing of apoptosis, temporal aspects of death, cell surface area assessment, cellular adhesion analysis, and genotoxicity-specific chromatin changes.

3.10.3 Necrosis

Necrosis (from the Greek "nekros" designating "to kill") has for long been seen upon as an uncontrolled, irreversible mode of cell death, while recent work suggests that necrosis is a tightly genetically regulated pathway yet triggering inflammatory and/or reparative reactions in the tissue [139]. Necrotic cell death can be classified into accidental cell death

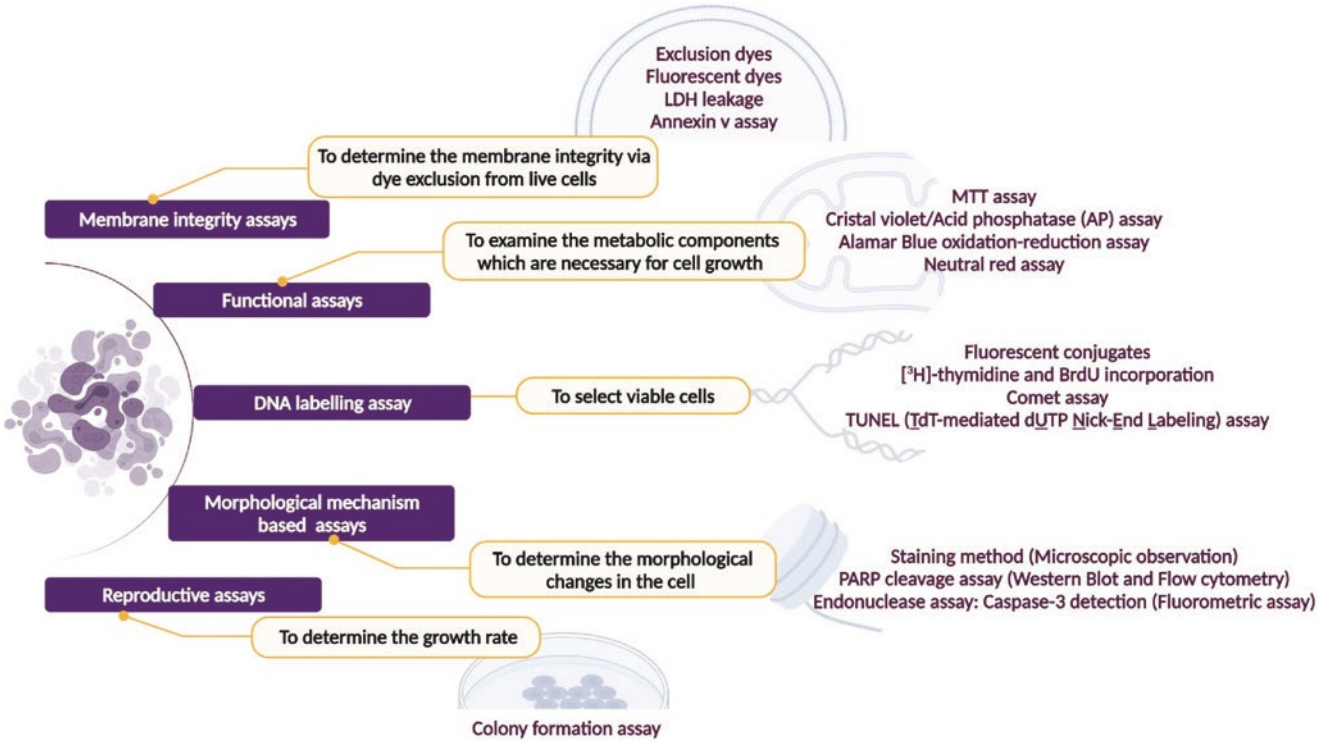

Fig. 3.40 Methods to detect cell death, in particular apoptotic cell death. The schematic diagram outlines various biological assays used to determine apoptotic cell death. Some of these assays can also be used to assess other types of cell death. These assays are based on the morphological criteria and distinguishing features of apoptotic pathways, e.g., staining for PS exposure on the outer plasma membrane (by annexin V assay) and caspase-3 activation or PARP cleavage (by, e.g., western blotting). Cell viability assays such as membrane integrity assays and reproductive assays are performed to monitor live cells in culture and measure an enzymatic activity as a marker of viable cells by using different classes of colorimetric reagents and substrates generating a fluorescent signal. Results from these assays do not always indicate apoptosis, but more about cell death in general. DNA labeling assay, functional assays, and morphological mechanism-based assays detect and quantify the cellular events, some of which are specifically associated with apoptotic cell death, such as formation of apoptotic antibodies, expression of apoptotic inhibitors, caspase activation in either intrinsic or extrinsic pathways, and DNA fragmentation. The principles for each assay are given in the respective yellow boxes. Abbreviations: *MTT* (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), *LDH* lactate dehydrogenase, *BrdU* bromodeoxyuridine, *PARP* polyadenosine diphosphate-ribose polymerase, *PS* phosphatidylserine

Table 3.11 Accidental and regulated necrosis, key features, and methods of detection

Type of cell death	Morphology	Detection methods
Accidental necrosis	Membrane disruption, mitochondria swelling (loss of organelle), cell swelling	LDH quantification, cell-impermeable DNA-binding dye, membrane integrity loss
Necroptosis	Membrane disruption, moderate chromatin condensation, cell swelling	Flow cytometry, western blot, immunohistochemistry—levels of biomarker proteins, mitochondrial depolarization detection, fluorescence microscopy for membrane loss, electron microscopy for morphology
Pyroptosis	Membrane disruption, bubbling, moderate chromatin condensation	LDH quantification, fluorescence microscopy for membrane integrity loss, western blot for GSDM D, IL-1β
Ferroptosis	Membrane disruption, iron accumulation, lipid peroxidation, diminutive mitochondria	Lipid peroxide quantification—flow cytometry and BODIPY-C11 probe
Methuosis	Membrane disruption, accumulation of large fluid-filled vacuoles, cell swelling	Electron microscopy, time-lapse fluorescence microscopy for morphology, metabolic flux analysis
NETosis	Membrane disruption, chromatin condensation	Fluorescence microscopy for morphology, flow cytometry, ELISA, western blot

(ACD) and regulated necrotic cell death (RNCD). RNCD can be further classified into necroptosis, pyroptosis, ferroptosis, NETosis, and methuosis given their molecular routes [139] (Table 3.11).

3.10.3.1 The Role of Necrosis in IR Cellular Responses

Necroptosis, pyroptosis, methuosis, and ferroptosis are all triggered in response to IR [124, 140]. In the context of RT

of cancer, necrosis can be induced either directly following DNA damage or indirectly by ROS formation that reacts with lipids generating lipid peroxides. IR has also been linked to lipid peroxidation and ferroptosis, and necroptosis together with ferroptosis was postulated to occur via ATM signaling.

Both ACD and RNCD trigger immunogenic cell death (ICD). In turn, ICD can stimulate an adaptive immune response after antigen is exposed by cells after RT or chemotherapeutics [141]. In case of immunogenic cell death, damage-associated molecular patterns (DAMPs) are delivered and identified by pathogen recognition receptors (PRRs) exhibited by intrinsic components of the immune system, conducting to the stimulation of an immune response [141]. ACD is an uncontrolled type of cell death which is activated by, e.g., physical damage, hypoxia, inflammatory toxins, and high doses of IR. The cells respond by morphological alterations, such as cytoplasmic swelling of the cell organelles, i.e., oncosis [142], which is a result of disturbance of ionic pumps causing Ca^{+} influx, plasma membrane disruption followed by the leakage of intracellular organelles with accidental deteriorated DNA, and absence of clear chromatin condensation [142]. RNCD comprises upregulation of diverse pro-inflammatory proteins and molecules such as nuclear factor-κB, leading to the rupture of the cell membrane causing leakage of the cellular debris, e.g., ATP, DNA, nuclear proteins, heat-shock proteins, and uric acid, into surrounding zones, provoking a cascade of inflammation and tissue injury. Thus, the release of proteins/molecules promotes inflammasome activation and production of pro-inflammatory cytokine interleukin-1 beta (IL1). The methods used to detect necrosis are lactate dehydrogenase (LDH) activity measurement and cell-impermeable DNA-binding dye. These techniques are based on the morphological characteristics proving the cellular release and membrane porosity (Table 3.12).

Table 3.12 Examples of some oncogenes in cancer from Weinberg [143] and Gillies et al. [144]

Oncogene	General function	Major tumor type with deregulation
K-ras	Guanine nucleotide-binding protein	Lung, ovarian, colorectal, bladder carcinomas
N-ras	Guanine nucleotide-binding protein	Head and neck cancers
H-ras	Guanine nucleotide-binding protein	Colorectal carcinomas
c-myc	Transcription factor	Various leukemias, carcinomas
L-myc	Transcription factor	Lung carcinomas
EGFR/HER2	Receptor tyrosine kinase	Glioblastomas, lung cancer, breast cancer
Src	Cytoplasmic tyrosine kinase	Colon cancer, head and neck cancers, chronic myelogenous leukemia
Sis/PDGF	Growth factor	Simian sarcoma

3.10.3.2 Necroptosis/Regulated Necrosis

Necroptosis, also known as a regulated necrosis, which works in a caspase-independent fashion, exhibits a necrotic morphology with membrane disruption and leakage of organelles (reviewed by Weinlich et al. (2017)). Different stimuli can elicit necroptosis: DRs, e.g., members of the TNFR superfamily, pattern recognition receptors (PRRs), Toll-like receptors (TLRs), T-cell receptors (TCRs), multiple chemotherapeutic drugs, and hypoxia. The process of necroptosis commences by the stimulation of receptor-interacting protein kinases (RIPKs) (Fig. 3.41).

RIPKs are stimulated to go into macromolecular complexes from the membrane receptors with the necrosome with RIPK1 and RIPK3 being the main components. RIPK3 subsequently stimulates mixed-lineage kinase domain-like protein (MLKL) through phosphorylation causing its oligomerization and relocalization, resulting in cell membrane permeabilization and subsequent cell death.

Different techniques can be used to identify necroptosis, e.g., flow cytometry, western blotting, and immunohistochemistry. Through these techniques, the expression levels of MLKL, RIPK3, and RIPK1 are evaluated as well as cell by electron microscopy (Table 3.11).

3.10.3.3 Pyroptosis and Ferroptosis: Triggers and Molecular Mechanisms

Pyroptosis, which is stimulated by IR as well as intracellular pathogenic factors in immune cells, follows a series of caspase-dependent events and is pro-inflammatory (reviewed by Yu et al. [146]). Thus, the NOD-like receptors (NLRs) of irradiated/infected macrophages/monocytes recognize cytoplasmic pathogen-associated molecular patterns (PAMPs) as well as DAMPs and trigger inflammasome complex production, which activates CASP1. CASP1 in turn activates gasdermin D, which mediates the plasma membrane rupture (Fig. 3.42) as well as the inflammatory cytokines interleukin 1β (IL-1β) and IL-18, which further regulate inflammation. Pyroptosis also involves cell swelling followed by disintegration of the plasma membrane and leakage of the pro-inflammatory contents, e.g., DAMPs, IL-1β, and IL-18, contributing to elimination of the immunologic challenges locally or systemically. Pyroptosis can be detected by LDH assay, fluorescence microscopy, western blot analysis (for identification of gasdermin D, IL-1β), and measurement of the cell intake of propidium iodide (Table 3.11).

Ferroptosis is a form of caspase-independent regulated necrosis and is distinguished by excessive iron-dependent lipid peroxidation. It presents a necrotic morphology with altered mitochondria, i.e., small mitochondria, fewer cristae, rupture of outer membrane, and an electron-dense ultrastructure. Execution of ferroptosis is decided by the equilibrium between ROS production due to iron increase and antioxidant protection mechanisms that impede lipid peroxidation.

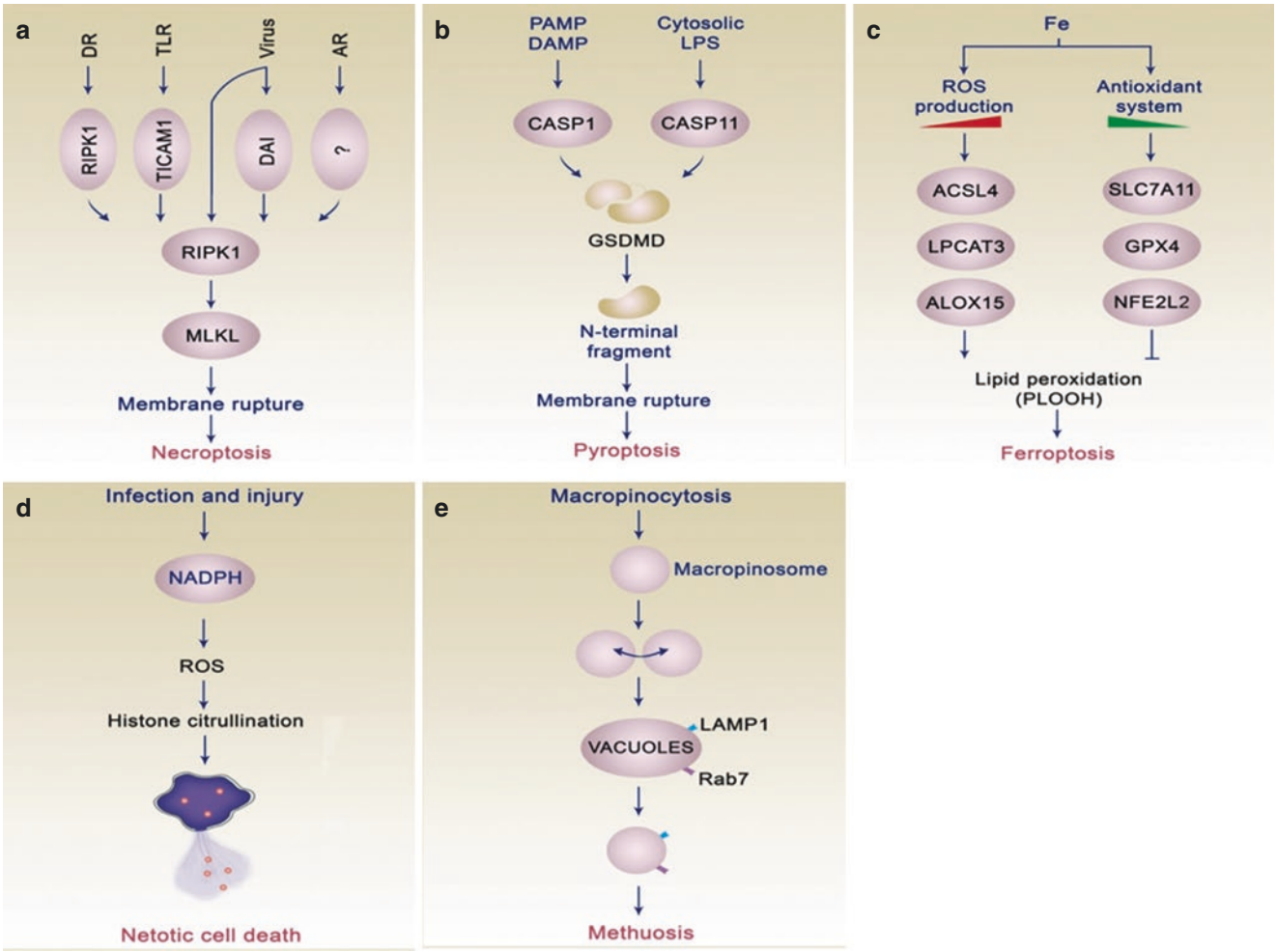

Fig. 3.41 Summary of regulated necrotic cell death. (**a**) Necroptosis elicited by DR, TLR, and viruses stimulates RIPK3 and then MLKL, which is required for membrane disruption. (**b**) Pyroptosis induced by GSDMD following its cleavage by CASP1 and CASP11. The main elicitors: PAMPs and DAMPs, or cytosolic LPS. (**c**) Ferroptosis is dependent on the balance between ROS production due to iron accumulation and antioxidant defense mechanisms that inhibit lipid peroxidation. The ACSL4–LPCAT3–ALOX15 pathway mediates lipid peroxidation, while system xc- (comprising SLC7A11, GPX4, and NFE2L2) impeded this process. (**d**) NETosis is triggered by NET leakage, which is mediated by ROS generation and histone citrullination. (**e**) Methuosis is associated with macropinocytosis. Nascent micropinosomes fused forming large vacuoles that contain late endosomal markers (LAMP1 and Rab7). These do not recycle or unify with lysosomes causing cell death. Reproduced with permission (CCBY) from Tang et al. [145]. *DR* death receptor, *TLR* Toll-like receptor, *RIPK3* receptor-interacting protein kinases 3, *MLKL* mixed-lineage kinase domain-like protein, *GSDMD* gasdermin D, *CASP1* caspase 1, *CASP11* caspase 11, *PAMPs* pathogen-associated molecular patterns, *DAMPs* damage-associated molecular patterns, or cytosolic, *LPS* lipopolysaccharide, *ACSL4* acyl-CoA synthetase long-chain family member 4, *LPCAT3* lysophosphatidylcholine acyltransferase 3, *ALOX15* arachidonate lipoxygenases (ALOXs, specifically ALOX15), *SLC7A11* the catalytic subunit solute carrier family 7 member 11, *GPX4* glutathione peroxidase 4, *NFE2L2* nuclear factor erythroid 2-like 2, *NET* NETosis extracellular trap, *ROS* reactive oxygen species, *LAMP1* lysosomal associated membrane protein 1, *Rab7* lysosomal Rab protein 7. (Adapted from Tang et al. [145])

Thus, ferroptosis is activated after lipid peroxidation in a process catalyzed by iron, either in a Fenton-like manner or through lipoxygenases (Fig. 3.41). Accordingly, the oxidation of polyunsaturated fatty acids (PUFAs), like arachidonic acid (AA), is necessary for lipotoxicity in ferroptosis, which takes place via a catalytic pathway comprising acyl-CoA synthetase long-chain family member 4 (ACSL4), lysophosphatidylcholine acyltransferase 3 (LPCAT3), and arachidonate lipoxygenases (ALOXs, specifically ALOX15) [147]. In addition, lipid peroxidation can be hindered by the various antioxidant systems such as the cystine/glutamate antiporter system, which consists of the catalytic subunit solute carrier family 7 member 11 (SLC7A11), glutathione peroxidase 4 (GPX4), and pro-survival proteins, like nuclear factor erythroid 2-like 2 (NFE2L2). System xc- facilitates the exchange of cystine and glutamate in and out of the cell. The cystine which is taken up is reduced to cysteine in cells, which is needed for the synthesis of glutathione GSH. GSH is used by

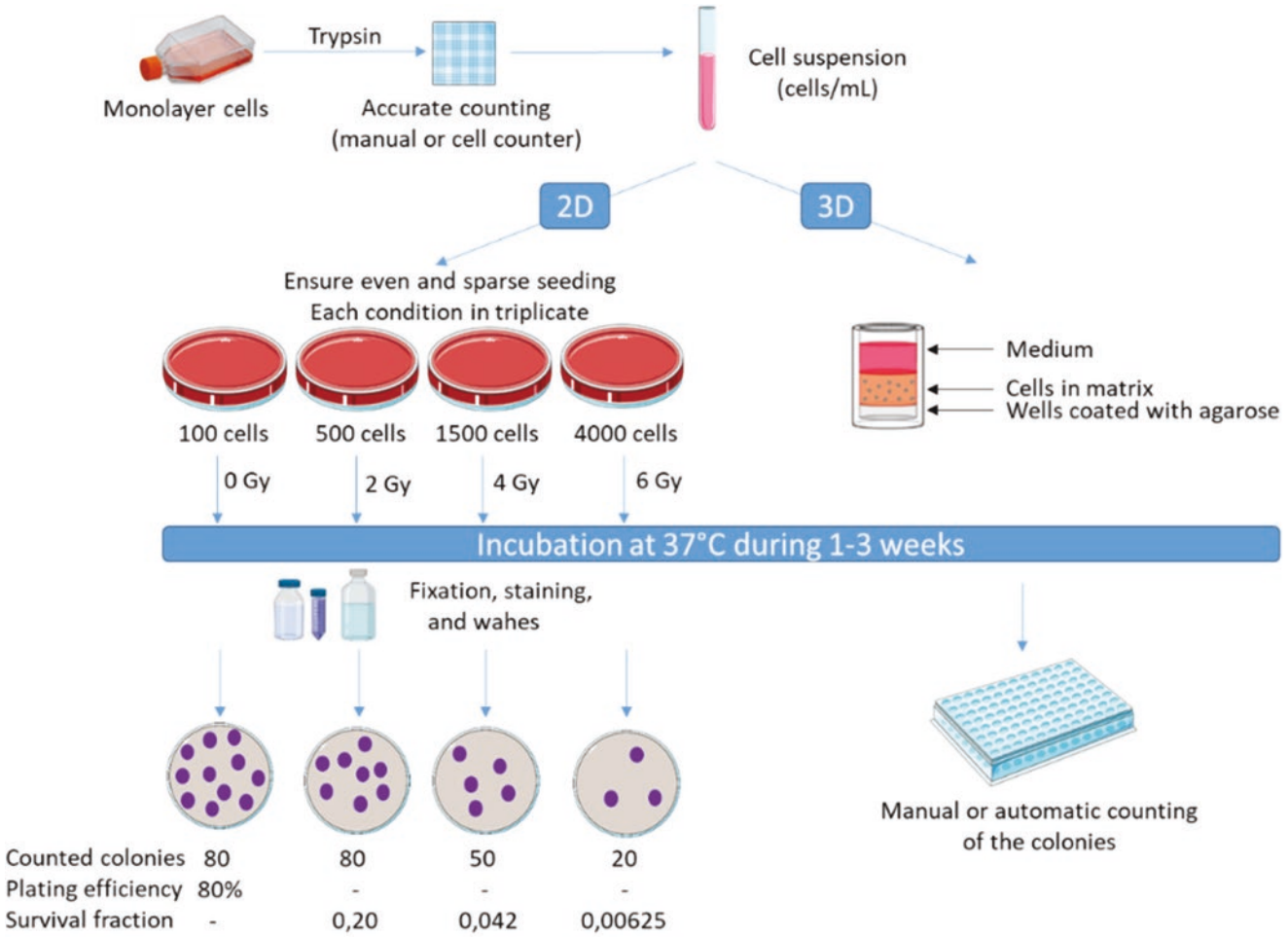

Fig. 3.42 Methodology for 2D (Puck) and 3D clonogenic curves. The clonogenic assay measures the ability of single cells to form colonies. A cancer cell that is not able to form a colony can be regarded as inactivated. Cellular monolayers are dissociated into single cells and counted and diluted to the required concentration, depending on the dose. The cells are then seeded in cell flasks/dishes for colony formation or in a 3D matrix for spheroid formation. After irradiation, the cells are incubated for 1–3 weeks depending on the cell doubling time of that particular cell line, before they are fixed, stained, and counted. The surviving fraction is calculated as the number of colonies in irradiated samples relative to the plating efficiency of unirradiated control dishes

GPX4 to stop the generation of phospholipid hydroperoxides (PLOOH), the key mediator of chain reactions in lipoxygenases. The induction of ferroptosis can be determined by measuring lipid peroxides coupled with flow cytometry (Table 3.11).

3.10.3.4 Neutrophil Extracellular Trap-Associated Cell Death (NETosis) and Methuosis

NETosis is stimulated by various pathogens or other stimuli, which release neutrophil extracellular traps of mainly DNA-protein structures [148] in a process dependent on NADPH oxidase 4 (NOX4), the principal source of ROS (Fig. 3.41). NETosis also comes along with important increase of ROS conducting to the stimulation of protein-arginine deiminase 4 (PAD4). Then, PAD4 citrullinates (converts arginine to citrulline via deamination) the histones, promoting the nuclear chromatin decondensation. Further, the NET is released into the cytosol leading to the disruption of the neutrophil membrane. Then, neutrophil breaks up and the NETs are released into the environment. NETs can be generated by other forms of immune cells, e.g., eosinophils, mast cells, basophils, macrophages, and also epithelial cells and cancer cells as a response to various injuries [145]. NETosis can be studied using various techniques: immunofluorescence, transmission electron microscopy, scanning electron microscopy, ELISA tests, flow cytometry, as well as western blot analyses of NETosis markers (Table 3.11).

Methuosis (from Greek methuo—"drink to intoxication") is another type of caspase-independent regulated necrotic cell death that is induced by exposure to heat, trauma, and infection and which lead to cell swelling, lysis

of plasma membrane, as well as inflammation. Methuosis is correlated to macropinocytosis (referred to as "cell drinking") and is associated with the extensive accumulation of fluid-filled cytoplasmic vacuoles stemmed from macropinosomes, which for example is observed in cancer cells driven by the oncoprotein Ras [149] (Fig. 3.41). Methuosis can be detected by electron microscopy, time-lapse fluorescence microscopy, visualization of vacuoles using fluorescent dyes, and metabolic flux analyses (Table 3.11) (Box 3.25).

Box 3.25 In a Nutshell: Necrosis

- Necrotic cell death is classified into accidental cell death and regulated necrotic cell death with different subtypes: necroptosis, pyroptosis, ferroptosis, NETosis, methuosis, etc.
- IR may stimulate necrosis via direct DNA damage response and via radical oxygen species.
- All types of necrosis are immunogenic cell death types.

3.10.4 Autophagy

Autophagy is an adaptive and catabolic process induced by various forms of cellular stress, intended to mitigate the impact of cell damage to avoid cell death, by recycling biomolecules and damaged organelles. This mechanism occurs via a self-digestion process involving the formation of double-membrane vesicles, called autophagosomes, that merge with lysosomes. Autophagy can be induced by nutrient deprivation (amino acids, in particular leucine and glutamine, and glucose) and cytotoxic insults such as IR or chemotherapy. The main function of autophagy is to provide nutrients and building blocks for vital cellular functions during different forms of stress. Therefore, this pathway is generally considered as a cytoprotective mechanism [150]. Autophagy is a complex mechanism involving several steps. First, the recruitment of autophagy-related proteins (ATG) to a specific subcellular location called the phagophore assembly site (PAS) allows phagophore nucleation (initiation and phagophore nucleation). During phagophore elongation, a portion of the cytoplasm is engulfed (cargo sequestration) and the autophagosome, a double-membrane vesicle, is being formed (autophagosome maturation). Fusion of the autophagosome with lysosome allows the degradation of the autophagic cargo.

A key regulator of autophagy is the mammalian target of rapamycin (mTOR) that exists in two distinct protein complexes, mTORC1 and mTORC2. In its active conformation, mTORC1 prevents autophagy by inhibiting the UNC51-like kinase 1 (ULK1) complex, composed of ULK1, the autophagy-related gene 13 (ATG13), ATG101, and the FAK family-interacting protein of 200 kDa (FIP200). Upon autophagic stimuli, mTORC1 is inhibited, leading to the activation of ULK1. Active ULK1 phosphorylates ATG13 and FIP200, which leads to the activation of the class III phosphoinositide 3-kinase (PI3K) complex, allowing phagophore nucleation. This triggers the production of phosphatidylinositol-3-phosphate (PIP3) at a characteristic ER structure called omegasome. PIP3 recruits WD repeat domain phosphoinositide-interacting proteins (WIPI2) and zinc finger FYVE domain-containing protein 1 (DFCP1) to the omegasome. By binding ATG16L1, WIPI2 recruits the ATG12-ATG5-ATG16L1 complex that allows the conjugation of ATG8 family proteins (including microtubule-associated protein light-chain 3 (LC3) and γ-aminobutyric acid receptor-associated proteins (GABARAPs)) to membrane-resident phosphatidylethanolamine (PE). By this process, LC3-I (diffuse form) is converted into LC3-II (membrane-anchored, lipidated form), a marker of autophagic membranes. The recruitment of ATG9-containing vesicles (coming from the plasma membrane, mitochondria, recycling endosomes, and Golgi complex), delivering additional lipids and proteins, further contributes to autophagosomal membrane expansion. Once the membrane is sealed, the autophagosome is formed and undergoes maturation. Then it can merge with the lysosome, where the autophagic cargo will be degraded by acidic hydrolases. For the molecular details, see Dikic et al. [151].

3.10.4.1 Role of Autophagy in IR Responses

Beyond apoptosis, the commonly studied IR-induced cell death mechanism, autophagy was shown to be frequently induced in response to IR. For example, autophagy can be triggered following DNA damage inflicted by IR or other agents. Indeed, DNA damage repair (DDR) is an energy-demanding process that consumes ATP but also NAD+ via the action of polyADP-ribose polymerase 1 (PARP1). Autophagy induction allows the recycling of metabolic precursors for ATP and provides energy for the DDR. ROS was also shown to trigger and regulate autophagy [152]. The function of IR-induced autophagy is still being debated. Results of in vitro and in vivo studies provided conflicting notions whether autophagy acts as a cytoprotective mechanism, promoting cell survival responsible for radioresistance. In that respect, radiosensitization strategies based on genetic or pharmacological autophagy inhibition led to different outcomes. Several studies also pointed out the non-cytoprotective function of IR-induced autophagy where autophagy inhibition failed to alter radiosensitivity. Although autophagic functions may vary depending on both cell type and treatment regimen applied, specific characteristics able to distinguish cytotoxic, cytoprotective, or non-cytoprotective forms of IR-induced autophagy have not yet been identified. There

are assumptions that autophagy duration may play a role in radiosensitivity, with radioresistance occurring in case of prolonged autophagy, while a transient form of autophagy will ultimately lead to apoptosis [150].

Outcomes of clinical trials conducted with approved autophagy inhibitors (e.g., chloroquine and hydroxychloroquine) were mitigated due to toxicity issues and unsatisfactory autophagy inhibition. Concerns were raised regarding the most probable non-tumor selectivity of autophagy inhibitors, off-target effects, effects on immune response, and difficulty to monitor autophagy inhibition in patients' tumors [153]. Further studies on the molecular mechanisms governing IR-induced autophagy may bring additional evidence on how to optimally modulate autophagy to produce favorable outcomes (Box 3.26).

Box 3.26 In a Nutshell: Autophagy

- Autophagy is triggered by IR and often considered as a cytoprotective mechanism.
- Autophagy inhibition as a radiosensitization strategy led to inconsistent results, suggesting an intricate role of autophagy, being regulated by many factors.

3.11 Clonogenic Cell Survival

As described in the sections before, cells damaged by radiation might suffer from genetic instability and/or die through, e.g., apoptosis or other types of cell death. These consequences of radiation exposure can be used to qualify and quantify the damage and draw conclusions on its severity. In this context, it is possible to look at not only the fatal outcome of radiation damage but also the capability of cells to survive IR. It is important to distinguish between cell survival and cell viability. In radiobiology, the term cell death is used also for cells that are inactivated, i.e., have lost their proliferation ability. Cancer cells and stem cells are characterized by their capacity for sustained proliferation. A cancer cell that has lost the ability to divide is by definition dead as a cancer cell even though it may still have an intact cell membrane and retained metabolic function. While non-proliferating cells retain their function even after radiation doses as high as 50–100 Gy, cancer cells may lose the capacity for uncontrolled cell division after doses in the order of 2 Gy.

There are several assays available to measure cell viability. Some use dye exclusion, such as trypan blue, to measure the proportion of cells with intact cell membrane. Others measure metabolic function through the activity of mitochondrial enzymes, such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay, cellular reducing conditions such as the Alamar Blue assay, or ATP production. Even though viability measurements over time can give an indication of cell proliferation, the only direct measurement of clonogenic function is the clonogenic assay, the gold standard for cell survival measurements. These assays can be performed in vitro with cultured cells or in vivo from biopsies.

3.11.1 *In Vitro* Dose-Response Assays

The first survival curve, i.e., the relation between survival and delivered doses, was established with HeLa cells cultivated in vitro and irradiated with X-rays by Puck and Marcus in 1956 [154]. A surviving cell is defined as a cell able to divide and form a colony composed of at least 50 cells. To find the surviving fraction, the capacity of nonirradiated and irradiated cells to form colonies is compared. Typically, in vitro cell survival is measured in adherent cells in monolayer culture. The day before the experiment, cells are trypsinized. Viable cells are counted with a hematocytometer or a cell counter. A determined number of cells in suspension is seeded in Petri dishes (or flasks) destined to be a control or irradiated before their first doubling time. Depending on the design of the experiments, the medium can be changed after irradiation. Then cells are incubated at 37 °C for 1–3 weeks according to the cell types (≥8 divisions). When the colonies grow to exceed 50 cells, observable by microscopy or visually detectable, they are fixated with methanol or ethanol and then stained with Giemsa, methylene blue, or crystal violet before several washes with water and drying [155]. After that, the clones formed are counted manually or with an automatic counter (Fig. 3.42).

All cells comprising each colony are the progeny of a single initial cell seeded, which survived irradiation. If we consider 100 untreated cells, the ideal number of colonies formed should be 100. However, this is never the case, depending on diverse factors (medium change, errors and uncertainties in counting the cell suspension, trauma of the detachment …), and in fact 50–90 colonies might be expected. Considering the outcome of the control conditions (nonirradiated), the term plating efficiency (PE) can be defined. This corresponds to the percentage of cells seeded, which grew into colonies. If 75 colonies are counted after seeding 100 cells, we talk about a PE of 75%. It must be noted that the PE may differ according to the number of cells seeded: this is the "feeder effect." This effect is attributed to the need of some cell types to be able to cooperate with neighboring cells [156]. If this communication is missing, the cells are not able to start proliferation. Therefore, the cell density seeded might play a role in the fraction of cells able

to form colonies. This might limit the robustness of the classical analysis of the colony-forming assay. In future, a different way of performing and analyzing this assay might be necessary [156].

In classical colony-forming assay parallel to the control samples, cells are irradiated, then incubated, fixed, and stained at the same time point as control cells. Different cases can therefore be observed: (1) some of the seeded cells being still single and not divided; (2) cells that managed one or two divisions to form a tiny abortive clone; and (3) cells able to form large colonies of at least 50 cells, corresponding to 5–6 cell divisions, but which can look like a little bit different from the untreated cells in terms of aspect and size. These latter cells, able to form colonies, are qualified of "survivors" and counted since they have retained their reproductive integrity. For example, if we seed 3000 cells followed by irradiation of 5 Gy, and if the PE previously determined is 0.75, then we can expect the attachment of 2250 cells (0.75 × 3000). If at 5 Gy 42 colonies grew up after incubation, the surviving fraction can be calculated at 1.9%: 42/(3000 × 0.75) = 0.019. In general, the plating efficiency (PE) and the surviving fraction (SF) are given by

$$\mathrm{PE} = \frac{\text{colonies counted}}{\text{cells seeded}} \times 100 \quad (2)\mathrm{SF} = \frac{\mathrm{PE}(\text{condition})}{\mathrm{PE}(\text{control})} \times 100. \tag{3.1}$$

Survival curves for mammalian cells are usually presented in a form with dose plotted on a linear scale and surviving fraction on a logarithmic scale and can be fitted by several models, as for example the linear-quadratic model (see Chap. 1). The form of the curves, as seen in Chap. 1, depends on the linear energy transfer and allows determining important biological parameters such as the surviving fraction, the ratio α/β, or the relative biological efficiency (RBE) for example (see Chap. 1 for details). The surviving fraction at 2 Gy (SF2) is often used to approximate cell radiosensitivity.

To obtain a survival curve, several doses of irradiation have to be applied. The number of cells seeded per dish needs to be accurate and often adjusted after preliminary experiments to count a significant number of colonies since these parameters are dependent on doses, cell lines, and type of radiation. At least a triplicate of different dilutions is realized for each condition tested (here each dose delivered). If colonies are few, the statistical significance is reduced. On the opposite, if the colonies are too many, some colonies can be merged with another one, and the counting is inaccurate. In some cases, cells could be irradiated first (one flask for one dose) and then detached to be seeded at different dilutions [157]. However, precautions need to be considered since some cells are sensitive to detachment after irradiation, which affects cell survival. In addition, colony-forming assays require very accurate cell counting, since the controls come from a separate trypsinization. Clonogenic curves cannot discriminate the type of cell death, but they give information about the radiosensitivity of the cells.

More recently, the literature showed that survival curves obtained with three-dimensional (3D) cell models more reliably reflect the cell response in vivo than the results obtained with 2D cell monolayer culture [158]. 3D cell models for cell survival can be obtained by embedding single cells in an extracellular matrix, put in 96-well plates pre-coated with agarose, covered with medium, and then exposed to radiations. Cells are grown for a few days until cell clusters reach 50 cells, and the number of colonies is microscopically counted (Fig. 3.42).

3.11.2 *In Vivo* Dose-Response Assays

An in vivo clonogenic assay allows measuring cell survival in an animal model, allowing the study of radiosensitivity of normal or tumor cells treated in vivo. These systems depend on the reproductive integrity of individual cells and allow the observation of a clone of cells regenerated in the irradiated tissue. There are assays developed for early-responding tissues, which divide rapidly and respond early to the effects of radiation, like bone marrow cells, skin, and intestinal epithelium, and assays for late-responding tissues, like lung, kidney, and spinal cord (Fig. 3.43).

The **spleen colony assay**, also called bone marrow stem cell assay, was first described by Till and McCulloch [159]. The basis of this assay relies on the use of one donor mouse and a group of recipient mice. Recipient mice are previously exposed to whole-body irradiation (9 Gy) to sterilize the spleen and suppress endogenous hematopoiesis. Then, from a donor mouse irradiated with a test dose, a cell suspension of bone marrow cells is taken and injected intravenously into the recipient donors. Some of these cells will lodge in the spleen, and after 10–11 days, single cell-derived clones will appear in the surface of the spleen. These colonies are usually called colony-forming units (CFUs). At this point of the experiment, the spleen of the recipient mouse is removed and the CFUs are counted. The surviving fraction is given by Eq. (3.2), similar to the one used for the in vitro assay. The experiment is then repeated for different radiation doses, enabling to trace a survival curve:

$$\text{Surviving fraction} = \text{colonies counted} / \left(\text{cells inoculated} \times \frac{\mathrm{PE}}{100} \right). \tag{3.2}$$

The **skin clone assay** is based on the formation of nodules of mouse skin regrowing from a single surviving cell. In a practical way, after shaving a small area on the back of one mouse, a ring of skin is irradiated with a massive dose of

Fig. 3.43 In vivo assays. Four in vivo animal assays to assess clonogenic capacity after irradiation have been important for radiobiology. (1) The jejunum crypt assay measures the regenerative ability of jejunal crypts after high doses of irradiation. The animals are sacrificed 3.5 days after irradiation, and the numbers of regenerating crypts per circumference are measured. One regenerating crypt corresponds to one surviving clonogenic cell. (2) The skin clone assay used pre-irradiation with a high dose in a ring (moat) around the test skin area to avoid migration of neighboring cells into the test area. The test area is then irradiated, and the number of regrowing skin nodules per cm^2 is counted. (3) The spleen colony assay uses transplants of bone marrow cells from an irradiated donor animal. These cells are transferred to recipient animals who have previously been irradiated with a high dose to kill all their own bone marrow cells. After 10–11 days, the recipient animals are sacrificed and their spleens are analyzed for colony-forming units arising from the implanted single cells. (4) The kidney assay uses the same animal for irradiation and control. One kidney of each animal is irradiated, and 60 weeks later, the animals are sacrificed. The number of intact kidney tubules is then counted in both kidneys, and the irradiated kidney can be compared to the unirradiated one

30 Gy to create a "moat" of dead cells. A small metal sphere is put in the central area to protect it from the radiation and create an isolated island of intact skin. This skin island is then irradiated with a test dose. Some days later, nodules of regrowing skin will be observed. The survival curve is obtained after repeating the experiment in different skin areas and by plotting the number of surviving cells per cm^2 of skin as a function of the radiation dose (Gy).

The **jejunal crypt stem cell assay** is based on the self-renewal system of the jejunum. Within this system, the stem cells in the crypts divide rapidly and move up to the villi where they undergo differentiation in functioning cells. For the assay, groups of animals are subjected to increasing doses of whole-body irradiation. The jejunal crypts will begin to regenerate after 3.5 days, time at each animal is sacrificed, and sections of the jejunum are imaged. One regenerating crypt corresponds to one surviving clonogenic cell. The survival curve is obtained by plotting the number of regenerating crypts per circumference of the sectioned jejunum as a function of the radiation dose (Gy).

The **kidney tubule assay** includes the irradiation of one kidney per mouse with a small field. As the kidney is a late-responding tissue, the assay is finished 60 weeks later, when unirradiated and irradiated kidneys are removed, and histologic sections are imaged. The number of intact kidney tubules is compared between the unirradiated and irradiated sides. The survival curve is obtained by plotting the number of tubule-regenerating cells in a defined number of tubule cross sections counted as a function of the radiation dose (Gy).

In addition, the tumor control dose assays (TCD_{50}) relate with tumor survival. During these assays, small parts of tumors (xenografts), which can be derived from tumor cell lines or from patient tumors, are implanted to nude mice. After they reach a desirable size, the tumors are irradiated by several doses and then the local control or recurrence is observed. A plot between the percentage of the controlled tumors versus the dose is made. TCD50 is then the dose to control 50% of the tumors [160] (Box 3.27).

Box 3.27 In a Nutshell: Cell Survival and Clonogenic Assays

- A surviving cell corresponds to a cell able to divide and form a colony.
- Clonogenic assay is based on the ability of a single cell to grow into a colony.
- The only direct measurement of clonogenic function is the clonogenic assay, the gold standard for cell survival measurements.
- Cell survival measurements allow to trace a cell dose-response curve, usually presented with dose plotted on a linear scale and surviving fraction on a logarithmic scale, and can be fitted by several models.
- An in vivo clonogenic assay allows measuring cell survival in an animal model, allowing the study of radiosensitivity of normal or tumor cells treated in vivo.

3.12 Oncogenes and Tumor Suppressor Genes

Transformation of a normal cell into a cancer cell is a multistep process where mutations or other genomic alterations, e.g., copy number alterations, deletions, and gene fusions, alter the normal gene coding sequence. These alterations can occur due to mis- or unrepaired IR damage. Not all alterations lead to the transformation of a normal cell to a cancer cell, called oncogenesis, as it is associated with alterations of specific places on DNA [143]. Cell transformation is mostly related to the activation of proto-oncogenes, which are then named oncogenes and the deactivation of tumor suppressor genes [143]. Proto-oncogenes are genes associated with the activation of cell proliferation and differentiation. When they mutate or are somehow pressed to overexpression, cells proliferate out of control [143]. On the other hand, tumor suppressor genes are genes that control cell proliferation, play significant roles during DNA repair, or activate cell death pathways, when it is needed. Mutations of tumor suppressor genes cause loss of control upon important pathways, which may again lead to unregulated cell proliferation [143]. Oncogenes and tumor suppressor genes can be affected genetically by mutations on the DNA or also switched on or off epigenetically. An overview is given in Fig. 3.44.

3.12.1 Proto-Oncogenes and Oncogenes

The discovery of proto-oncogenes came with investigation of the Rous sarcoma virus (RSV). This virus is able to transform normal chicken cells to cancer cells, and in its structure, the *src* gene was found, which as it was shown later was responsible for this transformation. The *src* gene was later also found in the normal chicken genome, but it was inactivated. These findings meant that the genomes of normal cells carry genes (proto-oncogenes) that have, under certain circumstances, the potential to induce cell transformation when activated [143]. For some time, biologists were convinced that cancer is caused by viruses which present into cells' genes (oncogenes) that activate uncontrolled cell proliferation. It was thus strange that people around these "infected" people do not suffer from the same cancer type as well, due to the fact that viruses are infectious. Indeed, viruses can include oncogenes into a cell's DNA, but viruses are not the main cancer cause. Viruses are responsible only for a minority of all cancers [143]. All this information led to new questions about proto-oncogenes and oncogenes. To find out if oncogenes exist in chemically or physically transformed cells, DNA from cancer cells was introduced to normal cells to see if they will be transformed. This gene transfer procedure is named transfection. Indeed, many other oncogenes were revealed using this method [143]. Another very important issue is that it is sufficient to activate only one of the alleles of a proto-oncogene to get oncogene upregulation [161]. Some of the most common oncogenes in human cancer are given in Table 3.12.

3.12.2 Tumor Suppressor Genes

In general, when a system has an activation "button," there has to be somewhere a deactivation "button" as well. Oncogenes are the genes activating uncontrolled cell proliferation, and on the other hand the deactivation/control of cell proliferation is associated with tumor suppressor genes.

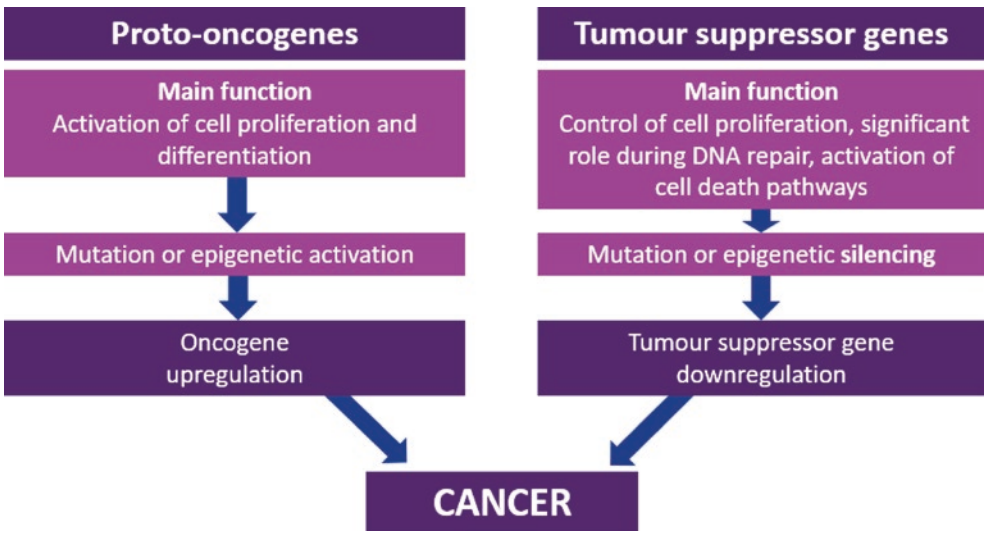

Fig. 3.44 Overview of oncogenes and tumor suppressor genes' function and regulation

Table 3.13 Examples of some tumor suppressor genes and familial cancer syndromes from Macleod [162] and Weinberg [143]

Tumor suppressor gene	General function	Types of cancer	Familial syndrome
TP53	Chromosome stability, transcriptional regulator, growth arrest, apoptosis	Many	Li–Fraumeni syndrome
p16	Cyclin-dependent kinase inhibitor	Many	Familial melanoma
BRCA1	Transcriptional regulator, DNA repair	Many, mostly breast and ovarian cancer	Familial breast cancer
BRCA2	Transcriptional regulator, DNA repair	Many, mostly breast and ovarian cancer	Familial breast cancer
RB1	Transcriptional regulator of cell cycle	Retinoblastoma, osteosarcoma	Familial retinoblastoma
E-cadherin	Cell adhesion regulator	Breast, colon, lung, skin carcinoma	Familial gastric cancer
APC	β-Catenin degradation	Colorectal, pancreatic, stomach, prostate cancer	Familial adenomatous polyposis coli
NF2	Cytoskeleton-membrane linkage	Schwannoma, meningioma, ependymoma	Neurofibroma-predisposition syndrome

Tumor suppressor genes were discovered much later than proto-oncogenes and oncogenes. Some of the tumor suppressor genes are listed in Table 3.13. One of the most important and known tumor suppressor genes is the TP53. A mutation of TP53 is associated with various tumor types. This gene codes the p53 protein, which is also sometimes called the "Master Guardian." p53 is responsible for activation of DNA repair as well as activation of cell cycle arrest, to enable DNA repair.

To deactivate a tumor suppressor gene, both alleles have to be damaged or switched off, because only one allele is enough for the production of a specific protein. Anyhow, if one allele of a tumor suppressor gene of a germ line cell is defective, then there is much higher probability of the born individual to suffer from cancer. This is because for this person, it becomes much more probable that the second allele will be damaged during life as well [143, 161, 162]. Since the defective allele in this case is genetically transferred to offspring, many familial syndromes were identified (Box 3.28).

Box 3.28 In a Nutshell: Oncogenes and Tumor Suppressor Genes

- DNA alterations in genomic or epigenetic level may cause proto-oncogenes to become oncogenes, disrupting normal cell division and causing cancers to form.
- Cell transformation is mostly related to the activation of proto-oncogenes, which are then named oncogenes, and deactivation of tumor suppressor genes.
- Proto-oncogenes are genes associated with the activation of cell proliferation and differentiation.
- Tumor suppressor genes are genes that control cell proliferation, play significant roles during DNA repair, or activate cell death pathways.
- Mutations of tumor suppressor genes cause loss of control upon important pathways, which may again lead to unregulated cell proliferation.
- Oncogenes and tumor suppressor genes can be affected genetically by mutations on the DNA or also switched on or off epigenetically.
- TP53 is one of the most important tumor suppressor genes.

3.13 Interconnectivity Between Cells

Cells are organized in complex cellular systems such as tissues or organs; therefore, it is crucial that they are able to communicate with each other. The most rapid way of communication is directly through cell-to-cell contact. There are various ways of direct interconnectivity of cells as shown in Table 3.14.

3.13.1 Gap Junctions

The most famous type of cell-to-cell connection is gap junction, which is the most direct manner of cell interconnectivity and forms the fastest communication channel. Gap junctions have a pore diameter of 2–3 nm and a length of 2–4 nm and are involved in the exchange of nutrients, ions, second messengers, and small metabolites up to ~1 kDa, allowing ionic and biochemical coupling between neighboring cells. These specialized structure membranes have a

short half-life of a few hours (~1–4 h), and their biosynthesis and assembly are firmly regulated [163]. These transmembrane structures are composed of connexons (Fig. 3.45) constituted of six connexin (Cx) subunits around a central pore, which allow communication between adjacent cells. These connexons could be made up of six similar Cx isoforms (homomeric) or a combination of six different Cx isoforms (heteromeric). To date, 21 Cx isoforms have been identified in human proteosome, each named according to its approximate molecular weight (in kDa), with Cx43 being the most studied till now [163]. According to electron microscopy analyses, all Cx share a common topology composed of four transmembrane proteins, with a cytoplasmatic C- and N-terminal domains, two extracellular loops, and an intracellular loop. In contrast to the transmembrane proteins and the extracellular loop which are highly conserved among the Cx family members, the intracellular loop and the C- and N-terminal showed high variability in terms of the length and amino acid sequence of each Cx. Thus, these regions play an important role in the modulation of the gap junction channel gating and in the intracellular trafficking of connexins, and consequently a variety in their biological roles and interactions [163].

Table 3.14 Summary of the size properties of the three main direct cell connections

Type of connection	Diameter	Length
Gap junctions	2–3 nm	2–4 nm
Tunneling nanotubes	50–1500 nm	Few to >100 μm
Epithelial bridges	1–20 μm	25–1000 μm

The spatial arrangements of Cx43 in breast cancer cells, fibroblasts, and internal mammary artery endothelial cells were studied by CLSM and super-resolution localization microscopy [164]. After radiation treatment (50 min postirradiation with a dose of 4 Gy), these cells behaved differently concerning the trafficking and response of Cx43. In breast cancer cells, high accumulations of Cx43 were found in the cytosol and along the membrane. The results did not significantly differ between non-treated and irradiated cells. In contrast to that, normal fibroblasts and endothelial cells revealed differences at the membrane and in the perinuclear cytosol after radiation exposure. In endothelial cells, a significant Cx43 accumulation and condensation were observed in the perinuclear region, whereas at the membrane, a signal reduction was found. In fibroblasts, Cx43 accumulations were found in the perinuclear region but also at the membrane.

Furthermore, as the Cx are phosphoproteins, they also play an important role in modulating the physiological properties and regulation responses of the channels, such as differentiation process, neuronal activity, development, cell synchronization, and immune response. Therefore, the pres-

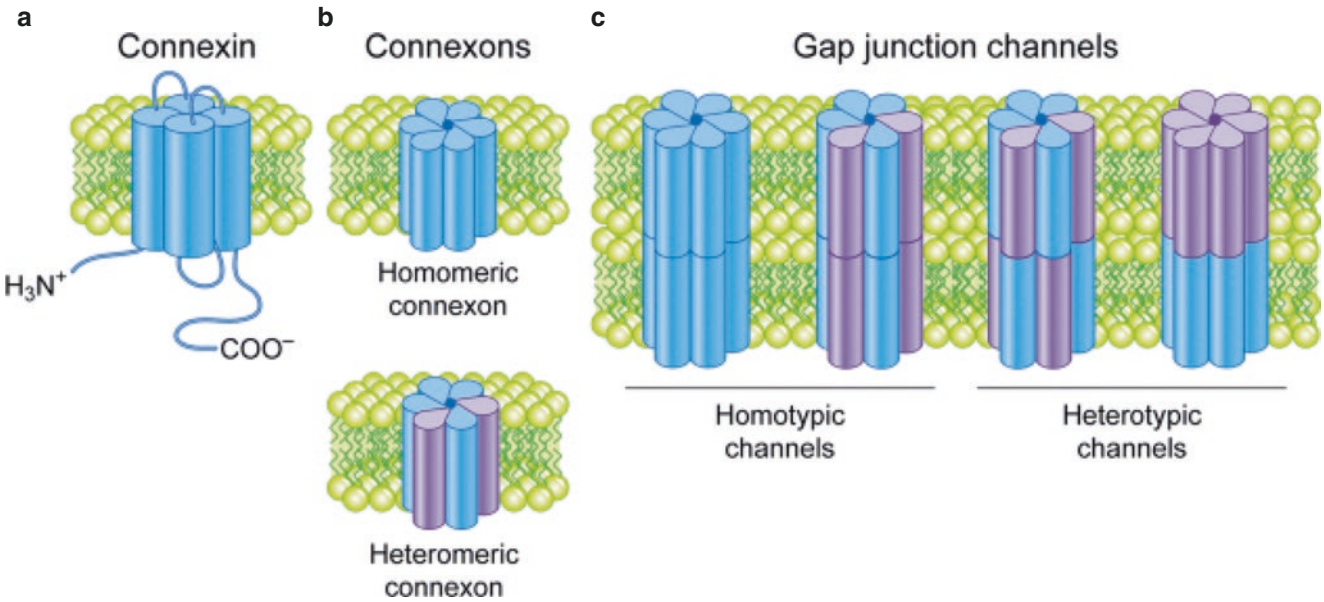

Fig. 3.45 Connexins and gap junctions. Each connexin (**a**) consists of four transmembrane domains. Six connexins form a hexameric torus called connexon (**b**). Depending on the composition, connexons are called homomeric (six equal connexins) or heteromeric (up to six different connexins). (**c**) When the cells form direct contact, the connexons stick together forming gap junctions. Here, the differentiation is made between homotypic channels (both connexons are the same) and heterotypic channels (different connexons). (Reproduced with permission (CCBY) from Totland et al. [163])

ence of mutation in these structures is associated with several human diseases, such as neurodegenerative and skin diseases, deafness, and developmental abnormalities [165]. Also, gap junctions have been described as having a selective permeability, dependent on the combination of Cx isoforms that are made, conferring a single gating, conductance, and permeability to specific molecules, which could allow the association of each channel to a specific disease.

3.14 Membrane Connections

Another type of intercellular communication is via membrane connections such as tunneling nanotubes (TNTs) and epithelial (EP) bridges, which can be distinguished through their structural composition. These connections serve as direct signaling path when cells are separated by greater distances, than necessary for gap junctions. A microscopic image of both connection types can be found in Fig. 3.47.

3.14.1 Tunneling Nanotubes (TNTs)

TNTs are thin cytoplasmic membrane bridges, which appear in straight lines in vitro but also with a curved shape in tissue or in vitro cultures in a three-dimensional extracellular matrix found in various mammalian cells [166]. Their diameter ranges from 50 nm up to 1.5 µm, and they can contact cells over long distances up to several cell diameter length. Even if an obstacle blocks the direct distance between two cells, TNTs, due to their flexible structure, can form a connection. The length of the TNTs dynamically varies when cells migrate up to a certain distance of several 100 µm, which is too large to keep the structure, and the tube disappears. The detailed structure of TNTs is very complex and not yet known in detail. Most TNTs consist of F-actin, and the thicker ones additionally contain microtubules and cytokeratin filaments. Further compounds are sequentially identified as more and more information about the responsibility of TNTs is gathered. TNTs are proven to serve as a highway for exchange of cellular compounds such as mitochondria, vesicles, and many more. Larger compounds are mainly transported along TNTs in so-called gondolas (see Fig. 3.46). Furthermore, TNTs play a key role in direct and active signal transduction including calcium and electric signals, which are known to occur in cells due to radiation stress. Overall, it can be said that the frequency of occurrence and also the complexity of TNT networks within a cell composite are connected to the stress this composite is exposed to. Under stress conditions, the networks are intensified, so that signal and compound exchange is enhanced and fastened. Furthermore, the TNT networks were identified to play a role in the bystander and also the rescue effect and other effects related to radiotherapy [166].

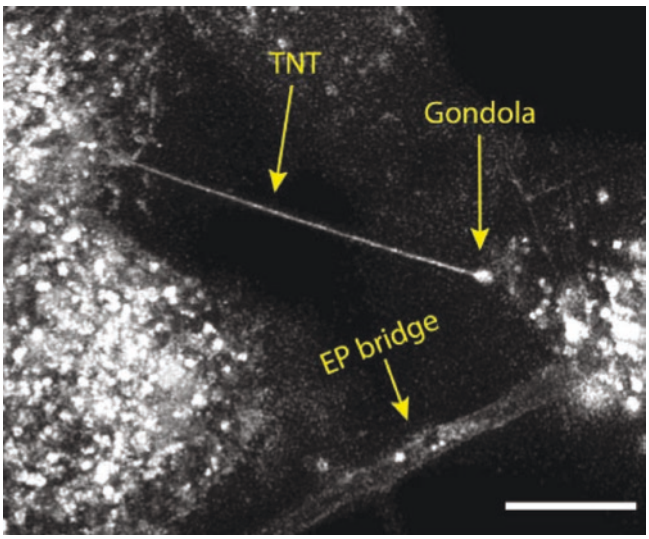

Fig. 3.46 Membrane connections. Microscopic image of membrane label of cells connected by a tunneling nanotube transporting a gondola and an epithelial bridge containing vesicles and cytoplasmic material. Scale bar: 10 µm. *EP* epithelial, *TNT* tunneling nanotube

3.14.2 Epithelial (EP) Bridges

In contrast to TNTs, EP bridges could, as also the name suggests, only be found in normal as well as cancerous human epithelial cells. They also differ from TNTs structurally, as they show a larger diameter of 1–20 µm and also a larger range from 25 µm to over a millimeter [166]. EP bridges consist of F-actin as well as microtubules, which promotes the structural stability allowing these connections to bridge such large distances. As TNTs, the EP bridges play a major role in cellular compound and signal transduction (Box 3.29).

Box 3.29 In a Nutshell: Interconnectivity Between Cells and Communication

- Cells communicate through direct cell-to-cell contact and for interconnectivity networks.
- Gap junctions, constituted by connexins, allow short-range ionic and biochemical coupling.
- TNTs and EP bridges are responsible for long-range signal and molecule transduction.
- Direct cellular communication plays a role in various diseases, spreading of pathogen and health signals, as well as stress and radiation response of cell composites.

3.15 Inflammation and Immunity

3.15.1 Basic Mechanisms of Inflammation

Since inflammation that can be induced by microbial infections and tissue damage is an essential mechanism of innate immune response, the terms "inflammation and immunity" are intrinsically linked [167]. The process of inflammation includes several biochemical events and multi-level cellular interrelationships. In a concerted action, inflammation is initiated, propagated, matured (effector phase), and finally resolved. This implies that radiation exposure under inflamed conditions affects several cell types including many immune cell (sub)types. Macroscopically, vasodilatation and extravasation of immune cells into the inflamed tissue occur that in sum results in the key characteristics of inflammation, namely swelling, redness, pain, loss of function, and increased temperature. The major immune cells involved in the inflammatory process are polymorphonuclear neutrophils (PMNs), which are the most abundant leukocytes in peripheral blood and are very quickly recruited to sites of inflammation, mononuclear monocytes that can differentiate into dendritic cells (DCs) and macrophages, and different subtypes of B and T lymphocytes mediating an antigen-specific adaptive immune response.

3.15.2 Radiation-Induced Modulation of Inflammation

The response of the key immune cells involved in inflammation is strongly dependent on the basal inflammatory status of these cells and the systemic inflammatory (micro)-environment. Further, the monocytic cells are central in all phases of the inflammatory process from initiation to termination and are characterized by an initial high plasticity that is weakened by prolonged tissue residency. Their phenotype is strongly influenced by the microenvironment, and radiation responses are therefore manifold and dose dependent [168]. Regarding inflammatory cytokine expression by macrophages, particularly TNF-alpha and IL1-beta, secretion is reduced following a single radiation exposure of 0.3–0.7 Gy without affecting the immune cell's viability. Further, decreased expression of the inducible nitric oxide synthase (iNOS) protein and, as a consequence, nitric oxide (NO) production in inflammatory macrophages after radiation exposure are observed in inflamed joints. Radiation exposure causes stress in cells via the production of reactive oxygen species (ROS), and a dose of 0.5 Gy, being routinely applied for low-dose radiotherapy of benign chronic inflammatory and destructive diseases, resulted in the strongest reduction of ROS by activated endothelial cells. Besides affecting immune cells and endothelial cells, low/intermediate-dose radiation exposure has osteoimmunological modes of action by reducing the activation of bone-resorbing osteoclasts and by fostering bone construction by osteoblasts [169]. Epidemiological, clinical, and experimental data regarding the effects of low-dose radiation on the homeostasis and functional integrity of immune cells was just recently comprehensively summarized [170]. Finally, particularly in the interactions of radiation with immune cells and cells of the inflammatory process, nonlinear dose relationships are prominent and may reflect a nonlinearity and complexity of immune responses. Figure 3.47 summarizes the key immune cells that are involved in inflammation and are modulated together with the endothelium by radiation in a dose range of 0.1–1.0 Gy. Finally, in polymorphonuclear leukocytes (PMN), irradiation with doses between 0.5 and 1.0 Gy resulted in a discontinuous reduction of chemokine CCL20 secretion that parallels a hampered PMN adhesion to endothelial cells [171].

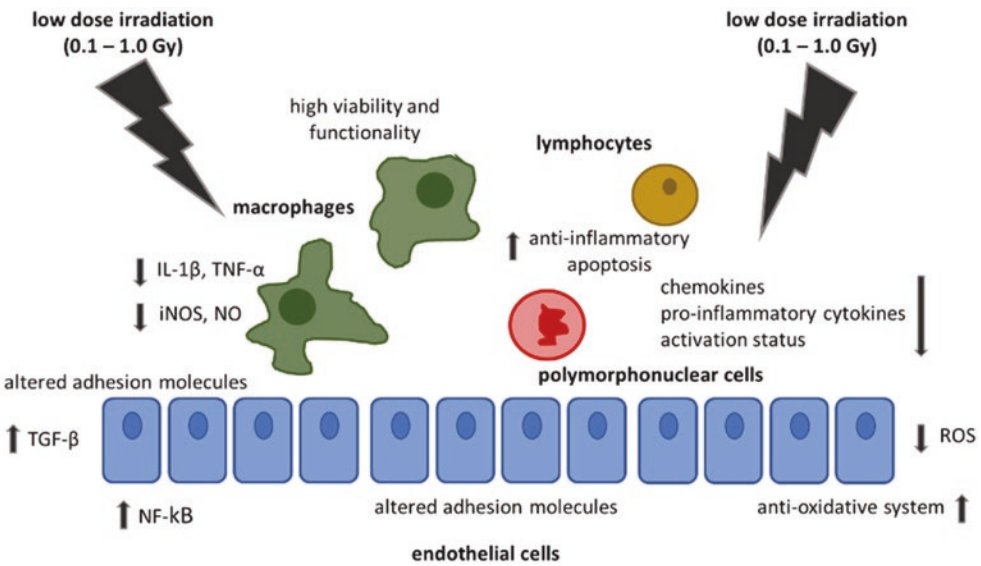

Fig. 3.47 Radiation affects key cells involved in initiation and maintenance of inflammation

3.15.3 Radiation and the Endothelium

Ionizing radiation causes phenotypic changes in endothelial cells, resulting in endothelial activation and ultimately endothelial dysfunction [172]. In vitro, this activation triggers an increase in the expression of the adhesion molecules vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), platelet endothelial cell adhesion molecule (PECAM-1), and E- and P-selectins involved in the recruitment of circulating lymphocytes. In vivo, increased expression of endothelial ICAM-1 and VCAM-1 was demonstrated in a model of radiation-induced intestinal inflammation. ICAM-1 knockout mice showed less severe pulmonary and intestinal inflammation than wild-type mice, suggesting that cellular infiltration may be deleterious in this situation. In humans, the endothelium may be activated by RT via the transcription factor nuclear factor kappa B (NF-kB) pathway, which is likely critical in the development of RT-induced cardiovascular diseases [173]. Overall, these studies demonstrated that radiation-induced increase in adhesion molecule expression by endothelial cells plays a crucial role in circulating cell recruitment and radiation-induced inflammation of the tissue and/or tumor, with a potential deleterious effect on normal tissues. Therefore, the vascular endothelium can be considered as a main control point of radiation-induced inflammatory and immune processes in normal tissues and tumors and may thus cover an ideal target to improve the therapeutic efficacy of radiotherapy of malign diseases. Furthermore, low-dose irradiation was demonstrated to result in a nonlinear expression and activity of major compounds of the antioxidative system in endothelial cells. This might contribute to anti-inflammatory effects in these stimulated cells and be beneficial in low-dose radiotherapy for benign diseases [174]. The effects of higher doses on the immune system in healthy tissue and tumors differ from those of low and intermediate doses and are covered in Chap. 4 (Box 3.30).

Box 3.30 In a Nutshell: Inflammation and Immunity

- Inflammation is intrinsincally linked to the immune response.
- Monocytes/macrophages are key immune cells in the initiation and resolution of inflammation.
- Radiation in a dose range of 0.1–1.0 Gy ameliorates inflammation by mainly affecting macrophages, PMN, lymphocytes, and endothelial cells.
- Ionizing radiation causes several phenotypic changes in endothelial cells
- Vascular endothelium can be considered as a main control point of radiation-induced inflammatory and immune processes.

3.16 CRISPR-CAS9

3.16.1 Definition

Clustered regularly interspaced palindromic repeats (CRISPR)-CAS (CRISPR-associated protein) system is a defense mechanism that has been identified in prokaryotes that effectively acts to fight viruses. The five homologous sequences of 29 nucleotides separated by spacers of 32 nucleotides were observed initially in 1987 by a Japanese research group. The group identified a gene responsible for the conversion of alkaline phosphatase isozyme in *Escherichia coli* [175]. In 2002, another grouping of genes adjacent to the CRISPR locus was revealed which was termed CRISPR-associated system, or Cas. The system has been found in diverse species of bacteria and archaea, however with slightly different composition and mechanism of action. Since this time, new forms of CRISPR systems have been discovered that can be classified into six types and grouped into two classes [176]. Types I–III are well studied, while other types IV–VI, which have more recently been discovered, need further research to fully understand their mechanism of action. These systems have now been realized to be important breakthroughs for modern genetic engineering and are revolutionizing science.

3.16.2 Mode of Action

CRISPR are fragments of RNA that are cloned from the DNA of viruses that have infected a bacterium. Together with other sequences, it forms an adaptive immune system that stores memory of viral DNA within the bacterial host chromosomes. It is comprised of three main components: an RNA sequence made from the relevant CRISPR gene (crRNA) that contains within it a 20-base pair-long sequence complementary to the target DNA sequence; a DNA endonuclease that can edit genes and is referred to as Cas9; and a tracrRNA that acts to help bind the crRNA and Cas9 together. All three components are well studied [177]. In concert, the CRISPR-Cas9 system works to fight virus invasion in prokaryotes. When a bacterium comes across a virus that it was previously exposed to, it produces an RNA copy of the CRISPR that contains that virus' genetic information. The crRNA then binds with the tracrRNA to form a single-guide RNA (sgRNA) that leads the enzyme Cas9 to the correct DNA sequence. The sgRNA binds to the target site in the genome that matches the viral sequence on the crRNA and directs the Cas9 protein to create a double-stranded break. Next to the viral sequence is a protospacer adjacent motif (PAM), which also helps to align the enzyme. Once broken, the strand will experience a change in the viral DNA sequence through the activation of a DNA repair method, either non-

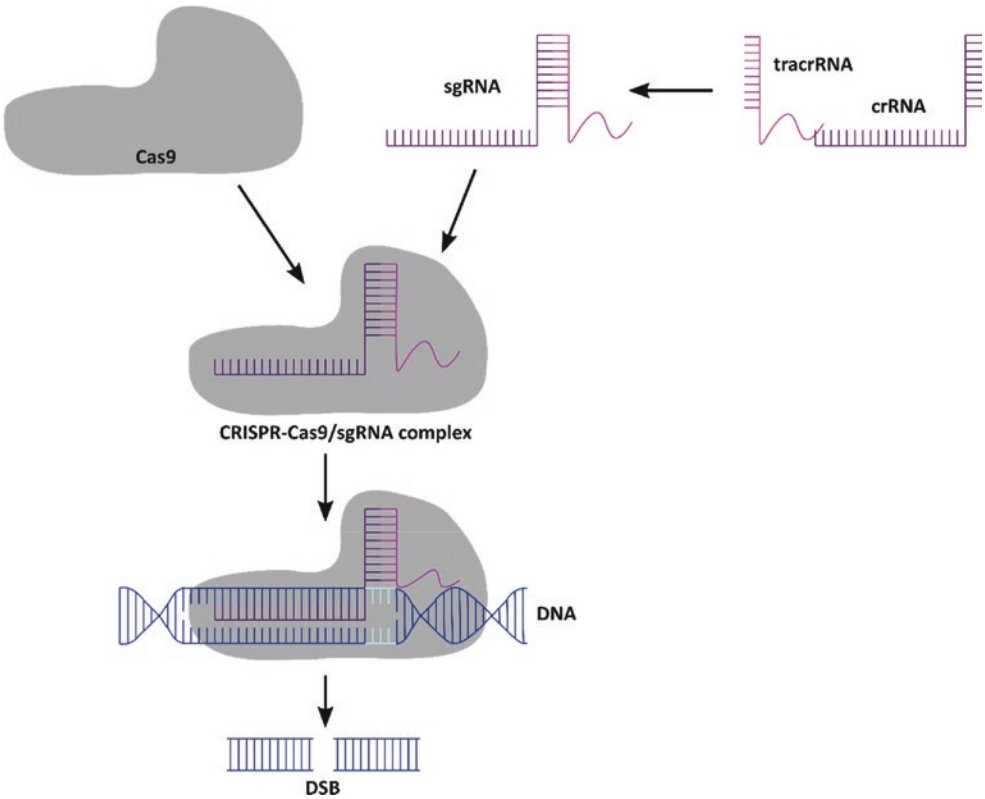

Fig. 3.48 Mechanism of CRISPR-Cas9 to produce a DNA double-strand break. The CRISPR-Cas9/single-guide RNA (sgRNA) complex consists of the Cas9 protein, which is coupled to the sgRNA, consisting of the transactivating crRNA (tracrRNA), responsible for binding of the RNA complex to Cas9 and the CRISPR RNA (crRNA) which encodes the target sequence. The CRISPR-Cas9/sgRNA complex binds to the specifically targeted DNA sequence and induces a DSB. (Adapted with permission (CCBY) from Zhao et al. [178])

homologous end joining or homology-directed repair [177, 178]. The process shown in Fig. 3.48 is very efficient and effective and shown to be a valuable tool for researchers to study gene function and uncover biological mechanisms.

3.16.3 Application

The CRISPR-Cas9 system is unique due to its ability to induce double-strand breaks in almost any type of organism or cell type. The system is more accurate, providing an alternative to previous genome editing tools, such as zinc finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs) [178]. The technology is an efficient genome editing system that can detect, manipulate, and annotate from diverse species-specific DNA sequences. The system is mainly used for studying DNA because manipulating RNA is difficult due to the lack of a PAM sequence, requiring efficient RNA targeting tools. The most widespread application of the CRISPR-CAS system has been in the context of genome editing of DNA, achieved through three mechanisms: (1) nonhomologous end joining, (2) single-base editing enzymes, and (3) homology-directed repair for DNA repair. The system can be delivered virally (adenovirus or lentivirus) or through nonviral mechanisms (hydrodynamic injection, electroporation, nanoparticles, and transposon carriers) and combined [178].

The technology can be applied to develop a better understanding of a specific gene function or the manipulation of genetic material, as genetic sequences can be removed or edited. For example, a select tissue type can undergo multiplex mutagenesis for high-throughput analysis to identify cancer drivers or correction of a loss-of-function mutation; likewise, gene knockout could be used to enhance a specific cell type. Beyond gene editing, researchers have also used the Cas9 unit for targeting purposes instead of catalytically, known as the dead Cas9 (dCas9) [179]. For instance, epigenetic editing involves the alteration of the chromatin structure without modifying the individual's genomic sequence. The dCas9 is fused to a functional DNA methylation or demethylation enzymes or DNA modifiers [179]. The same idea follows CRISPRi and CRISPRa, which repress and inhibit gene expression. The CRISPRi uses the dCas9 to bind to the DNA-blocking RNA polymerase and transcription factor binding, while CRISPRa combines the dCas9 unit and selects transcription factors targeting activating sequences.

Overall, these advancements provide new avenues to study genetic mechanisms and demonstrate the applicational value of CRISPR-Cas-based tools. It is being used with success in the field of agriculture, therapeutics, food industries, and more. The success of CRISPR has inspired efforts to discover new systems for targeting nucleic acids, including those from Cas9, Cas12, and Cas13 orthologues. The approach is gaining traction for use across multiple fields of research.

3.16.4 Challenges

As the field of CRISPR-Cas rapidly evolves, challenges have emerged which have also been the focus of much research. This is particularly in the context of development of treatment modalities for cancer. Some hurdles that have been identified are in relation to methods for effectively delivering the technology into the host that ensures suppression of the innate immune responses. Injection methods are traditionally used to deliver CRISPR-Cas9 components to cells via delivery vectors; however, the efficiencies of these injection methods are dependent on the target cells and tissues. Traditional delivery methods targeting cancer cells are not yet efficient enough to be applied clinically. For CRISPR-Cas9 to be applied as a therapeutic tool in cancer treatment, delivery must be more efficient and accurate which may require novel delivery methods [180].

Apart from limitations with delivery methods, the delivery vehicle itself also prevents a challenge, as delivery vectors hold a limited amount of genomic material. The most used delivery vehicle is adeno-associated virus (AAV) as it is relatively safe and effective; however, this method has a limited packaging capacity due to its size, which restricts the amount of genetic information that can be transferred to the target cell or tissue. AAVs can contain roughly 5 kB of information, while information for the Cas9 protein and the sgRNA which must be included on the plasmid is roughly 4.2 kB in size. To offset this, current research is being done to find smaller Cas9 orthologues, which in the future may allow for more helpful elements to be added such as reporter genes or fluorescent tags to support more successful gene editing [179].

Immune responses to the Cas9 protein have also been well documented in animal models, which presents an added challenge to the clinical application of the CRISPR-Cas system. A high prevalence of the human population has been exposed to the bacteria from which the Cas9 protein originates, meaning that there is likely a large population with preexisting immunity. While the implications of this are not yet entirely clear, testing of Cas9 orthologues may be required before CRISPR-Cas technology can be applied as a therapeutic to prevent T-cell responses. Alternatively, immunosuppressant drugs could potentially be used during treatment [179]. Off-target effects of the CRISPR-Cas system, such as mutations at undesired sites, also present a challenge. Extensive research has been done to minimize these effects; however, further investigation on increasing precision is required to improve safety [181]. As these hurdles become addressed, CRISPR-Cas9 will play a crucial role in medical treatments, including the treatment of cancers, and will effectively support gene therapy modalities (Box 3.31).

Box 3.31 In a Nutshell: CRISPR-Cas

- CRISPR-Cas system is a defense mechanism that has been identified in prokaryotes that effectively acts to fight viruses.
- CRISPR are fragments of RNA that are cloned from the DNA of viruses that have infected a bacteria.
- Cas9 is a DNA endonuclease that can edit genes.
- Together, CRISPR-Cas9 is an efficient genome editing system that can detect, manipulate, and annotate from diverse species-specific DNA sequences.
- It can be applied to develop a better understanding of a specific gene function or the manipulation of genetic material, as genetic sequences can be removed or edited.

3.17 Epigenetic Factors

DNA methylation, histone modifications, and incorporation of histone variants are chemical alterations of the cellular DNA. Such changes are not necessarily permanent and can be influenced by endogenous and exogenous stressors. One of these stressors is radiation. Radiation induces various alterations in these epigenetic modifications, mainly affecting gene expression and DNA repair.

MicroRNAs are small, highly conserved noncoding RNA molecules that regulate gene expression. They are single-stranded RNA transcripts with a length of 21–25 nucleotides that are derived from hairpin loop precursors. miRNAs affect the cellular radiation response via regulation of vital genes involved in DNA damage repair, cell cycle checkpoints, autophagy, and apoptosis.

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts with a length of more than 200 nucleotides missing a distinct protein-coding region. lncRNAs regulate gene expression on multiple levels, including transcription, RNA stability, and translation. Radiation exposure deregulates lncRNA expression, which affects radiosensitivity by interfering with canonical radiation response pathways, such as cell cycle control, DNA repair, and cell death induction.

Circular RNAs (circRNAs) are a recently described class of RNA molecules that are derived from precursor mRNA (pre-mRNA) in a process called backsplicing. Despite increasing attention, the number of studies investigating the direct effect of ionizing radiation on circRNA expression is still very limited. However, it is now evident that circRNAs are affected by irradiation and that they are important players in the cellular radiation response and sensitivity.

Extracellular vesicles (EVs) are generated by all cells within our body and are important communicators in normal and cancer cells. EVs have different sizes ranging from 40 nm up to several μm and are produced via different biogenesis routes. EVs' cargo contains various RNA species, DNA fragments, proteins, and lipids partly reflecting their cell of origin. EVs may influence neighboring cells via their cargo but also act in distant tissue as illustrated in cancer, where they play a role in both carcinogenesis and metastasis. Exosomes are a particular type of EVs formed by viable cells via the endosomal system, and there are specific cellular mechanisms that determine their cargo. Upon radiation, EVs are generated by both normal and cancer cells and transmit effects in irradiated and nonirradiated cells (e.g., bystander or non-targeted effects). EVs may constitute a source of biomarkers for diseases and stress conditions, including radiation.

3.17.1 DNA and Histone Modifications

Modifications of DNA bases and histone proteins, including the incorporation of histone variants, have important functions in the epigenetic control of gene expression. Both types of alterations add further information to the DNA molecule in addition to the genetic code, which contribute to phenotypic changes without altering the DNA sequence. Importantly, such changes are not necessarily permanent and can be influenced by endogenous and exogenous stressors. Enzymes that add, recognize, and dislodge DNA and histone modifications are called writers, erasers, and readers. The generation of modifications is facilitated by writers. Erasers modify and/or remove labels. Readers recognize and associate to modifications [182].

The methylation of DNA is a heritable epigenetic label in dividing cells. Methylation of DNA segments typically induces its silencing, while demethylation is characteristic for actively transcribed regions. Possible mechanisms for these effects are the binding of methyl-DNA-binding proteins, which affect gene activity or alterations of the chromatin structure. In mammals, DNA methylation patterns are retained or established by DNA methyltransferases (DNMTs) that catalyze the addition of methyl groups to nucleotides. S-Adenyl methionine (SAM) acts as a methyl group donor. DNMT1 function is the maintenance of methylation, and DNMT3a/b is responsible for de novo methylation. On the other hand, DNA demethylases can catalyze active demethylation. Most of DNA methylation takes place at cytosines, which are succeeded by a guanine nucleotide (CpG sites). Regions (>200 nucleotides and CG content >50%) with a high frequency of CpG sites are called CpG islands. Promotor sequences are often located within such CpG islands. Methylation of CpG islands silences gene expression, for example by impeding the binding of transcription factors or by recruitment of repressive methyl-binding proteins. The most common modification of DNA bases is the methylation of cytosine on carbon position 5, leading to 5-methylcytosine (5-mC). This modification accounts for approximately 1% of all bases and is therefore sometimes designated as the fifth base of the DNA. Further less abundant modifications are for example 5-hydroxymethylcytosine, 5-carboxycytosine, and 6-methyladenine.

Histone modifications are covalent modifications joined to histone proteins. These modifications impair DNA-histone interactions, thereby changing chromatin architecture and gene expression. Some reduce DNA-histone interactions leading to nucleosome unwinding, chromatin opening, and increased accessibility for the transcription machinery leading to the activation of gene expression (euchromatin). Others increase DNA-histone interactions, leading to tightly packed chromatin followed by reduced access of the transcription machinery and thus gene silencing (heterochromatin). Currently, acetylation, methylation, phosphorylation, and ubiquitination are the most well understood, while others, like GlcNAcylation, citrullination, crotonylation, and isomerization, are more recent discoveries. All of these modifications are highly dynamic and added to or removed from histone amino acid residues by specific sets of enzymes. A well-described posttranslational histone modification is the trimethylation of histone H3 on the lysine located at position 4 of the protruding N-terminal tail (H3K4me3), which is correlated with promoters of actively transcribed genes. In contrast, the trimethylation of lysine on positions 9 (H3K9me3) and 27 (H3K27me3) is a heterochromatin mark, associated with repressed genes.

In addition to posttranslational modifications, the histone structure of the chromatin can also be influenced by the incorporation of histone variants. Histone variants are low abundant and differ only in one or a few amino acids with their canonical counterparts. They are produced throughout the cell cycle and can be deposited into chromatin independent of replication by rapid exchange processes. Histone variants seem to be especially important for protecting genome integrity by the regulation of damaged chromatin accessibility and restoration.

3.17.1.1 DNA and Histone Modifications in the Context of Radiation

Both DNA methylation and histone modifications are essential components in the cellular stress response. Therefore, it is not surprising that various alterations are reported after radiation exposure. On a molecular level, radiation-induced alterations in histone and DNA modifications either are required for the efficient detection and repair of DNA damage to avoid chromosomal instability or lead to changes in transcriptional activity and thereby alter a variety of cellular

processes, including cell cycle regulation, DNA repair, and cell death induction. At organism level, epigenomic alterations were reported in various radiation-induced cancer and non-cancer diseases. As epigenomic alterations can be transferred to the offspring also, the contribution to radiation-induced transgenerational effects was recently suggested [183, 184].

Altered DNA methylation patterns were found in in vitro and in vivo studies in response to irradiation. The majority of studies showed global hypomethylation, which was often linked with a reduced expression of enzymes involved in DNA methylation. As global hypomethylation is connected to malignant transformations, radiation-impaired DNA methylation may contribute to cancer development. However, hypomethylation is not always evenly dispersed across the genome and also radiation-induced hypermethylation was reported for specific loci. Interestingly, new studies imply that low- and high-LET radiations affect methylation differentially.

A variety of radiation-induced histone modifications affecting transcriptional regulation and particularly DNA repair are described. In regard to DNA repair, an intensively studied histone modification is the phosphorylation of the histone H2A variant H2AX at serin-139 (phosphorylated H2AX is designated as γ-H2AX) at sites of DNA double-strand breaks (DSBs). Formation of γ-H2AX is an initial response after exposure and facilitates a cascade of further histone modifications, including ubiquitination of H2A/H2B as well as changes in the acetylation of H3 and H4. Together, these posttranslational histone modifications contribute to chromatin relaxation that enables the accession of DNA repair factors and influences the repair pathway choice. Moreover, the γ-H2AX modification is widely used as a biomarker for DSBs, and a lot of methods were developed to use γ-H2AX counting for DSB quantification.

With the knowledge about DNA methylation and histone modification in radiation response, the targeted modulation of these features is investigated as a novel strategy to radiosensitize tumor cells during radiotherapy. For example, radiosensitizing activity was shown for DNA demethylation agents, like cytidine analogs. In addition, small-molecule inhibitors of histone deacetylases changing histone acetylation showed the potential to alter radiosensitivity.

The first studies in this field also demonstrated an exchange of histone variants in response to radiation exposure. For example, it was shown that the histone variant H2A-Z.2 is incorporated into chromatin immediately after DSB induction, where it contributes to recombinational repair by assisting RAD51 foci formation. In line, H2A-Z.2 U2OS tumor cells were shown to be more radiosensitive than controls. H2A.J, another histone variant, accumulates during radiation-triggered senescence processes in the vicinity of 53BP1 foci and affects the expression of inflammatory genes (Box 3.32).

Box 3.32 In a Nutshell: DNA and Histone Modifications as Epigenetic Factors

- DNA methylation, histone modifications, and incorporation of histone variants are chemical alterations of the cellular DNA.
- Radiation induces various alterations in these epigenetic modifications, mainly affecting gene expression and DNA repair.
- Phosphorylation of histone H2AX (γ-H2AX) is the most prominent radiation-induced epigenetic alteration with significant impact on DNA repair.

3.17.2 MicroRNAs

MicroRNAs are small, highly conserved noncoding RNA molecules that regulate gene expression. They are single-stranded RNA transcripts with a length of 21–25 nucleotides that are derived from hairpin loop precursors. The basic mode of action of miRNAs is competitive partial binding with the 3′ UTR of the target mRNA, which inhibits translation and/or leads to mRNA destruction. MiRNAs have also been shown to interact with the 5′ UTR, coding regions, and gene promoters via binding complementary sequences [185]. Because each miRNA can act on multiple different target genes, and one target gene can be regulated by many different miRNAs, the miRNA-mediated regulation of cellular phenotype is highly complex. miRNA-mediated regulation is thought to affect roughly 60% of all protein-coding genes, according to estimations. To regulate miRNA abundance at the levels of transcription, maturation, and stability, cells have evolved various sophisticated methods to govern such extensive miRNA-mediated functions. miRNA actions have been linked to the regulation of a variety of cellular processes, including cellular homeostasis and stress responses. Furthermore, they have been linked to a variety of diseases. miRNAs, in addition to their intracellular roles, are also found in the extracellular environment. miRNAs can be identified in physiological fluids such as plasma, saliva, and urine. This extracellular miRNA population is varied and heterogeneous. Although the activities of extracellular miRNAs are not completely understood, it has been demonstrated that extracellular microvesicle-embedded miRNAs can be transferred and incorporated into destination cells [186].

3.17.2.1 Radiation Damage and miRNAs

Ionizing radiation (IR) disturbs cellular equilibrium in a variety of ways. Cellular stress pathways shield cells from the harmful consequences of genotoxic assault. Cells respond to ionizing radiation-induced stress by activating several pathways ranging from DNA damage processing, signal transmission, altered gene expression, cell cycle arrest, genomic

instability, and cell death. The available evidence implies that radiation exposure causes cellular responses that are influenced in part by gene expression networks. miRNAs govern several intracellular processes involved in the response to cellular stress and have been demonstrated to regulate gene expression [187].

Radiation exposure, whether accidental or intentional, is a serious public health issue that demands immediate attention for correct diagnosis and clinical planning. Exposure to large doses of ionizing radiation in a short time causes acute radiation syndrome (ARS), often known as radiation sickness or radiation poisoning. ARS involves a total dose of over 0.7 Gy (70 rad) from an external source, administered in a few minutes. Radiation sources might be accidental or deliberate. Several animal species were used to study the effects of radiation on miRNA expression. For ARS, miRNA analysis has been done in murine and nonhuman primate (NHP) models. Several studies employing different mouse strains (CD2F1, C57BL/6J, C57BL/6, and CBA/J) have identified miRNAs as biomarkers for radiation injury and countermeasure efficacy.

While several miRNAs have been proven to be modulated by radiation, not all studies have showed the same miRNAs. However, most studies have shown downregulation of miR-150 and overexpression of miR-30 and miR-126. Exposure to ^{60}Co γ-radiation, high LET, and high-energy particles reduced miR-150 expression (^{56}Fe, iron-56) [188]. In addition to total-body irradiation, miR-150 downregulation was observed in the lung and blood of female WAG/RijCmcr rats irradiated (15 Gy at 1.43 Gy/min), indicating the potential of employing miRNAs for partial-body exposure and the impact on miRNA expression in organs and biofluids [189]. A profile of seven significantly changed miRNAs (miR-150-5p, miR-215-5p, miR-30a-5p, miR-126-5p, miR-133a-3p, miR-133b-3p, and miR-375-3p) was discovered in rhesus macaques 24 h after exposure to ionizing radiation. Differences in the expression of three miRNAs (miR-133b, miR-215, and miR-375) were used to accurately discriminate between irradiated and nonirradiated NHPs. Two miRNAs (miR-30a and miR-126) were able to predict radiation-induced mortality in NHPs in this study. Another study utilizing rhesus macaques found miR-126-3p upregulated and miR-150-5p downregulated. Unlike rhesus macaques, miR-342-3p was shown to be most affected (tenfold persistent downregulation) at 24 and 48 h postirradiation in baboons [190].

miRNAs strongly affect the cellular radiation response via regulation of vital genes involved in DNA damage repair [187], cell cycle checkpoints [191], and apoptosis [187]. Several important miRNAs, as well as their mRNA targets and signaling pathways implicated in radioresistance and radiosensitivity, are depicted in Fig. 3.49a, b, respectively.

The expression of RAD51 and the subsequent formation of RAD51 foci in response to IR are a critical stage in HR. Following IR, RAD51 was revealed to be a direct target of miR-34a, miR-107, miR-155, and miR-222. Overexpression of miR-34a in lung cancer cells prevented the formation of radiation-induced RAD51 foci. Greater miR-155 levels were associated with lower RAD51 expression and better overall survival in a large dataset of triple-negative breast cancer patients. IR-induced damaged DNA is sensed by ataxia-telangiectasia mutated (ATM), which can initiate the signaling pathway, leading to checkpoint activation and DNA repair. ATM was shown to be downregulated by miR-18a in breast cancer, miR-26a in glioma, and miR-421 in squamous cell carcinoma (SCC), making the cells more sensitive to radiation.

Additionally, in response to IR, two miRNAs, miR-24 and miR-138, have been discovered to directly control H2AX. miR-182 suppressed BRCA1, another important protein in HR, in breast cancer cells. miR-875 also hampered the HR pathway by directly targeting the epidermal growth factor receptor (EGFR) and disrupting the EGFR-ZEB1-CHK1 axis.

PI3K/AKT is one of the key downstream targets of EGFR. The PI3-K/AKT pathway is crucial for establishing radiation resistance and intrinsic radiosensitivity of the cell. It is a critical regulator of normal and malignant development and cell fate decisions via activities such as proliferation, invasion, apoptosis, and activation of hypoxia-related proteins in cell signaling cascades. Several miRNAs are known to target and regulate key components of this pathway and help elicit the cellular response to radiation. AKT, an immediate downstream effector of the PI3K cascade, has been found to be directly targeted by miR-150 in natural killer (NK) and T-cell lymphoma cells. In a xenograft mouse model, miR-150 overexpression increased IR-induced apoptosis by decreasing PI3K/AKT signaling and sensitized NK/T-cell lymphoma cells to radiation. Furthermore, through blocking the AKT/GSK3/Snail signaling pathway, miR-203a-mediated ATM downregulation promoted apoptosis and cell cycle arrest in G1 phase in ovarian cancer cells. The tumor suppressor protein phosphatase and TENsin homolog (PTEN) is the central negative regulator of the PI3K/AKT pathway by dephosphorylation of PIP3 at the plasma membrane.

Several miRNAs generate pro-survival signals in response to IR by targeting PTEN. Activation of the PI3K/AKT pathway, suppression of apoptosis, and improved radioresistance were seen when miR-17, miR-20a, miR-106b, miR-205, miR-221, miR-222, and miR-498 were overexpressed. Regulation of PTEN expression is crucial for cell cycle maintenance. In colorectal cancer cells, miR-106b is known to target the CDK inhibitor p21 as well as

Fig. 3.49 (**a**) miRNAs and cellular radioresistance: a summary representation of miRNAs in different cancers (outer circle) that regulate various mRNA targets (middle circle). These mRNA targets in turn influence various crucial biological pathways (inner circle) responsible for cellular radioresistance. Data for the figure acquired and modified from Ebahimzadeh et al. [192] (data taken with permission); [193] (CCBY). Gene names: *P21* cyclin-dependent kinase inhibitor 1, *AIFM3* apoptosis-inducing factor mitochondria-associated 3, *APAF1* apoptotic peptidase-activating factor 1, *BRCA1* breast cancer gene 1, *p53* TP53 gene and tumor protein p53 gene, *RB* retinoblastoma protein, *TCEAL7* transcription elongation factor A-like 7, *PTEN* phosphatase and tensin homolog, *APAF1* apoptotic peptidase-activating factor 1, *MTOR* mechanistic target of rapamycin kinase. *miR* microRNA, *NSCLC* non-small cell lung cancer, *GBM* glioblastoma, *CRC* colorectal cancer, *HCC* hepatocellular carcinoma, *NPC* nasopharyngeal carcinoma, *OSCC* oral squamous cell carcinoma. (**b**) miRNAs and cellular radiosensitivity. A summary representation of miRNAs in different cancers (outer circle) that regulate various mRNA targets (middle circle). These mRNA targets in turn influence various crucial biological pathways (inner circle) responsible for cellular radiosensitivity. Data for the figure acquired and modified from Ebahimzadeh et al. [192] (data taken with permission); [193] (CCBY). Gene names: *STAT3* signal transducer and activator of transcription 3, *CDK4* cyclin-dependent kinase 4, *MCL1* MCL1 apoptosis regulator, *BCL2* family member, *SIRT1* sirtuin 1, *E2F1* E2F transcription factor 1, *P21* cyclin-dependent kinase inhibitor 1, *EGFR* epidermal growth factor receptor, *BCL2* BCL2 apoptosis regulator, *LDHA* lactate dehydrogenase A, *ATM* ataxia-telangiectasia mutated, *AKT* AKT serine/threonine kinase 1, *H2AX* H2A histone family, member X, *Beclin-1* coiled-coil, moesin-like BCL2-interacting protein, *ATG12* autophagy-related protein 12, *TP53INP1* tumor protein p53 inducible nuclear protein 1, *DRAM1* DNA damage-regulated autophagy modulator 1, *UBQLN1* ubiquilin 1, *DUSP10* dual-specificity phosphatase 10, *STMN1*, stathmin 1, *c-MYC* Myc-related translation/localization regulatory factor, *WNT2B* wingless-type MMTV integration site family, member 2B, *WNT* wingless-type MMTV integration site family, member, *PKM2* pyruvate kinase isozymes M1/M2, *LDHA* lactate dehydrogenase A, *MTOR* mechanistic target of rapamycin kinase. *miR* microRNA, *NSCLC* non-small cell lung cancer, *NK/T-cell lymphoma* natural killer/T-cell lymphoma, *SCC* squamous cell carcinoma, *ESCC* esophageal cancer, *GBM* glioblastoma; *CRC* colorectal cancer, *HCC* hepatocellular carcinoma, *NPC* nasopharyngeal carcinoma, *OSCC* oral squamous cell carcinoma, *DSB* double-strand breaks

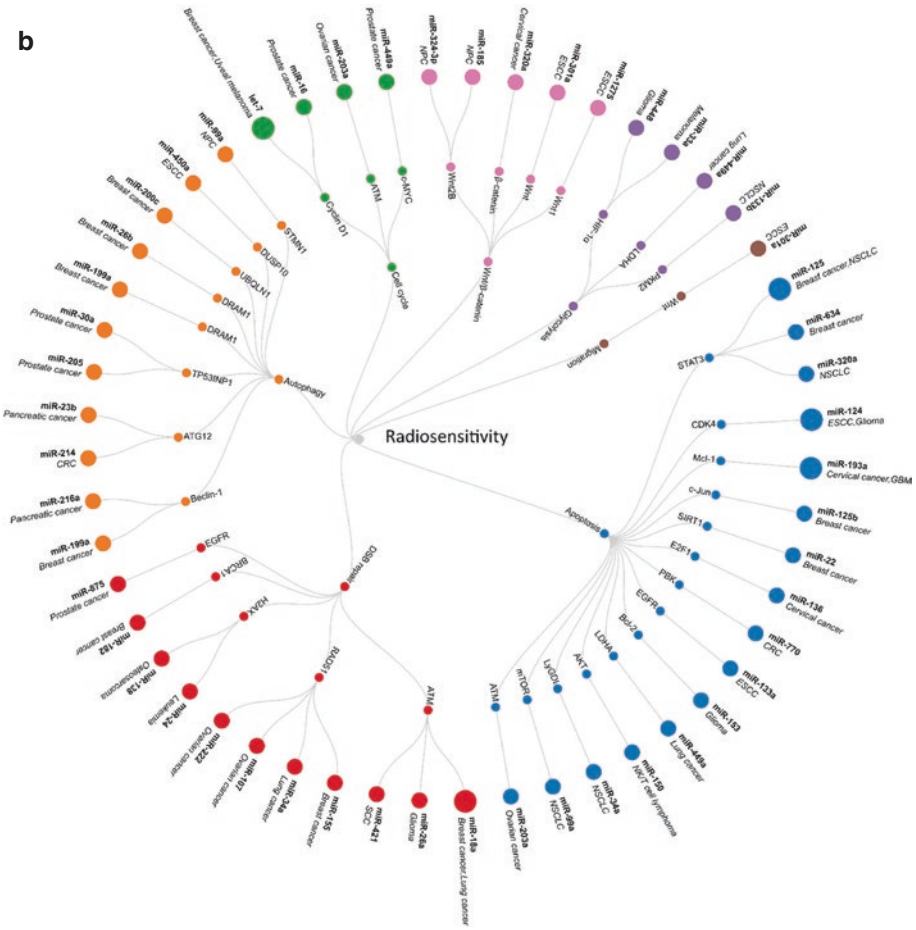

Fig. 3.49 (continued)

PTEN. Overexpression of miR-106b promoted the G1-to-S transition in CRC cells, which was blocked by overexpression of either PTEN or p21. miR-17-mediated PTEN inhibition, like miR-106b, boosted G2-to-M progression and increased NPC cell proliferation through its effects on AKT signaling.

After IR exposure, apoptotic regulatory pathways are activated to remove cells with a high burden of DNA damage. Several miRNAs, including miR-133a, miR-125b, miR-124, miR-320a, and miR-634, are known to exert their effects on IR response via targeting components of crucial survival pathways, i.e., extracellular signal-regulated kinase (ERK), Janus kinase/signal transducer, and activator of transcription (JAK/STAT) as well as PI3K/AKT pathway. These pathways are initiated in response to IR-dependent activation of EGFR. In the JAK/STAT pathway, STAT3 is a direct target of miR-124, 320a, and 634, and the regulatory effect of these miRNAs on STAT3 upon IR is known to promote radiosensitivity. p53 is a critical tumor suppressor that is activated in response to IR to cause cell cycle arrest or apoptosis. Apoptosis mediated by p53 was abolished in IR-treated gastric cancer cells when miR-375 was overexpressed. In lung cancer cells, miR-300 directly regulates Apaf-1, the structural core of the apoptosome. Ectopic miR-300 expression caused radioresistance via reduced Apaf-1-induced apoptosis. P21 (Waf1/Cip1) is a p53 transcription target implicated in both major functions of the tumor suppressor, apoptosis, as well as cell cycle arrest. In oral squamous cell carcinoma (OSCC) cells, miR-17 has been shown to inhibit p21. In xenograft tumors, suppressing miR-17 boosted p21 expression, apoptotic rate, and radiosensitivity. miR-210 improved radioresistance in hypoxic hepatoma cells by targeting AIFM3. The retinoblastoma (Rb) tumor suppressor protein is an important component in the protection of cells from apoptosis. miR-622 was shown to prevent apoptosis by inhibiting the Rb gene in colorectal cancer cells. Another miRNA that reduced IR-induced apoptosis was miR-212, which directly targeted BRCA1 in glioma cells.

IR-induced autophagy is important in defining cell fate and determining whether cells survive or die, and it also impacts radiosensitivity. A multitude of proteins, including Beclin-1, LC3B-II, mTOR, and other autophagy-related proteins, are crucial for the regulation of this multistep process. Beclin-1 is an autophagy central regulator that regulates autophagosome nucleation and maturation. Beclin-1 is known to be directly regulated by miR-216a and miR-199a, which inhibit autophagy and promote radiosensitivity in response to radiation. mir-199 has also been shown to regulate the DNA damage-regulated autophagy modulator protein 1 (DRAM1) in response to IR. In breast cancer cells, miR-26b also targets DRAM1. miR-23b reduced IR-induced autophagy by targeting ATG12, a ubiquitin-like protein involved in the production of autophagy vesicles. miR-214 also targets ATG12, which enhances radiosensitivity while blocking IR-induced autophagy in CRC both in vitro and in vivo. Several additional miRNAs that target autophagy activators have been demonstrated to suppress IR-induced autophagy. These include miR-200c, which targets ubiquilin-1 (UBQLN1), an autophagosome formation promoter; miR-101, which targets autophagy activator stathmin 1 (STMN1) in NPC cells; miR-30a and miR-205 in prostate cancer cells; and miR-450, which targets DUSP10. miR-1246 was one of the miRNAs that enhanced autophagy in NSCLC cells. In vitro and in vivo, ectopic expression of miR-1246 reduced mTOR activity and radiosensitivity in lung cancer cells.

miRNAs have been proven to be valuable diagnostic and prognostic biomarkers in the clinic for over three decades. miRNAs are found in plasma, serum, blood, and urine and even retrieved from formalin-fixed tissues. These benefits make it a biomarker that is persistent after IR exposure and allow for less invasive testing. Two miRNAs (miR-30a and miR-126) were found as predictors of radiation-induced death in nonhuman primates. Another study suggested that serum miRNAs could be utilized as functional dosimeters to detect early hematopoietic radiation harm. After 2 Gy total-body irradiation, miR-130a-3p expression increased, but miR-150-5p, -142-5p, -706, and -342-3p expression dropped. Determining the sublethal dose of 6.5 Gy required five miRNAs (miR-136-5p, -173p, -126-3p, -322-3p, and -34b-3p), while miR-30a-3p/30c-5p discriminated the lethal (8 Gy) and sublethal (6.5 Gy) groups. miRNAs can be used as clinical biomarkers to predict prognostic irradiation effects, in addition to radiation harm biomarkers. Some miRNAs show sensitivity or resistance to IR in cancer patients who have already received radiotherapy (Fig. 3.47). These miRNAs may be utilized as radiosensitivity or radioresistance biomarkers. miRNAs may soon be acknowledged as biomarkers at the level of proteins, which will be utilized to promptly classify harm from radiation exposure, as well as treatment responses, adverse reactions, and personalized radiotherapies.

Research conducted thus far shows a relevant role for miRNAs in the future of radiation oncology, which may offer the basis for predicting patient response to radiotherapy and aid in developing miRNA-based individualized treatments to improve radiosensitivity. Early research indicated that the use of miRNAs as a biomarker for therapeutic monitoring and prognosis, and hence for more precise and individualized patient treatment, is feasible. Applications of miRNA for treatment as radiosensitizers are currently limited to cell culture or xenograft model systems and will need to be expanded into in vivo applications in the future. The role of extracellular miRNAs is still unknown. A thorough examination of radiation-induced mechanisms for secretion, transfer, and activity in recipient cells may aid in the understanding of major RT issues such as abscopal effects and radiation-induced secondary cancers (Box 3.33).

Box 3.33 In a Nutshell: MicroRNAs as Epigenetic Factor

- miRNAs are small, highly conserved noncoding RNA molecules that regulate gene expression.
- miRNAs can be identified in physiological fluids such as plasma, saliva, and urine.
- miRNAs have been identified as biomarkers for radiation injury and countermeasure efficacy.
- miRNAs affect the cellular radiation response via regulation of vital genes involved in DNA damage repair, cell cycle checkpoints, autophagy, and apoptosis.

3.17.3 Long Noncoding RNAs

3.17.3.1 lncRNA basics

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts with a length of more than 200 nucleotides missing a distinct protein-coding region. In the genome, they are located in intergenic, intronic, and exonic regions as well as sense, antisense, and bidirectional with transcripts overlapping sometimes genes [194]. In humans, 30,000–60,000 long noncoding transcripts are estimated compared to 20,000–25,000 protein-coding mRNA transcripts. Details about the biogenesis and functions of lncRNAs are very well summarized by Statello and colleagues [195]. In principle, the biogenesis of most lncRNAs corresponds to the production of mRNAs with transcription by RNA polymerase II and subsequent 5′-end capping and 3′ poly-A-tailing. In comparison to mRNAs, lncRNAs are less efficiently processed and often remain in the nucleus. As mechanisms for nuclear retention, tethering, or degradation on chromatin, weak splicing signals and *cis*- and *trans*-acting motifs are suggested. However, a substantial proportion of lncRNAs is distributed to the cytoplasm, where they can be sorted to specific

organelles (e.g., mitochondria and exosomes) or they associate with diverse RNA-binding proteins. A considerable amount of lncRNAs assembles with ribosomes.

Initially, lncRNAs were considered as transcription by-products, but meanwhile important cellular functions are described for an accumulating number of lncRNAs. In general, lncRNAs are regulators of gene expression, which interact with DNA, RNA, and proteins on various levels. There are examples for both lncRNAs acting locally at the site of transcription (*cis*-acting) and lncRNAs leaving the site of transcription (*trans*-acting). To activate or suppress gene transcription, lncRNAs can regulate chromatin structure to change their accessibility or sequester chromatin-modifying proteins from or to the promoters of target genes. In addition to their roles in transcription regulation and nuclear organization, lncRNAs are involved in posttranscriptional regulation. This can occur by the association between lncRNAs and RNA processing proteins, resulting in altered mRNA splicing and turnover. Other lncRNAs can directly base pair with RNAs and subsequently recruit proteins involved in mRNA degradation or they support translation by promoting polysome association. Also, the binding between lncRNAs and microRNAs can regulate gene expression as miRNAs are sequestered from their target mRNAs by binding to an lncRNA [=sponge or competitive endogenous (ce) RNA] and thus abolish the inhibitory effect of miRNAs on mRNAs.

Through their manifold impacts on the regulation of gene expression, lncRNAs affect widespread aspects of physiology, including differentiation, growth, and responses to diverse stimuli and stresses.

3.17.3.2 lncRNAs in Radiation Response

lncRNAs are involved in many aspects of cellular response to radiation. For a detailed overview, see May et al. [196] and Podralska et al. [193]. Firstly, radiation affects the expression levels of a plethora of lncRNAs in cancer and non-cancer tissues and both up- and downregulation are reported. Radiation-triggered changes are also reported for a wide dose range including low doses (below 100 mGy) as well as therapeutically relevant doses and for single and chronic treatments. The functional relevance in radiation response was shown for a considerable number of lncRNAs, where some enhance radiosensitivity and others increase radioresistance. The affected pathways cover crucial pathways of cellular radiation response, such as cell cycle control, DNA damage repair, and apoptosis. As the mechanism during radiation response of action, frequently, the sponging of microRNAs by lncRNAs and thereby promoting of the expression of target genes are described.

The broad effects of irradiation on lncRNAs suggest valuable applications of this class of RNAs. Applications of biomarkers for radiation exposure may be important for biodosimetry or markers for normal tissue effects and radiotherapy response. Moreover, in vitro and in vivo studies demonstrated that modulation of the levels of lncRNAs can significantly enhance radiosensitivity of tumor cells. This suggests that lncRNAs may be used as targets to improve the outcome of radiotherapy in the future.

Prominent examples for lncRNAs with multiple roles in radiation response are HOTAIR, PVT1, and MALAT1. In breast cancer models, HOTAIR has been shown to increase radioresistance through interfering with DNA damage repair by targeting miR-218 and miR-449b-5p. In pancreatic ductal adenocarcinoma (PDAC), HOTAIR was induced by radiation, while a knockdown increased radiosensitivity. The knockdown increased the expression of Wnt inhibitory factor 1 (WIF-1), which was shown to enhance radiosensitivity. HOTAIR also promoted radiosensitivity of PDAC by increasing autophagosome formation through increasing LC3-II and ATG7A proteins. In cervical cancer, knockdown of HOTAIR increased radiosensitivity by the induction of a G1 cell cycle-phase arrest.

PVT1 contributes to NF90 transcription and HIF-1α stabilization in nasopharyngeal cancer, resulting in enhanced radioresistance. On the other hand, the knockdown of PVT1 resulted in reduced phosphorylation of ATM, p53, and CHk2 leading to increased radiosensitivity by decreased DNA damage signaling and increased apoptosis. In non-small cell lung cancer, PVT knockdown increases radiosensitivity by sponging miR-195.

lncRNA MALAT1 was downregulated after radiation in esophageal squamous cell carcinoma, and its overexpression enhanced radioresistance. It was shown that MALAT1 inhibited the downregulation of cyclin-dependent kinase subunit (Cks1), which resulted in a decrease in irradiation-induced apoptosis. MALAT1 also affected IR-induced apoptosis by interacting with miRNAs. In nasopharyngeal cancer cells, MALAT1 associated to miR-1, which led to increased levels of the anti-apoptotic protein SLUG. In cervical cancer cells, MALAT1 directly interacted with miR-145 to affect radiation-induced apoptosis (Box 3.34).

Box 3.34 In a Nutshell: Long Non-coding RNAs as Epigenetic Factor

- Long noncoding RNAs (lncRNAs) are transcripts >200 bp, which are not translated into proteins.
- lncRNAs regulate gene expression on multiple levels, including transcription, RNA stability, and translation.
- lncRNA expression is deregulated after radiation exposure, and they affect radiosensitivity by interfering with canonical radiation response pathways, such as cell cycle control, DNA repair, and cell death induction.

3.17.4 Circular RNAs

Circular RNAs (circRNAs) are a recently described class of RNA molecules that are derived from precursor mRNA (pre-mRNA) in a process called backsplicing. During this process, which is regulated by the spliceosome, a splice donor is joined to an upstream splice acceptor. This generates a covalently closed RNA molecule, which is typically resistant to degradation by exonucleases, and therefore circRNAs are in general biologically more stable compared to their linear counterparts. Although most circRNAs are expressed in relatively low levels, their increased stability can result in accumulation to levels far exceeding those of their cognate linear mRNAs [197]. This is for instance observed during aging, which led to the hypothesis that certain circRNAs may represent biomarkers for aging tissues (such as the brain) and aging-associated diseases. Recent studies even implicate circRNAs as causative factors in aging and cellular senescence [198]. Since irradiation and excessive DNA damage are often proposed as inducers of senescence and accelerated aging, radiation-responsive circRNAs may contribute to these longer term effects of radiation exposure.

3.17.4.1 Biogenesis and Functions

A detailed description of the biogenesis and function of circRNAs is beyond the scope of this chapter; we therefore refer the readers to some excellent reviews about these subjects [198] and will only briefly discuss matters that may directly relate to DNA damage and radiation.

Unlike original views that circRNAs are no more than aberrant by-products of normal splicing, it has become increasingly clear that they are often generated and function independently from their linear cognates. One important mechanism of circRNA biogenesis acts via the RNA-binding protein quaking (QKI). QKI is an alternative splicing factor that belongs to the STAR family of KH domain containing RNA-binding proteins and binds to specific sequences (QKI-binding motifs) in pre-mRNA [199]. The proposed mechanism for the role of QKI in circRNA biogenesis is that it binds motifs in introns adjacent to the circle-forming exons and subsequently forms a dimer to bring these exons into close proximity for further processing by the splicing machinery [200]. Importantly, QKI is expressed at low levels in epithelial cells but is increased during epithelial-to-mesenchymal transition (EMT), when cells reprogram their gene expression profiles resulting in the loss of intracellular junctions, polarity, and cytoskeletal organization, ultimately leading to a more migratory and invasive mesenchymal phenotype. The increase of QKI during EMT triggers the expression of hundreds of circRNAs [200]. EMT is a process which can be induced by irradiation, and very often the mesenchymal cells display a more radiation-resistant phenotype, highlighting the relevance of EMT, and therefore QKI-regulated circRNAs, for radiation and cancer biology.

Different functions for circRNAs have been identified, including (1) binding and transportation of RNA-binding proteins; (2) generation of protein isoforms; and (3) regulators of transcription and alternative splicing (e.g., Xiao et al. [198]). However, the most established and investigated function of circRNAs is the regulation of microRNA expression and subcellular localization via a sponging mechanism (competing endogenous RNA, ceRNA). However, since most circRNAs are expressed at only low levels, and they usually contain only a limited number of microRNA-binding sites, it is now clear that the function of microRNA sponges or ceRNAs to regulate the expression of microRNA targets cannot be generalized for many circRNAs [198].

An important consideration here is that studies often perform gene ontology enrichment analyses based on the functions of the host genes of differentially expressed circRNAs. However, since there is currently little evidence that circRNAs in general function in the same pathways as their hosts, such analyses should be critically interpreted.

3.17.4.2 circRNAs, Radiation Exposure, and Radiosensitivity

Despite increasing attention, the number of studies investigating the direct effect of ionizing radiation on circRNA expression is still very limited. On the other hand, there have been quite some studies in which the differential expression of circRNAs between radiation-sensitive and radiation-resistant cancer cell lines and patients was compared.

In HEK293-T cells, gamma irradiation (8 Gy, single dose) resulted in very big differences in the expression of circRNAs between control and irradiated cells. Here, the authors focused only on circRNAs detected under both experimental conditions and identified a total of 158 differentially expressed circRNAs. However, among 5592 detected circRNAs in total, 2205 were detected uniquely in control cells while 1026 circRNAs were uniquely found in irradiated cells. This indicates that the differences were actually larger than was reflected by the 158 that were considered to be differentially expressed.

A study by O'Leary and co-workers investigated differential circRNA expression at 4 h and 24 h after exposure of endothelial HUVECs to a medium (0.25 Gy) and high dose (2.5 Gy) of g-rays [202]. Radiation-responsive circRNAs were predominantly produced from genes involved in the p53 pathway, as is in general the case for the early transcriptional response to radiation. The authors furthermore focused on two circRNAs derived from the *WWOX* gene, showing that they are differently regulated by QKI in response to radiation depending on the cell type and that they are enriched in exosomes [202].

Another study focused on specific p53-dependent genes and their circRNA abundance in the embryonic mouse brain and primary neurons [203]. This study showed that the temporal induction of circRNA expression follows that of their linear mRNA hosts and that they remained more abundant for a longer time after irradiation compared to mRNA. This may have important implications for the use of circRNAs as long-term biomarkers of radiation exposure [203]. Indeed, gene expression changes at the level of mRNA are usually short-lived. Therefore, the increased stability of circRNAs may result in prolonged radiation-induced expression as was shown by Mfossa and co-workers [203].

3.17.4.3 Examples of Important circRNAs for Radiation Biology

circPVT1

One of the most extensively studied circRNA host genes related to radiation and cellular radiosensitivity is *PVT1*, a long noncoding RNA (lncRNA) gene from which different circRNAs can be generated. One of these, termed circPVT1 (CircBase ID: hsa_circ_0001821, consisting of the exon 2 of the PVT1 mRNA), is downregulated during both multiplicative and radiation-induced senescence in human diploid WI-38 fibroblasts. This leads to reduced sponging of the hsa-let-7 microRNA and a subsequent reduction of proliferative proteins encoded by let-7 targets (e.g., IGF2BP1, KRAS, and HMGA2) that prevent senescence. Thus, circPVT1 is a suppressor of (radiation-induced) senescence by acting as a decoy for let-7. Interestingly, linear PVT1 lncRNA was not decreased in senescent cells, indicating that the observed effects were exclusively regulated by circPVT1 [204].

Pvt1 was one of the p53 target genes investigated in the aforementioned study of Mfossa et al. [203]. Also, in human head and neck squamous cell carcinoma cells, circPVT1 expression was found to be dependent on p53 as it was enriched in tumors with p53 mutations and silencing of p53 resulted in a downregulation of circPVT1, but not linear PVT1 [205]. Several other studies have implicated circPVT1 as an oncogene in different cancers, and it enhances to chemotherapy resistance in gastric cancer cells and lung adenocarcinoma by acting as a ceRNA for miR-124-3p and miR-145-5p, respectively [201, 205]. In non-small cell lung cancer, circPVT1 expression is induced after irradiation, while it enhances radiosensitivity via inhibition of the PI3K/AKT/mTOR pathway through sponging of miR-1208 [206].

circ-AKT3

The PI3K/AKT/mTOR pathway plays a central role in cancer cell radioresistance in part via activation of EMT [207]. Inhibitors are currently being investigated as therapeutics to improve radiotherapy outcome. AKT is a serine-threonine kinase that exists in three isoforms, AK1, AKT2, and AKT3. The AKT3 gene hosts different circRNAs. Of these, circ-AKT3 (hsa_circ_0017250) is a protein-coding circRNA that competes with AKT phosphorylation, thereby reducing radiation resistance of different GBM cell lines. In contrast, another circ-AKT3 transcript (hsa_circ_0000199) increases chemoresistance of gastric cancer to cisplatin by upregulation of PIK3R1 (Huang et al. 2019). This suggests that different circRNAs originating from the same host gene can have opposite biological functions, as is sometimes also observed with linear splice variants. This furthermore highlights the importance of functional characterization of individual circRNAs. Several other circRNAs have been demonstrated to affect PI3K/AKT/mTOR signaling. Some of these have been described in the review papers by Cui et al. [201] and Jeyaraman et al. [208].

3.18 Future Perspectives

Altogether, it is now evident that circRNAs are affected by irradiation and that they are important players in the cellular radiation response and sensitivity. However, their exact functions in these processes, which furthermore may be cell type dependent, need to be investigated in a case-by-case manner. Novel methods for the genome-wide identification and functional characterization of circRNAs may prove to be useful tools for these future investigations [209].

3.18.1 Extracellular Vesicles

Extracellular vesicles (EVs) are particles generated by all cells in our body by different routes and differ in diameter from <50 nm up to several μm [210]. EVs can based on their physical and molecular characteristics be divided into exosomes, ectosomes, microvesicles, microparticles, oncosomes, and apoptotic bodies. Size and expression of certain proteins reflecting their biogenesis and cellular origin are used for their classification (Table 3.15) [211]. Physical properties, e.g., size, density, and solubility of EVs, are often used for the isolation by differential high-speed centrifugation, size-exclusion chromatography, and precipitation. However, due to overlapping characteristics, pure preparations of individual EV species are challenging.

EVs are enclosed by a lipid-bilayer membrane, and their cargo includes coding and noncoding RNAs, genomic and mitochondrial DNA fragments, proteins, metabolites, and lipids. Initially, EVs were discovered as "garbage bins" to remove unwanted materials. Now, it is clear that most of the cells in our bodies utilize EV secretion into its close or distant microenvironment as a way of communication [212]. Thus, EVs can transfer functional biological molecules to recipient cells either by direct fusion with the

Table 3.15 Characteristics of different extracellular vesicles (EVs)

Type of vesicle [size (nm)]	Description of characteristics
Microvesicles 100–1000 nm	A subgroup of EVs generated at the cell membrane. Found in both body fluids and tissues
Apoptotic bodies 500–2000 nm	A subgroup of EVs composed of cellular organelles and cytoplasm. Formed during apoptotic cell death by budding after the plasma membrane has undergone blebbing
Ectosomes 100–1000 nm	Membrane microvesicles produced by neutrophils or monocytes formed by direct budding from cell membrane. Vesicles larger than 350–400 nm are not always considered as true ectosomes
Oncosomes 100–500 nm Large oncosomes 1–10 µm	EVs of different sizes generated by tumor cells which function as transmitters of oncogenic signals (RNA, protein complexes) between cells
Exosomes 40–150 nm	Membrane-bound EVs formed by the endocytic pathway. These EVs are first formed at the plasma membrane and subsequently transformed into early endosomes. These subsequently mature into late endosomes where they bud off to the ER intracytoplasmic lumen. The formed multivesicular bodies thereafter are unified with the cell membrane, and exosomes are released to the extracellular surroundings of the cell. Exosomal markers include CD63, CD9, CD81, and TSG101 among others

plasma membrane or by internalization but also via interaction with cell surface receptors triggering downstream signaling (Box 3.35).

Box 3.35 In a Nutshell: Extracellular Vesicles

- Extracellular vesicles (EVs) can be of different sizes and are generated via different biogenesis routes from all cells within our body.
- EVs cargo RNA, DNA fragments, lipids, and proteins partly reflecting their cell of origin.
- EVs are important communicators in health and disease.
- EVs regulate carcinogenesis and metastasis.
- Exosomes are generated via the endosomal system and are released from viable cells.

EVs are found to be an integrated part of cell-to-cell communication, thereby contributing to regulation of the immune as well as the nervous system but also to tissue regeneration after damage [213]. Also, in the carcinogenesis and cancer metastasis fields, EVs have been demonstrated to be important communicators. Thus, EVs regulate the tumor and the tumor microenvironment signaling including angiogenic promotion, conversion of fibroblast into cancer-associated fibroblasts, and interplay with the immune system, thereby providing a good milieu for disseminated tumor cells to grow as well as establish themselves as metastases.

Given that EVs can influence a multitude of cell and tissue processes, it is not surprising that EVs today are considered an important source of biomarkers of different diseases including cancer. Thus, analyses of EVs and their cargo have been able to gain the US Food and Drug Administration (FDA) and international approvals to some extent [214]. With such diverse and varied roles of EVs in assisting cancer progression, it is essential that one can understand how IR, given its essential place in cancer therapy, can alter EVs cargo and/or function.

3.18.1.1 Exosomes

Exosomes are generated in the endosomal system of almost all cells (Fig. 3.50). These vesicles of nano size have membranes with parts from their cell of origin but also cargo membrane and cytosolic lipids, proteins, as well as various RNA species and DNA fragments [215].

When exosomes were identified in the 1980s, they were seen upon as "garbage bins," but later it was reported that exosomes generated from B lymphocytes could trigger a T-cell response. From that time, exosomes have been shown to participate in a multitude of cell–cell communication routes by carrying cargoes which are taken up by recipient cells close by or in a multicellular organism, in another tissue as exemplified in the cancer metastasis process [216]. The scientific community have gathered data on the exosome cargo, e.g., protein, lipid, and mRNA or miRNAs, into large databases, e.g., ExoCarta (http://www.exocarta.org/) and Vesiclepedia (http://www.microvesicles.org/), which are growing as more exosomes from cells of different origin and in different contexts are being deciphered and reported (Box 3.36).

Box 3.36 In a Nutshell: Exosomes

- Exosomes are EVs of endosomal origin, which contain nucleic acids, membrane and cytosolic proteins, metabolites, and lipids.
- Once released, exosomes may act on cells in close vicinity or in a distant tissue.
- Exosomes are involved in a multitude of human diseases including cancer where they regulate carcinogenesis, tumor-immune cell interplay, angiogenesis, and metastasis.

It is still to some extent difficult to sort out plasma membrane-derived EVs from exosomes as their sizes are similar and given that there is a cell heterogeneity in the expression of the protein markers that define exosomes

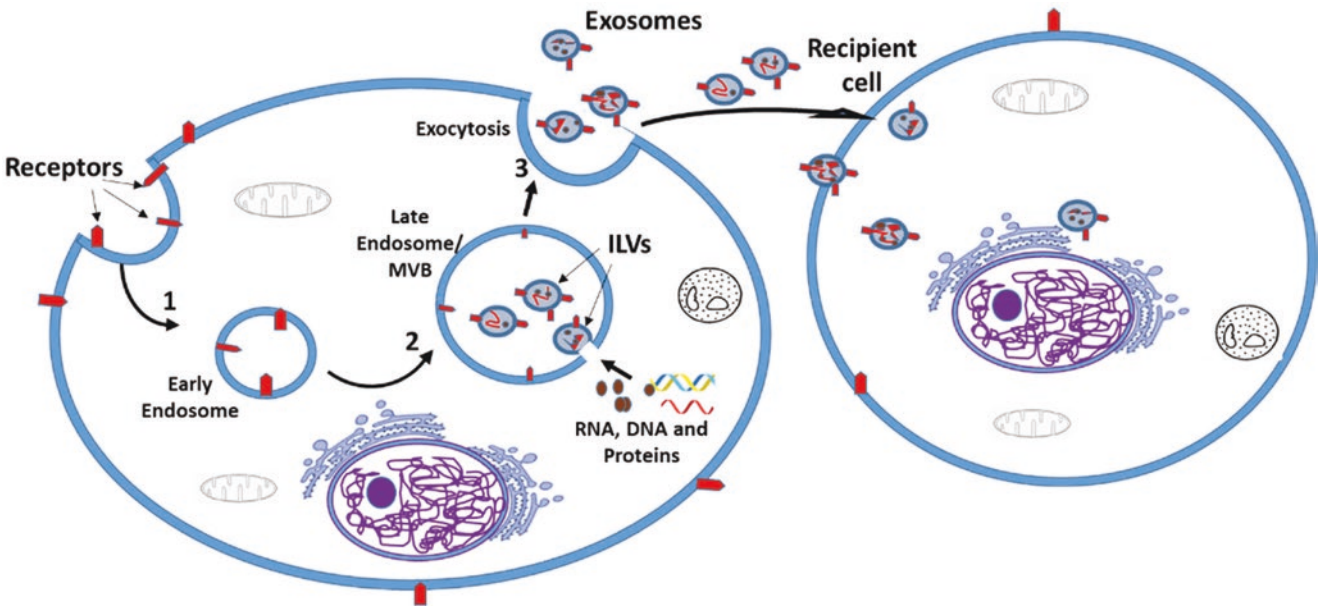

Fig. 3.50 Principal steps in exosome biogenesis. The early endosomes, which are generated at the plasma membrane (1), later undergo maturation, called late endosomes or multivesicular bodies (MVBs) (2). The MVBs' membrane invagination results in the formation of intraluminal vesicles (ILVs). During the invaginating process, particular proteins are incorporated into the invaginating membrane. Other cytosolic biomolecules, i.e., nucleic acids and proteins, are engulfed and enclosed within ILVs. The release of exosomes into the extracellular environment happens after fusion of the MVB with plasma membrane (3)

[210]. However, there are certain proteins that characterize exosomes including Rab GTPases, flotillin, heat-shock proteins (HSP70 and -90), tetraspanins (CD63, CD9, CD81, and CD82), Alix, flotillin, and TSG101. These markers are also suggested by the International Society for Extracellular Vesicles (ISEV) to be used to define exosomes [210].

The lipid membrane of EVs has a different composition relative to plasma membranes. Thus, EV membrane has higher level of sphingomyelin, cholesterol, ceramide, and phosphatidylserine while less expression of phosphatidylcholine. These lipids have been shown to have a profound effect on carcinogenesis and cancer progression including enhancing invasiveness, angiogenesis, and chemoresistance via transport of oncogenic elements.

There is clear evidence that cancer cells may have another rate of exosome release than non-transformed cells [217] while it is still a controversy as to what extent that is reflected in human liquid biopsies, e.g., plasma, and if it can be linked to therapy response. Also, it has been recognized that cancer and normal cells differ with respect to exosome cargo, e.g., miRNA, mRNA protein, and lipids. Exosomes have been found in plasma, serum, lymph fluid, bronchial fluid, cerebral spinal fluid (CSF), urine, saliva, tears, bile and gastric acid, amniotic fluid, breast milk, semen, and synovial fluid [218]. This has spurred an interest in their role as a source of biomarkers.

As indicated above, in human tissues, exosome can act near its cell of release or be transported in the blood to a distant tissue, e.g., site of metastasis in the context of cancer. It has been demonstrated in a large number of publications that once the exosome cargo reaches its target cell, several mechanisms cooperate for uptake as well as for altering signaling in the recipient cells [219]. Similar to EVs, exosomes participate in different processes of the immune system as well as in neurological signaling processes. Exosomes also have a clear function in cancer signaling. Exosomes are described to regulate tumor internal signaling but also tumor–tumor microenvironment interplay. For example, exosomes may promote angiogenesis as well as metastatic spread, and they are important communicators between tumor and different infiltrating immune cells.

Exosomes may exert these events by modulating paracrine, autocrine, and endocrine pathways in different cell types via their cargo. The exosome surface proteins are reported to resemble those of plasma membrane and endosome of a given cell yet with minor contribution of proteins from nucleus or Golgi. It has also been reported that EV membrane composition differs from plasma membrane concerning their lipids. Thus, EV membrane has higher level of sphingomyelin, cholesterol, ceramide, and phosphatidylserine while less expression of phosphatidylcholine.

3.18.1.2 Exosome RNA Loading

Exosomes carry a wide range of cargoes, and it is currently thought that such cargoes, e.g., RNA species, are selectively loaded into exosomes and that loading is not a random pro-

cess. This is supported by observed differences in miRNA abundances in cells compared to exosomes, which have been linked to 3′ uridylation in miRNAs of exosomes, while 3′ adenylated miRNAs are enriched in cellular fractions [220]. Moreover, certain sequence motifs are recognized by the nuclear ribonucleoprotein A2B1 (hnRNPA2B1), which then dictates the miRNA loading process into exosomes, possibly via interaction with cytoskeletal components. The protein AGO2 has also been shown to selectively package exosomes with miRNAs specifically miR-451. In addition, overexpression of the protein neutral sphingomyelinase 2 has been associated with an increase in exosome-associated miRNA. It has also been demonstrated that 3′ mRNA fragments are enriched in exosomes. The conserved 25-nucleotide sequence (also known as a zip code-like 25 nucleotide) is usually incorporated into mRNA's 3′-untranslated region and expressed in many types of cells, leading to mRNA enrichment in the MVs/exosomes. It has been suggested that miR-1289 plays a crucial role in MV enrichment of the mRNA via binding to zip code sequence directly.

3.18.1.3 Exosome Release and Functional Effects

The release of exosomes requires the movement of late endosomes/multivesicular bodies (MVBs) to the cell surface, where they fuse with the cell membrane (Fig. 3.50). The actin cytoskeleton and microtubule network have been shown to be important in facilitating MVB movement towards the cell surface, while Rab GTPases facilitate the release of exosomes into the extracellular space. Interestingly, certain Rabs have been demonstrated to preferentially export exosomes with certain phenotypes; for example, Rab27A/B have been shown to release exosomes positive for CD63-, TSG101-, and Alix expression [221].

Exosomes may, via their cargo, induce both pro-survival and pro-death signaling in recipient cells. Thus, exosomes may promote tumor growth as well as induce inflammation through activation of mesenchymal stem cells (MSCs) and the subsequent secretion of IL-6 as well as IL-8. There is also evidence that exosomes carry inhibitors of apoptosis proteins (IAPs), e.g., survivin, XIAP, cIAP1, and cIAP2, the delivery of which is postulated to offer protection from a continually changing microenvironment, thereby helping tumor progression.

In the context of tumor and immune cell interplay, there is growing substantiation that tumor cells of different origin can via their exosome cargo impair infiltrating T-cell function as illustrated by PD-L1-expressing exosomes [222]. Similarly, CD73, an ecto-5′-nucleotidase and a master regulator in the tumor-immune microenvironment, has been reported in exosomes and also to have functional activity in this context, e.g., impairing T-cell function [222]. The effect exosomes have on tissue poses a number of interesting questions when it comes to radiation biology as radiation has tumor growth-inhibiting effects yet may negatively influence certain normal tissue in the radiation therapy field. Exosomes are also thought to offer some beneficial properties against different tissue damages. Thus, exosomes from MSCs have been reported to offer protection against diabetic nephropathy in the renal system by blocking apoptosis as well as promote vascular regeneration. Moreover, acute kidney injury caused by the DNA-damaging agent cisplatin was found to be blocked by microvesicles as a result of inhibition of apoptosis. Exosomes have moreover been shown to enhance recovery from ischemic brain injury through promoting angiogenesis and providing an extracellular milieu for appropriate brain remodeling.

3.18.1.4 Extracellular Vesicles in Radiation Responses

As EVs are important regulators of multiple cellular signaling events, it is not surprising that EVs are also important communicators in the context of IR [223]. EVs are affected by IR on multiple levels, including alterations in subtype/size, release, cargo, uptake, and function. These changes facilitate the dissemination of IR signals to neighboring cells and to distant sites, which contributes to systemic effects in irradiated and nonirradiated areas. Therefore, EVs are potential mediators of IR-targeted and non-targeted effects, e.g., bystander and abscopal effects.

Several studies suggest increased EV release after irradiation in *in vitro* and *in vivo* models. As an example, it has been shown that IR may increase EV release in different tumor models including head and neck cancer and glioblastoma. Moreover, also in normal tissue after partial-body irradiation of mice, it has been reported that the EV content is altered in different tissues including the liver, brain, and heart. As the potential mechanism for the IR-increased EV release, p53-mediated induction of genes involved in the EV biogenesis and altered MAPK signaling were suggested. It was also shown that the cellular uptake of EVs is affected by radiation exposure. In mesenchymal stem cells, irradiation induced changes in the formation of cell surface CD29/CD81 complexes, which increased the cellular uptake of EVs [224].

IR also induces changes in the composition of EVs released from cancer and non-cancer cells. Alterations seem to be highly related to cell type, radiation dose, and also time postradiation exposure where both microRNA and protein changes have been described. Additionally, changes in lipids and metabolites in EVs from irradiated donor cells are reported. EV cargo changes were also shown for EVs isolated from blood during or after tumor RT. For example, differential expression of serum EV miR-

NAs was monitored in prostate cancer or glioma patients after RT. In the serum of HNC patients, it was shown that tumor-derived exosome (TDE) amount relative to total exosomes increased in patients that were refractory to a combination of radio-, targeted, and immune therapy while the opposite was found in the patients that responded. Moreover, in the same study, results demonstrated an increase in regulatory T-cell (Treg)-derived exosomes as well as in CD3(−)PD-L1+ exosomes in serum after treatment if the patients were refractory. Such alterations in EV cargo open up for potential applications of EVs/exosomes as a source of biomarkers for radiation exposure as well as for prognostic or predictive biomarkers of RT response in the context of cancer.

A substantial amount of studies suggest that EVs play a role in the progression, RT resistance, and metastasis of cancer cells. In glioblastoma, Mrowczynski et al. demonstrated a pro-survival function of EVs derived after IR, which may be triggered by elevated cargo levels of oncogenic miRNAs and mRNAs, while tumor-suppressive RNAs were reduced [225]. In the same cancer type, a pro-migratory role of radiation-related EVs was reported. Likewise, EVs from irradiated HNC and neuroblastoma cells were shown to stimulate survival, migration, and invasiveness. However, there are also studies reporting on an induction of harmful effects of EVs from irradiated cancer cells into recipient cells, like chromosomal damage and increased ROS levels.

EVs are also involved in the communication of radiation signals among normal cells. Early work by Jella et al. showed the transmission of cytotoxic effects between irradiated and nonirradiated keratinocytes in an *in vitro* model system. Thus, EVs from irradiated mice were able to increase DNA damage and reduce viability in co-cultivated mouse embryonic fibroblasts. On the other hand, several reports found beneficial effects of EVs released from irradiated human PBMCs. For example, EVs from irradiated blood cells were shown to reduce radiation-induced apoptosis in endothelial cells [226]. Accordingly, pro-angiogenic and tissue-regenerative capacities were attributed to EVs from irradiated PBMC. In this regard, it was shown that EVs (especially from mesenchymal cells) could be used for the treatment of radiation injury [227].

In summary, current knowledge indicates a vital role of EVs in the IR response of cancer and non-cancer cells. IR not only affects the production and the composition of EVs, but also alters the phenotypes of recipient cells. Therefore, these mechanisms can contribute to the communication between irradiated cells as well as to the systemic distribution of local radiation effects throughout an organism. Moreover, EVs may offer a source of biomarkers for monitoring RT responses in cancer patients (Box 3.37).

Box 3.37 In a Nutshell: Extracellular Vesicles as Epigenetic Factor

- EVs, including exosomes, act as intercellular signaling components in response to IR.
- IR may influence the EV release/uptake as well as cargo in normal as well as tumor cells contributing to both direct and bystander effects of IR.
- EVs/exosomes may contribute to the distribution of systemic IR effects and offer a source of IR response biomarkers.

3.19 Omics

3.19.1 Proteomics

The term proteome was created to describe the set of proteins expressed by the genome [228]. Proteomics analyzes the proteome at a specific time and in a specific state. Proteome profiling provides information not only about the protein expression, but also about the function, structure, and interactions of proteins.

In the well-established paradigm of proteomics, protein mixture will be separated before digestion either by gel electrophoresis (gel-based approaches) or using liquid chromatography (gel-free approaches) to resolve the complexity of the protein mixture [228]. In the next step, proteins were fragmented into smaller units called peptides during digestion. The generated peptides were further separated and sorted in the mass spectrometry system based on the mass and charge, where the abundance of each peptide is translated into numerical values called intensity. To identify a protein, a certain number of good-quality peptides must be detected. Quantitative proteomics compares the peptide intensities for each protein between treated (e.g., irradiated) and non-treated (e.g., nonirradiated) samples. The alterations in peptide intensities represent the changes in the expression level of corresponding protein.

Protein quantification can be performed in two ways: either label based or label free. In label-free methods, protein expression in several samples is compared by measuring the intensity of the corresponding peptides or counting the number of correlated spectra for each protein. Label-based quantification is performed by labeling peptides or proteins with fluorescent dyes, chemical isotopes, radioisotopes, or affinity tags before mass spectrometry. Label-based proteomic approaches are classified into chemical labeling (ICPL, iTRAQ, and iCAT) and metabolic labeling techniques (SILAC).

Advanced proteomics approaches also offer an accurate platform to identify and quantify the posttranslational modifications (PTMs) such as phosphorylation, acetylation, methylation, or ubiquitination [229]. These modifications are crucial for the stability, localization, and conformation and functions of proteins. The analysis of phosphoproteome, acetylome, or ubiquitinome has revealed the regulatory role of these PTMs in cellular function and homeostasis [229].

A comprehensive combination of proteomics and advanced bioinformatics makes the complex biological processes in cells understandable. The bioinformatics tools provide a broad spectrum of information on protein functions, protein-protein interactions, protein interactions with other biomolecules (genes and metabolites), contribution to the signaling pathways, and predictions of diseases [230].

3.19.1.1 Proteomics in Radiation Research

Different proteomics approaches were applied to investigate the biological effects of radiation exposure on normal and tumor tissues, cancer radiotherapy outcome, individual sensitivity, risk assessment, biodosimetry, and biomarker discovery; an extensive review can be found in the work of Azimzadeh et al. [231].

One of the main goals of cancer proteome profiling in radiation research has been to identify biomarkers that predict the tumor's response to radiation exposure. The proteomes of different cancer cell lines such as nasopharyngeal carcinoma, head and neck cancer, oral squamous cell carcinoma, laryngeal cancer, breast cancer, and lung cancer have been analyzed in radiobiological studies to identify signatures of cancer radioresistance and potential prognostic markers for radiotherapy. Although the results of these studies are not uniform, the proteins identified and quantified belong mainly to the family of antioxidant proteins, heat shocks, and structural proteins.

The most challenging aspect of radiotherapy for cancer is to select the radiation dose so that the tumor is killed but the surrounding normal tissue is harmed as little as possible. The effect of radiation on normal tissue has also been analyzed by proteomics approaches. A number of studies have been carried out on in vitro and in vivo models to simulate the effects of radiation on normal tissue such as the heart, brain, and liver. These studies underlined the adverse effects of irradiation on tissue structure and function. The mitochondrial proteins, the metabolic enzymes, and the oxidative stress response proteins are the main groups of proteins affected in the irradiated heart. The structural proteins, proteins involved in cognition and learning function, and inflammatory response were impaired in the irradiated brain.

Biofluids such as serum, plasma, and urine are optimal biomaterials for biomarker discovery, mainly because of the relatively noninvasive collection methods. However, proteomic profiling in biofluids is still an analytical challenge due to the complexity and variable spectrum of protein abundance. Several studies have compared the biofluid proteome before and after radiation exposure. These studies provide a panel of proteins that serve as biomarkers of radiation exposure, radiation damage, cancer radiosensitivity and radiotherapy outcome, and biodosimetry.

Since cellular responses to irradiation are tightly regulated by PTMs, the analysis of these changes is becoming increasingly important in radiation research. PTM profiling is still a young field in radiation research, and only a few studies have analyzed the change in protein phosphorylation, acetylation, and ubiquitination in the context of cancer and normal tissue response to irradiation.

Archival formalin-fixed, paraffin-embedded (FFPE) tissues are the invaluable alternative of fresh frozen biomaterial in radiation research. Proteomic analysis of these samples is challenging, mainly due to the harsh conditions of tissue fixation and, in particular, biomolecule extraction method. The proteomics studies conducted on FFPE tissues from radiobiology archives have mainly investigated the predictive marker for radiotherapy resistance of cancer or adverse effect on normal tissue. They demonstrated the compatibility and applicability of FFPE tissues for proteomics studies [231].

3.19.2 Lipidomics

The study of cellular lipid pathways and networks in biological systems is known as lipidomics [232]. Lipids are a necessary component of biological membranes and play essential roles in biological systems, such as the plasma membrane bilayer structure that separates the cell cytoplasm from the extracellular microenvironment, the provision of a hydrophobic medium for the functional performance and interactions of membrane proteins, and the generation of second messengers through enzyme reactions [233]. Lipidomics refers to the analysis of all lipids present in a sample using liquid chromatography (LC) and mass spectrometry (MS) techniques.

Glycerolipids, saccharolipids, sphingolipids, glycerophospholipids, sterols, polyketides, fatty acyls, and prenols are the eight types of lipids that can be classified based on their chemical structures and hydrophobic and hydrophilic aspects [233]. The most prevalent phospholipids (PLs) are glycerophospholipids, found in biological membranes and essential for numerous cellular activities. PCs and other related phospholipid derivatives like lysophosphatidylcholines (LPCs) are signaling molecules that play a role in regulating cellular death and proliferation. Triacylglycerides (TGs), sphingomyelins (SMs), phosphatidylinositols (PIs), diacylglycerides (DGs), and cholesteryl esters are also among lipids with key roles in cell physiology [234].

Reactive oxygen/nitrogen species (ROS/RNS) react extensively with lipid molecules following irradiation, causing lipid breakdown and eliciting both direct and indirect inflammatory responses. Lipid peroxidation and pro-inflammatory lipid intermediates can have immediate impacts on physiology and can lead to long-term consequences like CVD, lung damages, and even carcinogenesis. Apoptosis can also be triggered by the direct action of radiation or by lipid intermediates, such as the activation of sphingomyelinase, which produces ceramide from the hydrolysis of sphingomyelin. Ceramide is a direct apoptotic cell death [234]. Post-ionizing irradiation (IR) changes affecting lipids have been proven in preclinical investigations and may have biological consequences such as the acute radiation sickness (ARS) or lead to delayed effects of acute radiation exposure (DEARE).

When comparing sham or pre- and post-IR specimens, lipids examined in blood, such as PCs, LPCs, TGs, SMs, and CEs, exhibit modifications.

The link between lipid levels in serum/plasma and radiation has been studied in animal models in several publications. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) levels in rat plasma following gamma irradiation exposure increased dramatically, thus indicating that IR may disrupt phospholipid metabolism [233]. Fatty acids, such as linoleic acid and palmitic acid, were found to be present at reduced levels in the blood following 137Cs exposure in mice, while phosphatidylcholines were among the most disturbed molecules in 137Cs-exposed mouse serum. A total of 67 biomarkers were discovered in some tissues and biofluids of mice exposed to radiation (6 Gy) (serum and urine). Among these, 3-methylglutarylcarnitine was found to be a unique metabolite seen in the liver, serum, and urine that might be employed as a marker of early radiation response.

Changes in lipid metabolism, including key lipid species such as free fatty acids, glycerolipids, glycerophospholipids, and esterified sterols, have also been observed in nonhuman primates exposed to IR. The results show that diacylglycerides decreased 1 day after IR, but triacylglycerides and lysophosphatidylcholines increased from 2 to 7 days after IR. At 7 days, after 10 Gy irradiation, the amount of polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid, increased significantly in the nonhuman primate model. Between 2 and 3 days after irradiation, an increase in LysoPCs and a decrease in SMs could be regarded as viable indicators (6.5 Gy). Compared to nonirradiated controls, recent research in nonhuman primates has discovered plasma-derived exosomal indicators of IR exposure related to the enrichment of N-acyl-amino acids, fatty acid esters of hydroxyl fatty acids, glycolipids, and triglycerides.

Radiation therapy caused blood lipidome disturbances, which were corrected within 1–2 months after IR treatment, according to lipid species quantification in individuals receiving radiation therapy. As a result, radiation-induced lipidome modifications could indicate changes in early time points and, perhaps, alternative damage pathways. Patients undergoing a complete body irradiation at the MSK Cancer Center (NYC), before hematopoietic stem cell transplantation, showed seven urine indicators with changes between pre- and postexposure at 4–6 h (1.25 Gy) and 24 h (three fractions of 1.25 Gy each) postirradiation, which involved trimethyl-*l*-lysine, acetylcarnitine, decanoylcarnitine, and octanoylcarnitine (carnitine conjugates involved in fatty acid transport), as well as hypoxanthine, xanthine, and uric acid (purine catabolism pathway end products).

During the period 2015–2019, the National Cancer Institute's Radiation Research Program, in partnership with the Small Business Innovation Research Development Center, financed four small firms. This led to the development of a metabolomic/lipidomic assay for predicting late effects that have a negative impact on the quality of life in prostate cancer patients treated with radiation. Shuttle Pharmaceuticals (Rockville, MD) intended to move forward by developing and validating a metabolomic/lipidomic biomarker panel that could predict radiation toxicity. In a phase I experiment, metabolites in plasma from 100 patients were examined in order to develop a kit that could support metabolomic analysis and act as a biomarker panel to predict sensitivity to radiation late effects. A phase II SBIR project was set up for the multi-site analytic validation of the metabolite panel kit created in the phase I SBIR project [235].

Lipidomics has then emerged as a reliable technique for lipid identification and quantification and the search for biomarkers that can be used in radiation-related incidents such as nuclear and radiological hazardous occurrences. In this sense, easily available biofluids are critical, especially in the case of irradiated victims, as well as the use of biodosimetry techniques that are both quick and accurate. Huang et al. [233] discovered seven radiation-responsive lipids in the serum of mice and showed their utility in dose calculation. Lipid changes after whole-body exposures have been thoroughly documented in a variety of animals, including atomic bomb survivors [234]. Indeed, estimating the radiation dose has always been a priority in the medical treatment of these events.

Because of the combined effects of neutrons and photons, shielding from structures, and closeness to the epicenter, among other factors, radiation exposures from an IND can be complicated. Using lipidomics, Laiakis et al. [234] evaluated serum samples from mice exposed to varying percentages of neutrons and X-rays to a total dosage of 3 Gy. Several lipids including triacylglycerides, phosphatidylserines, lysophosphatidylethanolamines, lysophosphatidylcholines, sphingolipids, and cholesteryl esters exhibited a delayed increase in mixed exposures, while diacylglycerides declined and phosphatidylcholines (PCs) remained virtually unaltered.

The mammalian lipidome's structural variety is so great that it necessitates a systematic nomenclature for precise categorization (identifying lipid subclasses, or the total number of carbons in the fatty acid chain and the number of double bonds). Because numerous lipid classes showed differences when comparing sham or pre- to post-IR samples, the variable sums and ratios within each metabolite class can increase and must be carefully considered.

3.19.3 Metabolomics: Metabolites

Radiation exposure can cause a complex molecular and cellular response having an impact in metabolic processes and consequently change metabolite levels [236]. This approach aims to detect small molecules (<1000 Da) in biological samples, which occur downstream of genomic, transcriptomic, and proteomic processes, constituting a more complete picture of the system's response to insult even prior to the onset of clinical symptoms [237]. The first use of metabolomics concerning radiation exposure studies was reported in the 1960s according to the experiences developed in human and animal samples; however, the understanding of cellular and molecular effects of ionizing radiation and the identification of radiation exposure biomarkers remain a challenge [236]. To obtain metabolic information, different methodologies can be used as nuclear magnetic resonance (NMR) or mass spectrometry (MS), including gas chromatography (GC) and liquid chromatography (LC). After sample collection, processing, and data acquisition, data analysis is the second step using principal component analysis (PCA) to have an initial perception about patterns and outliers, and if there are any easily discernible biomarkers. After, complex statistical data analysis should be employed in order to at the end perform biomarkers data interpretation and validation [236]. However, it is important to consider that there exist multiple analytical and clinical challenges that constitute an impediment for the successful translation of these biomarkers for clinical use [237].

Despite the few existing studies, some metabolite changes in small-molecule metabolites remain underexplored and underexploited concerning ionizing radiation effects [236]. The role of polyamine metabolism has been studied related to ionizing radiation effects. Present in all cells, polyamines, as putrescine, spermidine, and spermine, are aliphatic polycations with multiple functions related to cell metabolism, cell proliferation, and cell differentiation. These molecules have pleiotropic effects that allow their linkage to DNA, RNA, and proteins, identifying a regulatory role in cell metabolism [236]. Besides this, increased levels of these molecules are reported as healthy cell protectors against oxidative stress. Different studies using animal and patient samples have been performed reporting altered metabolites in response to ionizing radiation, namely creatine, creatinine, carnitine, hypoxanthine, citric acid, taurine, xanthine, threonine, uric acid, and citrulline. Besides these metabolites with high alterations, 2′-deoxyuridine, arginine, glycine, glutamine, hippuric acid, inositol, palmitic acid, uridine, lactic acid, leucine, linoleic acid, methionine, tyrosine, and sebacic acid are described as metabolites with moderate alterations in consequence of ionizing radiation. Therefore, and considering experimental data, among them exist strong candidates for diagnostic biomarkers being considered time- and dose-dependent measures [232]. Data obtained using T cells demonstrated that different metabolic pathways related to amino acid, nucleotide, fatty acid, and glutathione metabolism can be affected by in vivo radiation. Related with cancer and ionizing radiation response, metabolic profiling may help identify metabolites responsible for response to therapy, being the alteration of metabolite production, a feature that can influence tumor microenvironment and consequently cancer progression [237]. The complete characterization of the metabolome can be an opportunity to influence prognostic, predictive, and even pharmacodynamic biomarkers contributing to an individualized and targeted treatment [232]. Notwithstanding the capacity of metabolomics to detect alterations, it is necessary to be aware of the tumor-related responses, namely to the therapy, as inflammation and altered energy metabolism. Besides that, and considering cancer as a syndrome, there are also other cancer-associated conditions such as weight loss that can influence metabolism [238]. Having in consideration that ionizing radiation triggers a complex response influencing molecular and cellular processes and alteration of the metabolic processes and metabolite levels, more research work is necessary to identify biomarkers related to specific type and dose of radiation, genotypic differences, pathological conditions, and specific organs or tissues (Box 3.38).

Box 3.38 In a Nutshell: Omics

- Omics might provide biomarkers of high sensitivity and specificity for radiation research.
- Omics can provide a qualitative and quantitative overview of the global perturbations induced by IR in cells and biological fluids.
- Proteomics provides a comprehensive analytical platform to study the molecular mechanisms of the biological effects of radiation on normal tissues and tumours.
- Proteomic profiling is used to identify biomarkers of radiation exposure, radiation-induced damage, individual sensitivity, and biodosimetry.
- Proteome analyses of cancers deliver information on the outcomes of cancer radiotherapy.

- Metabolomics is used to detect small molecules (<1000 Da) in biological samples.
- To obtain metabolic information, different methodologies can be used as nuclear magnetic resonance (NMR) or mass spectrometry (MS).
- Ionizing radiation triggers a complex response influencing molecular and cellular processes that can be characterized using metabolomics.

3.19.4 Transcriptomics

Molecular signatures provide a composite inventory of radiation-specific responses as changes in the composition of these molecules and their abundance. Transcript levels of signature genes in various tissues, notably blood, saliva, skin, and tumor samples, can discriminate and, in some instances, quantify irradiated from unexposed samples [239]. Expression of coding genes and noncoding miRNAs, and lncRNAs, has been implicated in radiation responses, in some cases, which can be precisely calibrated to dose. For individual genes, e.g., FDXR, alternative splice isoforms have also been demonstrated to arise in a radiation-specific manner.

Transcriptomic approaches to classify radiation response can be based on the evaluation of many known genes, which minimizes technical bias in the selection of optimal combinations of gene subsets. Detection and quantification of ionizing radiation have been based on changes in the expression of a set of genes, primarily in blood from multiple individuals. Generally, signatures selected genes with largest average differences and combined changes in gene expression levels, to predict ionizing radiation exposure in humans and mice [240]. Genes previously implicated or established from genetic evidence and biochemical pathways that are altered in response to these exposures can be used to predict radiation exposure by supervised machine learning (ML) [241] (Fig. 3.51). Termed biochemically inspired ML, diagnostic gene signatures for radiation and chemotherapy have been proven accurate on clinical samples. However, typical sample sizes of typical datasets have limited the effective ML methods for deriving gene signatures.

To establish the reproducibility of these signatures, radiation exposures are predicted with data from independently exposed individuals. Consistent performance on independent dataset depends on the composition of the genes, how genes are ranked, how they validate the response, and how they account for the amplitude of the radiation response. Other important variables include how well signatures differentiate irradiated from unirradiated samples, or even different levels of absorbed radiation from each, or different radiation qualities (energy levels and source, particle types). Transcriptomics can integrate different genes/transcripts in the induced and repressed biochemical pathways that constitute these responses. There is an enormous range of accurate gene signatures that can be derived in many independently derived datasets. Interestingly, there are some core sets of genes and pathways that are present from different studies.

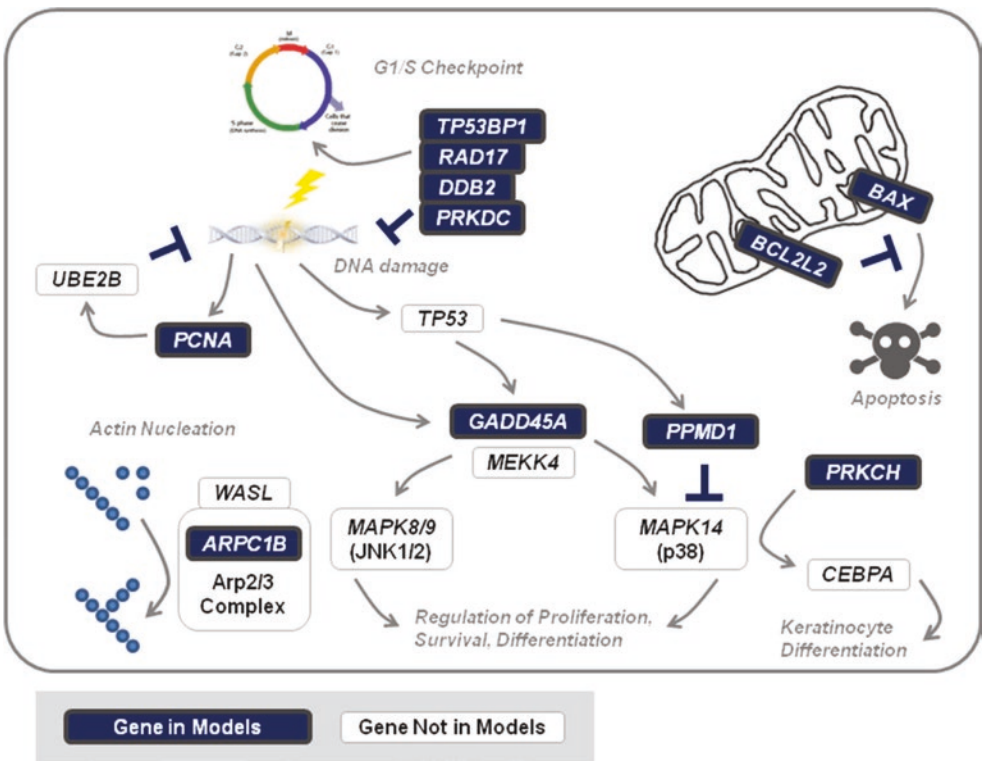

Fig. 3.51 Graphical depiction of major cellular functions containing the most frequently appearing genes of the highest performing human signatures adapted with permission (CCBY) from Zhao et al. [241]. Genes common among these signatures (white lettering) are indicated in pathways which contain products that these genes interact with (black lettering)

Many radiation response genes were frequently selected for multiple blood signatures, which include genes with roles in DNA damage response (*CDKN1A*, *DDB2*, *GADD45A*, *LIG1*, *PCNA*), apoptosis (*AEN*, CCNG1, *LY9*, *PPM1D*, *TNFRSF10B*), metabolism (*FDXR*), cell proliferation (*PTP4A1*), and immune system (*LY9* and *TRIM22*). While biochemical pathways comprising the best performing radiation gene signatures are often shared between human and other species, there are some distinctive differences, notably genes associated with immune cell communication (mice) and redox response (humans).

Expression of gene combinations that detect radiation exposure can also exhibit similar expression patterns in infectious diseases and other blood disorders [242]. Underlying pathways activated by radiation effects, for example DNA damage response and apoptosis, appear to be activated in some individuals affected with other conditions The genes involved are commonly present in multiple published radiation gene signatures and assays. For example, a 74-gene radiation signature comprised of 16 genes present in the human signatures was developed as reported in Zhao et al. [241], including *CDKN1A*, *DDB2*, and *PCNA*. Misclassification of radiation exposures in unexposed individuals with other blood disorders might be mitigated by reevaluating false-positive predictions with signatures containing radiation-responsive genes explicitly derived from other unrelated biochemical pathways, including those encoding secreted proteins.

3.20 Cellular Hyper-radiosensitivity

An important factor in the cellular response to radiation is the ability to repair DNA damage. In some individuals, hereditary mutations in genes involved in DNA repair result in a high sensitivity to irradiation [243]. The A-T syndrome is an example of one such mutation and is described in detail below. However, even with intact DNA repair pathways, it turns out that for small doses, cells refrain from the cell cycle arrest that would give time for repair. This results in a much higher cell kill per unit dose for small doses than higher doses, a phenomenon called low-dose hyper-radiosensitivity (HRS). An explanation to the presence of HRS could be that sacrificing a few cells may be advantageous to the risk of misrepair.

3.20.1 Repair-Deficient Cells (AT)

Ataxia-telangiectasia mutated (ATM) is a serine/threonine protein kinase with a key role in repairing double-strand DNA breaks (DSBs) and is also involved in the regulation of oxidative stress, metabolic syndrome, and neurodegeneration, among others reported [244].

The human ATM gene is located at 11q22-23, contains 66 exons, covers 160 kb of genomic DNA, and encodes the 370 kDa ATM protein. Germline mutation in ATM gene, either loss or inactivation of both copies, leads to the autosomal recessive ataxia-telangiectasia (A-T) syndrome, a devastating childhood condition characterized by chromosomal instability, neurological degeneration, immune dysfunction, premature aging, and high cancer risk. In fact, ATM has a critical role in the activation of the cell cycle progression and checkpoint activation in response to DSBs, as well as in the repair of these lesions through homologous recombination (HR) and nonhomologous end joining (NHEJ) pathway [245]. Thus, these mechanisms need to be active during DNA replication to maintain genome integrity in cells. When disruptions occur in these mechanisms, the cells are more susceptible to damage induced by the exposure to endogenous or exogenous agents, such as radiation exposure. Considering the well-known fact that exposure to IR activates ATM kinases to mediate the cellular response, individuals with these defective mechanisms, like A-T patients, will be more sensitive to the same IR exposure than non-A-T patients. Several published studies have corroborated these hypotheses, showing that failures in cell cycle checkpoints lead to a failure in the arrest in G1/S, S, or G2/M allowing the cells to escape from the proper DSB repair, among other signaling pathways including apoptosis and chromatin remodeling process [243]. Therefore, these non-repaired or misrepaired DBSs are responsible for chromosomal instability also in daughter cells, increasing the radiation sensitivity in these individuals as well as the carcinogenesis risk. It is important to elucidate that this cellular radiosensitivity is not unique for A-T syndrome being also observed in other individuals carrying mutations in other genes related to DNA damage repair and response pathways, namely in FANC, BRCA 1/2, MRE11, and DNA Lig4 genes, among others (Box 3.39).

Box 3.39 In a Nutshell: Cellular Hypersensitivity in AT Repair Deficient Cells

- Individuals carrying mutations in genes related to DNA damage repair are hypersensitive to radiation.
- A-T syndrome is connected to mutations in ATM, which has a critical role in checkpoint activation and DNA damage recognition and repair.
- HRS/IRR has been attributed to the early G2 checkpoint, which is only activated at a certain level of phosphorylated ATM.

3.20.2 Low-Dose Hyper-radiosensitivity

As described in Sect. 3.19.1, cells with defects in DNA DSB repair pathways are very sensitive to radiation. However,

even repair-competent cell lines can be hypersensitive to radiation within a certain low-dose range. This is called low-dose hyper-radiosensitivity (HRS) and is characterized by a high sensitivity to radiation doses below about 0.5 Gy (depending on the cell line and radiation quality) [246], which is followed by a more radioresistant response per unit dose in the dose range of ~0.5–1 Gy. This transition towards radioresistance is described by the term *induced radioresistance* (IRR) (Fig. 3.52) and occurs at doses corresponding to about ten double-strand breaks.

HRS [247] was first identified in vitro in 1993 after having been observed in mouse skin in 1986 and in mouse kidney in 1988. HRS has been observed in cells given acute proton and pi-meson irradiation as well as in cells given high-LET neutrons at a low dose rate and appears to be the default response for all radiation qualities in both tumor and normal cell lines. IRR, on the other hand, is only observed after low-LET irradiation and only in repair-competent cell lines [247].

In 2003, a mechanism explaining the basis for the HRS effect was proposed [248]. A second radiation-induced G2 checkpoint was discovered to be associated with HRS/IRR [249]. The radiation-induced G2 checkpoint that was known earlier, often denoted the Sinclair checkpoint, does not arrest cells irradiated while in G2, because it takes some time for the checkpoint to be activated. Only cells irradiated while in G1 or S phase are accumulated in G2 by the Sinclair mechanism. The newly discovered G2 checkpoint, also called the early G2 checkpoint, is induced immediately after irradiation and therefore arrests cells that were irradiated while in G2.

Contrary to the dose-dependent mechanisms by which cells irradiated in G1 or S phase accumulate in the Sinclair checkpoint, the "early" G2 checkpoint is ATM dependent [249] and independent of dose over the range of 1–10 Gy. Activation of the critical damage sensor molecule ATM by an autophosphorylation event at serine 1981 is detectable at doses of 0.1 Gy with a gradual increase until phosphorylation of more than 50% of the ATM molecules in the cell after

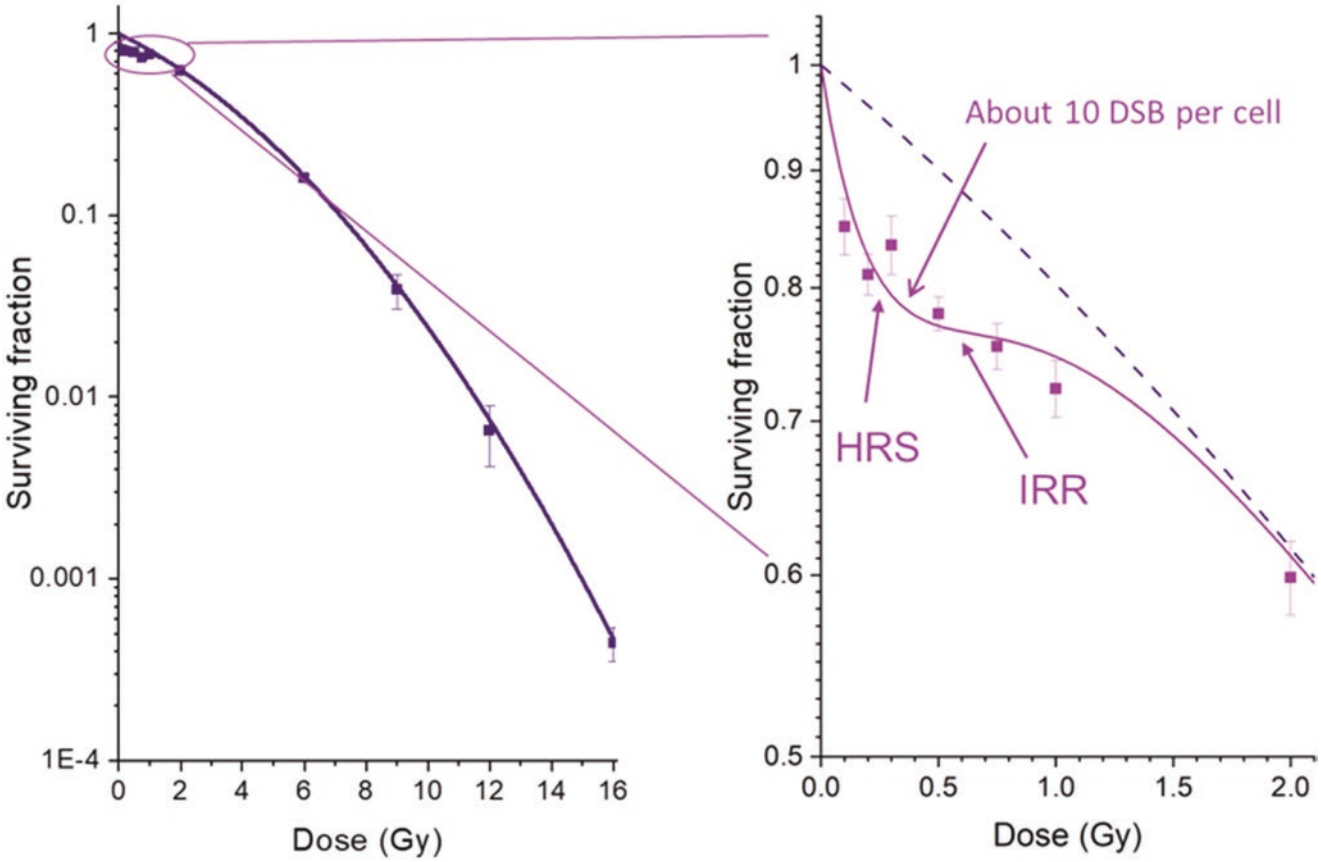

Fig. 3.52 Low-dose hyper-radiosensitivity (HRS) and increased radioresistance (IRR) in T-47D breast cancer cells. The left panel shows a full dose-response curve. The right panel shows the low-dose region. Below about 0.3 Gy, the cells appear to proceed from G2 to mitosis without repair of DNA damage, leading to a steep decrease in survival with dose. For doses above a threshold around 0.3 Gy, the damage is repaired increasingly with dose until the surviving fraction follows the linear-quadratic response curve. The transition dose corresponds to approximately 8–10 double-strand breaks. The dashed line shows a curve fit by the linear-quadratic model, and the solid line by the induced repair model (see Chap. 4)

Fig. 3.53 Path to "increased radioresistance" or "hyper-radiosensitivity." Cells irradiated with doses below about 0.3 Gy while in G2 will not have enough ATM activated by serine 1981-phosphorylation to reach the threshold level for activation of the early G2 checkpoint. They therefore follow the alternative in the left column, which does not give extra time for repair before mitosis resulting in "hyper-radiosensitivity" (HRS). Cells irradiated with doses above 0.3 Gy while in G2 follow the alternative in the right column and thereby are given more time for repair before mitosis resulting in "increased radioresistance" (IRR)

a dose of about 0.5 Gy and saturated expression at larger doses. Thus, the HRS/IRR transition appears to be coincident with both the induction of the early G2-phase checkpoint and activation of ATM.

The hyper-radiosensitivity is thus a result of the progress into mitosis with repairable but unrepaired DNA damages for cells that were irradiated while in G2 but received doses below the threshold for checkpoint activation (see Fig. 3.53).

It has been demonstrated that HRS-negative cell lines have the same ATM activation pattern as cells with HRS, whereas they show an early G2 arrest even after low radiation doses that produce insufficient damage to induce full ATM activation. It has therefore been suggested that the dose-dependent ATM regulatory control is evaded by aberrant early G2-checkpoint response in HRS-negative cell lines caused by dissociation between ATM activity and early G2-checkpoint function [250].

The existence of HRS appears in some cell lines to be associated with an elevated level of caspase-3-mediated apoptosis after low-dose exposures [250], suggesting that the radiation-damaged G2-phase cells that evade the early G2 checkpoint are disposed of by this mechanism when entering mitosis. However, also cell lines deficient in TP53 induction after irradiation or with mutated TP53 have been shown to display HRS/IRR, and no increase in apoptosis in response to HRS-inducing doses was observed in BMG-1 cells with wild-type TP53. In addition, no connection between HRS and apoptosis in the six HRS-competent cell lines investigated measured by DNA-PKcs as early apoptosis marker was found. This corroborates the hypothesis that the transition from HRS to IRR primarily is related to induction of the "early" G2 checkpoint and that the death process of the cells entering mitosis with damages is cell line dependent.

Early G2-checkpoint activation as the underlying mechanism for HRS/IRR is supported by priming experiments where a dose of 0.2–0.5 Gy removes the HRS response to subsequent irradiation given within 6–8 h after the priming. This timing corresponds to the duration of the early G2-checkpoint activation. Surprisingly, the HRS/IRR response can be permanently eliminated by priming irradiation if it is given with a low dose rate of 0.1–0.3 Gy/h for 1 h (shown in T98G glioblastoma and T-47D breast cancer cells). The low dose rate primed cells activated the early G2 checkpoint for all doses. The response was shown to involve transforming growth factor beta 3 (TGF-β3) and inducible nitric oxide synthase (iNOS) activation. The HRS phenotype could be reinstated by inhibition of iNOS [251].

It has been suggested that HRS is a protective mechanism and that it is advantageous to sacrifice a small fraction of cells rather than risking the development of genomic instability and mutations. While cells without the IRR are deficient in DNA repair or checkpoint activation, cells without HRS may have been "turned off" by mechanisms similar to the ones induced by low dose rate priming (Box 3.40).

Box 3.40 In a Nutshell: Low Dose Hyper-Radiosensitivity

- Cells with HRS are very sensitive to doses below about 0.5 Gy.
- Above 0.5 Gy, the survival per dose increases until it reaches the LQ curve.
- HRS/IRR has been attributed to the early G2 checkpoint, which is only activated at a certain level of phosphorylated ATM.

3.20.3 HRS and Bystander Signaling (Cytotoxic or Adaptive)

As described in Chap. 2, irradiated (donor) cells can secrete signals which, when transferred to unirradiated (reporter) cells, make these respond as if they had been irradiated themselves. This is called the bystander effect. For a while, it was believed that HRS and cell kill by the bystander effect were mutually exclusive, but when doses in the HRS dose range were tested on HRS-proficient cells, these were able to induce strong bystander signals. However, when doses reached the level where IRR was dominating, there was no bystander signaling.

Bystander signals may also be protective. Transfer of medium from HRS-deficient HaCaT normal human epithelial cells to HRS-proficient T98G glioblastoma cells increased the survival above the normal plating efficiency for these cells [252]. In addition to the effect on cell survival, bystander signals can also moderate the HRS/IRR response to subsequent irradiation. Medium transferred from cells, in which the HRS/IRR response was permanently eliminated by priming with 0.1–0.3 Gy for 1 h, has been seen to remove the HRS response in recipient cells for 8–12 h [251] (Box 3.41).

Box 3.41 In a Nutshell: Hyper-Radiosensitivity and Bystander Signaling

- HRS-proficient cells irradiated with doses in the HRS range produce bystander signals that reduce the survival of reporter cells.
- HRS-proficient cells, in which the HRS response has been removed by low dose rate priming, produce bystander signals that remove the HRS response to subsequent irradiation in recipient cells.

3.20.4 HRS and Clinical Relevance

The presence of HRS may have implications for cancer radiotherapy in which the aim is to control the eradication of tumor tissue while minimizing the damage to normal tissue. The introduction of intensity-modulated radiation therapy (IMRT) in cancer treatment results in irradiation of a larger proportion of normal tissue but at lower doses when compared to conventional treatment. In some situations, one could fear that HRS will tend to increase the effect of low doses in normal tissue and thus negate the benefits of using IMRT, in particular in tissues with a pronounced volume effect [253].

Since HRS is related to the fraction of cells in G2 phase, it may be of more consequence for early-responding proliferating tissues, such as skin, than for slowly proliferating normal tissues with a small fraction of cells in G2. In support, evidence of HRS has been demonstrated in studies with human skin using basal cell density or skin erythema as endpoint. On the other hand, with the HRS effect being more pronounced in fast-dividing tumor cells than slowly or nondividing normal tissues, it may be possible to exploit HRS clinically using dose fractions within the HRS dose range. However, to obtain the same cell kill as with 2 Gy fractions, more fractions are needed. Increasing treatment time would give the tumor more time to grow; therefore, the time between fractions has to be decreased, but that could be a problem: Experiments with ultrafractionation of 0.4 Gy per fraction, three fractions per day in murine DDL1 lymphoma or in human A7 glioblastoma xenografts, did not show evidence of HRS. This could be because with three fractions per day, the timing between fractions would have been too short for the cell to be released from the early G2-checkpoint arrest induced by the previous dose. Since HRS affects cells in G2, another approach is to synchronize the cells to improve the therapeutic potential of ultrafractionation. A protocol using a taxane (paclitaxel), which synchronizes cells in G2 phase, in combination with carboplatin and low-dose fractionated radiation, was extremely well tolerated by the patients and showed a synergistic effect in patients with squamous cell cancer of the head and neck [254] (Box 3.42).

Box 3.42 In a Nutshell: Hyper-Radiosensitivity and Clinical Relevance

- Attempts to exploit HRS in the clinic using hyperfractionation have not been successful.
- Combination of low radiation doses with chemotherapeutics synchronizing cells in G2 phase has shown promise.

3.21 Induced Radiation Resistance

3.21.1 Basic Mechanisms Leading to Radiation Resistance

By the term radiation resistance, we refer to the inherent ability of specific types of cells and tissues (usually of malignant origin) to show a differential response to ionizing radiation overcoming its damaging effects like cell killing or inactivation. The amount of energy (for example level of dose in Gy) and consecutive damage that each organism can withstand is a characteristic of the organism's ability to respond to radiation by a variety of mechanisms often called as DNA damage response (DDR) mechanisms. At the organism level, usually humans are more sensitive compared to other primates or mammals. In nature, there is a great variety of resistance to radiation with the extreme case of certain extremophiles, such as the bacteria *Deinococcus radiodurans* and the tardigrades, to be able to withstand large doses of ionizing radiation on the order of 5000 Gy. Although none of the strategies discussed in various studies on extreme radioresistance appear to be universal against ionizing radiation, a general trend was found. There are two cellular mechanisms by which radioresistance is accomplished: (a) protection of the proteome and DNA from damage by scavenging and regeneration strategies and (b) recruitment of advanced and highly sophisticated DNA repair mechanisms, in order to reconstruct a fully functional genome [255].

While normal (nonmalignant) mammalian cells compared to tumor ones are usually less radioresistant, one cannot exclude the opposite possibility. Elucidation of the molecular mechanisms and pathways related to radioresistance of tumor cells is of major importance in order to develop strategies maximizing tumor control during chemo- or radiation therapies. Many studies using a wide range of *in vitro*, *ex vivo*, and *in vivo* models as well as bioinformatics have fingerprinted the main pathways leading to cellular radioresistance, and these are primarily implicated in DNA damage repair, oxidative stress, cell pro-survival, hypoxia, cell cycle control, and apoptotic pathways [231].

Another important factor contributing to resistance of tumors is the existence of cancer stem cells (CSCs) as a distinct subpopulation within a tumor. CSCs are able to self-renew and differentiate while showing a high proficiency to repair DNA damage, reveal low levels of reactive oxygen species (ROS), and proliferate at a slower rate compared to other tumor cell populations. These features render CSCs resistant to various therapies, including radiation therapy (RT) [256]. The results of such studies can serve as potential diagnostic/prognostic markers of cancer cell resistance to radiation treatment, as well as for therapy outcome and increase of cancer patient survival.

3.21.2 Adaptive Response

The radiation-induced adaptive response was first described by Olivieri et al. in 1984 [257] as the reduced sensitivity to a challenge irradiation induced by a previous small priming dose. Radio-adaptive responses have been observed in vitro and in vivo using various endpoints, such as cell lethality, chromosomal aberrations, mutation induction, radiosensitivity, and DNA repair [258]. Adaptation is most efficiently induced by doses of 0.01–0.5 Gy at dose rates from 0.01 to 1.0 Gy/min (Tapio und Jacob 2007) with challenge doses in the range of 0.5–2 Gy. The protective effect has been reported to last for about three generations following the priming irradiation. The molecular mechanisms underlying the adaptive response are not well understood, but data indicate involvement of DNA damage repair, antioxidant production (NRF2 pathway), NF-κB inflammatory pathway, MAPK pathway, autophagy, cell cycle regulation, apoptosis, and bystander signaling [258] (Box 3.43).

Box 3.43 In a Nutshell: Induced Radiation Resistance

- Cellular radioresistance can be modulated either through protection against DNA damage or through DNA repair.
- Pre-exposure to a low dose can induce protection against a subsequent high dose.

3.21.3 Cancer Stem Cells

The continuous advances and improvements in anticancer therapies using IR has significantly increased the treatment efficacy and quality. However, radioresistance is still one of the major problems of radiation oncology, since it leads to tumor locoregional recurrence and disease progression. One plausible cause of tumor radioresistance is the failure of the current treatments in eradicating a subpopulation of cells intrinsically more resistant to multiples therapies, the cancer stem cells (CSCs). The biological characteristics and radioresistance mechanisms of CSCs are shown in Table 3.16. Targeting CSCs and controlling their behavior is an approach to overcome radioresistance and to improve on the efficacy of cancer treatments (Box 3.44).

Box 3.44 In a Nutshell: Cancer Stem Cells

- Cancer stem cells are a radioresistant tumor subpopulation due to high DNA repair proficiency, low ROS generation and high ROS scavenging, and slow proliferation (giving time for repair).

Table 3.16 Biological characteristics and radioresistance mechanisms of cancer stem cells

Biological characteristics	Radioresistance mechanisms
Are long-lived and have tumorigenic abilities	To activate pro-survival pathways
Are able to proliferate, maintain their growth indefinitely	To improve DNA repair ability through the activation of DNA damage checkpoint proteins, such as ATM, Chk1, Chk2, SMC1, and TP53
Differentiate, generating different cell populations inside the tumor	To defend against oxidative stress, since CSCs present lower levels of ROS and overexpress ROS scavengers that protect them from ROS produced in response to radiation
Have long-term repopulation potential	To indefinitely self-renew, through the activation of cell signaling pathways, such as Wnt/β-catenin, notch, TGF-β, and PI3K/AKT/mTOR
Have a flexible phenotype (plasticity), since a conversion of a CSC into a non-CSC phenotype can be reversed, a process highly dependent on the epithelial-mesenchymal transition (EMT)	To overcome the cell cycle control by the abnormal expression of cell cycle-related proteins
Can adapt to the tumor microenvironment	To inhibit cell death pathways after radiation exposure, through the upregulation of anti-apoptotic proteins (like BCL-2 and survivin) and the inhibition of autophagy-related proteins (like Beclin-1 and ATG-5)

3.21.4 Hypoxia

Hypoxia refers to conditions with low oxygen. Hypoxia induces radiation resistance by preventing the sensitizing effect of the presence of oxygen during or within microseconds of radiation exposure. Oxygen has high affinity for electrons. It may therefore react with radiation-damaged biomolecules as well as with radiation-induced water radicals (see Chap. 1).

3.21.4.1 The Direct Effect of Oxygen

Radiation creates radicals either directly in a biomolecule (e.g., DNA) or indirectly through water radicals: MH + radiation → M° + H°, where MH is an intact biomolecule while M° is the radical after loss of one hydrogen atom. Oxygen can sensitize by a direct interference with the primary radiation process. This can take place because deposit of radiation energy not always completes the dissociation. Often, the large biomolecule is just polarized as follows: MH + radiation $\rightarrow {}^{+}MH^{-}$

This process is however reversible. Thus, a **spontaneous restitution** can take place by the electron falling back to its normal position in the molecule and losing the excitation energy. Due to its great affinity for electrons, oxygen may however "steal" the excited electron before it gives away the excitation energy:

$${}^{+}MH^{-} + O_2 \rightarrow MH^{+} + O_2^{-}$$

By this process, oxygen creates a biomolecule radical after the following dissociation:

$$MH^{+} \rightarrow M^{\circ} + H^{+}$$

In this way, oxygen increases the gain of biomolecule radicals.

3.21.4.2 The Indirect Effect of Oxygen

Due to its great affinity for electrons, oxygen will easily react with both radiation-damaged biomolecules and radiation-induced water radicals. When oxygen reacts with the biomolecule radical, it forms a stable bond as follows:

$$M^{\circ} + O_2 \rightarrow MO_2^{\circ}$$

Thereby, oxygen fixates the damage and prevents restitution by hydrogen donors (antioxidants), which is a natural protective means of the cells:

$$M^{\circ} + RSH \rightarrow MH + RS^{\circ}$$

where RSH represents hydrogen donors, of which glutathione is one example.

In cells, the concentration of SH compounds is normally high, and they represent a fundamental protective means against harmful radicals if not outcompeted by oxygen. Hypoxia thus protects against radiation damage through restitution by hydrogen donors because oxygen is not present to outcompete restitution and fixate the damage (Box 3.45).

Box 3.45 In a Nutshell: Hypoxia and Radiation Resistance

- Hypoxia induces radioresistance by preventing the radiosensitizing effect of oxygen.
- Oxygen radiosensitizes by fixating DNA damage and thus preventing restitution by hydrogen donors.
- Oxygen can also increase the amount of radicals through the direct effect.

3.22 Exercises and Self-Assessment

Q1. Which of the following effects of IR produce free radicals within the cell, which can damage the cellular macromolecules?
(a) Double ionization
(b) Direct action
(c) Indirect action
(d) Single ionization

Q2. What are the most significant differences between the two repair patterns of the base excision repair (BER) repair mechanism?

Q3. Which repair pathway provides a "backup" to the replicative proofreading carried out by most (but not all) DNA polymerases during DNA replication?
(a) Base excision repair
(b) Nucleotide excision repair
(c) Nonhomologous end joining
(d) Mismatch repair

Q4. Compare the two principal DNA DSB pathways. What is similar and what is different in these?

Q5. Complex translocation to some extent depends on the radiation quality. Please indicate when they most often occur. Complex translocation types are characteristic especially for cellular exposure to:
(a) Low-LET radiation
(b) High-LET radiation
(c) Photonic radiation
(d) LET of radiation has no effect on the character of chromosomal translocation

Q6. Pick one incorrect statement for completing the sentence "The superoxide anion is …"
(a) Produced by mitochondria
(b) A free radical reactive oxygen species
(c) Converted to water by superoxide dismutase
(d) Able to react with hydrogen peroxide producing hydroxyl radicals
(e) Less lipid soluble than hydrogen peroxide

Q7. For cell transition in the cell cycle, in which phase do the CDK1/cyclin B complex plays a significant role?
(a) G2 into M
(b) G1 into S
(c) S into G2
(d) G0 into G1
(e) M into G1

Q8. What will the cycling cells do when They get a "go-ahead" indication at the checkpoint?
(a) Directly progress into the telophase
(b) Finish the cell cycle and finally divide
(c) Leave the cell cycle and modify to a nondividing state
(d) Demonstrate a fall in M phase-promoting factor
(e) Finish cytokinesis and generate new cell membranes

Q9. Which is the most radiosensitive cell cycle phase, and which is the most resistant one?

Q10. Why is immortalization so important for cancer cells?

Q11. Cells may execute cell death in different ways in response to IR. Please discuss the factors that may influence the pathway elicited.

Q12. What is the main reason for activation of cell death in response to IR in solid tumor cells?
(a) DNA damage-induced apoptosis
(b) Initiation of senescence as a result of DNA damage
(c) Mitotic catastrophe following improper segregation of genetic material
(d) Oxidation-triggered damage to proteins
(e) Generation of ceramide at the plasma membrane via sphingomyelinase

Q13. Which of the following pathways has been implicated in cellular response to IR:
(a) Autophagy
(b) Apoptosis
(c) Necrosis
(d) Mitotic catastrophe
(e) All of a–d

Q14. Apoptosis can proceed by two main routes, intrinsic and extrinsic signaling. Describe the initial triggers for these two pathways and how they lead to apoptosis.

Q15. Cite the different steps of the autophagy process.

Q16. Considering that 800 colonies have grown at 0 Gy for 1200 cells seeded, and that 126 colonies are counted at 2 Gy for 2000 cells seeded, which of the following statements are correct?
(a) The plating efficiency at 0 Gy is 66.6%.
(b) The plating efficiency at 2 Gy is 6.4%.
(c) The plating efficiency at 2 Gy is 9.5%.
(d) The surviving fraction at 2 Gy is 9.5%.
(e) The surviving fraction at 1 Gy is 100%.

Q17. Which alteration is more likely to lead to the death of an embryo? Alteration of the function of an oncogene or a tumor suppressor gene?

Q18. Which cells are mainly involved in inflammation and modulated by low to medium doses of IR?

Q19. Are the following statements regarding epigenetic DNA alterations true or false?
(a) 5-Methylcytosine is a common DNA modification.
(b) DNA methylation is equally common in all four nucleotides.
(c) Histone variants are only synthesized during S phase.
(d) The amino acid lysine in a histone protein is a target for acetylation.

Q20. Is this statement true?
(a) One miRNA regulates only one mRNA target.

Q21. Is the following statement true or false: ARS involves a total dose of over 0.7 Gy (70 rad) from an external source, administered in a few minutes.

Q22. Is the following statement true or false: PTEN is a central positive regulator of the PI3-K/AKT pathway.

Q23. Which of the following statements are correct about lncRNAs?
(a) lncRNAs are translated into regulatory proteins.
(b) lncRNAs are short RNA transcripts of around 20 nucleotides.
(c) lncRNAs can interact with other RNA subtypes to regulate gene expression.

Q24. Which of the following statements are correct about extracellular vesicles?
(a) Extracellular vesicles have a size range of 40 nm to several μm.
(b) Extracellular vesicles cargo only proteins.
(c) Extracellular vesicles can indicate cell death.
(d) Extracellular vesicles are only formed by cells and tissue undergoing cell death.
(e) Radiation effects on cells and tissue only generate extracellular vesicles to protect against radiation-induced cell death.

Q25. In the field of lipidomics or metabolomics, what is the accurate method to achieve the comprehensive metabolite of a sample using LC-MS/MS?
(a) MRM method
(b) PCR methods
(c) Elisa methos
(d) Dicentric assay

Q26. What happens to cells irradiated while in G2 with (a) 0.1 Gy and (b) 1Gy?

Q27. What is the challenge when exploiting HRS in radiotherapy?

Q28. What is the radiation adaptive response?

Q29. Please refer two mechanisms responsible for the increased radioresistance of cancer stem cells.
Q30. Explain why hypoxic cells are more radioresistant than oxygenated cells.

3.23 Exercise Answers

SQ1. Alternative (c). Free radicals are formed after IR by indirect action.
SQ2. The two BER mechanisms are SP-BER and LP-BER. SP-BER involves replacing the damaged base only. It requires DNA synthesis to replace the missing bases by DNA polymerase β, and to finalize the process, it uses ligase 3. In LP-BER, up to ten nucleotides are cut out and replaced, and the polymerases used are DNA polymerases δ and ε and ligase 1 to finalize the process.
SQ3. Alternative (d). Mismatch repair.
SQ4. Common: End termini protection is used to avoid extensive exonuclease activity, but different proteins are important for HR and NHEJ for this purpose. Differences: HR is operative only when there is an undamaged chromosome to work with, i.e., in late S or G2, while NHEJ can operate on DNA DSBs in all cell cycle phases. The fidelity in repair is higher in HR, while NHEJ may cause alterations in DNA sequence as a consequence of the repair which can result in mutations/chromosomal aberrations and which may cause oncogenic transformation of cells.
SQ5. Alternative (b). High-LET radiation.
SQ6. Alternative (c). Superoxide dismutase converts superoxide anions to hydrogen peroxide.
SQ7. Alternative (a). It works in the G2 into M transition.
SQ8. Alternative (b). Complete the cycle and divide.
SQ9. The mitosis is most sensitive, and early G1 and late S are most resistant.
SQ10. Because otherwise they would reach their Hayflick limit and proceed to senescence, and thus they would not be able to divide continuously to form large tumors.
SQ11. The type of radiation quality, dose, and dose rate as well as the cellular threshold for DNA damage and repair largely influence the cell death route. The position in the cell cycle when the damage is inflicted as well as functionality of DNA damage sensors, e.g., TP53, influence the decision.
SQ12. Alternative (c). Mitotic catastrophe.
SQ13. Alternative (e). Cell death after IR can take place via several routes, including mitotic catastrophe, autophagy, apoptosis, and necrosis.
SQ14. The triggers and execution of the two pathways, intrinsic and extrinsic, are depicted in Fig. 3.38. The answer can be found in the legend of the figure.
SQ15. Initiation and phagophore nucleation-phagophore elongation-cargo sequestration-autophagosome maturation-fusion of the autophagosome with the lysosome.
SQ16. Alternatives (a, b, d, and e). The plating efficiency at 0 Gy is 66.6%, the plating efficiency at 2 Gy is 6.4%, the surviving fraction at 2 Gy is 9.5%, and the surviving fraction at 1 Gy is 100%.
SQ17. The alteration of an oncogene because it then affects the normal embryonic development and causes embryonic lethality. The alteration of a tumor suppressor gene does not affect the embryogenesis; it increases the probability of cancer during life.
SQ18. From the table, it can be seen that the radiosensitivity is correlated to the existence of TNTs and their density and the complexity of networks formed. If all other properties are the same, the hypothesis which can be formulated is the following: The ability of cells to avoid death after irradiation is connected to the ability of the cells to communicate in a direct and fast manner through TNTs. This might be linked to the rescue effect, where less damaged cells are able to send components needed for the damaged cells to survive.
SQ19. Macrophages, endothelial cells, lymphocytes, and PMN.
SQ20. Answers:
(a) True, 5-mC accounts for about 1% of all bases within DNA.
(b) False, guanine is the predominantly modified base.
(c) False, histone variants are synthesized throughout the cell cycle.
(d) True, lysine and arginine are the most frequently acetylated amino acids.
SQ21. No, each miRNA can act on multiple different target genes, and one target gene can be regulated by many different miRNAs.
SQ22. True.
SQ23. False: PTEN is a central negative regulator of the PI3K/AKT pathway.
SQ24. a. Wrong, lncRNAs lack protein-coding sequences and they are not translated.
b. Wrong, lncRNAs are defined as transcripts longer than 200 bp; microRNAs around 20 nucleotides in size.
c. Correct, for example lncRNAs can interact with mRNAs and microRNAs for regulatory purposes.
SQ25. a. correct. The different sizes of extracellular vesicles are given in Table 3.14.

b. Wrong. Extracellular vesicles cargo in addition to proteins also mRNA/miRNA, long noncoding RNAs, DNA fragments, and lipids.
c. Correct. In particular apoptotic bodies; see Table 3.14.
d. Wrong. Exosomes are generated by viable cells (Fig. 3.52) albeit they may act as communicators in cell death.
e. Wrong. Extracellular vesicles may via their cargo participate in both cell pro-survival and pro-death signals.

SQ26. (a) MRM method.

SQ27. (a) The low dose will not phosphorylate enough ATM to activate the early G2 checkpoint, and the cells will proceed to mitosis with unrepaired damage and die. (b) The cells will be arrested in G2, and the DNA damage that is repairable will be repaired before the cells enter mitosis.

SQ28. The timing between doses may coincide with the duration of early G2 arrest.

SQ29. A protection against high radiation doses induced by a low "priming" dose.

SQ30. For example, to activate pro-survival pathways and to improve DNA repair ability through the activation of DNA damage checkpoint proteins.

SQ31. Oxygen fixates DNA damage and sensitizes the cells to radiation.

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