Role of Rad51 and DNA repair in cancer: A molecular perspective

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A B S T R A C T

The maintenance of genome integrity is essential for any organism survival and for the inheritance of traits to offspring. To the purpose, cells have developed a complex DNA repair system to defend the genetic information against both endogenous and exogenous sources of damage. Accordingly, multiple repair pathways can be aroused from the diverse forms of DNA lesions, which can be effective *per se* or via crosstalk with others to complete the whole DNA repair process. Deficiencies in DNA healing resulting in faulty repair and/or prolonged DNA damage can lead to genes mutations, chromosome rearrangements, genomic instability, and finally carcinogenesis and/or cancer progression. Although it might seem paradoxical, at the same time such defects in DNA repair pathways may have therapeutic implications for potential clinical practice. Here we provide an overview of the main DNA repair pathways, with special focus on the role played by homologous repair and the RAD51 recombinase protein in the cellular DNA damage response. We next discuss the recombinase structure and function *per se* and in combination with all its principal mediators and regulators. Finally, we conclude with an analysis of the manifold roles that RAD51 plays in carcinogenesis, cancer progression and anticancer drug resistance, and conclude this work with a survey of the most promising therapeutic strategies aimed at targeting RAD51 in experimental oncology.

Contents

Abbreviations: 4-HNE, 4-hydroxynonenal; 5'-AMP, 5'-adenosine monophosphate; 5′-dRP, 5′-deoxyribose phosphate; 53PB1, TP53-binding protein 1; 6,4PP, pyrimidine-6,4-pyrimidone photoproduct; 8-oxoG, 8-oxoguanine; 17-AGG, 17-allylamino-17-demethoxygeldanamycin; AAG/MPG, alkyladenine/methylpurine DNA glycosylase; ABL1, tyrosine-protein kinase ABL1 (aka c-ABL); ADS, asynchronous DNA synthesis; AGO2, argonaute-2; AKT1, RAC-alpha serine/threonine-protein kinase; AML, acute myeloid leukemia; AP, apurinic/apyrimidinic; aNHEJ, alternative non-homologous end joining; APE1, DNA-(apurinic or apyrimidinic site) lyase; APLF, aprataxin and PNK-like factor; APTX, aprataxin; ASR, asymmetric self-renewal; ATG5, autophagy related 5; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related homolog protein; ATRIP, ATR-interacting protein; BARD1, breast cancer type 1 susceptibility protein-associated RING domain protein 1; BCCIP, BRCA2 and CDKN1A-interacting protein; BCDX2, RAD51B/RAD51C/RAD51D/XRCC2 complex; BCL-2, B-cell lymphoma 2; B-CLL, B-cell chronic lymphocytic leukemia; BCR, breakpoint cluster region; BER, base excision repair; BET, bromodomain and extraterminal domain; BIR, break-induced replication; BLM, Bloom syndrome protein; BM, brain metastasis; BPDE, benzopyrene-7,8-dihydrodiol-9,10-epoxide; BRCA1, breast cancer type 1 susceptibility protein; BRCA2, breast cancer type 2 susceptibility protein; CTCF, CCCTC-binding factor; CD24, signal transducer CD24; CDK, cell cycle dependent kinase; CDKN3, cyclin dependent kinase inhibitor 3; ceRNA, competitive endogenous RNA; CETN2, centrin 2; CHK1, serine/threonine protein kinase CHK1; CML, chronic myeloid leukemia; CMS4, consensus molecular subtype 4; cNHEJ, classical NHEJ; CPD, cyclobutane pyrimidine dimer; CRC, colorectal cancer; cryo-EM, cryogenic electron microscopy; CSC, cancer stem cell; CSM2, chromosome segregation in meiosis protein 2; CST, CTC1-STN1-TEN1 complex; ctIP, DNA endonuclease RBBP8; CT/RT, chemoradiation therapy; CUL1, cullin1; CX3, RAD51C/XRCC3 complex; cyPu, cyclopurine; DCIS, ductal carcinoma in situ; dG, deoxyguanine; dHJ, double Holliday junction; diRNA, DSB-induced siRNA; DMC1, meiotic recombination protein DMC1/LIM15 homolog; DNA2, ATP-dependent helicase/nuclease 2; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DDR, DNA damage response; DSB, double-strand break; dsDNA, double-strand DNA; DDT, DNA damage tolerance; DSS1, deleted in split hand/split foot syndrome; EGFR, epidermal growth factor receptor; EMD, Electron Microscopy Data Bank; EMT, epithelial-mesenchymal transition; EOC, epithelial ovarian cancer; ER, endoplasmic reticulum; ERCC1, DNA excision repair protein ERCC-1; ERCC4, DNA repair endonuclease XPF; ERK1/2, extracellular signal-regulated protein kinase 1/2; ESA, epithelial specific antigen; ESC, embryonic stem cell; ESCA, esophageal cancer; EXO1, exonuclease-1; FA, Fanconi anemia; FAN1, Fanconi-associated nuclease 1; FANCD2, Fanconi anemia group D2 protein; FANCI, Fanconi anemia group I protein; FBH1, F-box DNA helicase 1; FEN1, flap structure-specific endonuclease 1; FIGNL1, fidgetin-like 1; FLIP, fidgetin-like 1 interacting protein, FMS-like tyrosine kinase 3; GB, glioblastoma; GBM, glioblastoma multiforme; GSC, glioblastoma stem cell; GCR, gross chromosomal rearrangements; GG-NER, global genome repair; γH2AX, histone variant H2AX; GPx2, hydroperoxide-scavenging enzyme glutathione peroxidase-2; H3K4, histone H3 lysine 4; HAT, histone acetyltransferase; hCAS/CSEIL, cellular apoptosis susceptibility/chromosome degregation1-like (aka exportin 2); HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HELB, DNA helicase B; HER2, receptor tyrosine-protein kinase erbB2; HGFR, hepatocyte growth factor receptor (aka c-MET); HIF1-α, hypoxia-inducible factor 1-α; HLX1, homeobox gene HLX1; HNC, head and neck cancer-deoxyguanine; HPV, human papilloma virus; HR, homologous recombination; Hsp90, heat shock protein 90; ICL, interstrand covalent crosslink; I-D, FANCI-

https://doi.org/10.1016/j.pharmthera.2020.107492

1. Introduction

To our cells, DNA damage is daily bad news. Thus, to preserve genomic integrity cells have developed an arsenal of DNA healing strategies that detect different types of damage and initiate the appropriate repair pathway or, if irreparable, induce cell cycle arrest and/or apoptosis. Some methods simply trim the nucleic acid broken ends and then reconnect them together. These procedures are fast and easy but, unfortunately, have the intrinsic disadvantage of possibly incorporating errors during the repair. Cells also have more accurate methods to repair DNA breaks; however, these responses need to be tightly regulated to ensure that repair pathways are only activated by genuine DNA damage and not, for example, in response to nucleic acid breaks deliberately generated by the cell for specific biological purposes (e.g., to initiate recombination between homologous chromosomes during meiosis or as intermediates during developmentally regulated rearrangements). This control is achieved by multiple levels of regulation, including checkpoint signaling, non-coding RNAs and post-translational modifications such as ubiquitylation.

One of such methods - namely homologous recombination - involves the exchange or replacement of a segment of parental DNA with a segment having the identical or very similar (i.e., homologous) sequence from a partner DNA, and the recent identification of the DNA repair protein RAD51 homolog 1 (RAD51) recombinase represents a landmark discovery in our understanding of the key reactions in this repair pathway in eukaryotes. With the aid of a plethora of accessory proteins, RAD51 performs the tricky task of surrounding the single DNA broken strand, and then captures the backup DNA copy, matching the sequence of the broken strand with a homologous sequence in the intact DNA double helix. Inside the RAD51 complex, the single strand is then exchanged for one of the strands in the duplex DNA, powered by ATP. Finally, a host of other proteins fill in all the missing sections of DNA, ultimately restoring two matching copies.

Although, as mentioned above, a tight control is imposed by the cell on each DNA break-and-repair event, sometimes one or more of these occurrences - homologous repair and its molecular commander RAD51 being no exception - can go awry, with potentially devastating consequences for the cell itself and/or the whole organism. Accordingly,

FANCD2 complex; IDBC, invasive ductal breast cancer; IDC, invasive ductal carcinoma; IGF, insulin and insulin-like growth factor; IGF-1R, IGF type 1 receptor; IR, ionizing radiation; ITD, internal tandem duplication; JAK2, tyrosine-protein kinase JAK2; KRAS, GTPase KRAS; KU, Ku70/Ku80 complex; LACC, locally advanced cervical cancer; LD_{50}, lethal dose; LIG, DNA ligase 1; LIG4, DNA ligase 4; LIGIIIα, DNA ligase IIIα; lncDNA, long non-coding DNA; lnc-RI, ionizing rasiation-inducible lncRNA; LOH, loss of heterozygosity; LP-BER, long-patch BER; LSD1, lysine-specific histone demethylase 1A; M1G, pyrimido[1,2-α]purin-10(3H-)one; m7G, 7-methylguanine; MAD2B, mitotic spindle assembly checkpoint protein MAD2B; MAP2K1, mitogen-activated protein kinase kinase 1 (aka MEK1); MAPK, p38 mitogen-activated protein kinase; MAT1, CDK-activating kinase assembly factor MAT1; MATR3, matrin3; MCM, minichromosome maintenance protein homolog; MDA, malondialdehyde; MDB4, methyl1-CpG-binding domain 4; MDC1, mediator of DNA damage checkpoint protein 1; MED1, mediator of RNA polymerase II transcription subunit 1; mFaPy-G, 2,6-diamino-4-hydroxy-5N-methyl-formamidopyrimidine; MET, mesenchymal-epithelial transition; MGMT, O^6-methylguanine-DNA-methyltransferase; MLH1, mutL homologue 1; miRNA, microRNA; MMR, mismatch repair; MMS22L, MMS22-like; MND1, meiotic nuclear division protein 1 homolog; MRE11, double-strand break repair protein MRE11; MSH2, mutS homologue 2; MSH6, mutS homologue 6; MYC, MYC proto-oncogene protein; MYH, MutY homolog; NBS1, Nijmegen breakage syndrome protein 1; NEIL, NEIL endonuclease VIII-like; NER, nucleotide excision repair; NHA, normal human astrocytes; NHEJ, non-homologous end joining; NHT1, endonuclease three homolog 1; NMR, nuclear magnetic resonance; NPC, nasopharyngeal carcinoma; NRP, neuropilin; NSB1, DNA repair and telomere maintenance protein NSB1; NSCLC, non-small-cell lung cancer; OGG1, 8-oxoguanine glycosylase 1; OS, overall survival; p130, retinoblastoma-like protein 2; p21, cyclin-dependent kinase inhibitor 1 protein; p400, E1A-binding protein p400; PALB2, partner and localizer of BRCA2; PANCC, pancreatic cancer cell; PARP, poly (ADP-ribose) polymerase; PARPi, PARP inhibitor; PC, prostate cancer; PCNA, proliferating cell nuclear antigen; PDB, Protein Data Bank; PDGF-B, platelet-derived growth factor subunit B; PHA, polycyclic aromatic hydrocarbon; PI3K, phosphoinositide-3-kinase; PIF1, ATP-dependent DNA helicase PIF1; PLK1, serine/threonine-protein kinase PLK1; PMS2, postmeiotic segregation increased 2; PNKP, bifunctional polynucleotide phosphatase/kinase; POF, premature ovarian failure; Polα, DNA polymerase α; Polβ, DNA polymerase β; Polδ, DNA polymerases δ; Polε, DNA polymerase ε; Polζ, DNA polymerase ζ; Polη, DNA polymerase η; Polθ, DNA polymerase θ; Polι, DNA polymerase ι; Polκ, DNA polymerase κ; Polλ, DNA polymerase λ; Polμ, DNA polymerase μ; Polν, DNA polymerase ν; POT, protection of telomers protein1; PSY3, platinum sensitivity protein 3; p-TEFb, positive transcription elongation factor b; PTEN, phosphatase and tensin homolog; PUFA, polyunsaturated fatty acid; RAD17, cell cycle checkpoint protein RAD17; RAD18, E3 ubiquitin-protein ligase RAD18; RAD23B, UV excision repair protein RAD23 homolog B; RAD50, DNA repair protein RAD50; RAD51, DNA repair protein RAD51 homolog 1; RAD52, DNA repair protein RAD52 homolog; RAD54, DNA repair and recombination protein RAD54; RAD51AP1, RAD51-associated protein 1; RADX, RPA-related protein RADX; RAF, proto-oncogene serine/threonine-protein kinase RAF; RCC, renal cell carcinoma; RAP1, telomeric repeat-binding factor 2-interacting protein; REV1, DNA repair protein REV1; RFC, replication factor C; RFWD3, E3 ubiquitin-protein ligase RFWD3; RIF1, telomere-associated protein RIF1; RIP-1, receptor-interacting serine/threonine-protein kinase 1; RISC, RNA-induced silencing complex; RMI, recQ-mediated genome instability protein; RNAi, RNA interference; RNAPII, RNA polymerase II; RNS, reactive nitrogen species; RPA, replication protein A; ROS, reactive oxygen species; RUVBL1, RuvB-like 1; S5S1, SWI5/SFR1 complex, S-adenosyl-L-methionine methyl transferase; SAHA, suberoyl anilide hydroxamic acid (aka vorinostat), stem cell; SCC, squamous cell carcinoma; SCF, SPK1/CUL1/F-box protein; SDSA, synthesis-dependent strand annealing; SCLC, small cell lung cancer; SFR1, SWI5-dependent recombination DNA repair protein 1 homolog; SH2/3, Scr homology 2/3; SDSA, synthesis-dependent strand annealing; SHU1/2, suppressor of HU sensitivity involved in recombination protein 1 and 2; SIM, SUMO-interacting domain; siRNA, small interfering RNA; SKP1, S-phase kinase-associated protein 1; SL, synthetic lethality; SMARCAL1, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1; SMI2s, singleminded homolog 2s; SMN, survival motor neuron; SMUG1, single-strand-selective monofunctional uracil DNA glycosylase 1; SNP, single nucleotide polymorphism; snRNA, small nuclear RNA; snRNP, spliceosomal small nuclear ribonucleoprotein; SP-BER, short-patch BER; SPIDR, scaffolding protein involved in DNA repair; SSA, single-strand annealing; SSB, single-strand break; ssDNA, single-strand DNA; STAT5, signal transducer and activator of transcription 5; STS, soft tissue sarcoma; SUMO, small ubiquitin-like modifier; SWI5, DNA repair protein SWI5 homolog; SYCP3, synaptonemal complex protein 3; t-AML, therapy-related AML; TA-HRR, transcription-associated homologous recombination repair; TAZ, transcriptional co-activator with PDZ-binding motif; TC-HR, transcription homologous recombination; TC-NER, transcription-coupled repair; TDG, thymine DNA glycosylase; TDP1, tyrosyl-DNA phosphodiesterase 1; TFIIH, transcription factor II human; ThyC, thyroid cancer; TIF1-β, transcription intermediary factor 1-β; TIN2, TRF1-interacting nuclear protein 2; TKI, tyrosine kinase inhibitor; TLS, translesion DNA synthesis; TNBC, triple-negative breast cancer; TONSL, Tonsoku-like; TOP1, DNA topoisomerase 1; TOPBP1, DNA topoisomerase 2-binding protein 1; TPP1, adrenocortical dysplasia protein homolog; TS, template switching; UPR, Unfolded protein response; TMZ, temozolomide; TRF, telomeric repeat-binding factor; UAF1, USP1-associated factor 1; UNG, uracil DNA glycosylase; UNRIP, unr-interacting protein; USP1, ubiquitin carboxyl-terminal hydrolase 1; UTR, untranslated region; UV, ultraviolet; VEGF, vascular endothelial growth factor; VHL, von Hippel-Landau tumor suppressor gene; WT, wildtype; WRN, Werner syndrome ATP-dependent helicase; WRNIP1, WRN interacting protein 1; XLF, non-homologous end-joining factor 1; XPA, DNA repair protein complementing XP-A cells; XPC, xeroderma pigmentosum complementation group C protein; XPC-C, XPC complex; XPG, DNA repair protein complementing XP-G cells; XRCC1, X-ray repair cross-complementing protein 1; XRCC4, X-ray repair cross-complementing protein 4; YAP, yes-associated protein.

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in this review we initially explore the main pathways that detect and repair different types of DNA damage, highlighting the special role played by homologous repair and RAD51 in the cellular DNA damage response. We next focus on the recombinase structure and function *per se* and in combination with all its principal mediators and regulators, and proceed with analyzing the multifaceted roles that RAD51 plays in carcinogenesis, cancer progression and anticancer drug resistance. Finally, we conclude this work with a survey of the most promising therapeutic strategies aimed at targeting RAD51 in experimental oncology. Our intention is to provide the readers not only with all basic aspects of the topics just mentioned above and, likely for the first time, with a molecular structure perspective of the main actors involving in DNA repair (and specifically in RAD51 functions) when available, but also with the most updated literature references on these subjects which appeared in the last 10 years. Nonetheless, given the wealth of data currently available, we regretfully had to perform a choice, the responsibility of which relies entirely on us. Therefore, we offer our sincere apologies to those colleagues whose excellent work has not been directly cited due to space limitation.

2. The DNA damage challenges

Maintenance of genetic integrity is critical for the survival of any organism. Yet, every day human cells may experience up to 10^5 spontaneous DNA lesions (Hoeijmakers, 2009) provoked by both endogenous and exogenous threats. The former may result from several cellular processes including, e.g., mismatches of nucleobases due to errors in DNA replication, epigenetic modifications, nucleobase oxidation, alkylation, hydrolysis, and bulky adduct formations. Damages caused by exogenous agents come in a multiplicity of forms, and include, among others, ultraviolet (UV) light, ionizing radiations, hydrolysis or thermal disruption, and industrial and environmental chemicals, as briefly discussed below.

2.1. Endogenous DNA damage

2.1.1. Spontaneous hydrolytic DNA decay

As a chemical entity in an aqueous environment, DNA can undergo spontaneous reaction with water, ultimately leading to the formation of an apurinic/apyrimidinic (AP) site, due to hydrolysis of the glycosidic bond between the sugar and the base (Fig. 1, left). As AP sites are non-coding templates, their presence can cause mutagenic endpoints or can effectively halt the DNA replicative processes performed by DNA or RNA polymerases (Loeb & Preston, 1986). In addition, AP sites can potentially react with guanine residues located at the opposite strand, resulting in even more dangerous DNA damages known as interstrand covalent crosslinks (ICLs) (Deans & West, 2011) (Fig. 1, middle).

DNA hydrolysis can also generate inappropriate base entities, such as uracil and thymine formation from cytosine or 5-methylcytosine via a deamination reaction (Liu & Meng, 2018). Recent studies have shown that unexpected uracil or thymine deriving from deamination reactions are major drivers of mutagenic events associated with carcinogenic processes (Olinski, Jurgowiak, & Zaremba, 2010). Indeed, as uracil base-pairs with adenine when copied, the original information coded by cytosine are ultimately changed into those of thymine in what constitutes a common cancer-associated C>T mutational signature (Rogozin et al., 2018).

2.1.2. DNA base alkylation

In addition to spontaneous hydrolytic decay, endogenously generated intracellular metabolites are an important source of DNA lesions. For instance, the S-adenosyl-L-methionine methyl transferase (SAM) can modify bases to form pre-mutagenic lesions such as 7-methylguanine (m7G) at very high daily rate (~4000/day) (Ames, 1989). Although m7G does not appear to alter the genetic information, it is subjected to high risk of conversion into an AP site or into the corresponding replication-arresting 2,6-diamino-4-hydroxy-5N-methylformamidopyrimidine (mFaPy-G) (Fig. 1, right). SAM also methylates all three remaining DNA bases at position 3, and the corresponding modified nucleotides act as potent replication blockers (Rydberg & Lindahl, 1982). Finally, mutagenic O-alkylated adducts (such as O^6-methylguanine, O^4-methylthymine, and O^4-ethylthymine) are generated by N-nitroso compounds and may result into GC>AT and TA> GC transitions during DNA replication (Du, Wang, Li, & Wang, 2019).

2.1.3. Reactive oxygen species

Reactive oxygen species (ROS) - including the superoxide anion radicals ($O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen ($^1O_2$) - are other sources of endogenous DNA lesions produced by e.g., mitochondria (where O_2 acts as a terminal electron acceptor for electron transport chain), NADPH oxidase (a cell membrane bound enzyme), peroxisomes (which contain enzymes that produce H_2O_2 e.g. polyamine oxidase), endoplasmic reticulum (that produces H_2O_2 as a byproduct during protein folding (Kalyanaraman et al., 2018; Srinivas, Tan, Vellayappan, & Jeyasekharan, 2018)) or by specific chemistry such as the Fe^{2+}-catalyzed reduction of H_2O_2 to $OH^{\bullet}$ (Fenton reaction). Cells cope with ROS production exploiting different chemistries, e.g., the reduction of $O_2^{\bullet-}$ to H_2O_2 (via the enzymatic action of superoxide dismutase) and its further degradation to water by glutathione reductase (Jezek, Cooper, & Strich, 2018). Yet, ROS eluding cell surveillance can induce extensive DNA damages, which include base oxidation, AP sites formation (§2.1.1), and single-strand breaks (SSBs) generation (see below) (Cadet & Davies, 2017) (Fig. 2, left). 8-oxoguanine (8-oxoG), 8- or 2-hydroxyadenine and cyclopurines (cyPus) are all ROS-induced mutagenic base lesions that act either as miscoding templates or replication blockers. 8-oxoG is one of the best characterized oxidative DNA lesions, and it can give rise to point mutations due to its miscoding potential that instructs most DNA polymerases to preferentially insert adenine opposite 8-oxoG instead of the correct cytosine. If uncorrected, A:8-oxo-G mispairs can give rise to CG>AT transversion mutations (Markkanen, 2017) (Fig. 2, middle).

On the other hand, bulky cyPus adducts such as 8,5'-cyclopurine-2'-deoxynucleotides - generated by the attack of $OH^{\bullet}$ to 2′-deoxyribose units followed by C5′ radical generation and cyclization with the C8 position of the purine base (Chatgilialoglu et al., 2019) - when

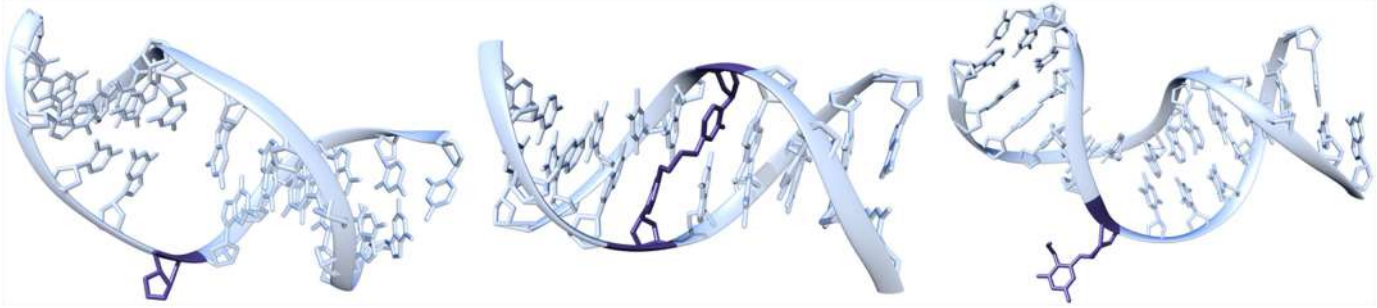

Fig. 1. Models of DNA (light blue) carrying an AP basic site (left), an alkyl ICL (middle), and a mFaPy-G alkylation (right). All lesions are highlighted in a dark slate blue color.

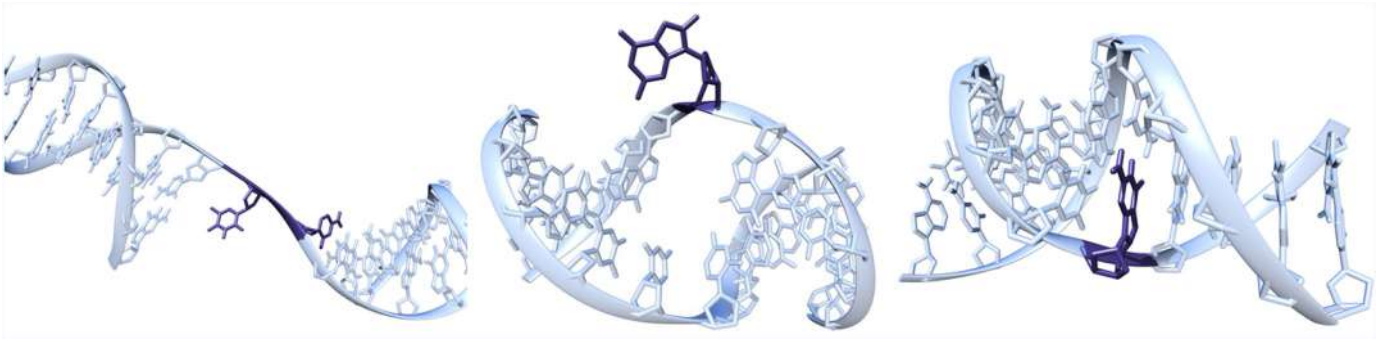

Fig. 2. Models of a DNA SSB (left), a DNA carrying an 8-oxoG lesion (middle), and a DNA with a cyPu adduct (right). Colors as in Fig. 1.

incorporated cause substantial structural changes in DNA that include displacement of the purine base, an unusual sugar pucker, deformation of the sugar-phosphate backbone, and alterations in the base stacking with adjacent nucleotides in DNA (especially 5′ to the lesion). As such, cyPus are strong blockers of DNA transcription (Brooks, 2017). ROS are one of the most common sources of DNA SSBs, i.e., discontinuities in one strand of the DNA double helix usually accompanied by loss of a single nucleotide and by damaged 5′- and/or 3′-termini at the site of the break, which can occur directly by disintegration of the oxidized sugar or indirectly during DNA repair of oxidized bases (§4.1.1), AP sites, or bases that are damaged or altered in other ways (Caldecott, 2008). If not repaired rapidly or appropriately, chromosomal SSBs pose a serious threat to genetic stability and cell survival, the most likely consequence of unrepaired strands in proliferating cells being the blockage or collapse of DNA replication forks during the S-phase of the cell cycle, possibly leading to the formation of double-strand breaks (DSBs, §5.2) (Abbotts & Wilson III., 2017). Finally, ROS can generate other forms of endogenous genotoxins which, in turn, may exert DNA damage. A prototypical example is constituted by lipid peroxidation, a process involving the oxidation of polyunsaturated fatty acids (PUFAs), which are basic components of biological membranes, to give lipid hydroperoxides as the main primary products (Farmer & Mueller, 2013). Among the many different aldehydes which can be formed as secondary products during lipid peroxidation - malondialdehyde (MDA), propanal, hexanal, and 4-hydroxynonenal (4-HNE) - MDA appears to be the most mutagenic whereas 4-HNE the most toxic (Srinivas et al., 2018). In particular, MDA can react with all DNA bases, the major product being the bulky adduct pyrimido[1,2-α]purin-10(3H-)one (M1G). In the absence of repair, M1G and other MDA-DNA adducts may lead to mutations (point and frameshift), strand breaks, cell cycle arrest, and induction of apoptosis, and all this MDA-induced DNA alteration may contribute significantly to cancer and other genetic diseases (Ayala, Munoz, & Arguelles, 2014).

2.1.4. Base/nucleotide mismatch, indels, and epigenetic modifications

The molecular machinery that is responsible for replicating almost all chromosomal DNA sequences has a remarkable low rate of error by virtue of the high fidelity of nuclear DNA polymerases such as DNA polymerase α (Polα), δ (Polδ), and ε (Polε). While advancing down single-strand DNA templates and extending nascent DNA strands in a 5'-3' direction, these polymerases continuously look backward scanning the stretch of DNA they have just synthesized in a "watch over your shoulder" process called *proofreading*. Should these enzymes detect a copying error, they will use their 3'-5' exonuclease activity to move backward, digest the erroneous new DNA segment, and copy this segment again confiding in a correct outcome during the second round. Notwithstanding this evolved strategy, erroneous nucleotide incorporation takes place with the frequency of 1 out every 100 millions of insertional events (Bebenek & Ziuzia-Graczyk, 2018), the inadvertent insertion of a ribonucleotide being more probable by virtue of their higher physiological concentration with respect to deoxyribose-based ones (Cerritelli & Crouch, 2016; Williams, Lujan, & Kunkel, 2016). Both these incidents can change the original genome coding, induce structural modifications in the DNA regions deputed to protein binding, or alter the epigenetic landscape, all events potentially leading to cancerous outcomes.

Some DNA regions found in many places along the entire genome carry short mononucleotide repeats (e.g., AAAAAAA), dinucleotide repeats (e.g., AGAGAGAG) or repeats of greater complexity, globally called *microsatellites*. Because of strand slippage, an event that may occur when the parental and nascent strand slip out of proper alignment (Sinden, Pytlos-Sinden, & Potaman, 2007), DNA polymerases may occasionally 'stutter' while copying these repeats, resulting in the incorporation of longer or shorter versions of the microsatellites into the newly formed daughter strands. The resulting insertions or deletions (*indels*) may elude detection by the proofreading components of the DNA polymerases, thereby permitting chromosome duplication to proceed (Madireddy & Gerhardt, 2017). Failure in detecting and removing indels will result in the expansion or shrinkage of the microsatellite sequences in progeny cells. This creates the genetic condition known as *microsatellite instability*, which can cause a range of disorders, including Huntington disease, various ataxias, motor neuron disease, frontotemporal dementia, fragile X syndrome, other neurological pathologies and cancer (Cortes-Ciriano, Lee, Park, Kim, & Park, 2017; Hannan, 2018; Maruvka et al., 2017).

A type of inheritable DNA alteration similar in some ways to a mutation is an epigenetic change, which refers to a functionally relevant modification of DNA or of the histone proteins controlling the winding/relaxation of the nucleic acid within the nucleosome structures. The information conveyed by epigenetic modifications such as DNA methylation, histone modification, nucleosome remodeling, and RNA-mediated targeting plays a critical role in the regulation of all DNA-based processes, such as transcription, DNA repair, and replication. Consequently, abnormal expression patterns or genomic alterations in chromatin regulators can have profound results and can lead to the induction and maintenance of various cancers (Dawson & Kouzarides, 2012). A typical epigenetic pathway connected to cancer is DNA methylation at particular sites (CpG islands) which are present in ~70% of all mammalian promoters (Koch et al., 2018). CpG hypermethylation of promoters (between 5%-10% in various cancer genomes) not only affects the expression of protein coding genes but also the expression of various noncoding RNAs like microRNAs (miRNAs), some of which have a role in malignant transformations (§8.7) (Peng & Croce, 2016). Other epigenetic changes involve modifications of histones associated with particular regions of DNA. Although the great diversity in histone modifications introduces a remarkable complexity that is slowly beginning to be elucidated (Fig. 3, right), it is now clear that these modifications have a major influence, not just on transcription, but in all DNA-templated processes (Gates, Foulds, & O'Malley, 2017).

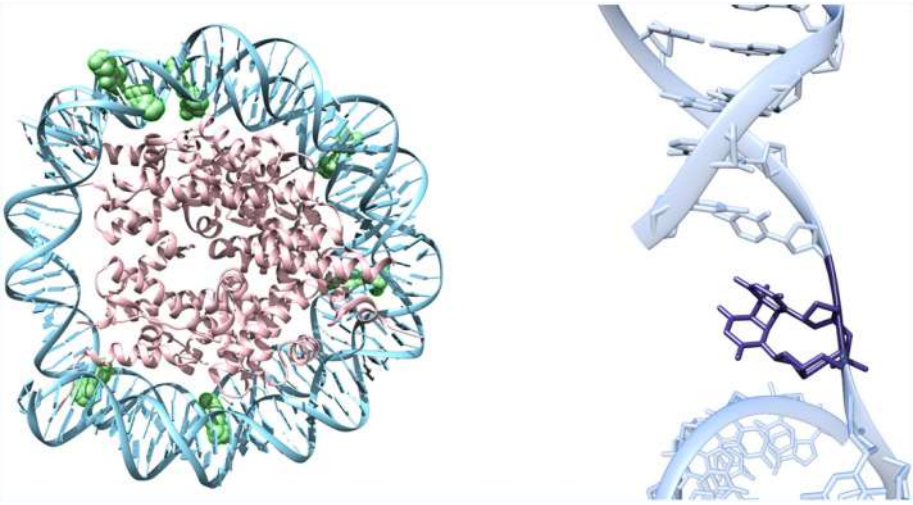

Fig. 3. (Left) Structure of a nucleosome (a fundamental unit of DNA packaging in eukaryotes, consisting of a segment of DNA wound in sequence around eight histone protein cores, pink ribbons) showing the human DNA satellite 2 sequence (light blue) methylated at CpG sites (green spheres) in the pericentric region of human chromosome 1 (Protein Data Bank (PDB): 5CPK (Osakabe et al., 2015)). (Right) Model of a damaged DNA structure (light blue) carrying a CPD lesion (dark slate blue).

2.2. Exogenous DNA damage

2.2.1. Ultraviolet radiations

Sunlight is a very strong, unavoidable genotoxic stressor. Although the ozone layer absorbs the most dangerous part of the solar UV spectrum (UVC), residual UVA and UVB in strong sunlight can induce up to 10^5 lesions per exposed cell per hour (Mullenders, 2018). As generally known, this natural threat can cause pathologies ranging from mild sunburns to skin cancer induction (Cadet & Douki, 2018). In particular, UVB damages DNA directly by promoting covalent linkage formation between adjacent pyrimidine bases (*photodimers*), creating primarily cyclobutane pyrimidine dimers (CPDs) (Fig. 3, right) and pyrimidine-6,4-pyrimidone photoproducts (6,4PPs). In contrast, UVA damage DNA indirectly via interactions with cellular chromophores that act as photosensitizers that generate DNA-damaging ROS (§2.1.3). Depending on the distance between the chromophore and the target, UVA irradiation can also result in one-electron abstraction and the formation of a reactive radical cation. Importantly, UVA-generated ROS damage other biomolecules including proteins and lipids, and this non-DNA photodamage may be a further contributor to carcinogenesis (Brem, Guven, & Karran, 2017).

2.2.2. Ionizing radiations

IRs (e.g., natural radioactive decay, cosmic radiation, or different radiation sources used in medical imaging) also contribute DNA damage by direct ionization or indirect ROS production via water radiolysis (Sage & Shikazono, 2017; Vignard, Mirey, & Salles, 2013). The main lesions induced by IR include base modifications, SSBs and DSBs, depending on the time and type of radiation exposure, which can ultimately lead to cancerous malignancies. For example, uranium spontaneous decay produces radioactive radon gas associated with lung-cancer incidence (Shankar et al., 2019), while radiation-induced second malignancies following exposure to natural or synthetic radioisotopes (e.g., ^{18}F, ^{68}GA, ^{90}Y, ^{99m}TC, ^{131}I, ^{152}Tb, and ^{177}Lu) are important late side effects having an impact on optimal cancer theranostics (Dracham, Shankar, & Madan, 2018; Yordanova et al., 2017). Finally, the aftermaths of nuclear detonations over Hiroshima and Nagasaki, and the two major nuclear disasters of Chernobyl (Ukraine) and Fukushima (Japan) have provided lessons about the health consequences following prolonged/excessive radiation exposure (Kamiya et al., 2015).

2.2.3. Environmental and lifestyle-related threats

Man-produced chemicals present in our environment and that are part of our lifestyle mimic the action of IRs and thus are also effective in inducing DNA damages (Tiwari & Wilson, 2019). Typically, compounds that damage DNA and inhibits its repair - such as polycyclic aromatic hydrocarbons (PHAs), N-nitroso molecules, aldehydes, and benzene among others - are generated in tobacco smoke (Weng et al., 2018). The most important tumorigenic compound in tobacco smoke is acrolein, an α,β-unsaturated aldehyde that efficiently reacts with

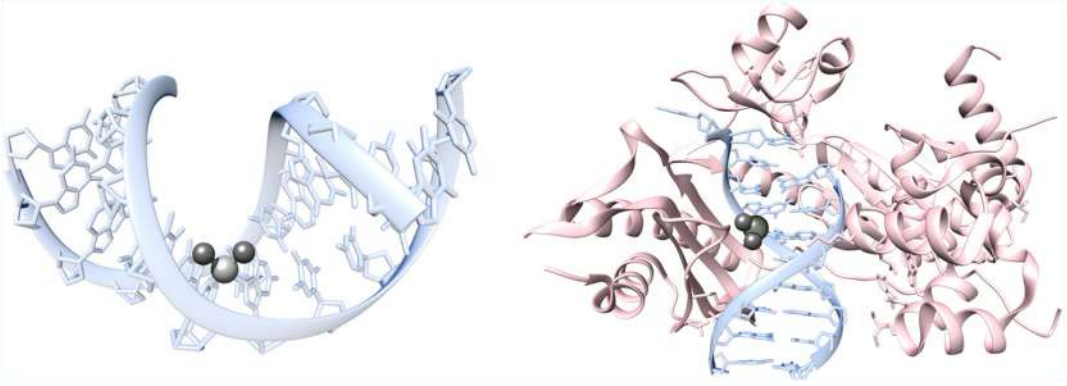

Fig. 4. (Left) Crystal structures of a double-stranded DNA (light blue) containing an intrastrand adduct of the anticancer drug cisplatin (gray spheres, PDB: 3LPV (Todd & Lippard, 2010)). (Right) Human DNA polymerase η (pink ribbons) failing to extend the primer (light blue) 2 nucleotide after a cisplatin crosslink (gray spheres) as captured by X-ray spectroscopy (PDB: 4DL7 (Zhao et al., 2012)).

deoxyguanine (dG) to give two exocyclic DNA adducts known as HOPdGs (hydroxy-propano-deoxyguanines), which are the major type of mutagenic DNA damage caused by this social attitude (X. Y. Liu, Zhu, & Xie, 2010). PHAs like benzopyrene and anthracene, that are produced during standard energy source consumption (oil & gas) and can be also found in food, are well known carcinogens and indeed several PHAs leave molecular signatures in the form of characteristic patterns of mutations in the tumor suppressor gene TP53, the genome master guardian controlling cell proliferation, differentiation, apoptosis and DNA repair regulation (§7.2.10) (Hafner, Bulyk, Jambhekar, & Lahav, 2019). As an example, benzopyrene is metabolized via enzymatic reactions involving cytochrome P450 to a major carcinogenic metabolite (benzopyrene-7,8-dihydrodiol-9,10-epoxide or BPDE) that binds DNA to give BPDE-N^2-dG adducts. These, in turn, promotes G>T transversion mutations that negatively affect the replication process (Seo, Jelinsky, & Loechler, 2000). Also, DNA damaging chemicals can be found in foods including aflatoxin-contaminated peanuts (Bedard & Massey, 2006), overcooked meat containing heterocyclic amines, and a plethora of other dietary risks extensively reviewed in a recent global study published on Lancet (Collaborators, 2019).

Several distinct classes of DNA damaging compounds are intentionally used as frontline cancer chemotherapeutics in clinical oncology (Guichard, Guillarme, Bonnabry, & Fleury-Souverain, 2017), including base-alkylating agents (Fu, Calvo, & Samson, 2012), and crosslinking molecules that introduce covalent bonds between the DNA bases of the same strand (*intrastrand*) or ICLs (Deans & West, 2011; Yu, Wang, Cui, & Wang, 2018) (Fig.4). Prototypical examples are i) platinum-based molecules like cisplatin and carboplatin (Sarkar, 2018), which prevalently stall DNA replication and transcription by forming ICL adducts, thereby eliciting cell death responses (Dasari & Tchounwou, 2014); ii) nitrogen mustards such as cyclophosphamide, ifosfamide, melphalan and chlorambucil (Singh, Kumar, Prasad, & Bhardwaj, 2018), which mainly alkylate the N^7-position of the base guanine; iii) antimetabolites including methotrexate, 5-fluorouracil, and gemcitabine (Parker, 2009) that, being synthetic purines and pyrimidines, interfere with DNA replication by either direct substitution during DNA or RNA copying or promoting nucleotide pool imbalance that arrests chromosome duplication (Pai & Kearsey, 2017); and iv) natural products like mitomycin C and psoralens that induce DNA intra/interstrand links (Huang & Li, 2013). Additionally, inhibitors of *topoisomerases* (those ubiquitous enzymes controlling DNA supercoiling and entanglements that are essential during transcription and replication) like camptothecin, etoposide, daunorubicin and doxorubicin induce the formation of both DNA SSBs and DSBs by trapping topoisomerase-DNA intermediates during isomerization reactions (Liang et al., 2019; Pommier, 2013).

Finally, bacteria and viruses can also contribute to DNA damage and, ultimately, cancer (Krump & You, 2018; van Elsland & Neefjes, 2018). For example, *Helicobacter pylori* infection increases both ROS and reactive nitrogen species (RNS) production in the human stomach which, in turn, induces significant DNA damage to the gastric epithelial cells (Butcher, den Hartog, Ernst, & Crowe, 2017). In the case of human papilloma virus (HPV), while healthy cervical cells can deal with RNS-induced stress, when infected by HPV the relevant production of RNS in the same cells results in higher levels of DNA mutations and DSBs (Cruz-Gregorio, Manzo-Merino, & Lizano, 2018).

3. The DNA damage response

Because DNA normal functions demand structural and sequence integrity over many hundreds of millions of non-redundant base pairs, the chromatin proteins in which DNA is embedded might afford some damage protection. Yet, the vital process of replication, transcription and even repair itself required chromatin rearrangement, implying periods in which DNA vulnerability might be enhanced. The eukaryotic strategy to deal with damaged DNA and secure somatic cell homeostasis is the DNA damage response (DDR), a complex network of cellular pathways that masters injured DNA recognition, damage assessment (enforced by checkpoints), coordination of DNA repair or commitment of the unrepairable cells to senescence or apoptosis. This DDR signal transduction cascade includes specialized sensor proteins that recognize DNA damage, and transducer proteins that in turn recruit effectors responsible for the ultimate decisional steps (Fig. 5) (Ciccia & Elledge, 2010; Goldstein & Kastan, 2015; Jackson & Bartek, 2009). Accurate DNA repair is a key part of the DDR, and its loss leads to genome instability (Shen, 2011), which is a hallmark of cancer development (Andor, Maley, & Ji, 2017; Negrini, Gorgoulis, & Halazonetis, 2010; Turgeon, Perry, & Poulogiannis, 2018; Yao & Dai, 2014).

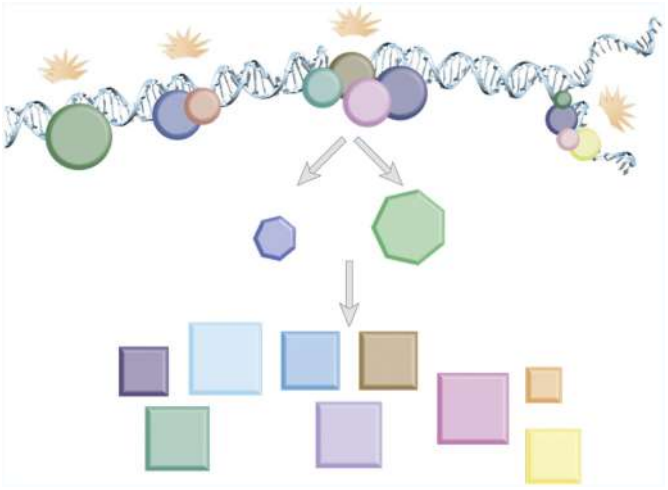

Fig. 5. Schematic representation of cellular response to DNA damage. Multiple sensor proteins (colored circles) are initially involved in recognizing the different nucleic acid lesion (light orange pointed symbols). Other transducer proteins (colored hexagons) then amplify and diversify the DNA-damage signal, and a plethora of downstream effectors (colored squares) regulate various aspects of the cellular function, including replisome stability, transcription, cell cycle, energy and autophagy, chromatin remodeling, repair, RNA processing, apoptosis and senescence.

Under normal physiological conditions, this sophisticated orchestra processes six major DNA repair pathways to heal any damaged DNA prior to trigger checkpoint control: base-excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), translesion DNA synthesis (TLS), non-homologous end joining (NHEJ), and homologous recombination (HR). The major determinant of the DDR repair pathway choice is the initiating lesion itself, i.e., "the wand chooses the wizard" (Rowling & GrandPré, 1998). For those DNA damages that do not alter substantially the nucleic acid double helical structure and/or interfere with the DNA/RNA polymerase progression - recently termed *idle* lesions (Tiwari & Wilson 3rd., 2019) - DDR usually relies on MMR and BER. *Active* lesions that explicitly modify the duplex or impede polymerase activity require more complex pathways like NER, stimulate TLS mechanisms or eventually activate more generalized responses ultimately leading to cell senescence or death. Finally, SSB and DSB modifications naturally halt DNA replication processes and, being *severe* nucleic acid lesions, require resolution via dedicated DDR pathways like NHEJ and HR.

4. DNA idle and active lesions repair pathways

4.1. DNA idle lesions repair pathways

4.1.1. Base-excision repair

BER, a highly conserved pathway from bacteria to humans, can be considered as the cellular workhorse repair mechanism coping with the vast majority of idle lesions resulting from, e.g., metabolic ROS production, environmental stress, alkylating agents and spontaneous reactions such as deaminations and depurinations (Wallace, 2014). BER

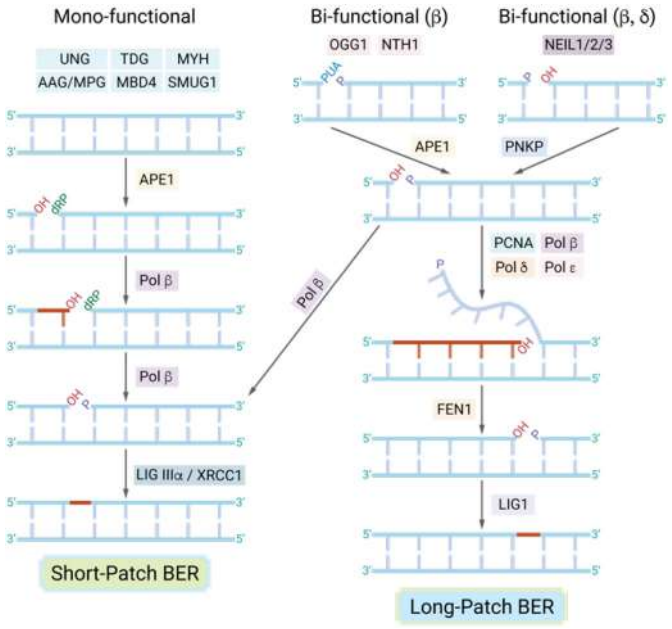

Fig. 6. Schematic representation of the human BER pathway.

response involves five key enzymatic steps: i) excision of damaged or inappropriate base; ii) incision of the phosphodiester backbone at the resulting abasic site; iii) termini clean-up to permit unabated report synthesis and/or nick ligation, iv) gap-filling to replace the excised nucleotide, and v) sealing of the final, remaining DNA nick. These repair steps are executed by a pool of enzymes that include DNA glycosylases, AP endonucleases, phosphatases, phosphodiesterases, kinases, polymerases and ligases (Kim & Wilson 3rd., 2012) (Fig. 6).

The initial step in BER is the search for DNA lesions by DNA glycosylases, enzymes that first recognize the damaged or inappropriate base by stably flipping it into the compatible enzyme active site and then catalyze the cleavage of the N-glycosidic bond between the substrate base and the 2'-deoxyribose creating an abasic site. Of these eleven enzymes, four (i.e., uracil DNA glycosylase (UNG), thymine DNA glycosylase (TDG), single-strand-selective monofunctional uracil DNA glycosylase 1 (SMUG1), and methyl1-CpG-binding domain 4 (MDB4)) preside over the removal of mispaired uracil and thymine, one processes the removal of alkylated bases (the alkyladenine/methylpurine DNA glycosylase (AAG/MPG)), and six are involved in oxidative damage repair (MutY homolog (MYH), 8-oxoguanine glycosylase 1 (OGG1), endonuclease three homolog 1 (NHT1), and NEIL endonuclease VIII-like 1, 2 and 3 (NEIL1/2/3)). The first five glycosylases are monofunctional enzyme (Drohat & Maiti, 2014) that recognize and remove inappropriate bases to form AP sites (i) (Fig. 6, left). These AP sites are, in turn, recognized by the DNA-(apurinic or apyrimidinic site) lyase (APE1), which incises the damaged strand leaving 3′-OH and 5′-deoxyribose phosphate (5′-dRP) groups at the margins (Fig. 7, left).

A DNA polymerase β (Polβ)-mediated DNA synthesis step fills the single-nucleotide gap (Fig. 7, right), and the cytotoxic 5′-dRP group is removed by the 5′-dRP lyase activity of the same enzyme (iii, iv). Alternatively, DNA polymerase λ (Polλ) or DNA polymerase ι (Polι) - both encoding a 5′-dRP lyase activity - may also participate in BER to remove this toxic repair intermediate. Finally, DNA ligase I (LIG1) or a complex of DNA ligase IIIα (LIGIIIα) and the X-ray repair cross-complementing protein 1 (XRCC1) conduct the final, nick-sealing step in the pathway (v). Since Polβ operates on short nucleotide gaps, this pathway typically involves the incorporation of a single nucleotide and is therefore known as the short-patch BER (SP-BER) (left pathway in Fig. 6).

The glycosylases that recognize oxidative lesions are bifunctional and not only excise the damaged base but also cleave the DNA backbone, leaving either an α,β unsaturated aldehyde (OGG1 and NTH1) or a phosphate (NEIL1/2/3) attached to the 3′ side of the nick (Fig. 6, right) (Whitaker, Schaich, Smith, Flynn, & Freudenthal, 2017). The sugar is then removed by the phosphodiesterase activity of APE1, and the phosphate group by the polynucleotide phosphatase/kinase PNKP (PNKP). Distinctly from SP-BER, in this case multiple nucleotides (2 to 12) are usually incorporated via synthesis mediated by Polδ and Polε, in cooperation with the proliferating cell nuclear antigen (PCNA). Accordingly, this pathway is termed long-patch BER (LP-BER) (Fig. 6, right pathway). The downstream 5' DNA end of the damage-containing strand is next displaced to form a flap intermediate, which is subsequently removed by the flap structure-specific endonuclease 1 (FEN1). Finally, the nick is sealed by a ligase, usually LIG1.

4.1.2. Mismatch repair

MMR removes base mismatches and small indels generated by replication errors, spontaneous/induced base modifications such as methylation or oxidation, and repairs DNA adducts like those formed by platinum-based chemotherapeutics (Jiricny, 2006; Kunkel & Erie, 2015). It is a very highly conserved cellular process that plays additional roles in DSB repair (§5.2), apoptosis and recombination. Four MMR key proteins have been identified so far: mutL homologue 1 (MLH1), mutS homologue 2 (MSH2), mutS homologue 6 (MSH6), and postmeiotic segregation increased 2 (PMS2). MSH2 and MSH6 form a heterodimeric complex (mutSα), which identifies and binds the damaged DNA forming a sliding clamp (Fig. 8, left).

Upon an ATP-dependent conformational change, mutSα recruits and binds mutLα (the MLH1/PMS2 heterodimer) (Fig. 8, right). Another ATP-driven conformational switch releases mutSα/mutLα complex from the mismatch site and, if this tetrameric sliding clamp moves

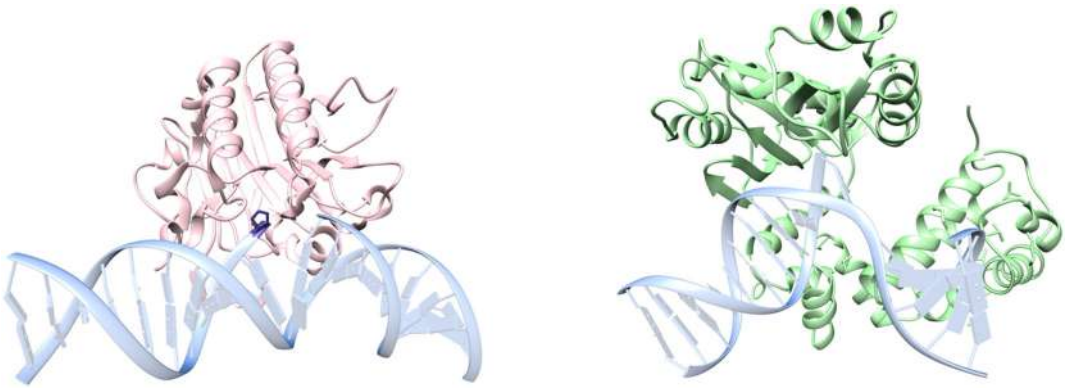

Fig. 7. (Left) Crystal structure capturing APE1 (pink ribbons) while processing an AP site (dark slate blue, PDB: 5DFF (Freudenthal, Beard, Cuneo, Dyrkheeva, & Wilson, 2015)). (Right) Crystal structure representing the intermediate in the 1-nucleotide gap-filling reaction of Polβ (green ribbons) during BER (PDB: 1BPZ (Sawaya, Prasad, Wilson, Kraut, & Pelletier, 1997)).

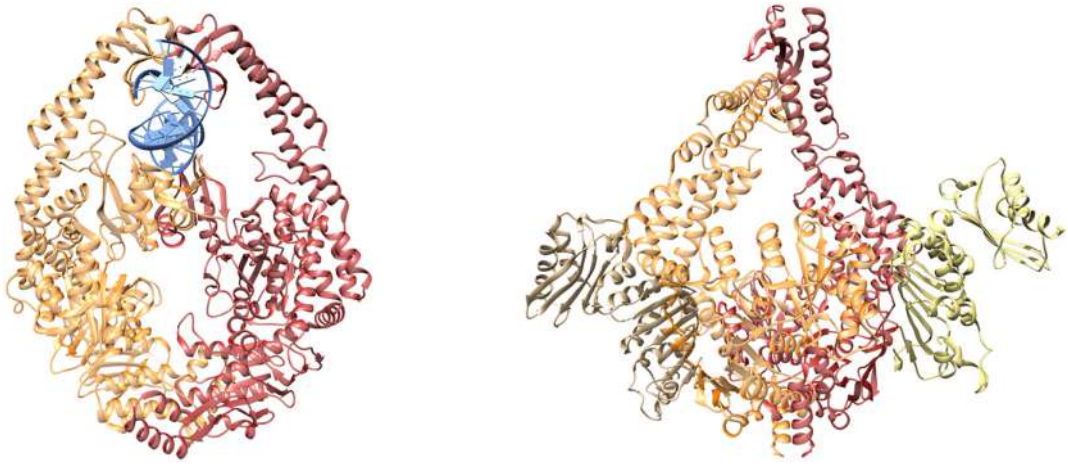

Fig. 8. (Left) Crystal structure of the mutSα sliding clamp (orange and firebrick ribbons) bound to DNA (light blue) upon mismatch identification during MMR (PDB: 3ZLJ (Groothuizen et al., 2013)). (Right) Crystal structure of the mutSα/mutLα tetrameric sliding clamp (mutSα: orange and firebrick ribbons; mutLα, tan and khaki ribbons; PDB: 5AKB (Groothuizen et al., 2015)).

upstream, it encounters and displaces the replication factor C (RFC) that is located at the 5' terminus of the strand break, and loads the exonuclease-1 (EXO1). The activated EXO1 starts strand degradation in the 5'-3' direction. The replication protein A (RPA) then stabilizes the single-stranded gap, while a complex of Polδ and PCNA fills the gap. Finally, LIG1 seals the remaining nick to complete the repair process (Fig. 9, left pathway). Conversely, if the mutSα/mutLα sliding clamp migrates downstream, it encounters PCNA bound at the 3' terminus of the strand break. This recruits and activates EXO1, resulting in the degradation of the DNA region up to the RFC complex, which prevents further degradation in the 5'-3' direction (i.e., away from the mismatch). Once the mismatch is removed, the action of EXO1 is inhibited by the bound RPA and mutLα and, as in the alternative pathway, Polδ fills the gap and LIG1 seals the nick to finalize repair (Fig. 9, right pathway).

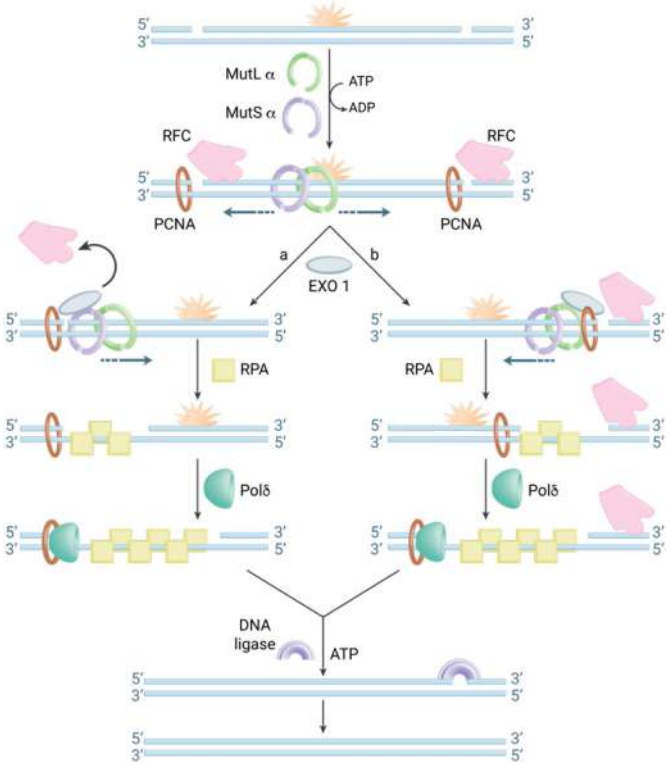

Fig. 9. Schematic representation of the human MMR pathway.

4.2. DNA active lesions repair pathways

4.2.1. Nucleotide excision repair

NER is a versatile mechanism that removes a variety of helix-distorting DNA lesions and structures caused mostly by exogenous sources such as UV-induced damage and bulky chemical adducts (Spivak, 2015). NER actually consists of two distinct yet ultimately converging pathways: the transcription-coupled repair (TC-NER) and the global genome repair (GG-NER) (Fig. 10). The former is a highly efficient repair of DNA damage that specifically blocks the progression of RNA polymerase II (RNAPII) along the DNA strand while GG-NER is a slow, transcription-independent random process of inspecting the entire genome for damage.

In analogy with other DNA repair pathways, a complex set of NER proteins cooperate to i) sense and recognize the nucleic acid fault; ii) perform DNA unwinding and a dual incision of the damaged DNA strand, one on either side of the lesion; iii) remove the lesion; iv) synthesize a patch using the undamaged complementary strand as a template, and v) ligate the patch to the contiguous strand.

TC-NER removes DNA damage from the transcription units of actively expressed genes; hence, this pathway, unlike GG-NER, operates on very specific regions of the genome. In TC-NER (Fig. 10, upper right), the first step for damage recognition consists in the arrested transcription by RNAPII through a suspected sequence. TC-NER factors are then recruited, which remove or backtrack the RNAPII to allow access to the transcription factor II human (TFIIH) complex (constituted by its 7 subunits and the CDK-activating kinase assembly factor MAT1 (MAT1), Fig. 11, left) and other NER repair enzymes.

In GG-NER (Fig. 10, upper left), damage recognition is performed by the Xeroderma pigmentosum complementation group C protein (XPC, Fig. 11 middle), in synergy with the UV excision repair protein RAD23 homolog B (RAD23B) and centrin 2 (CETN2). This XPC complex (XPC-C), together with other core recognition factors as the DNA repair protein complementing XP-A cells (XPA) and RPA, recognize a wide spectrum of damaged DNA characterized by distortions of the DNA helix such as single-stranded loops, mismatched bubbles or single-stranded overhangs. Of note, CPDs formed upon UV-induced DNA damage escape detection by the XPC-C due to a low degree of structural perturbation. However, CPDs are first detected by an E3 ubiquitin-protein ligase complex that initiates NER by recognizing damaged chromatin with concomitant ubiquitination of core histones at the lesion (Yeh et al., 2012). Once the damage has been located and identified, the XPC-C interacts with the undamaged DNA strand opposite the lesion, promotes DNA melting and recruits the TFIIH complex.

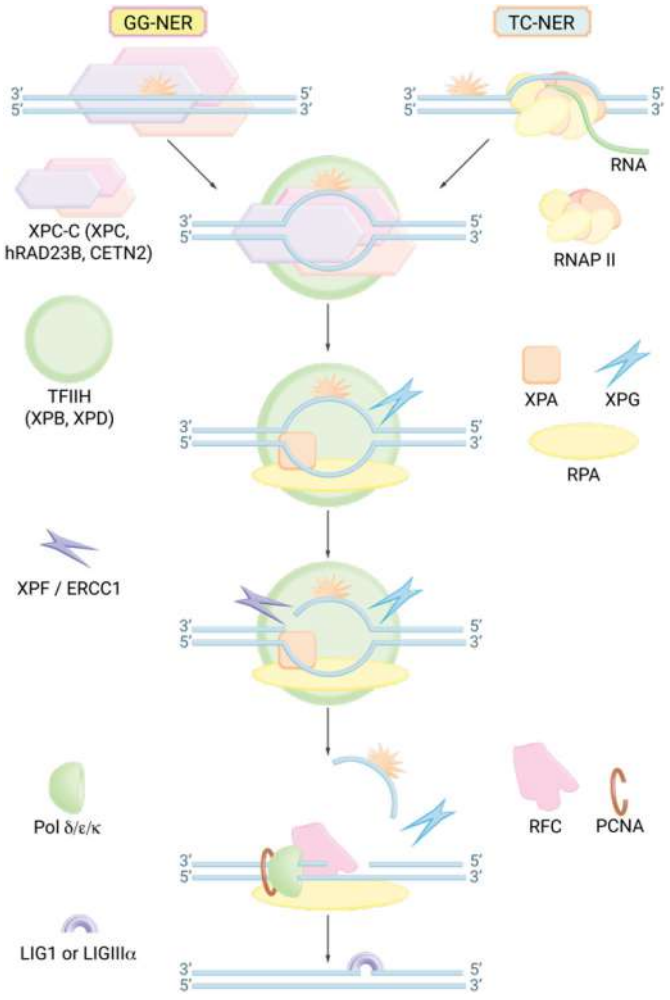

Fig. 10. Schematic representation of the human NER pathway.

At this point, TC-NER and GG-NER converge (Fig. 10), and repair proceeds following a unique pathway. Specifically, the TFIIH helicase subunits XPB and XPD unwind the DNA to create a ~30 nucleotide bubble. Once the pre-incision complex is assembled, XPA, RPA and the DNA repair protein complementing XP-G cells (XPG) are recruited and the XPC-C is released (Fig. 11, right). XPA binds near the 5' side of the bubble, while RPA bind the single-strand DNA (ssDNA) opposite the lesion with the twofold purpose of protecting it from degradation and coordinating excision and repair events. In the next step, the DNA excision repair protein ERCC-1 (ERCC1)/DNA repair endonuclease XPF (ERCC4) complex and XPG associate with TFHII, ERCC1-ERCC4 make the first incision, and repair synthesis operated by the DNA replication machinery Polδ/ε/κ-PCNA-RPA proceeds for several nucleotides displacing the damaged strand. Ultimately, the repair process ends with the incision of the 3' single/double strand junction by XPG and DNA sealing carried out by LIG1 or LIGIIIα and its cofactor XRCC1.

4.2.2. Translesion DNA synthesis

To overcome the challenge of replicating damaged DNA, cells have developed DNA damage tolerance (DDT) pathways (aka *post replication repair*) that enable the replication machinery to bypass sites of damaged DNA by initiating DNA synthesis downstream of the lesion and allowing for its repair after DNA replication (Chang & Cimprich, 2009; Marians, 2018). Two major pathways are available for DTT in mammalian cells: TLS and the damage avoidance by template switching (TS) (Fig. 12).

The replicative DNA polymerases are particularly specific for normal DNA base pairs, and cannot accommodate damaged bases or bulky adducts into their active site, as these result in blocked replication forks. Thus, during TLS stalled replicative polymerases are replaced by TLS polymerases, which are a class of specialized proteins with low-processivity but can replicate over distortions in DNA and directly bypass lesions. Mammalian cells have at least seven enzymes with TLS activity. These include four Y-family polymerases (Polη, Polι, Polκ, and the DNA repair protein REV1 (REV1)), one B-family polymerase (Pol ζ), and two A-family polymerases (Polθ and Polν) (Fig. 13).

Each of the TLS polymerases has different substrate specificities for different types of DNA damage (Yang & Gao, 2018). Therefore, depending on the TLS polymerase that is recruited, lesions can be bypassed

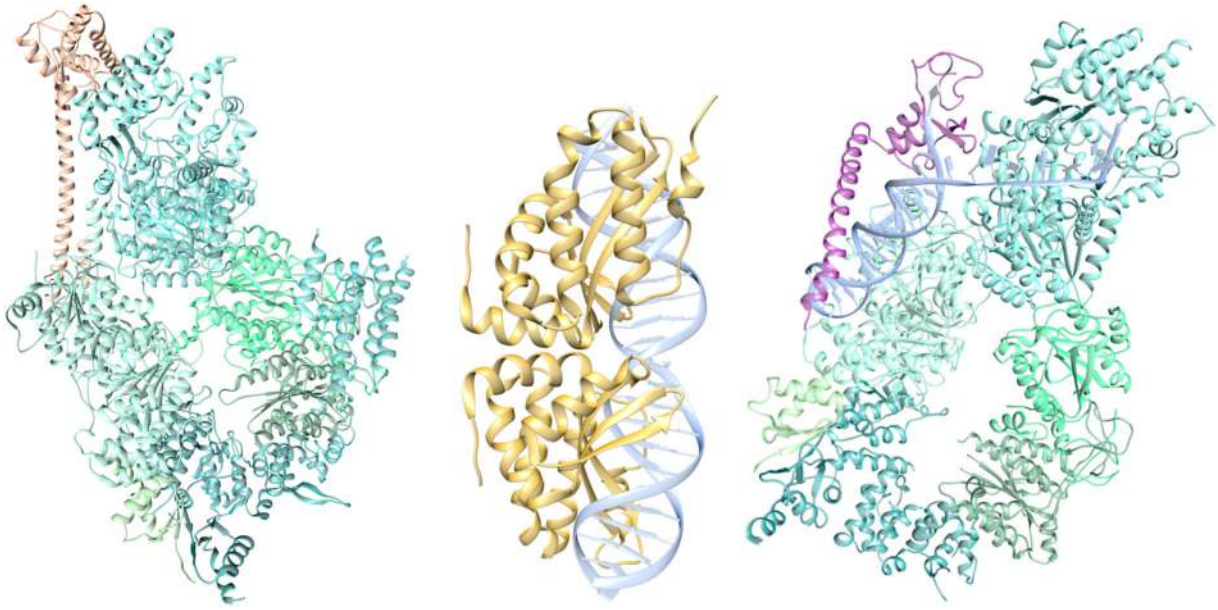

Fig. 11. (Left) Cryo-EM structure of the TC-NER key factor, the human TFIIH core complex (PDB: 6NMI (Greber, Toso, Fang, & Nogales, 2019). The 7 TFIIH subunits are colored in green shades while MAT1 is in sandy brown. (Middle) Crystal structure of XPC (light gold) recognizing damaged DNA (light blue) in GG-NER (PDB: 2VBJ (Redondo et al., 2008). (Right) Cryo-EM structure of the human core TFIIH-XPA-DNA complex operative in TC-/GG-NER shared pathway (PDB: 6RO4 (Kokic et al., 2019)). The TFIIH subunits are in light green shades while XPA is in magenta.

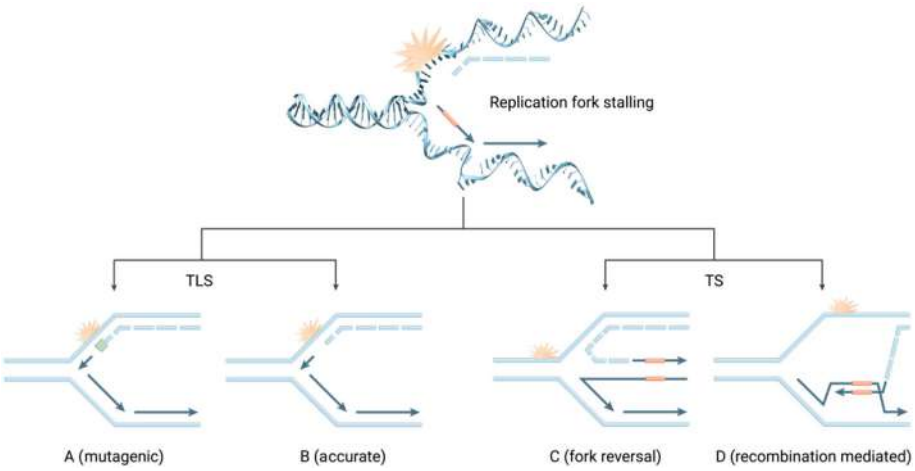

Fig. 12. Overview of the DDT pathways.

either in a relatively error-free mode (e.g., when using Polη - Fig. 12, path B), or by an error-prone mechanism when Polζ and REV1 are in charge of TLS (Fig. 12, path A).

The mechanism of lesion bypass by TS, which uses the undamaged sister chromatid, is still not entirely clear. Briefly, TS involves a structural rearrangement of the replication fork for which two models have been proposed: i) fork reversal, involving the formation of a four-way junction (or *chicken foot*) intermediate (Fig. 12, path C), and ii) recombination-mediated TS, implicating strand invasion (Fig. 12, path D). In any case, the key regulator of the DDT pathway is the modification of PCNA. While under undamaged conditions replicative DNA polymerases bind to unmodified PCNA during DNA replication, in the presence of genotoxic stress PCNA is ubiquitinated at a specific residue (K164) to initiate DDT pathways. Specifically, PCNA monoubiquitination activates TLS while its polyubiquitination promotes TS (Ghosal & Chen, 2013).

5. The single-strand and double-strand break repair pathways

Once an insult has provoked an SSB or DSB, the DDR signaling pathway recruits and activates different specialized, multimeric protein sensor complexes to facilitate the recognition of these qualitatively different lesions. These sensors in turn recruit and activate a group of serine/threonine kinases, belonging to the phosphoinositide-3-kinase (PI3K) family, which are placed at the apex of the DDR signaling pathway (Blackford & Jackson, 2017; Goldstein & Kastan, 2015; Scully, Panday, Elango, & Willis, 2019) (Fig. 14).

Specifically, DSBs are detected by the heterotrimeric complex MRN - composed by the double-strand break repair protein MRE11 (MRE11), the DNA repair protein RAD50 (RAD50) (Fig. 15, top left) and the Nijmegen breakage syndrome protein 1 (NBS1, aka nibrin) - (Syed & Tainer, 2018), which acts as the recruitment/activation platform for the DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM) (Shiloh & Ziv, 2013).

Once at the lesion site, the noncovalent dimeric ATM (Fig. 15, top right) undergoes trans-autophosphorylation at a specific serine residue (S189), which disrupts the inactive dimer and convert it into active monomers. Activated ATM monomers specifically target the histone variant H2AX (known as γH2AX) for phosphorylation at S139 at the break site or its flanking regions. This entails DNA damage signal spreading along the chromatin via γH2AX binding to DNA damage mediators like the mediator of DNA damage checkpoint protein 1 (MDC1) and the DNA repair and telomere maintenance protein NSB1 (NSB1), which further promotes ATM binding and H2AX phosphorylation in a positive feedback loop (Marechal & Zou, 2013; Paull, 2015). Furthermore, ATM phosphorylate a large number of substrates (of the order

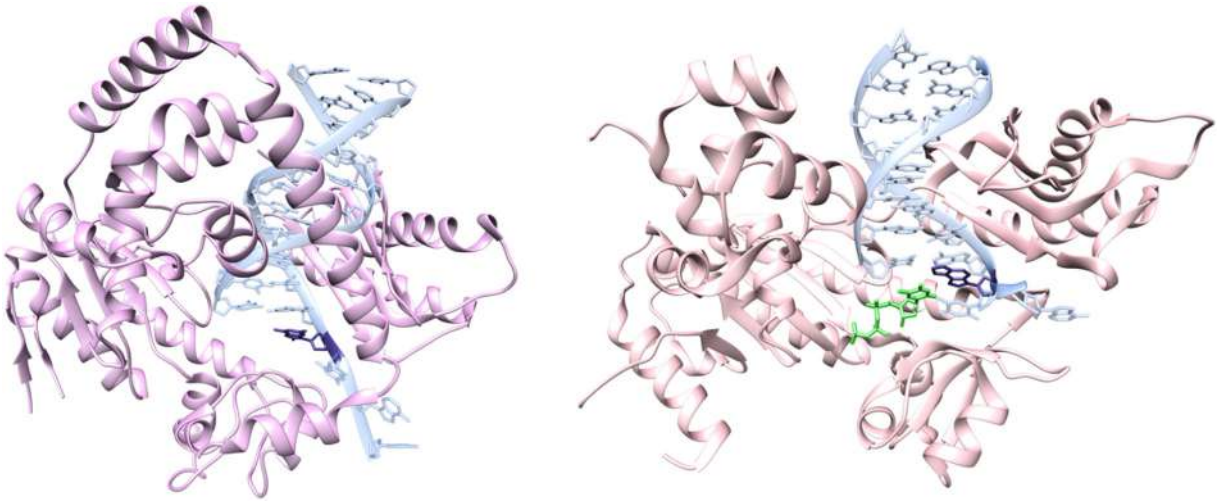

Fig. 13. (Left) Crystal structure of human Polκ (plum ribbons) while bypassing opposite the major oxidative DNA adduct 8-oxoG (dark slate blue) in TLS (PDB: 2W7O (Irimia, Eoff, Guengerich, & Egli, 2009)). (Right) Crystal structure of human Polη (pink ribbons) inserting 1 nucleotide (thymidine triphosphate) across a DNA template containing a 1,N^6-ethenodeoxyadenosine lesion (green) during TLS (PDB: 5DG7 (Patra et al., 2016)).

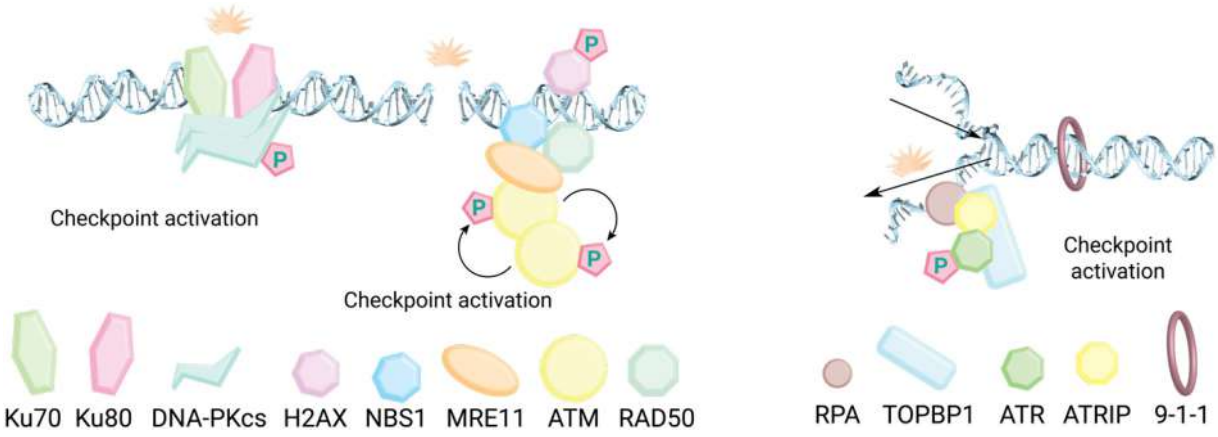

Fig. 14. Recognition of DNA DSBs (left) and SSBs (including stalled replication forks, right) and activation of the DNA damage checkpoint kinases ATM, DNA-PKcs, and ATR.

of hundreds), thereby propagating the damage signal into numerous cellular processes and pathways, setting the cell into the 'alarm mode' at the beginning of the HR pathway (§5.3) (Matsuoka et al., 2007).

DSBs also activate another DNA damage-response kinase, the DNA dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs is recruited at the DSB by the KU protein sensor complex, a heterodimer of two subunits (i.e., the ATP-dependent DNA helicase 2 subunits Ku70 and Ku80, respectively (Shibata, Jeggo, & Lobrich, 2018)), which binds free DNA ends and is subsequently activated (Fig. 15, bottom left). With respect to ATM, DNA-PKcs appears to regulate a smaller number of targets, playing a primary role in the initiation of DNA repair along the fast and efficient NHEJ pathway (§5.2.1).

When cells face a DNA SSB or a replication error (e.g., stalled replication forks), the master transducer of the DNA signal is the ataxia telangiectasia and Rad3-related protein (ATR) (Lecona & Fernandez-Capetillo, 2018; Saldivar, Cortez, & Cimprich, 2017). The canonical ATR

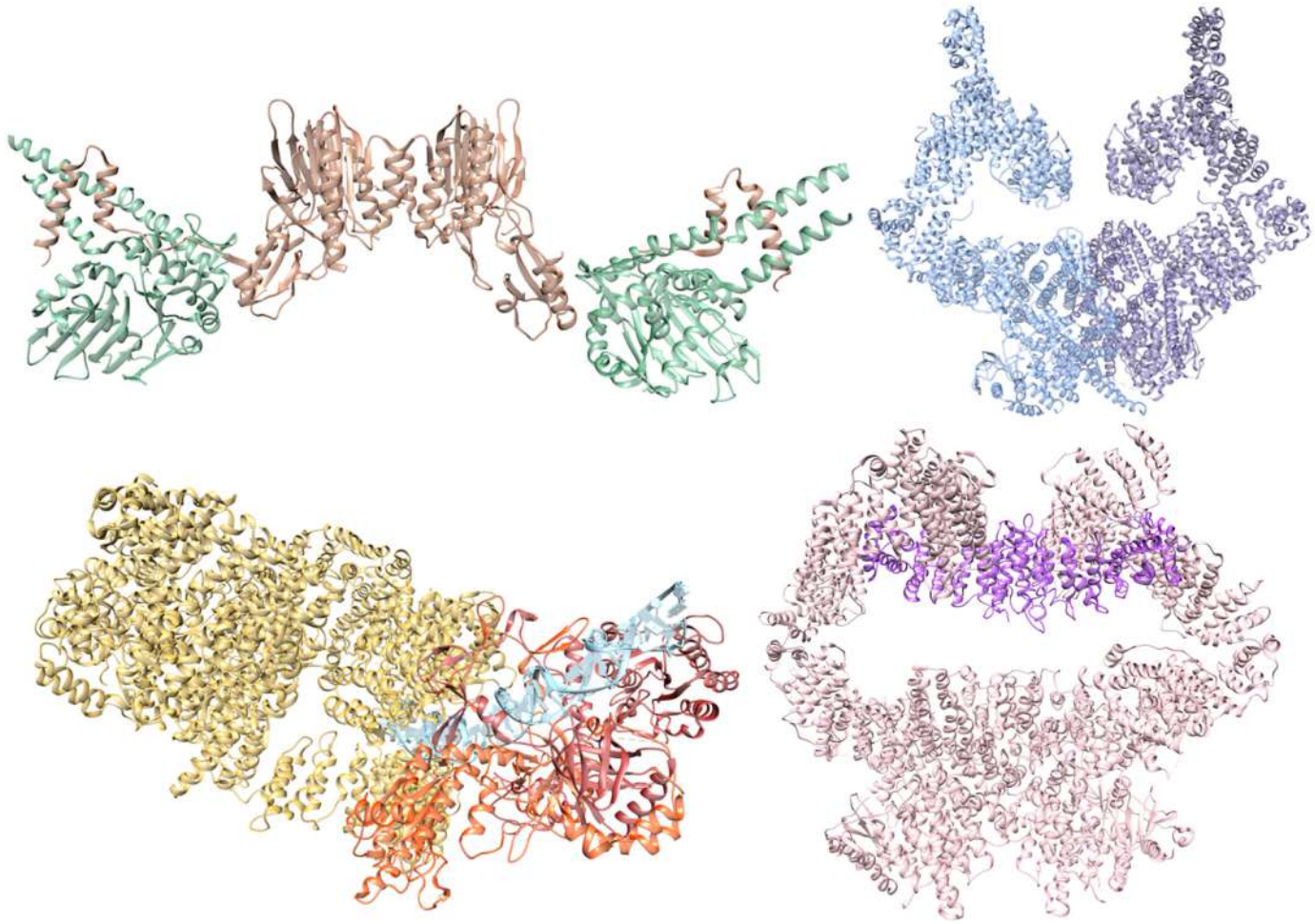

Fig. 15. (Top, left) The crystal structure of the MRE11 dimer (sienna) and the two Rad50 nucleotide-binding domains (sea green) forming the catalytic head that harbors ATP-stimulated nuclease and DNA-binding activities (left, PDB: 3QG5 (Lammens et al., 2011)). Cryogenic electron microscopy (cryo-EM)-derived structures of (top, right) the closed ATM dimer (PDB: 5NP0 (Baretic et al., 2017)), (bottom, left) the DNA-PKcs (goldenrod) in complex with Ku70/Ku80 (orange red/ firebrick) and a DNA duplex (light blue, PDB: 5Y3R (Yin, Liu, Tian, Wang, & Xu, 2017)), and (bottom, right) the dimeric ATR/ATRIP complex (pink/ purple, PDB: 5YZ0 (Rao et al., 2018)).

pathway is triggered by the binding of RPA to ssDNA. RPA-ssDNA interactions serve as a platform for the binding of many proteins, including the ATR-interacting protein (ATRIP), which facilitates the recruitment of ATR to the lesion. Localization of the ATR-ATRIP complex (Fig. 15, bottom right) to the site of damage is not sufficient for ATR kinase activation, which takes place upon ATR conformational change promoted by the DNA topoisomerase 2-binding protein 1 (TOPBP1). Next, activated ATR triggers a cell cycle checkpoint by stimulating serine/threonine-protein kinase CHK1 (CHK1). This results in cell cycle arrest in the G2/M phase, and provide the damaged cell with time to proceed with DNA repair.

However, the role of ATM and ATR is not limited to the early stages of DSB and SSB DNA repair. For instance, ATM is involved as an activator of some HR mediators at later time points along this repair pathway (§5.3) (Ahlskog, Larsen, Achanta, & Sorensen, 2016; Bakr et al., 2015) while, although ATR is mainly associated with SSBs and replication stress, ATM/ATR cross-talks occur during DSB repair (Smith, Tho, Xu, & Gillespie, 2010), e.g., at IR-induced DSB repair during 5'-end resection, where ATM is required to recruit ATR to RPA-coated single-strand overhangs (Jazayeri et al., 2006).

5.1. Single-strand break repair

DNA SSBs arising from endogenous oxidative damage (§2.1.3), necessary enzymatic reactions (e.g., obligate intermediates of APE1 activity during BER (§4.1.1) (Whitaker & Freudenthal, 2018)), strand incisions by RNase H2 during resolution of erroneous incorporation of ribonucleotides into DNA (§2.1.4) (Cerritelli & Crouch, 2016), transient nicked DNA intermediates created by DNA topoisomerase 1 (TOP1) to relax supercoiled DNA during transition or replication (§2.2.3 and §5.2) (Sassa, Yasui, & Honma, 2019)), and defective activity of cellular enzymes are all repaired by a specialized sub-pathway of BER (Abbotts & Wilson III., 2017). This activates most of the BER protein pool (APE1, Polβ, LIGIIIα), in tandem with the nick sensors poly(ADP-ribose) polymerase 1 and 2 (PARP1/2) (Pascal, 2018) (Fig. 16, left) and XRCC1. Importantly, PARP1 also promotes the repair of some lesions (e.g., 8-oxo-7,8-dihydroguanine) in Polβ deficient cells along the LP-BER pathway (Ray Chaudhuri & Nussenzweig, 2017).

Both chemical and enzyme-derived SSBs commonly harbor non-conventional termini, such as a 5'-adenosine monophosphate (AMP), 3'-phosphate, 3'-phosphoglycolate, and 3'-protein adducts. Such SSBs demand DNA end processing to convert them to the necessary 5'-phosphate and 3'-OH ends required for gap-filling synthesis and nick ligation (§4.1.1). 3'-phosphate are routinely processed by the bifunctional PNKP (Jilani et al., 1999) (Fig. 16, right), 3'-α,β-unsaturated aldehydes are the substrate of APE1 (Whitaker & Freudenthal, 2018), 5'-AMP ends are handled by aprataxin (APTX) (Kijas, Harris, Harris, & Lavin, 2006), while TOP1-DNA intermediated are resolved by the tyrosyl-DNA phosphodiesterase 1 (TDP1) (R. Gao, Huang, Marchand, & Pommier, 2012).

5.2. Double-strand breaks repair

Of the many types of DNA damage that exist within the cell, DSBs are probably the most deleterious, as even a single unrepaired DSB may result in cell death (Rich, Allen, & Wyllie, 2000). DSBs are generated when the two complementary strands of the DNA double helix are broken simultaneously at sites that are sufficiently close to one another that base-pairing and chromatin structure are inefficient to keep the two nucleic acid ends juxtaposed. This allows for the eventual physical dissociation of the duplex into two separate chains, making ensuing repair difficult to perform and providing opportunity for the inappropriate recombination with other sites of the genome. Moreover, erroneous rejoining of broken DNA DSBs may occur, leading to the loss or amplification of chromosomal material or, under certain circumstances, to translocations in which segments of chromosomal arms are exchanged, sometimes in a reciprocal fashion. This can lead to tumorigenesis if, for example, the deleted chromosomal region encodes a tumor suppressor or if the amplified region encodes a protein with oncogenic potential. In the case of chromosomal translocation, this can sometimes lead to a gene fusion that dysregulates or alter the functions of a proto-oncogene (Shibata & Jeggo, 2014).

Among the exogenous agents causing DNA DSBs, the most pervasive is UV (§2.2.1), although IRs (§2.2.2) also contribute to DSB formation. As mentioned above (§2.2.3), i) chemicals that mimic the action of IRs (Tiwari & Wilson 3rd., 2019), ii) topoisomerase inhibitors (Pommier, 2013), iii) compounds generated in tobacco smoke (Weng et al., 2018) and in foods (Bedard & Massey, 2006) can all contribute to the generation of DSBs.

DSBs are also generated during cellular metabolism. For example, i) endogenous ROS can trigger both SSBs and DSBs upon DNA base oxidation (Woodbine, Brunton, Goodarzi, Shibata, & Jeggo, 2011), ii) mechanical stress on the chromosomes may also result in these dangerous nucleic acid lesions (Gelot, Magdalou, & Lopez, 2015), and iii) defective telomere metabolism may originate DSBs at chromosome termini (Aksenova & Mirkin, 2019). In proliferating cells, DNA replication is thought to be the major source of DSBs, as DNA intermediates at replication forks are fragile and susceptible to breakage. Notably, breaks can occur following polymerase stalling, which leads to the generation of persistent ssDNA intermediates. Broken or collapsed replication forks containing ssDNA also resemble DSBs at different stages of processing, and are also a source of genomic instability if not properly repaired (Ait Saada, Lambert, & Carr, 2018).

Necessarily, DSBs are in some instances deliberately generated by the cell for a specific biological purpose like, for instance, to initiate

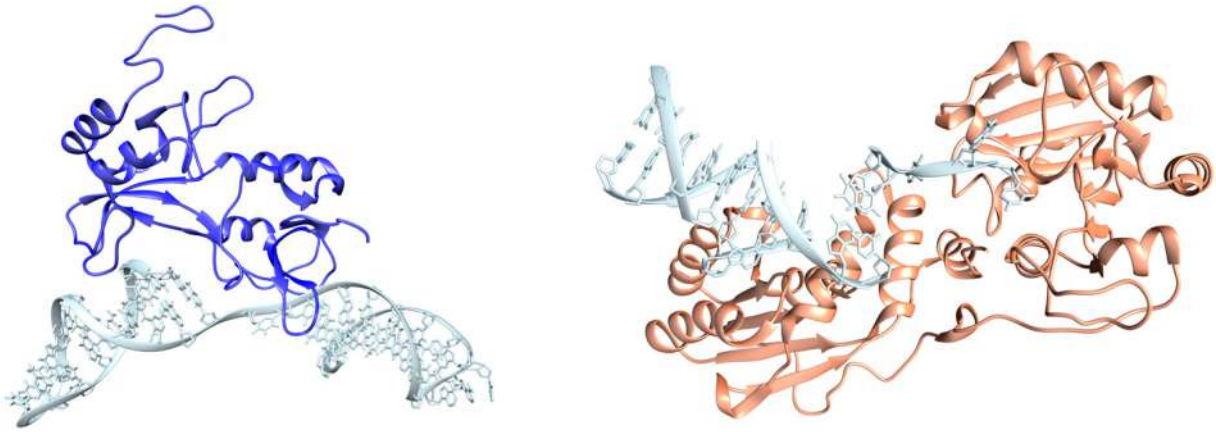

Fig. 16. (Left) Nuclear magnetic resonance (NMR)/X-ray derived structure of the PARP1 N-terminal zinc finger domain (blue) sensing a DNA SSB (light blue, PDB: 2N8A (Eustermann et al., 2015)). (Right) Crystal structure of 3'-phosphate DNA end (light blue) recognized by the mammalian PNKP 3' phosphatase (coral, PDB: 3ZVN (Garces, Pearl, & Oliver, 2011)).

recombination between homologous chromosomes during meiosis (Murakami & Keeney, 2008). Also, DSBs naturally occur as intermediates during developmentally regulated rearrangements, such as V(D)J recombination (Roth, 2014) and immunoglobulin class-switch recombination (Nicolas, Cols, Choi, Chaudhuri, & Vuong, 2018). The former is a process by which T and B cells randomly assemble different gene segments - known as variable (V), diversity (D), and joining (J) genes - in order to generate unique receptors (the antigen receptors) that can collectively recognize many different types of molecule. The latter (aka *isotype switching* or *isotypic commutation*), is a biological mechanism that induces B cells to switch the production of a given immunoglobulin isotype (e.g., IgM) to another one (e.g., IgG). Although a tight control is imposed by the cell on such events, sometimes they can go awry with potentially devastating consequences for the cell itself and/or the whole organism.

Two pathways dominate the repair of DSBs: NHEJ and HR (Scully, Panday, Elango, & Willis, 2019). The former is a fast, high-capacity pathway that joins two DNA ends with minimal reference to DNA sequence. By contrast, HR is an error free pathway that uses a sister chromatid or homologue to patch up the damage. Therefore, NHEJ is not restricted to any stage of the cell cycle, whereas HR is active during the S and G2 phases due to the requirement of a homologous partner. Before directing the lesion to either NHEJ or HR, however, the DDR must analyze the DNA end structures at the damage. Accordingly, short, blunt or minimally recessed DNA ends can be directly subjected to DSB repair. On the contrary, those that are chemically blocked or that originate within compact chromatin may require extensive processing/chromatin remodeling before entering either repair process (Hauer & Gasser, 2017; Stadler & Richly, 2017), while the presence of long ssDNA tails or complex patterns of ssDNA gaps must also be directed to further processing prior to any repair (Bonetti, Colombo, Clerici, & Longhese, 2018; Symington, 2016).

Most importantly, the cell cycle phase in which the damage is discovered governs the pathway choice between NHEJ and HR (Her & Bunting, 2018). In fact, HR requires 5'end resection at the break, a process promoted during the S and G2 phases of the cell cycle but inhibited in G1 phase by the TP53-binding protein 1 (53BP1)/telomere-associated protein RIF1 (RIF1, (Fig. 17))/mitotic spindle assembly checkpoint protein MAD2B (MAD2B) pathway, which directs DDR towards NHEJ (Escribano-Diaz et al., 2013; Xu et al., 2015).

The cell cycle dependent kinase (CDK) activity, which increases as cells enter the S phase, also provides activating signals to the resection machinery and to proteins that act later in HR. As an example, upon phosphorylation by CDK, the DNA endonuclease RBBP8 (ctIP) both senses the cell phase and transduces this information to initiate DNA resection (Ira et al., 2004). In addition to CDK-mediated action, other mechanisms communicate cell cycle status to the DSB repair machinery

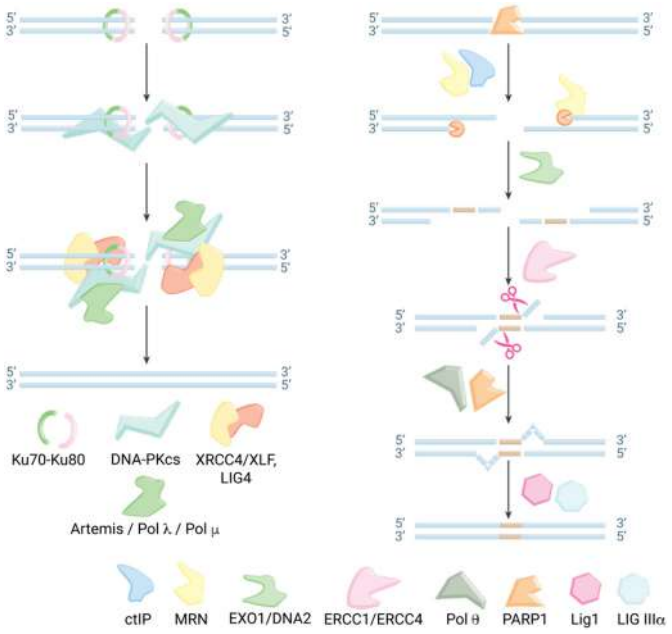

Fig. 18. The pathways of cNHEJ (left) and aNHEJ (right).

(Her & Bunting, 2018; Shibata, 2017; Zhao et al., 2017). For instance, HR gene expression becomes upregulated as the cell transits for G1 to S phase moreover, DNA helicase B (HELB), which is inactivated as cells progress to phase S, prevents DNA end resection in mammalian cells in G1 phase, thereby directing DDR to NHEJ (Sung & Klein, 2006; Tkac et al., 2016).

5.2.1. Classical non-homologous end joining

The classical NHEJ (cNHEJ, Fig. 18, left) is an error-prone DSB repair pathway that, although particularly common in the G_0, G_1, and early S-phase of mitotic cells, can take place along the entire cell cycle since it does not require a homologous sequence located on the sister chromatid (H. H. Y. Chang, Pannunzio, Adachi, & Lieber, 2017). The starting step in cNHEJ consists in the rapid binding of KU to both ends of the broken DNA molecule to prevent promiscuous end resection.

Once bound, KU recruits and activates DNA-PKcs and this, in turn, triggers an extensive signaling cascade that orchestrates downstream repair. Briefly, binding of KU to blunt DNA ends requires minimal DNA processing, and repair is directly assisted by two scaffold proteins - the X-ray repair cross-complementing protein 4 (XRCC4) and the non-homologous end-joining factor 1 (XLF, also known as Cernunnos)

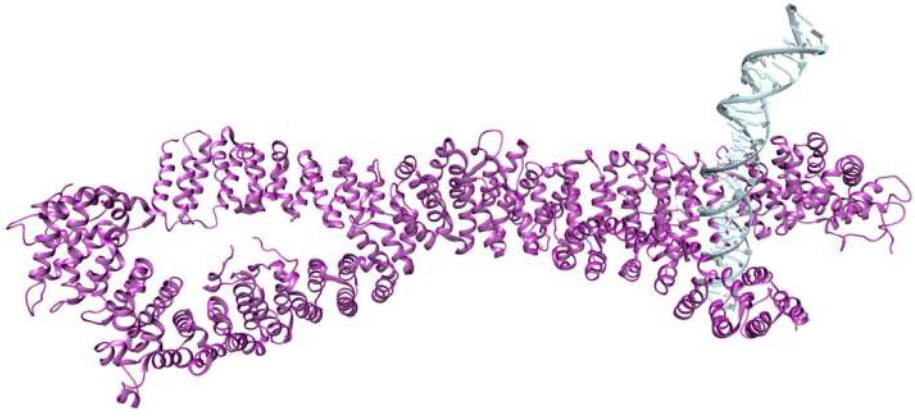

Fig. 17. Crystal structure of the N-terminal domain of RIF1 (magenta) binding to DNA (light blue) and suppressing its end resection, promoting repair by NHEJ (PDB: 5NW5 (Mattarocci et al., 2017)).

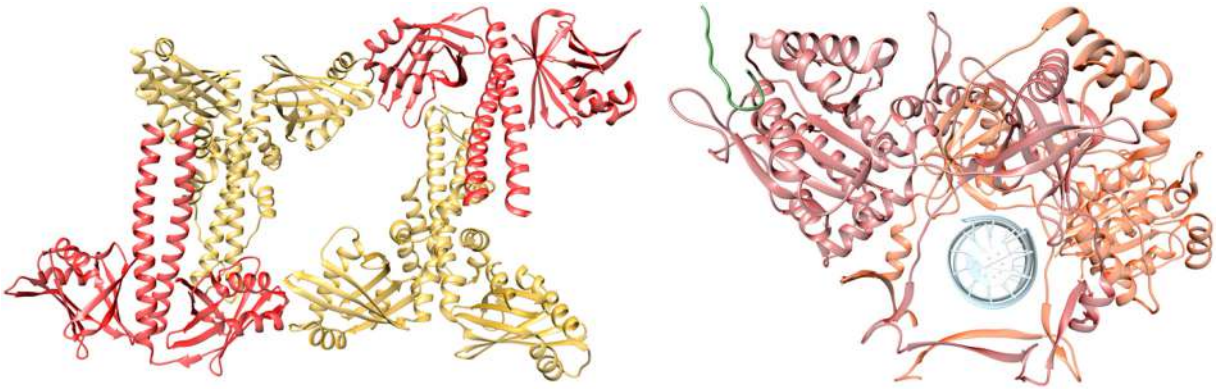

Fig. 19. (Left) Crystal structure of the cNHEJ DNA repair pathway core XRCC4/XLF (Cernunnos) homodimers (light red/gold, PDB: 3Q4F (Ropars et al., 2011)). (Right) Crystal structure of the KU-binding motif of the cNHEJ protein APLF (sea green) bound to a KU/DNA complex (KU: orange red/firebrick; DNA: light blue, PDB: 6ERF (Nemoz et al., 2018)).

- that bind to DNA ligase 4 (LIG4) responsible for the break sealing (Fig. 19, left).

In all other cases (e.g., incompatible 5' overhanging ends, resection-dependent compatible ends, incompatible 3' ends, and 3'-phosphoglycolate ends), end resection processes are carried out by the endonuclease Artemis (activated upon interaction with DNA-PKcs) or by specialized DNA polymerases like Polλ and the DNA polymerase μ (Polμ) (Fig. 7, left). A number of accessory factors - and likely more yet are still to be discovered - support or otherwise regulate cNHEJ. These include the multifunctional complex MRN involved in

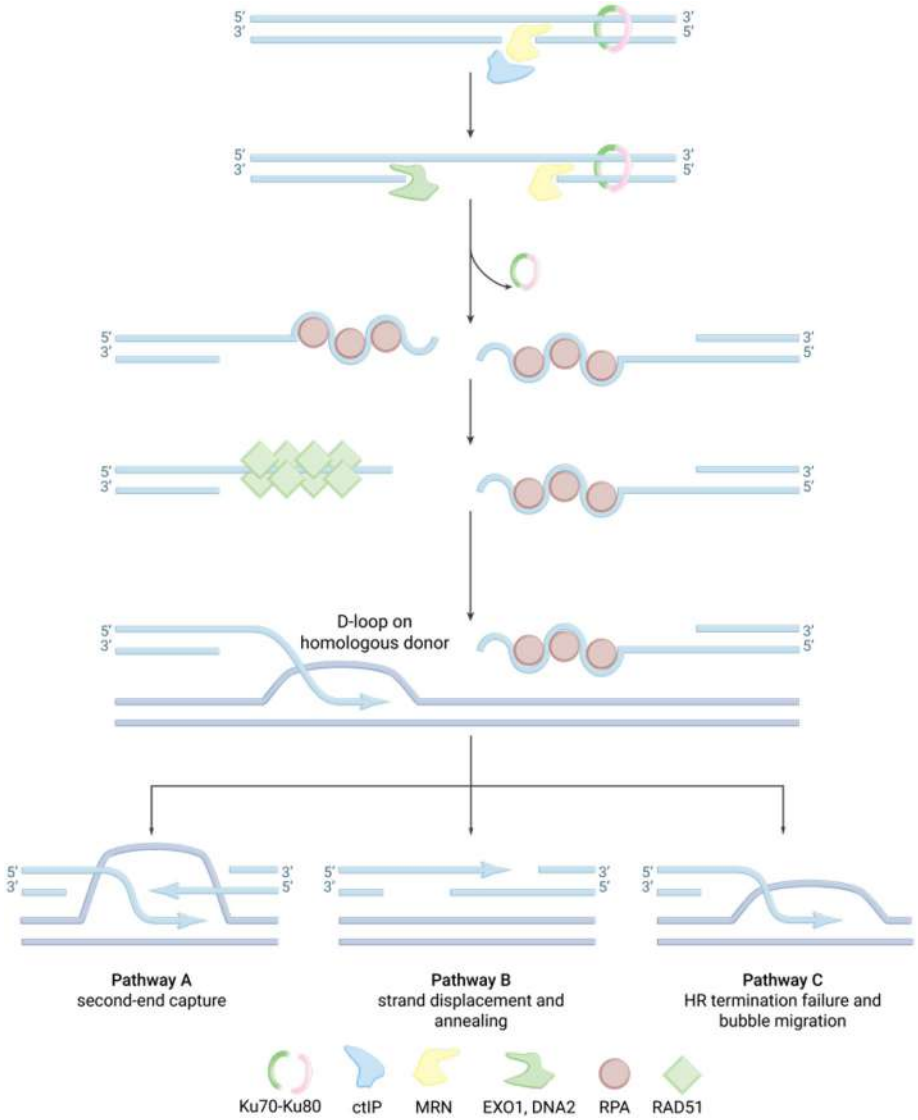

Fig. 20. Schematic view of the human HR pathway.

DNA end recognition in HR (§5.2) (Dinkelmann et al., 2009) and the aprataxin and PNK-like factor (APLF) (Fig. 19, right), which interacts with Ku80 and PARP proteins in the vicinities of the DSB (Hammel et al., 2016). Several additional positive and negative regulators of Ku70/Ku80 have been identified, as a KU binding motif in a number of these proteins is thought to mediate their NHEJ regulatory role (Grundy et al., 2016).

5.2.2. Alternative non-homologous end joining

Alternative non-homologous end joining (aNHEJ, Fig. 18, right) is another direct end joining repair pathway that mainly operates during the S and G2 phases of the cell cycle on 3' ssDNA ends in the absence of cNHEJ proteins (Chang et al., 2017; Nagaria & Rassool, 2018). aNHEJ engages the MRN complex, ctIP, ERCC4/ERCC1, PARP1 and LIG1 or LIGIIIα. PARP1 plays the initial role of recognizing and tethering either ssDNA nicks or blunt DS ends. Resection is next performed by the MRN complex, which can directly bind DSB ends. As a component of MRN, MRE11 generates 3'ssDNA overhangs through a combination of endonucleolytic cleavage followed by 3'-5' exonucleolytic processing. In addition, the NSB1 subunit of MRN recruits ctIP that is essential for resection by MRE11. Once MRN/ctIP initiate resection, the loading of the EXO1 or the DNA replication ATP-dependent helicase/nuclease 2 (DNA2) generates longer stretches of ssDNA, likely contributing to the larger deletion events associated with aNHEJ. After the annealing of the microhomology exposed by DNA resection or unwinding, the remaining non-complementary DNA segments form 3′ flaps that must be removed before ligation. Removal of 3′ flaps also assists in stable association of DNA ends and provides a proper substrate from which DNA synthesis can initiate for gap filling. ERCC4/ERCC1 cleaves 3′ flaps from an annealed intermediate. At this point, PARP1 plays another role in DNA end bridging and alignment, at which time non-complementary 3' tails are removed by not yet unequivocally identified nucleases. Gap filling is performed most likely by Polθ, and nick sealing is carried out by LIG1 or LIGIIIα. Whether aNHEJ is a genuine DSB repair pathway is still a matter of debate, but it may act as a backup plan for both cNHEJ and HR, when both pathways are overwhelmed with too many DSBs.

5.3. Homologous repair

As mentioned above, at variance with NHEJ the HR pathway (Fig. 20) requires the use of homologous sequences to align DSB ends prior to ligation. Accordingly, in vertebrate cells HR occur largely during the S phase of the cell cycle, where there is a replicated sister chromatid that can be used as a homologous template to copy and restore the DNA sequence missing on the damaged chromatid. The search for sequence homology to template HR requires the presence of ssDNA at the DSB end. This intermediate can be generated by the nucleolytic degradation of the 5'strand of a DNA DSB. Resection is initiated by the MRN complex, which can directly bind DSB ends and via the action of MRE11 generates 3'ssDNA overhangs through a combination of endonucleolytic cleavage followed by 3'-5' exonucleolytic processing. In addition, the NSB1 subunit of MRN recruits ctIP that mediates 5' to 3' end resection (Fig. 20).

Once MRN/ctIP initiate resection, the EXO1 and DNA2 nucleases perform the bulk of end resection required for HR. In this process, DNA2 acts in complex with the Bloom syndrome protein (BLM) and/or the Werner syndrome ATP-dependent (WRN) RecQ helicases (Fig. 21, left and middle). ATM is also required for proper DSB resection. To the purpose, ATM targets for phosphorylation and removes the transcription intermediary factor 1-β (TIF1-β) from the heterochromatin environment of the DNA lesion and promotes the recruitment and activity of several resection factors, including the MRN complex, ctIP, EXO1 and DNA2.

The ssDNA overhangs generated by DSB resection are rapidly coated by the RPA complex to allow extensive resection by EXO1/DNA2 and to prevent degradation or self-annealing of the nascent ssDNA (Fig. 21, right). Subsequently, various mediator proteins, including the DNA repair protein RAD52 homolog (RAD52), the breast cancer type 2 susceptibility protein (BRCA2), the partner and localizer of BRCA2 (PALB2), and the breast cancer type 1 protein (BRCA1)-associated RING domain protein 1 (BARD1) promote nucleation, displacement of RPA, and assembly of the RAD51 recombinase, resulting in the so-called *pre-synapsis* phase of HR. The RAD51-ssDNA nucleoprotein filament thus formed is a dynamic structure subjected to competing activities that promote its stability and disassembly. Accordingly, under physiological conditions several RAD51 paralogs are involved in assisting RAD51-ssDNA nucleoprotein filament stability, optimizing its efficiency and, at the same time, restricting RAD51 function to appropriate DNA substrates (§7.4). During *synapsis*, the RAD51-ssDNA complex facilitates the formation of a physical connection between the invading DNA substrate and homologous duplex DNA template, leading to the generation of heteroduplex DNA called *D-loop*. Here, RAD51/double-strand DNA (dsDNA) filaments are formed by accommodating both the invading and donor DNA strands within the filament. Finally, in a Ca^{2+}-dependent manner during the *post-synapsis* stage RAD51 stimulates the ATPase activity of the DNA repair and recombination protein RAD54 (RAD54) to promote intertwining of the 3'invading strand and the complementary template strand to generate a primer-template junction suitable for DNA synthesis by a polymerase, usually Polδ although some TLS polymerases have also been shown to play a role in this process.

After the formation of the D-loop, the intermediate can proceed along two distinct sub-pathways: the double Holliday junction (dHJ) formation (Fig. 20, lower part, pathway A) and the synthesis-dependent strand annealing (SDSA) (Fig. 20, lower part, pathway B). The replication activity along the dHJ sub-pathway is prominent in meiotic recombination and occurs when the D-loop captures also the second end of the break not involved in strand invasion (Wright, Shah, & Heyer, 2018). Accordingly, its 3'ssDNA overhang forms a dHJ (Fig. 22, left) with the homologous chromatid (*second-capture*), which can be processed by a resolvase complex (formed by the crossover junction endonucleases MUS81 and EME1 (Fig. 22, middle left), the structure-specific endonuclease subunits SLX1 and SLX4, and the flap

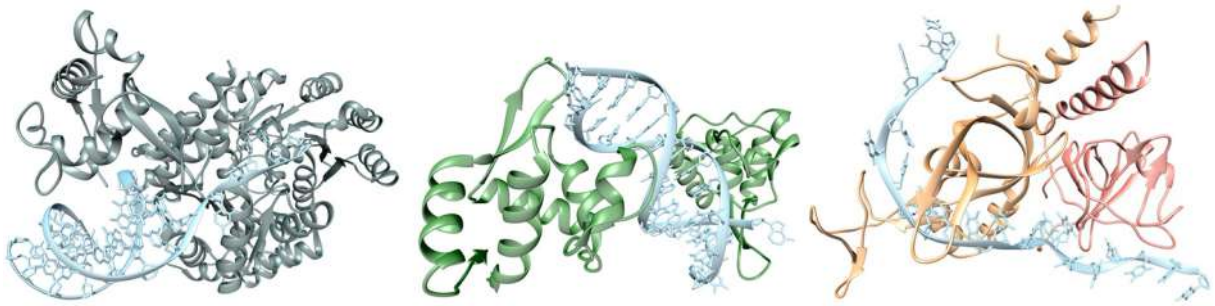

Fig. 21. Crystal structures of the BLM (left, dark slate gray, PDB: 4O3M (Swan et al., 2014)) and the WRN (middle, green, PDB: 3AAF (Kitano, Kim, & Hakoshima, 2010)) helicases in complex with a double-strand DNA (light blue). (Right) Dimeric RPA (light brown shades) in complex with a ssDNA (light blue) (PDB: 6I52 (Yates et al., 2018)).

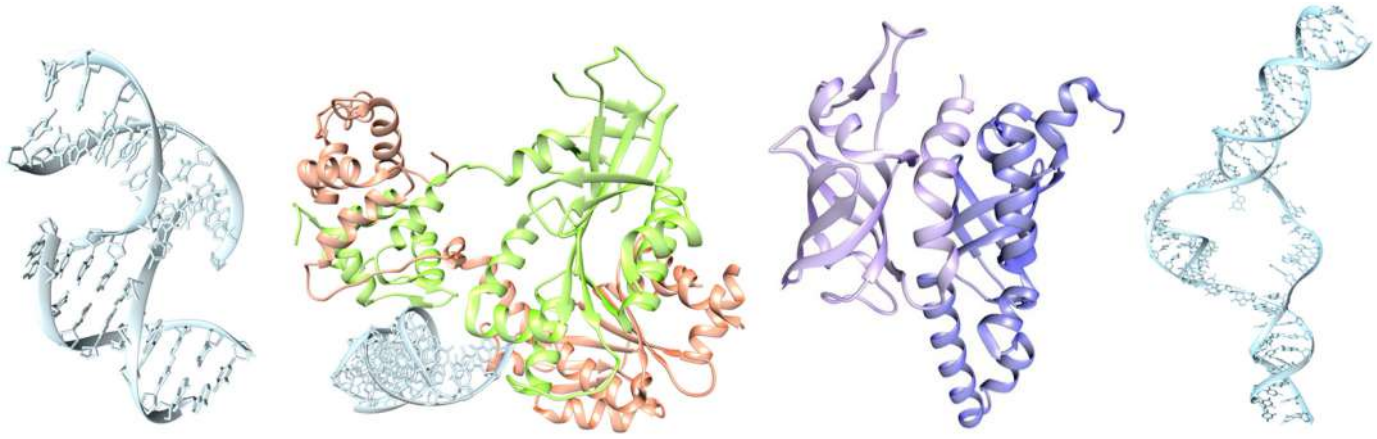

Fig. 22. (Left) Crystal structure of a DNA Holliday junction (PDB: 6GDH (Haider et al., 2018)). (Middle left) Crystal structure of the structure-selective MUS81-EME1 nuclease (chartreuse-coral) bound to a 5'-flap DNA substrate (light blue) (PDB: 4P0P (Gwon et al., 2014)). (Middle right) Crystal structure of the RMI complex (RMI1: medium blue; RMI2, light purple) (PDB: 3MXN (Hoadley et al., 2010)). (Right) Structure of a DNA bubble.

endonuclease GEN homolog 1) into either non-crossover or crossover products (Boddy et al., 2001; Fricke & Brill, 2003; Ip et al., 2008). Alternatively, the dHJ can be dissolved by a mechanism involving the BLM-mediated branch migration and their subsequent catalyzed dissolution via the DNA topoisomerase IIIα, stimulated by the RMI complex formed by the recQ-mediated genome instability proteins 1 and 2 (RMI1/2) (Singh et al., 2008; Wu & Hickson, 2003; Xu et al., 2008) (Fig. 22, middle right), generating exclusively non-crossover products.

SDSA is the major HR sub-pathway that takes place in somatic cells (Verma & Greenberg, 2016). This route occurs without the formation of a dHJ, so that the two processes of homologous recombination are identical until just after D-loop formation assisted by RAD51. At this point, a DNA helicase like the ATP-dependent DNA helicase Q5 prevents formation of the dHJ (Paliwal, Kanagaraj, Sturzenegger, Burdova, & Janscak, 2014); the invading 3' strand is thus extended along the recipient homologous DNA duplex by DNA polymerase in the 5' to 3' direction, so that the D-loop physically translocates - a process referred to as *bubble migration* DNA synthesis (Fig. 22, right). The resulting single Holliday junction then slides down the DNA duplex in the same direction in a process called *branch migration*, displacing the extended strand from the template strand. This displaced strand pops up to form a 3' overhang in the original double-stranded break duplex, which can then anneal to the opposite end of the original break through complementary base pairing. Therefore, although SDSA produces non-crossover products because flanking markers of heteroduplex DNA are not exchanged, gene conversion does occur, wherein nonreciprocal genetic transfer takes place between two homologous sequences (San Filippo, Sung, & Klein, 2008).

In case of defective dHJ or SDSA pathways, other (error-prone) replicative HR responses such as break-induced replication (BIR, Fig. 20, lower part, pathway C) (Kramara, Osia, & Malkova, 2018; Sakofsky & Malkova, 2017) can take over. With respect to SDSA, BIR mostly differs in its DNA synthesis mode being an asynchronous process; accordingly, the leading strand primed at the 3'-OH end accumulates as ssDNA while the D-loop (or bubble) migrates. To complete the repair, the lagging strand utilizes the leading one as the template, resulting in conservative inheritance of the newly synthesized DNA (Donnianni & Symington, 2013). The identity of the main helicases driving BIR progression is not fully determined. At the moment, the ATP-dependent DNA helicase PIF1 (PIF1) has been shown to be essential in BIR, although its specific role - whether ahead of the progressing D-loop to unwind the DNA duplex or behind the migrating bubble, to extract the newly synthesized DNA strand from the replication machinery - has still to be conclusively determined (Saini et al., 2013; Wilson et al., 2013). A major challenge for the DSB repair system is also posed by sites with broken replication forks or collapsed replication forks, since one-ended DSB or solitary DNA ends can arise in these contexts. In this case there is no immediate partner for end joining, and the absence of a second DNA end, failure to engage the second end of the break or to displace the nascent strand favors do not allow the possibility of engaging error-free SDSA and favor the error-prone replicative HR responses of BIR (Scully, Panday, Elango, & Willis, 2019).

All of the above pathways require RAD51, with the exception of some forms of BIR (Kramara et al., 2018) and single-strand annealing (SSA), a non-conservative, RAD51-independent DSB repair pathway (Bhargava, Onyango, & Stark, 2016) that does not entail the presence of a sister chromatid. Yet, SSA is initiated by end resection, which can only occur during the late S and G2 phases of the cell cycle, as it in part depends on CDK activation of ctIP (Sartori et al., 2007; You et al., 2009). SSA joins direct repeat sequences (e.g., tandem repeats) at 3'ssDNA end through annealing at the cost of deletion of the intervening sequence between the repeats. RAD52 is responsible for the annealing of the flanking repeats resulting from the end resection (Hanamshet, Mazina, & Mazin, 2016). ERCC1 in complex with ERCC4 then removes the non-homologous 3'ssDNA tails. Polymerases and ligases are in charge of the final steps - gap filling and ligation - although the exact players remain still poorly understood (Bhargava et al., 2016). In order to reveal complementary homologous sequences, SSA requires extensive DNA end resection and RPA displacement; moreover, sequence information can be lost or rearranged if overlapping ends by as little as 30 base pairs are unsuitably joined. Therefore, SSA is considered to be an obligatorily error-prone pathway.

6. The interstrand crosslink repair pathway

Like DSBs, ICLs are quite dangerous because they present an absolute block to DNA replication. ICLs can be repaired by replication-dependent and replication-independent mechanisms. ICL repair in the G1/0 phase involves dual incisions flanking the ICL, repair via NER, and DNA synthesis to fill the gap (Fig. 23, left). ICL repair in the S phase is similar, yet it calls for HR to provide an accurate template for repair synthesis across the excised lesion (Fig. 23, right) (Deans & West, 2011).

When replication forks converge on an ICL, both the breast cancer type 1 susceptibility protein 1 (BRCA1) and RAD51 protect the stalled fork by MRE11-mediated degradation, and the Fanconi anemia (FA) repair pathway (Niraj, Farkkila, & D'Andrea, 2019) adjusts the crosslink (Fig. 23, top right). In a simplified view, the FA repair pathway is initiated with the ubiquitination of the Fanconi anemia group D2 protein (FANCD2) (Liang et al., 2016), which recruits the endonuclease pool constituted by ERCC4, the crossover junction complex endonuclease MUS81/EME1, and the structure-specific endonuclease subunit SLX1

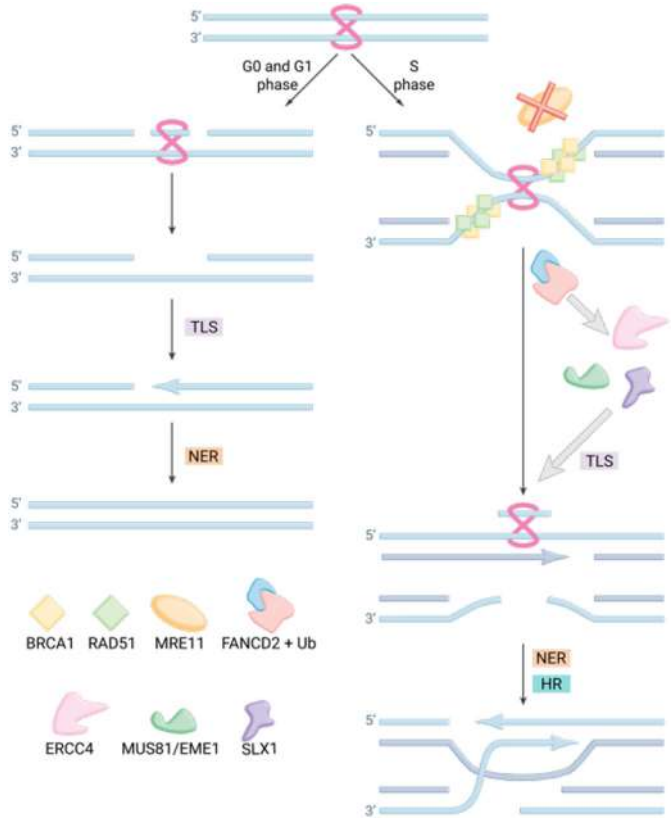

Fig. 23. Schematic representation of the ICL repair pathway.

to incise the crosslink, followed by TLS across the lesion, NER to remove the lesion, and HR to repair the replication fork along with BRCA1 and RAD51 (Taniguchi et al., 2002). Both mechanisms are error-free, except for the TLS step, where mutations may be introduced depending on the operational DNA polymerase (§4.2.2).

7. At the core of HR: RAD51 and its mediators in recombination

7.1. The structure of the RAD51 nucleoprotein filament

As mentioned in §5.3, during the HR presynaptic phase ATP binds to RAD51 and activates the formation of the presynaptic filament structure, that is the assembly of the recombinase promoters into a right-handed helical filament onto ssDNA (the invading strand) (Fig. 24, left) (Brouwer et al., 2018; Forget & Kowalczykowski, 2010; Hilario, Amitani, Baskin, & Kowalczykowski, 2009; Subramanyam, Kinz-Thompson, Gonzalez Jr., & Spies, 2018; Xu et al., 2017). The presynaptic complex engages and sample the dsDNA while searching for a homologous region within the duplex with the assistance of the recombinase co-factor HOP2/MND1 complex, constituted by the homologous-pairing protein 2 homolog (HOP2) and the meiotic nuclear division protein 1 homolog (MND1). Specifically, this heterodimer stabilizes the RAD51 presynaptic filament via the C-terminal binding domain of HOP2, while the N-terminal DNA binding domains of HOP2-MDI work in conjunction with the presynaptic filament to assemble the synaptic complex (Zhao & Sung, 2015). Successful homology search is followed by limited base-pairing between the invading and complementary strand in the dsDNA during the so-called synaptic complex formation phase (Mani, Braslavsky, Arbel-Goren, & Stavans, 2010). More extensive DNA-strand exchange follows, and the newly formed DNA joint molecule is bound by the recombinase filament within the postsynaptic complex (Fig. 24, right). This complex is subsequently resolved with the disassembly of the recombinase filament and the recruitment of a DNA polymerase that extends the 3'end of the invading DNA strand, as discussed in §5.3.

The presynaptic and postsynaptic complexes have comparable helical parameters of 15.8Å rise and 56.77° twist, corresponding to a helical assembly of 6.3 RAD51 protomers per turn with a pitch of approximately 100Å. Accordingly, the overall protein conformation and promoter-promoter interactions are also very similar, with an N-terminal domain composed of 5 α-helices and a β-strand responsible for promoter-promoter interactions, and a classical α/β ATPase core domain featuring both nucleotide-binding Walker motifs and 2 DNA interacting loops L1 and L2 (Fig. 24) (Xu et al., 2017). RAD51 promoters

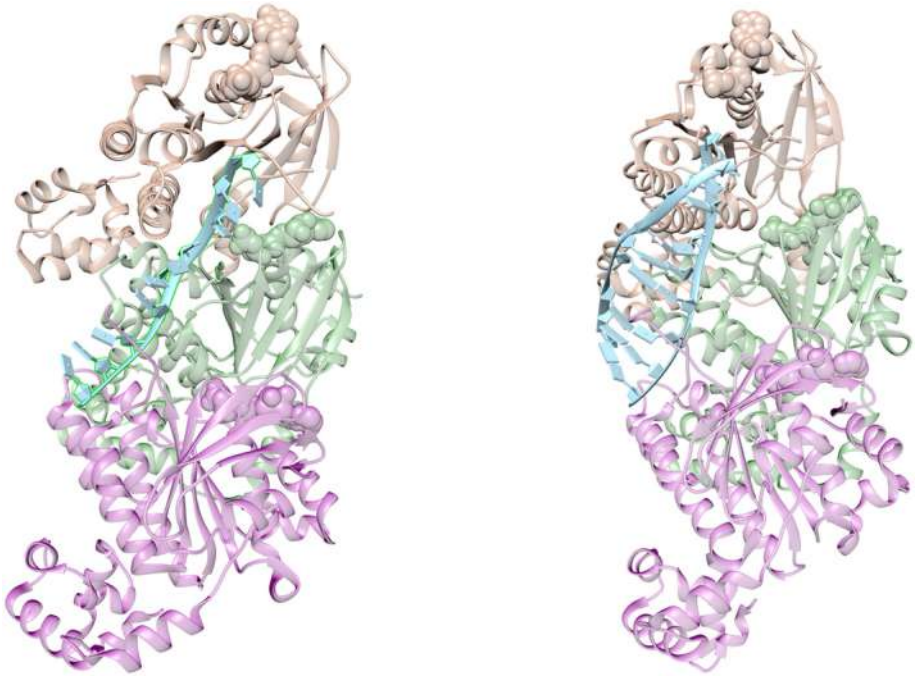

Fig. 24. Cryo-EM structures of the RAD51 presynaptic (left, PDB: 5H1B) and postsynaptic (right, PDB: 5H1C) complexes (Xu et al., 2017). The three RAD51 protomers are colored in light magenta, light green and light sienna, respectively; the DNA is in light blue, and the three RAD51-bound adenosine monophosphate molecules are highlighted as spheres.

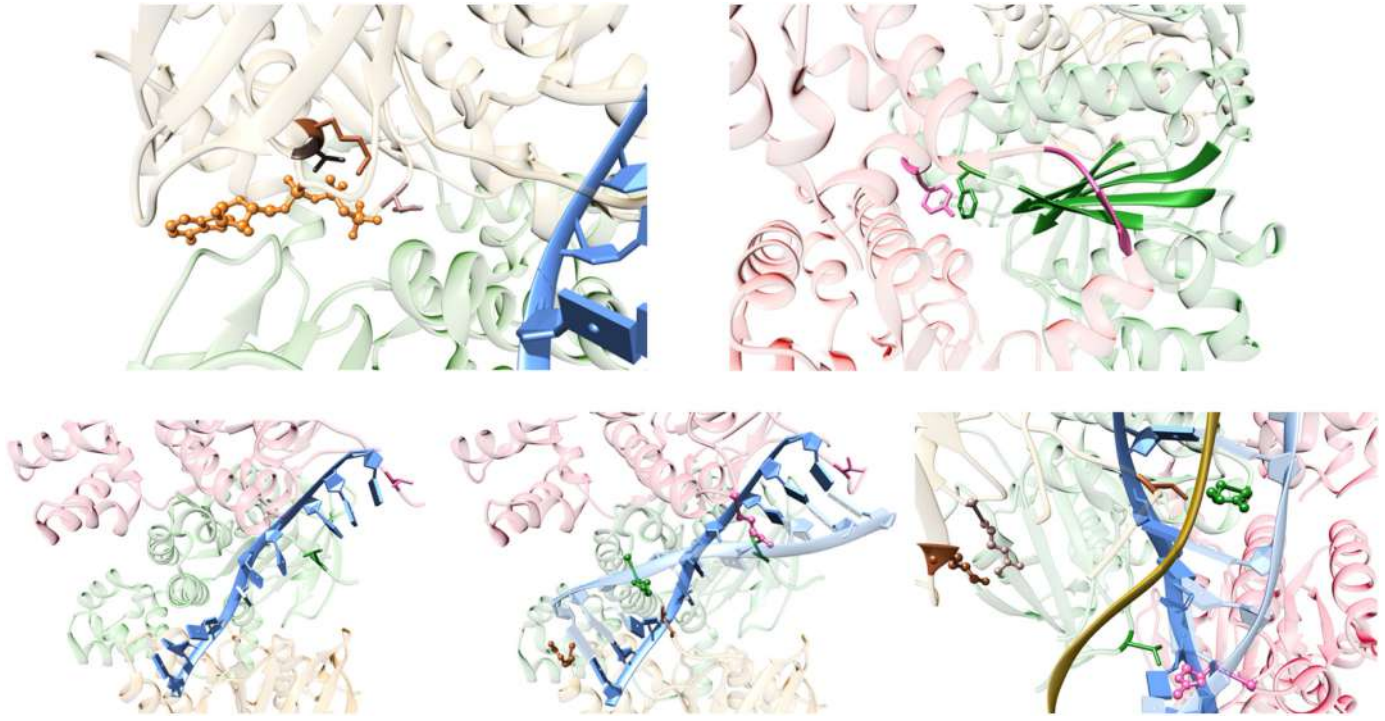

Fig. 25. Zoomed views of the RAD51/RAD51 and RAD51/DNA interfaces in the recombinase nucleoprotein filament. Top panel: (left) the first RAD51 protomer-protomer interface showing the nucleotide (in orange sticks-and-balls) encased within two adjacent RAD51 chains, portrayed as transparent sand and green ribbons. The three key promoter residues stabilizing the nucleotide binding are shown as colored sticks (K133, sienna; T134, dark brown; and E163, rosy brown). (Right) the other two protomer-protomer interfaces constituted by the small β-strand (hot pink) of one promoter (transparent light pink ribbon) packed against the central β-sheet (forest green) of the ATPase domain of the adjacent promoter (transparent light green ribbon), and the aromatic packing between Y54 of the N-terminal domain of one promoter (hot pink sticks) and F195 in the ATPase domain of the adjacent promoter (forest green sticks), respectively. Bottom panel: (left) the RAD51/DNA interaction in the presynaptic complex. The ssDNA is shown in cornflower blue. The three RAD51 protomers are shown as transparent pink, green and sand ribbons, while the stabilizing V273 residues are shown as sticks colored according to the corresponding chain (hot pink, forest green and sienna, respectively). (Middle) the RAD51/DNA interaction in the postsynaptic complex. The complementary DNA strand is in cornflower blue while the invading strand is in light blue. The three RAD51 protomers and the V273 residues are shown as in the left panel, while the further R235 residues are shown as color-matching sticks-and-balls. (Right) the proposed RAD51-mediated interstrand exchange, showing the intermediate state with the displaced strand depicted in gold and the two residues R130 and K304 (sienna sticks-and-balls) of one protomer (PDBs: 5H1B and 5H1C (Xu et al., 2017)).

form the helical assembly mainly via three interfaces: the first, mediated by an ATP molecule sandwiched between two neighboring promoters (Fig. 25, top left), involving the conserved residues K133, T134 and E163, consistently with the known role of the former two amino acids in ATP-mediated binding and hydrolysis (Flott et al., 2011; Forget, Loftus, McGrew, Bennett, & Knight, 2007) and of the latter residue in recombinase activity (Amunugama et al., 2012). The second and third interfaces are formed by the packing of a short β-strand linking the N-terminal and ATPase domains of one promoter against the central β-sheet of the ATPase domain of the adjacent promoter, and by an aromatic packing between Y54 of the N-terminal domain of one promoter and F195 in the ATPase domain of the adjacent promoter, respectively (Fig. 25, top right).

From the nucleic acid perspective, the solved crystal structures of the RAD51-DNA nucleofilament have revealed that the recombinase engages both ssDNA and dsDNA in nucleotide triplet clusters via similar interactions of two main protein loops that play an important role in strand exchange (Xu et al., 2017). Specifically, in the presynaptic complex each nucleotide triplet connects with three RAD51 chains. Adjacent triplets are separated and stabilized by a specific residue, that is V273 belonging to the L2 in the center of each RAD51 promoter while RAD51 mainly interacts with the backbone of the ssDNA (Fig. 25, bottom left). In the postsynaptic complex, the complementary strand adopts a conformation very similar to the ssDNA; moreover, besides the preserved stabilizing role of the V273 residues in each protomer a further amino acid - R235 in the L1 of each RAD51 chain - plays a role in facilitating the separation of the neighboring triplets (Fig. 25, bottom middle), in agreement with the evidence that R235E RAD51 mutants fail to perform strand-exchange while the same substitution does not affect ssDNA binding (Prasad, Yeykal, & Greene, 2006; Reymer, Frykholm, Morimatsu, Takahashi, & Norden, 2009).

Using a specifically-designed DNA-substrate combination and cryo-EM, Xu et al. were also able to capture the structure of an arrested strand-exchange synaptic complex, which allowed these authors to propose a mechanistic model for the RAD51-DNA strand exchange (Xu et al., 2017). According to this model, the displaced strand locates near the C-terminal part of a RAD51 potential secondary DNA binding site encompassing residues R130 and K304, which facilitates strand separation in the homologous dsDNA partner, while the invading strand and the complementary strand are again stabilized by V273 and R235, respectively (Fig. 25, bottom right).

7.2. Key RAD51 mediators and regulators

The presence of multiple RAD51-dependent pathways and other alternative mechanisms suggest the existence of sophisticated regulatory mechanisms that influence the choice of pathways and their execution manner. Many important decisions need to be made to control the repair of different types of lesions. For example, whether both ends of the DBSs are used to repair, how DNA synthesis is initiated and terminated, whether SSA and BIR pathways are used only when other repair attempts fail. Considering the central role of RAD51 in HR, it is only logical that much of the regulation impinges on this protein and its modulators (Godin, Sullivan, & Bernstein, 2016; Krejci, Altmannova, Spirek, & Zhao, 2012; Schild & Wiese, 2010; Sung & Klein, 2006; Sung, Krejci, Van Komen, & Sehorn, 2003). Below, we will focus on how this multi-layered control affects the formation, maintenance and disassembly of RAD51 nucleofilaments. There are both positive and negative regulators

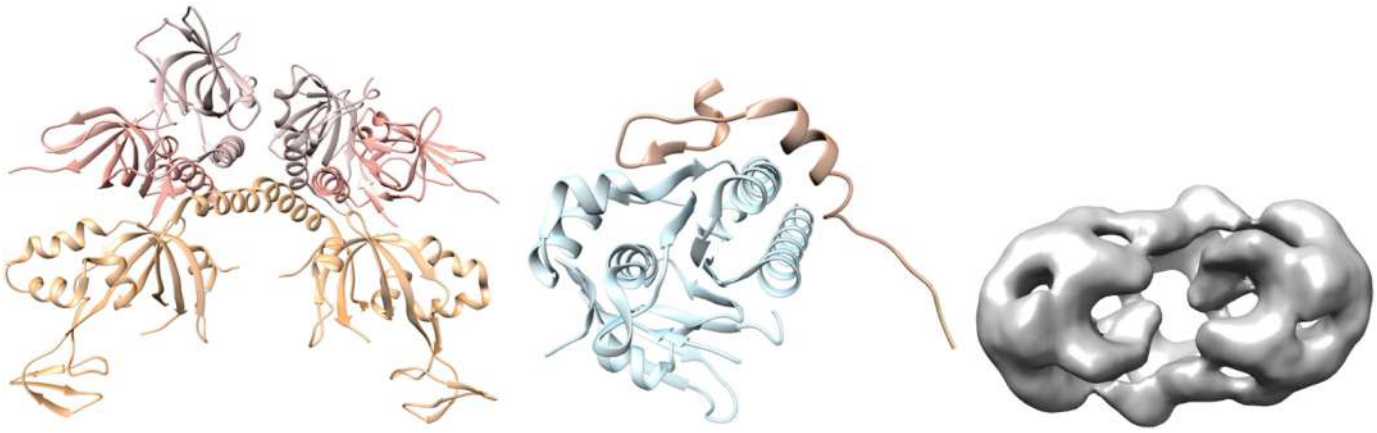

Fig. 26. (Left) The structure of the RPA trimerization core comprising the C-terminal DNA-binding domain of subunit RPA70 (DBD-C), the central DNA-binding domain of subunit RPA32 (DBD-D) and the entire RPA14 subunit (PDB: 1L1O (Bochkareva et al., 2002)). (Middle) The structure of a complex between an evolutionarily conserved sequence in BRCA2 (the BRC repeat, sienna) and the core domain of RAD51 (light blue) (PDB: 1N0W (Pellegrini et al., 2002)). (Right) Surface view of the 3D reconstruction of the BRCA2 dimer as obtained from EM (Electron Microscopy Data Bank (EMD): 2779 (Shahid et al., 2014)).

of RAD51 functions, some of which play a general role in both mitotic and meiotic cells, whereas others are specific to only one of those (Krejci et al., 2012). In addition, HR regulation employs protein post-translational modifications such as phosphorylation and SUMOylation (Andriuskevicius, Kotenko, & Makovets, 2018; Godin, Sullivan, & Bernstein, 2016; Oberle & Blattner, 2010), to provide the required RAD51 filament flexibility, dynamics and even end capping, which is thought to stimulate strand exchange (Taylor et al., 2015; Taylor et al., 2016).

7.2.1. RPA

As described in §5.3, at the beginning of HR endonucleolytic activity produces a long 3' ssDNA overhang that is rapidly coated by the heterotrimeric protein RPA (Fig. 26, left) (Bochkareva, Korolev, Lees-Miller, & Bochkarev, 2002; Yates et al., 2018). Displacement of RPA from the nucleic acid is a rate-limiting step to RAD51 filament formation and, since RPA binds ssDNA with higher affinity than the recombinase (Ma, Gibb, Kwon, Sung, & Greene, 2017), RAD51 mediator proteins are required to assemble RAD51 on the ssDNA by nucleating, elongating, and stabilizing the relevant nucleoprotein filament. On the other hand, RPA not only promotes recombination by removing secondary structures on ssDNA that could impede RAD51 filament formation (Sung et al., 2003) but also aids RAD51 in its action by preventing the reversal reaction of recombinase-mediated D-loop formation. This action takes place by the scavenging and sequestration of free ssDNA, thereby preventing DNA from entering the second DNA binding site of RAD51 (Eggler, Inman, & Cox, 2002; Van Komen, Petukhova, Sigurdsson, & Sung, 2002). An updated and detailed discussion on RPA and RAD51 co-operation in preserving genome stability can be found in the recent work by Bhat and Cortez (Bhat & Cortez, 2018).

7.2.2. BRCA2

In humans RAD51 nucleoprotein filament formation is mainly mediated by the RAD51 BRCA2 loader (Holloman, 2011), which binds RAD51 through the its BRC repeats and C-terminal domain (Fig. 26, middle). The BRC repeats in BRCA2 mimic the oligomerization interface of RAD51, thus enabling RAD51 loading. BRCA2 delivers RAD51 monomers to ssDNA rather than dsDNA, allowing filament formation and ultimately promoting RAD51 strand exchange activity (Pellegrini et al., 2002). Besides BRC domains, BRCA2 also interacts via its C-terminal domain with RAD51 only in its nucleoprotein filament form and in a cell cycle-dependent fashion (Davies & Pellegrini, 2007; Esashi, Galkin, Yu, Egelman, & West, 2007). In particular, the work of Ayoub and collaborators revealed that BRCA2 carrying mutations in its C-terminus that prevent interaction with RAD51 do not affect RAD51 foci formation or HR repair but result in rapid foci disassembly and mitotic entry (Ayoub et al., 2009). In 2010 Thorslund et al confirmed that BRCA2 directs the binding of RAD51 to ssDNA, reduces the binding of the recombinase to dsDNA, and stimulates RAD51-mediated strand exchange (Thorslund et al., 2010). Contextually, another study showed that this BRCA2 C-terminal is essential for fork protection by preventing MRE11-mediated RAD51 filaments degradation (Schlacher et al., 2011). The BRCA2 mechanism of action was elucidated in 2014 by Shahid et al., who showed that BRCA2 facilitated nucleation of RAD51 filaments at multiple sites on ssDNA (Shahid et al., 2014). In this work, three-dimensional EM reconstructions revealed that BRCA2 forms dimers (Fig. 26, right), and that two oppositely-oriented sets of RAD51 proteins bind the dimer. ssDNA binds along the long axis of BRCA2, such as only one set of recombinase monomers can form a productive complex with DNA, originate the nucleoprotein filament and mediate HR.

BRCA2 also binds and coordinates the activity of several other recombinator mediators including the deleted in split hand/split foot syndrome protein (DSS1, §7.2.3) and PALB2 (§7.2.4) to promote RAD51 loading and function. In 2017 Kolinjivadi et al. also demonstrated that BRCA2 prevents ssDNA gap accumulation at replication fork junctions and behind them by promoting RAD51 binding to replicating DNA (Kolinjivadi et al., 2017). In the absence of BRCA2, they showed that forks with persistent gaps are converted by the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1, an ATP-dependent annealing helicase that binds selectively to fork DNA relative to ssDNA or dsDNA and catalyzes the rewinding of the stably unwound DNA (Lugli, Sotiriou, & Halazonetis, 2017)) into reversed forks, thereby triggering MRE11-dependent nascent DNA degradation. They also verified that RPA or impaired RAD51 mutants could not prevent MRE11-dependent DNA degradation, while MRE11 inhibition promoted reverse fork accumulation in the absence of BRCA2. Contextually, RAD51 was found to interact with the N-terminal domain of Polα, supporting the binding of the same polymerase as well as Polδ to stalled replications forks and, hence, promoting replication fork restart and gap avoidance. In essence, BRCA2 and RAD51 cooperate to prevent the generation of DNA abnormal intermediates which, if processed by SMARCAL1 and MRE11, may lead to genome instability.

Notably, Badie et al. showed that BRCA2 associates with telomeres during the S and G2 phases of the cell cycle and facilitates the loading of RAD51 onto telomeres (Badie et al., 2010). Conditional deletion of BRCA2 and inhibition of RAD51 in mouse embryonic fibroblasts, but not inactivation of BRCA1, leads to shortening of telomeres and accumulation of fragmented telomeric signals - a hallmark of telomere fragility that is associated with replication defects. These findings suggest that

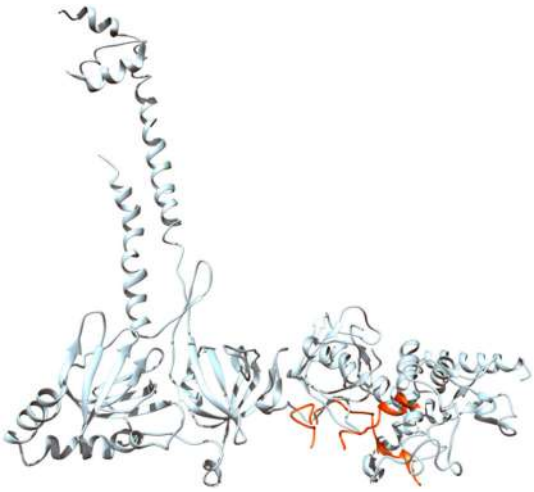

Fig. 27. Overall view of the COOH-terminal domain of BRCA2 (light blue) bound to DSS1 (orange) (PDB: 1MIU (H. Yang et al., 2002)).

BRCA2-mediated HR reactions contribute to the maintenance of telomere length by facilitating telomere replication and imply that BRCA2 has an essential role in maintaining telomere integrity during unchallenged cell proliferation. Indeed, according to this study mouse mammary tumors that lack BRCA2 accumulate telomere dysfunction-induced foci, and human breast tumors in which BRCA2 is mutated have shorter telomeres than those carrying BRCA1 mutations, suggesting that the genomic instability in BRCA2-deficient tumors is due in part to telomere dysfunction.

7.2.3. DSS1

The DSS1 gene (P. H. Duijf, van Bokhoven, & Brunner, 2003), a biomarker for different cancers (Ma et al., 2013; Rezano et al., 2013; Wei, Trempus, Cannon, Bortner, & Tennant, 2003), encodes the small (70 residues), highly acidic DSS1 protein. In 2015, Zhao et al. reported that RPA-RAD51 exchange requires the BRCA2 partner DSS1 (Zhao et al., 2015). Biochemical, structural, and *in vivo* analyses revealed that DSS1 allows the BRCA2-DSS1 complex (Fig. 27) to physically and functionally interact with RPA. Mechanistically, DSS1 acts as a DNA mimic to attenuate the affinity of RPA for ssDNA, and a mutation in the solvent-exposed acidic domain of DSS1 compromises the efficacy of RPA-RAD51 exchange. Thus, by targeting RPA and mimicking DNA, DSS1 functions with BRCA2 in a two-component homologous recombination mediator complex in genome maintenance and tumor suppression.

7.2.4. PALB2

PALB2 plays a critical role in HR. Specifically, PALB2 colocalizes with BRCA2 in nuclear foci, promotes its localization and stability in key nuclear structures (e.g., chromatin and nuclear matrix), and enables its recombinational repair and checkpoint function (Xia et al., 2006) (Fig. 28, left). Also, Sy et al. proved that both PALB2 chromatin association and its oligomerization serve to secure the BRCA2 x RAD51 repair machinery at the sites of DNA damage (Sy, Huen, Zhu, & Chen, 2009). Accordingly. these attributes of PALB2 are likely instrumental for proficient homologous recombination DNA repair in the cell. The same group reported that PALB2 binds directly to BRCA1, and serves as the molecular scaffold in the formation of the BRCA1-PALB2-BRCA2 complex (Sy, Huen, & Chen, 2009). They showed that the association between BRCA1 and PALB2 is primarily mediated via apolar bonding between their respective coiled-coil domains. More importantly, BRCA1 mutations identified in cancer patients disrupted the specific interaction between BRCA1 and PALB2. Consistent with the converging functions of the BRCA proteins in DNA repair, cells harboring mutations with abrogated BRCA1-PALB2 interaction resulted in defective HR repair. Thus, the authors proposed that, via its direct interaction with PALB2, BRCA1 fine-tunes recombinational repair partly through its modulatory role in the PALB2-dependent loading of BRCA2-RAD51 repair machinery at DNA breaks, and suggested that impaired HR repair is one of the fundamental causes for genomic instability and tumorigenesis observed in patients carrying BRCA1, BRCA2, or PALB2 mutations. Buisson et al. showed that human PALB2 binds DNA, preferentially D-loop structures, and directly interacts with RAD51 to stimulate strand invasion (Buisson et al., 2010). This stimulation occurs through reinforcing biochemical mechanisms, as PALB2 alleviates inhibition by RPA and stabilizes the RAD51 filament. Moreover, PALB2 can function synergistically with a BRCA2 chimera (termed piccolo, or piBRCA2) to further promote strand invasion. In a successive effort, Buisson and Masson discovered that the N-terminal coiled-coil motif of PALB2 regulates its self-association and HR (Buisson & Masson, 2012). Thus, monomeric PALB2 shows higher efficiency to bind DNA and promotes RAD51 filament formation with or without the inhibitory effect of RPA. Moreover, overexpression of the PALB2 coiled-coil domain severely affects RAD51 loading to DNA damage sites suggesting a competition between PALB2 self-interaction and PALB2-BRCA1 interaction. In the presence of DNA damage, the switch between PALB2-PALB2 and PALB2-BRCA1 interactions allows the activation of HR. Therefore, controlling HR via PALB2 self-interactions could be important to prevent aberrant recombination in normal conditions and activate DNA repair when required. In addition, although PALB2 alone stimulates D-loop formation, it has a cooperative effect with RAD51AP1, an enhancer of RAD51, in promoting D-loop formation and in assembling the synaptic complex (Dray et al., 2010).

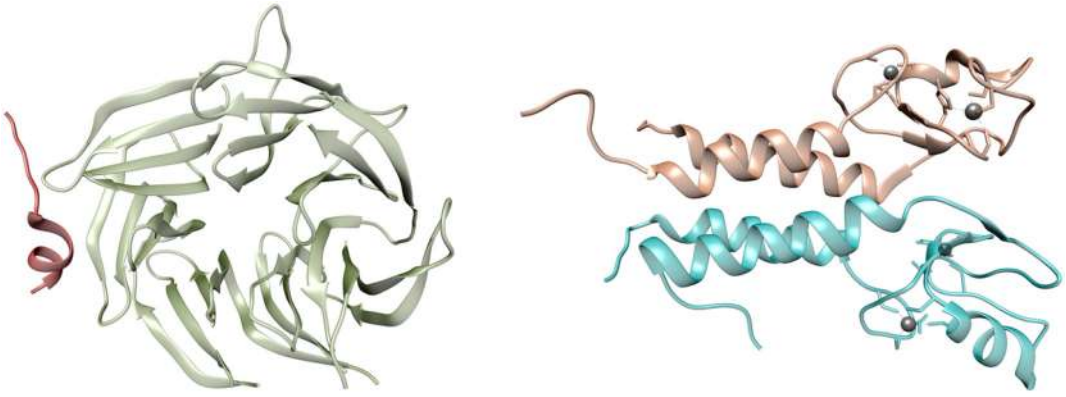

Fig. 28. (Left) Crystal structure of the PALB2 carboxy-terminal domain (olive green) in complex with a BRCA2 peptide (dark red) (PDB: 3EU7 (Oliver, Swift, Lord, Ashworth, & Pearl, 2009)). (Right) NMR-derived solution structure of the heterodimer formed between the RING domains of BRCA1 (cyan) and BARD1 (sienna). The zinc ions (spheres) and the coordinating residues (sticks) are highlighted (PDB: 1JM7 (Brzovic, Rajagopal, Hoyt, King, & Klevit, 2001)).

7.2.5. BARD1

BARD1 is a multifunction, 777-residue protein that, together with BRCA1, is synthesized during the S phase of the cell cycle (Irminger-Finger, Ratajska, & Pilyugin, 2016). The two proteins form the tumor suppressor complex BRCA1-BARD1, that colocalizes at nuclear foci and functions in DNA DSB repair during HR (§5.3). BRCA1 and BARD1 form stable heterodimers via their RING-finger domains (Fig. 28, right) implicating residues 1-109 of BRCA1 and residues 26-119 of BARD1 (Brzovic et al., 2001). The role of BRCA1-BARD1 during this process is to facilitate the nucleolytic resection of DNA ends to generate a single-stranded template for the recruitment of the other tumor suppressor complex BRCA2-PALB2 described above (§7.2.4), and RAD51. In a recent work, Zhao et al. demonstrated that the heterodimer BRCA1-BARD1 binds DNA and interact with RAD51, thereby enhancing the recombinase activity (Zhao et al., 2017). From a mechanistic viewpoint, the authors showed that the BRCA1-BARD1 ensemble promotes the assembly of the synaptic complex, and are indispensable for RAD51 stimulation. Using mutant BARD1 isoforms defective for RAD51 binding they further proved that the relevant heterodimer exhibited compromised DNA joint formation and impaired mediation of homologous recombination and repair, and suggested that targeting the BRCA1-BARD1 interaction could constitute a novel strategy in cancer therapy.

7.2.6. RAD52

As reported in (§5.3), RAD52 is one of the proteins assisting RAD51 in its nucleoprotein filament formation. In this respect, Ma et al. further showed that RAD52 binds tightly to the RPA-ssDNA complex and imparts an inhibitory effect on RPA turnover (Ma, Kwon, Sung, & Greene, 2017). They also found that during presynaptic complex assembly, most of the RPA and RAD52 is displaced from the ssDNA, but some RAD52-RPA-ssDNA complexes persist as interspersed clusters surrounded by RAD51 filaments. Once assembled, the presence of RAD51 restricts formation of new RAD52-binding events, but additional RAD52 could bind once RAD51 dissociated from the ssDNA. Together, these results provide new insights into the behavior and dynamics of human RAD52 during presynaptic complex assembly and disassembly. Early this year Malacaria and coworkers demonstrated that RAD52 prevents MRE11-mediated excessive degradation of reversed replication forks by binding to the stalled replication fork and promoting its occlusion (Malacaria et al., 2019). RAD52-inhibited cells rely on RAD51 for completion of replication and viability upon replication arrest. In aggregate their data suggest a novel gatekeeper mechanism by which RAD52 limits excessive remodeling of stalled replication forks, thus indirectly assisting RAD51 and BRCA2 in protecting forks from unscheduled degradation and preventing genome instability.

The RAD52 ssDNA annealing activity is also responsible for the RAD51-independent DSB repair pathway through SSA between repeated DNA sequences, as mentioned in §5.3. The detailed mechanism of DNA annealing promoted by RAD52 has remained elusive until Saotome and coworkers reported two crystal structures of human RAD52-ssDNA complexes that probably represent key reaction intermediates of RAD52-mediated DNA annealing (Saotome et al., 2018). The first structure (Fig. 29, left) shows a "wrapped" conformation of ssDNA around a homo-oligomeric RAD52 ring, in which the edges of the bases involved in base pairing are exposed to the solvent, while the second structure (Fig. 29, right) reveals a "trapped" conformation of ssDNA between two RAD52 rings. This conformation is stabilized by a different RAD52 DNA binding site, which promotes the accumulation of multiple RAD52 rings on ssDNA and the aggregation of ssDNA.

It must be said here that RAD52 has been implicated in a much wider range of pathways throughout the cell cycle as a RAD51-independent protector of the genome instability (Jalan, Olsen, & Powell, 2019). Thus, RAD52 has not only been found to have a role in alternative pathways such as RNA-template repair (McDevitt, Rusanov, Kent, Chandramouly, & Pomerantz, 2018; Storici, Bebenek, Kunkel, Gordenin, & Resnick, 2007), but it may also play a larger role in HR as previously thought. For instance, transcriptionally active loci are particularly fragile and it is critical for the maintenance of homeostasis that any DNA damage within such transcriptionally active regions undergoes accurate repair. To this purpose, further dedicated DDR pathways have evolved, such as the transcription-coupled homologous recombination (TC-HR) and the transcription-associated homologous recombination repair (TA-HRR) (Aguilera & Gaillard, 2014; Marnef, Cohen, & Legube, 2017), in which RAD52 is vital for the DSB repair at actively transcribed genes (Yasuhara et al., 2018). Further research is required into the newly described functions of RAD52, and the interested reader is referred to the most recent review works on this fascinating and challenging subject (Ghosh et al., 2017; Hanamshet et al., 2016; Jalan et al., 2019).

7.2.7. RAD54

DNA transcription, replication, repair and recombination are all events that require direct access to DNA. This process is facilitated by the SWI2/SNF2 family of ATPases, which detach DNA from histones and other bound proteins (Clapier, Iwasa, Cairns, & Peterson, 2017). As a SWI2/SNF2 enzyme, RAD54 can translocate along dsDNA in an ATP hydrolysis dependent manner, generate superhelical torsion, and promote chromatin remodeling, thereby enhancing the accessibility to nucleosomal DNA (Amitani, Baskin, & Kowalczykowski, 2006). As reported in §5.3, RAD54 interacts physically and functionally with RAD51 and

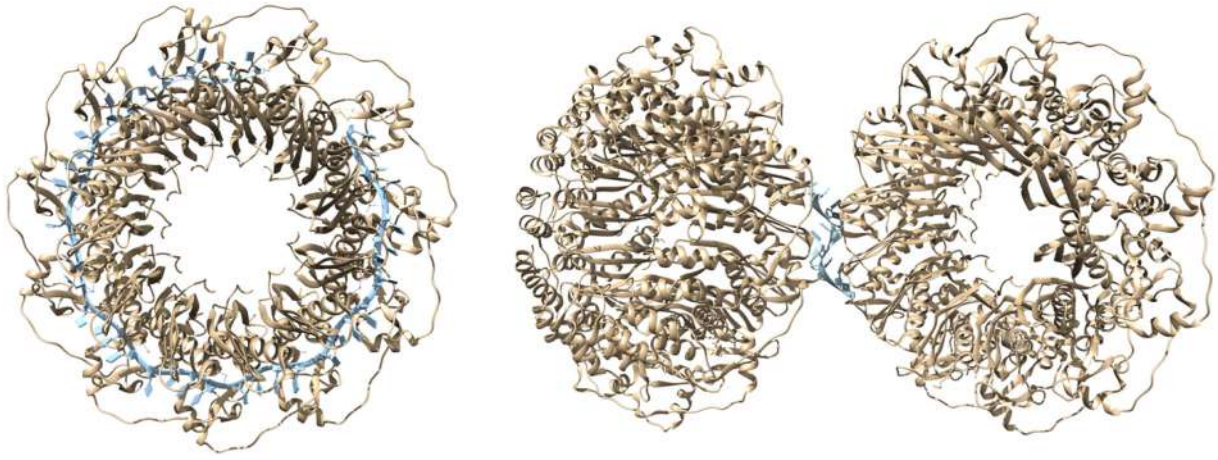

Fig. 29. (Left) Crystal structure of the RAD52-ssDNA complex in the “wrapped” ssDNA conformation, viewed down the central channel of the ring (the 40-nucleotide ssDNA (light blue) spans across 10 RAD52 subunits (tan) (PDB: 5XRZ (Saotome et al., 2018)). (Right) Crystal structure of ssDNA bound to the outer DNA binding site of RAD52. The ssDNA (light blue) is “trapped” between two RAD52 rings (shown in tan) (PDB: 5XS0 (Saotome et al., 2018)).

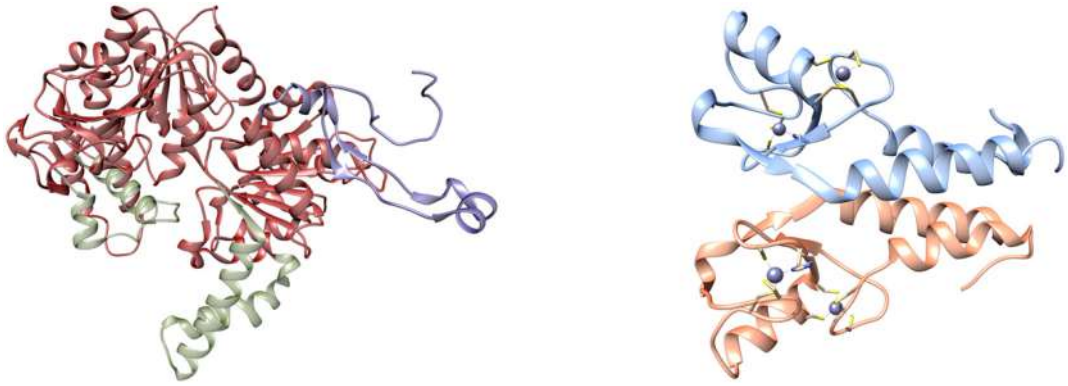

Fig. 30. (Left) Crystal structure of RAD54 showing the SWI2/SNF2 specific elements in olive green and the primary RAD51 binding site within the N-terminal domain in dark slate blue (PDB: 1Z3I (Thoma et al., 2005)). (Right) Crystal structure of the RAD18 homodimer. The zinc ions and their coordinating residues are highlighted as spheres and sticks, respectively (PDB: 2Y43 (A. Huang et al., 2011)).

stimulates the recombinase DNA strand exchange activity (Kiianitsa, Solinger, & Heyer, 2006). Also, RAD54 binds Holliday junctions and drives their branch migration (Goyal et al., 2018), and interacts with MUS81-EME1 (§5.3 and §6) stimulating its DNA cleavage activity (Mazin, Mazina, Bugreev, & Rossi, 2010). RAD54 has a primary RAD51-binding site within a ~90-residue N-terminal region that is unstructured (Raschle, Van Komen, Chi, Ellenberger, & Sung, 2004) (Fig. 30, left), and a second weaker site in the remainder of the protein (Golub, Kovalenko, Gupta, Ward, & Radding, 1997). The N-terminal region is expendable for the nucleosome-remodeling activity of RAD54 but is important for its RAD51-specific functions (Alexiadis, Lusser, & Kadonaga, 2004).

Very recently, using EM Tavares et al. demonstrated that RAD54 is crucial for RAD51-mediated synaptic complex formation and homology search (Tavares, Wright, Heyer, Le Cam, & Dupaigne, 2019). They showed that the K341R RAD54 ATPase-deficient mutant protein promotes formation of synaptic complexes but not D-loops and leads to the accumulation of stable heterologous associations, suggesting that the RAD54 ATPase is involved in preventing non-productive intermediates. Accordingly, the authors proposed that RAD51 and RAD54 form a functional unit operating in homology search, synaptic complex and D-loop formation. In another recent effort based on a combination of molecular dynamics simulations and living-cell fluorescence experiments, Lengert and coworkers revealed that phosphorylation of RAD54 is a critical event in balancing the DNA binding strength and mobility of RAD54 and might therefore provide optimal conditions for DNA translocation and subsequent removal of RAD51 during HR (Lengert, Spies, & Drossel, 2019).

Centromeres consist of DNA repeats in many eukaryotes, and nonallelic HR between them can result in gross chromosomal rearrangements (GCRs). In their work Onaka et al. provided evidence that RAD51 and RAD54 promote non-crossover recombination between centromere repeats on the same chromatid, thereby suppressing crossover between nonallelic repeats on sister chromatids and preventing GCRs (Onaka et al., 2016). They also reported that both RAD54 and RAD51 are required for gene silencing in centromeres, suggesting that HR plays a role also in the structure and function of centromeres. All these studies make clear that RAD54 is not merely a multifunctional protein; by acting through virtually all steps of HR and interacting with different protein partners RAD54 is becoming a main player in DNA repair, and further investigations are indeed required to uncover its multifaceted contributions in genome integrity preservation.

7.2.8. RAD18

The E3 ubiquitin-protein ligase RAD18 (RAD18) is well-known for its function in DNA damage bypass and TLS in yeast and vertebrates via its ability to facilitate PCNA mono-ubiquitination at stalled replication forks (Fig. 30, right). However, emerging evidence has also indicated that, in mammalian cells, RAD18 plays an important role in HR (Ting, Jun, & Junjie, 2010). Mechanistically, Ting and coworkers showed that, in response to DSBs, RAD18 functions as an adaptor protein by binding directly to RAD51C (§7.4), and this allows the accumulation of RAD51C at DNA damage sites and thus facilitates RAD51 foci formation and HR repair. Recently, Tripathi et al. confirmed that RAD18 functions upstream in the HR pathway as its downregulation prevents activation of FANC2, and diminished BRCA2 and RAD51 protein levels, formation of nuclear foci of all three proteins, and recovery of stalled or collapsed forks in response to DSB-induced formation.

7.2.9. RADX

The group of Cortez identified the RPA-related protein RADX (RADX) as an RPA-like, ssDNA binding protein, which is recruited to replication forks to prevent fork collapse by modulating RAD51 activity (Bhat et al., 2018; Dungrawala et al., 2017). When RADX is inactivated, excessive RAD51 activity slows replication elongation and causes DSBs. By antagonizing RAD51 at forks, RADX allows cells to maintain high HR capacity while ensuring that replication functions of RAD51 are properly regulated. This renders RADX a key factor in preserving the correct RAD51 balance and, hence, genome integrity.

7.2.10. RAD51AP1

A key HR protein downstream of RAD51 filament formation is the RAD51-associated protein 1 (RAD51AP1), which is highly conserved among vertebrates (Pires, Sung, & Wiese, 2017). Of the 3 RAD51AP1 splice variants described, only isoforms 1 and 2 (352 and 335 amino acids, respectively) have been shown to code for a functional protein (https://www.uniprot.org/uniprot/Q96B01-2). Rich in hydrophilic residues (aspartic acid, arginine, glutamic acid, and lysine constituting 1/3 of the protein primary sequence), both isoforms behave very similarly in biochemical and cell-based assays (Dunlop et al., 2012; Modesti et al., 2007); accordingly, the relevant literature generally refers to RAD51AP1 without isoform distinction. Discovered at the end of the '90s as a RAD51-interacting protein, its RAD51 binding domain consists of 26 residues located in the C-terminal portion (Kovalenko, Golub, Bray-Ward, Ward, & Radding, 1997; Kovalenko, Wiese, & Schild, 2006); also, consistent with its role in HR DNA repair, RAD51AP1 and RAD51 foci both co-localize spontaneously and after DNA damage induction (Wiese et al., 2007). In particular, RAD51AP1 binds both ssDNA and dsDNA, yet it presents the highest affinity for branched DNA substrates and D-loops, pointing to its role in the DNA intermediate formation during HR. RAD51AP1 possesses two DNA binding domains - one mapped onto the N-terminal region and the other located in proximity of the protein C-terminus - both required for promoting D-loop formation by RAD51 (Dunlop et al., 2012). As discussed in §5.3, during D-loop formation the RAD51 presynaptic filament engages with a dsDNA partner molecule to conduct homology search and

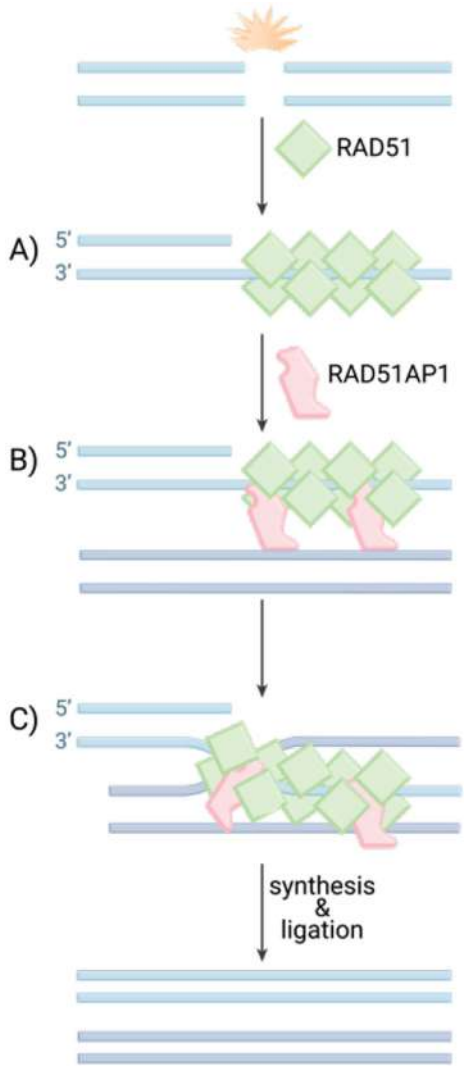

Fig. 31. Schematic role of RAD51AP1 during some of the steps in HR: A) presynaptic filament formation; B) synaptic complex; C) D- loop formation.

synaptic complex formation, in which homologous DNA sequences are aligned to allow base switching. Based on the evidence that mutated RAD51AP1 isoforms either lacking the RAD51 binding domain or carrying missense mutations in the same region are impaired for RAD51 interaction and the related ability to stimulate D-loop formation (Kovalenko et al., 2006; Modesti et al., 2007), a mechanistic hypothesis was proposed according to which RAD51AP1 acts by bridging the incoming duplex DNA with the presynaptic filament, as exemplified in Fig. 31 (Pires et al., 2017). Nonetheless, substantial work lies ahead to fully determine the spatial and temporal organization of this RAD51 mediator in relation to HR.

7.2.11. p53

The transcription factor p53 plays a central part in the cell cycle and is arguably the most important tumor suppressor, so that it is often referred to as the guardian of the genome (Joerger & Fersht, 2016). p53 can be activated by a variety of stress signals, including DNA damage, ribonucleotide depletion, hyperploidy, hypoxia, activated oncogenes and loss of cell adhesion. Upon stimulation, p53 can initiate cell cycle arrest, DNA repair, premature senescence, or apoptosis by activating transcription of a number of downstream genes (Hafner et al., 2019). Mutations in the p53 gene are common in a variety of cancers, and its inactivation leads to cancer predisposition (Mantovani, Collavin, & Del Sal, 2019). In the context of DNA damage, p53 was found to play an important role in several of the DDR mechanisms including NER, BER, MMR, HR and NHEJ (Menon & Povirk, 2014; Sengupta & Harris, 2005; Williams & Schumacher, 2016). In HR, independently of its cell cycle checkpoint controller p53 has both trans-activation-independent and -dependent functions. Specifically, transcriptional regulation of DSB repair by p53 has been verified via the interaction between p53 and the RAD51 promoter, with corresponding changes in RAD51 gene expression (Arias-Lopez et al., 2006; Fong et al., 2011; Lazaro-Trueba, Arias, & Silva, 2006), although the direct contribution of p53 to RAD51 gene regulation is limited when compared with other transcription factors (Gong et al., 2015; Hine et al., 2014). In addition to regulating RAD51 expression, p53 also appears to modulate HR via direct interaction with RAD51 and RAD54 proteins. Saintigny et al. (Saintigny, Rouillard, Chaput, Soussi, & Lopez, 1999) originally demonstrated that the frequency of both spontaneous and radiation-induced HR between repeated DNA sequences increases upon p53 inhibition. This interaction of p53 with RAD51 and subsequent inhibition of recombination was further confirmed by studies involving wildtype (WT) and mutant isoforms of both RAD51 and p53. Thus, for instance, a 2/3-fold increase in HR was seen following overexpression of the p53 non-binding L186P RAD51 mutant (Linke et al., 2003). At the same time, RAD51 was find to bind WT and (albeit to a lesser extent) H173Y, R249S, and R273H p53 mutants in the absence of DNA or RNA intermediates (Buchhop et al., 1997). Further experiments in this study carried out using a panel of p53 deletion mutants suggest that RAD51 could bind two regions of p53: one between residues 94 and 160 and another between amino acids 264 and 315, while the p53 binding domain on RAD51 locates between residues 125 and 220. Linke et al. further reported an interaction between p53 and RAD54, mainly occurring via the extreme C-terminal domain of p53, which is involved in sensing mispaired homologous recombination intermediates (Linke et al., 2003). This led the authors to conclude that p53 likely prevents illegitimate recombination by inhibitory interactions with RAD51 and RAD54, suggesting yet another mechanism by which p53 could suppress genomic instability. Using fluorescence and NMR spectroscopy, the Fersht group identified that peptides corresponding to residues 179-190 of RAD51 bind to the core domain of p53 in a promiscuous site that overlaps with its specific DNA binding site and the binding region for peptides derived from other proteins, including 53BP1 (§8.2.8 and Fig. 45). Binding is mediated mainly by a strong, nonspecific, electrostatic component and is fine-tuned by specific interactions (Friedler, Veprintsev, Rutherford, von Glos, & Fersht, 2005). Importantly, the p53-RAD51 complex was found to inhibit branch migration after the crossing-over or postsynaptic phase of recombination (Yoon, Wang, Stapleford, Wiesmuller, & Chen, 2004), thereby establishing another p53/RAD51direct functional link and unveiling the additional transcription-independent modulation of homologous recombination of p53 via prevention of detrimental genome rearrangements promoted by RAD51. Finally, experiments have shown that RAD51-p53 contacts play a role in targeting p53 to heteroduplex joints and indicates an involvement in recombination immediately following RAD51-mediated strand transfer (Susse, Janz, Janus, Deppert, & Wiesmuller, 2000). Specifically, RAD51 stimulates the 5' to 3' exonucleolytic DNA degradation by p53, when it generates strand transfer intermediates, supporting a bidirectional influence between the genome guardian and the recombinase.

7.2.12. p21

In 2011 Raderschall and coworkers proposed that, besides its established role in DNA HR, RAD51 could be further involved in regulatory aspects of the cell cycle and apoptosis (Raderschall et al., 2002). By using RAD51-overexpressing cells they found that the overexpressed recombinase forms foci and higher-order nuclear structures even in the absence of DNA damage. This correlates with increased expression of the cyclin-dependent kinase inhibitor 1 protein (p21), a major regulator of cell cycle progression at the G1-S phase checkpoint (Georgakilas, Martin, & Bonner, 2017) and a regulator of replication-coupled DSB repair fidelity/chromosome maintenance stability

(Mauro et al., 2012). Interestingly, while upon DNA damage upregulation of RAD51 shows a reduced number of DNA breaks/chromosome aberrations and a greater resistance to apoptosis as expected, downregulation of RAD51 results in decreased levels of p21 while inhibition of p21 reduces RAD51 foci formation in both normal and RAD51-overexpressing cells. Accordingly, the authors proposed an interrelation between RAD51 foci formation and p21 expression levels, suggesting a functional link between RAD51 and the p21-mediated cell cycle regulation, which could contribute to a highly effective HR in cell cycle-arrested cells and protection against DNA damage-induced apoptosis (Raderschall, Bazarov, et al., 2002).

7.2.13. c-ABL

BCR-ABL, and BCL-2. The tyrosine-protein kinase ABL1 (ABL1 or c-ABL) is activated by different genotoxic insults, including IR. After IR damage, c-ABL is directly phosphorylated by ATM which, via its pathway activation, promotes cell growth arrest and apoptosis as a response to the insult (Matt & Hofmann, 2016; Meltser, Ben-Yehoyada, & Shaul, 2011). Specifically, RAD51 is phosphorylated by c-ABL at Y54, possibly via the formation of a tripartite complex with ATM (Chen et al., 1999), and this inhibits binding of RAD51 to DNA and the function of the recombinase in ATP-dependent DNA strand exchange reactions (Yuan et al., 1998). IR-activated c-ABL also phosphorylates RAD51 at Y315, which increases its association with RAD52 and chromatin in the recombination complex in an ATM dependent manner (Mahajan & Mahajan, 2015). Shimizu and coworkers also showed that c-ABL associates with chromatin after DNA damage in a kinase-activity-dependent manner (Shimizu et al., 2009). Then, using RAD51 mutants unable to oligomerize to form nucleoprotein filaments, they separated RAD51 assembly on DNA to form foci into two steps, namely stable chromatin association and subsequent oligomerization. They next verified that phosphorylation of RAD51 Y315 promoted by c-ABL is required for chromatin association of oligomerization-defective RAD51 mutants, but is not sufficient to restore oligomerization, suggesting a new model for HR early step regulation. On the other hand, by combining biochemical and single-molecule analysis Subramanyam et al. proved that while Y54 phosphorylation enhances RAD51 DNA strand exchange activity by altering the nucleofilament properties, the recombinase Y315 phosphorylation has only limited effect on the RAD51 activities (Subramanyam, Ismail, Bhattacharya, & Spies, 2016).

Translocation of the ABL1 gene located on chromosome 9 to the breakpoint cluster region (BCR) gene located on chromosome 22 results in a BCR-ABL1 fusion gene on the Philadelphia chromosome, the hallmark of chronic myeloid leukemia (CML) (Kurzrock, Gutterman, & Talpaz, 1988). The BCR-ABL fusion-produced proteins (p230, p210 or p185) exhibit constitutive tyrosine kinase activity, which is the cause of the resistance of BCR-ABL-expressing tumors to DNA damage induced by therapeutic drugs. The expression of BCR-ABL has been shown to increase the cellular levels of RAD51 by a series of combined mechanisms (Slupianek et al., 2001; Slupianek et al., 2002). Firstly, signaling from the Src homology 3 (SH3) and 2 (SH2) domains of BCR-ABL (Fig. 32, left) stimulates RAD51 transcription via activation of the signal transducer and activator of transcription 5 (STAT5) (Levy & Darnell Jr., 2002) (§8.3). Concomitantly, transcription of the RAD51 paralogs RAD51B, RAD51D, and XRCC2 is also stimulated whereas transcription of the paralogs RAD51C and XRCC3 is decreased (§7.4). Secondly, BCR-ABL inhibits caspase 3 activation and, thus, RAD51 degradation. Furthermore, Slupianek et al. proved that, when overstimulated by the BCR-ABL-mediated phosphorylation at Y315, RAD51 promotes pairing between nonallelic DNA sequences that share high sequence identity (divergent sequences) resulting in nonallelic HR (Slupianek et al., 2011), also called *homeologous recombination* (HomeoRR) (D. Yang & Waldman, 1997). Recombination between sequences other than those in equivalently positioned sister chromatids can lead to genome instability by promoting unequal sister chromatid exchange, which results in sequence deletions and expansions (*intrachromosomal recombinations*), and by generating translocations when nonhomologous chromosomes are involved (*interchromosomal recombinations*).

BCL-2 derives its name from B-cell lymphoma 2, as it is the second member of a range of proteins initially described in chromosomal translocations involving chromosomes 14 and 18 in follicular lymphomas (Delbridge, Grabow, Strasser, & Vaux, 2016) (Fig. 32, right). The oncogenic role of BCL-2 is generally attributed to its protective effect against apoptosis (Kale, Osterlund, & Andrews, 2018); however, Saintigny et al. also reported a novel role for BCL-2: the specific inhibition of the conservative RAD51 recombination pathway (Saintigny, Dumay, Lambert, & Lopez, 2001). Their data showed that BCL-2 overexpression inhibits UV-C-, γ-ray- or mutant p53-induced HR. Moreover, BCL-2 recombination inhibition is independent of the role of p53 in cell at G1 arrest. At an acute DSB in the recombination substrate, BCL-2 specifically inhibits RAD51-dependent gene conversion without affecting the mutagenic SSA pathway. According to these authors, BCL-2 consistently thwarts recombination stimulated by RAD51 overexpression and alters RAD51 protein by post-translation modification. The inhibition of error-free repair pathways by BCL-2 thus resultes in elevated frequencies of mutagenesis. The authors hence proposed that BCL-2 combines two separable cancer-prone phenotypes: apoptosis repression and a genetic instability/mutator phenotype.

7.3. Other RAD51 mediators and interactors

7.3.1. MATR3

Matrin3 (MATR3) is a highly conserved inner nuclear matrix protein involved in multiple stages of RNA metabolism. Although Salton at al.

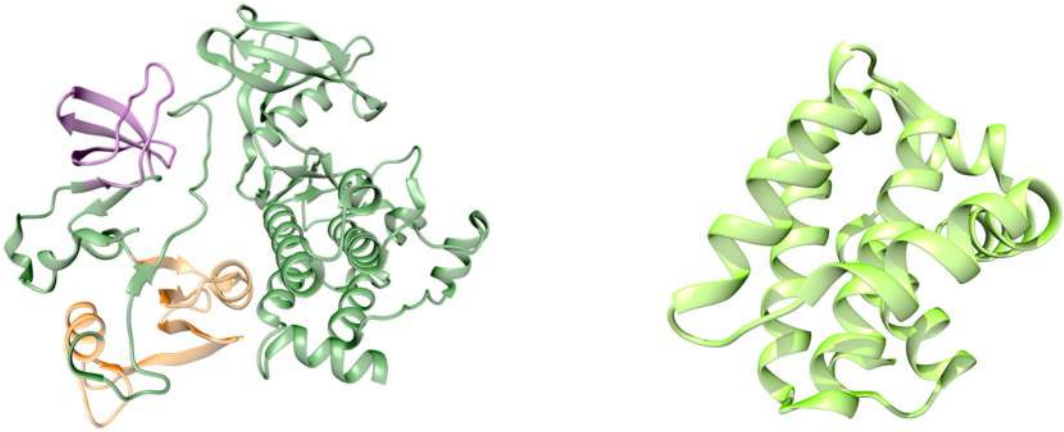

Fig. 32. (Left) Crystal structure of the common BCR-ABL and c-ABL kinase domain showing the SH2 and SH3 domains in orange and magenta, respectively (PDB: 2FO0 (Nagar et al., 2006)). (Right) Crystal structure of BCL-2 (PDB: 6O0K (Birkinshaw et al., 2019)).

found that MATR3 is phosphorylated by ATM (which activates the cellular response to DNA DSBs, §5) (Salton et al., 2011), its precise role in DDR is still unclear. However, very recently Shi et al. showed the depletion of MAT3 leads to increased cell HR efficiency and IR sensitivity by impairing the formation of RAD51 nuclear foci (Shi et al., 2019). According to the authors, these results suggest that MAT3 promotes HR by regulating RAD51.

7.3.2. RFWD3

The E3 ubiquitin-protein ligase RFWD3 (RFWD3) is a recently identified FA protein that mediates RPA ubiquitination and is required for replication fork restart, normal repair kinetics during replication stress, and HR at stalled replication forks (Elia et al., 2015). Mechanistically, RFWD3 associates to PCNA at the fork, enabling ubiquitination of RPA and its subsequent removal to facilitate DNA replication (Lin et al., 2018). Inano et al. identified RAD51 as another target of RFWD3 (Inano et al., 2017). In particular, they showed that RFWD3 polyubiquinates both RPA and RAD51 *in vitro* and *in vivo*, thereby facilitating timely removal of both these proteins from DNA damage site, a crucial step for progression to the late-phase HR.

7.3.3. FIGNL1

Yuan and Chen reported the identification of the RAD51-binding protein fidgetin-like 1 (FIGNL1), which specifically interacts with RAD51 through its conserved RAD51 binding domain (J. Yuan & Chen, 2013). Cells depleted of FIGNL1 show defective HR repair. Interestingly, FIGNL1 is recruited to sites of DNA damage in a manner that is independent of BRCA2, RAD51, and probably, RAD51 paralogs. Conversely, FIGNL1 depletion does not affect the loading of RAD51 onto ssDNA. Their additional analysis uncovered KIAA0146, also known as scaffolding protein involved in DNA repair (SPIDR), as a binding partner of FIGNL1 and established that SPIDR acts with FIGNL1 in HR repair, thereby uncovering a new protein complex in DNA repair that could provide potential directions for cancer diagnosis and therapy. Last year, Fernandes et al. identified the fidgetin-like 1 interacting protein (FLIP) as a new partner of FIGLN1, and suggested that these two proteins form a conserved complex that regulate the crucial step of strand invasion during HR (Fernandes et al., 2018).

7.3.4. BLM

Beside its roles in assisting EXO1 and DNA2 nucleases to perform the bulk of end resection required for HR in tandem with WRN, and dHJs dissolution by a mechanism involving the BLM-mediated branch migration mentioned in §5.3 (Fig. 21), in 2017 Patel and coworkers uncovered an anti-recombinase activity of BLM (Patel, Misenko, Her, & Bunting, 2017). They found that ablation of BLM rescues genomic integrity and cell survival in the presence of DNA DSBs, and this is linked to a substantial increase in the stability of the RAD51 at DSB sites and the overall HR efficiency. Ablation of BLM also rescues RAD51 foci and HR in cells lacking BRCA2 or the RAD51 paralog XRCC2 (§7.4), suggesting that the anti-recombinase activity of BLM is of general importance for normal retention of RAD51 at the DSBs and HR regulation.

7.3.5. The MMS22L-TONSL complex

The MMS22-like (MMS22L) and the Tonsoku-like (TONSL, Fig. 33, left) proteins are components of the MMS22L-TONSL complex, required to maintain genome integrity during DNA replication by promoting HR-mediated repair of replication fork-associated DSBs (Duro et al., 2010; Piwko et al., 2016). Duro et al. reported that both proteins accumulate at stressed replication forks, and depletion of MMS22L or TONSL from cells causes hypersensitivity to agents that generates S phase-associated DSBs, such as topoisomerase inhibitors; accordingly, MMS22L and TONSL are required for the HR-mediated repair of replication fork-associated DSBs (Duro et al., 2010). Further, these authors discovered that in cells depleted of either protein DSBs induced by the TOP1 inhibitor camptothecin are resected normally, but the loading of the RAD51 recombinase is defective, suggesting that MMS22L and TONSL are required for the maintenance of genome stability when unscheduled DSBs occur in the vicinity of DNA replication forks. A few years later, using *in vitro* and *in vivo* approaches, Piwko and collaborators showed that the MMS22L-TONSL heterodimer localizes to replication forks under unperturbed conditions and its recruitment is increased during replication stress in human cells (Piwko et al., 2016). MMS22L-TONSL is found to associate with RPA-coated ssDNA, and the MMS22L subunit directly interacts with RAD51. MMS22L is required for proper RAD51 assembly at DNA damage sites *in vivo*, and HR-mediated repair of stalled forks is abrogated in cells expressing a MMS22L mutant deficient in RAD51 interaction. Recombinant MMS22L-TONSL is shown to limit the assembly of RAD51 on dsDNA, which stimulates RAD51-ssDNA nucleoprotein filament formation and RAD51-dependent strand exchange activity *in vitro*. Thus, according to these authors, by specifically regulating RAD51 activity at uncoupled replication forks MMS22L-TONSL stabilizes perturbed replication forks by promoting replication fork reversal and stimulating their HR-mediated restart *in vivo*. In 2018 the group of Tyler at Cornell reported that blocking chromatin assembly via knockdown of the histone chaperones ASF1A or CAF-1 hinders RAD51 loading onto ssDNA during HR (Huang et al., 2018). They verified this is a consequence of reduced recruitment of the RAD51 MMS22L-TONSL loader to the ssDNA, resulting in persistent RPA foci, extensive DNA end resection, persistent activation of the ATR-CHK1 pathway, and cell cycle arrest. They hence proposed that the transient assembly of newly synthesized histones onto ssDNA serves to recruit the MMS22L-TONSL complex to efficiently

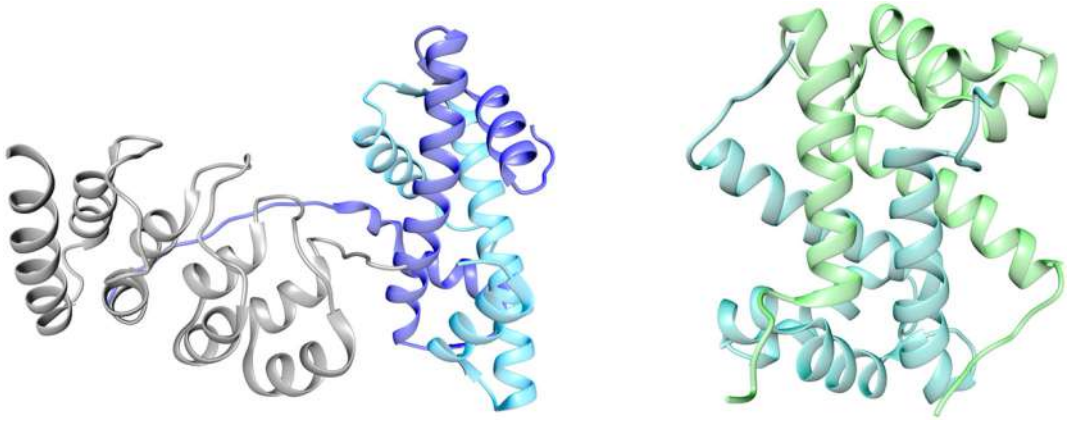

Fig. 33. (Left) Crystal structure of the human TONSL (gray) binding to a histone H3 (sky blue)-H4 (medium blue) tetramer (PDB: 5JA4 (Saredi et al., 2016)). (Right) NMR-derived structure of the SA100-A11 homodimer in solution (PDB: 2LUC (Hung, Chang, & Yu, 2012)).

form the RAD51 nucleofilament for strand invasion, supporting an active role for chromatin assembly in HR.

7.3.6. S100-A11

S100-A11 (or S100C, Fig. 33, right) is a member of the S100 protein family, composed of 21 members that exhibit a high degree of structural similarity, but are not functionally interchangeable. This family of proteins modulates cellular responses by functioning both as intracellular Ca^{2+} sensors and as extracellular factors (Donato et al., 2013). Dysregulated expression of multiple members of the S100 family is a common feature of human cancers, with each type of cancer showing a unique S100 protein profile or signature. that acts in different tumors by regulating a number of biologic functions (Bresnick, Weber, & Zimmer, 2015). Foertsch and coworkers identified an endogenous complex of RAD51 and S100-A11 which localizes at DNA repair sits both in immortalized and normal human epidermal keratinocytes synchronized in the S phase (Foertsch et al., 2016). Using biochemical assays this group verified that S100-A11 enhances RAD51 strand exchange activity; on the contrary, in cells expressing a mutant S100-A11 isoform defective for Ca^{2+} binding, prolonged persistence of the recombinase at the DNA repair sites and increased γH2AX nuclear foci are observed, suggesting incomplete repair. S100-A11 silencing via RNA interference (RNAi) produces the same effects and results in reduced sister chromatid exchange and increased chromosomal aberrations. Accordingly, these data support the involvement of S100-A11 in HR as a regulator of the presence of RAD51 at DSBs and as a contributor of the genomic stability.

7.3.7. The CST complex

Telomere replication is a multistep process that has evolved to prevent the telomere shortening that would otherwise occur because DNA polymerase is unable to replicate the DNA 5′ end (Wu, Upton, Vogan, & Collins, 2017). Shortening of telomeres has two opposing effects during cancer development: on the one hand, it can exert a tumor-suppressive effect through the proliferation arrest induced by activating the kinases ATM and ATR at unprotected chromosome ends. On the other hand, loss of telomere protection can lead to telomere crisis, which is a state of extensive genome instability that can promote cancer progression (Maciejowski & de Lange, 2017). While the ribonucleoprotein telomerase is central to this process because it elongates the G-overhang through addition of TTAGGG repeats, other players are also required, including the ssDNA-binding trimeric complex CTC1-STN1-TEN1 (CST) (Fig. 34), which participates in multiple aspects of telomere replication (Feng, Hsu, Kasbek, Chaiken, & Price, 2017).

Beside its role in telomere maintenance, the CST complex has been implicated in promoting efficient replication in difficult-to-replicate sequences in the genome (Kasbek, Wang, & Price, 2013), and deficiencies in CST components have been shown to reduce cell viability after exposure to replication fork stalling reagents, e.g., hydroxyurea and camptothecin (F. Wang, Stewart, & Price, 2014; Zhou & Chai, 2016). In this respect, Chastain et al. reported that STN1 is enriched at GC-rich repetitive sequences genome-wide as a response to replication stress induced by hydroxyurea (Chastain et al., 2016). Specifically, STN1 deficiency aggravates the fragility of these sequence under replication stress, leading to chromosome fragmentation. They also found that, upon fork stalling, the CST proteins form nuclear foci that colocalize with RAD51 and that replication stress further induces the ATR-mediated physical association of the ternary complex with the recombinase. Interestingly, CST deficiency decreases RAD51 hydroxyurea-induced foci formation and reduces RAD51 recruitment to telomeres and non-telomeric GC-rich fragile sequences. Thus, according to this study, in response to replication stress CST promotes RAD51 recruitment at fragile genome GC-repetitive sequences to facilitate replication restart, thus contributing to genome stability maintenance.

7.3.8. BCCIP

The BRCA2 and CDKN1A-interacting protein (BCCIP), expressed by the BCCIP gene in the two isoforms BCCIPα and BCCIPβ, is required during interphase for microtubule organizing and anchoring activities and, during mitosis, for the organization and stabilization of the spindle pole (Huhn et al., 2017). However, it has also been shown that both BCCIP isoforms coprecipitate and colocalize with BRCA2 and RAD51, while their RNAi-mediated silencing results in reduced BRCA2 and RAD51 foci formation, decreased HR and accumulation of spontaneous DNA damage (Lu et al., 2005; Lu, Yue, Meng, Nickoloff, & Shen, 2007). In this respect, Wray and collaborators investigated the colocalization of RAD51 with BCCIP (both isoforms) and RAD52 in human cells, and proposed that the BCCIP-dependent repair of DSBs by HR is an early RAD51 response to IR-induced DNA damage, and that RAD52-dependent HR occurs later to restart a subset of blocked or collapsed replication forks (Wray, Liu, Nickoloff, & Shen, 2008). More recently, Kelso et al. investigated the biochemical role of the BCCIP β-isoform in relation to RAD51, and demonstrated that this protein binds DNA and physically and functionally interacts with the recombinase to stimulate its homologous DNA pairing activity (Kelso et al., 2017). Remarkably, this stimulatory action is not the result of a RAD51 nucleoprotein filament stabilization but is a consequence of a BCCIPβ-induced conformational change of the RAD51 filament which, in turn, promotes ADP release to help maintaining an active presynaptic filament. So, this study revealed a functional role for BCCIPβ as a RAD51 accessory factor in HR.

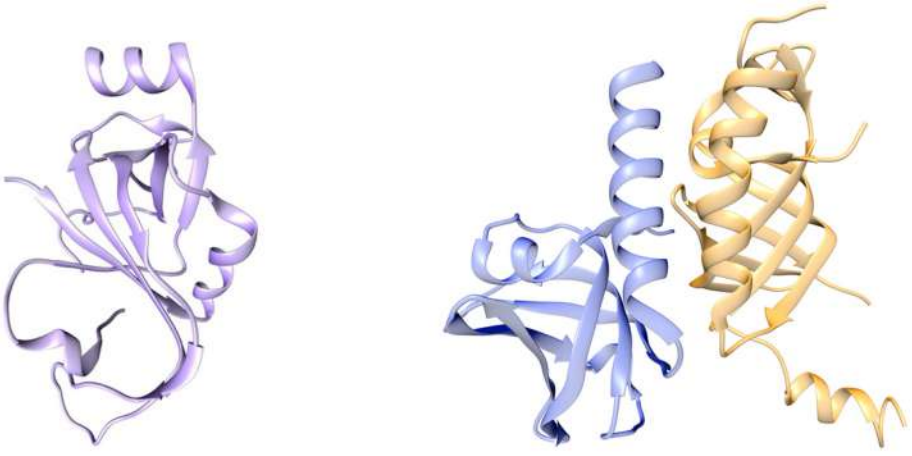

Fig. 34. Crystal structures of the central domain of CTC1 (left, PDB: 5W2L (Shastrula, Rice, Wang, Lieberman, & Skordalakes, 2018)) and the complex between STN1 (khaki) and TEN1 (medium blue) (right, PDB: 4JOI (Bryan, Rice, Harkisheimer, Schultz, & Skordalakes, 2013)), the component of the trimeric CST complex.

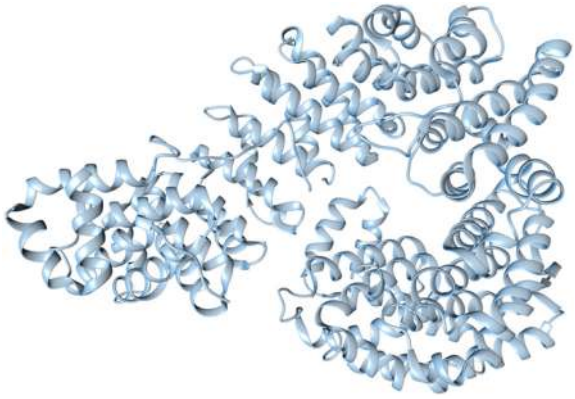

Fig. 35. Crystal structure hCAS/CSE1L (PDB: 1Z3H (Cook et al., 2005)).

7.3.9. hCAS/CSE1L

The cellular apoptosis susceptibility/chromosome segregation1-like (hCAS/CSE1L, or exportin 2, Fig. 35) was initially identified as a protein involved in the resistance of breast cancer cells to apoptosis (Tai, Hsu, Shen, Lee, & Jiang, 2010). However, this protein performs other, multiple functions, including playing a role in the regulation of p53 target genes and affecting cell cycle (Ewings & Ryan, 2007), and transporting importin α (or karyopherin α, a class of adaptor proteins that are involved in the import of proteins into the cell nucleus) from the nucleus to the cytoplasm in human cells (Cautain, Hill, de Pedro, & Link, 2015). As RAD51 concentration in the cell nucleus must increase after DNA damage induction to foster HR, Okimoto et al investigated the mechanism that regulates the RAD51 intracellular distribution and found that hCAS/CSE1L associates with the recombinase in human cells (Okimoto et al., 2015). In particular, it negatively regulates the nuclear protein levels of RAD51 under normal conditions, and is required to repress DNA damage-induced RAD51 foci formation. They also found that hCAS/CSE1L is involved in HR activity and chromosome stability, highlighting a role for this exportin in mediating HR activity by directly interacting with RAD51.

7.3.10. FBH1

The F-box DNA helicase 1 (FBH1) is a 3′-5′ DNA helicase with a putative function as a negative regulator of HR (Fugger et al., 2009). Using a combination of molecular genetics, biochemical, and single-molecule biophysical techniques, Simandlova and coworkers confirmed this FBH1 function and provided mechanistic insight into the mode of action of the FBH1 helicase as a regulator of RAD51-dependent HR in mammalian cells (Simandlova et al., 2013). This group showed that FBH1 binds directly to RAD51 and is able to disrupt RAD51 filaments on DNA through its ssDNA translocase function. In line with this, a mutant FBH1 isoform carrying a deletion in the helicase domain fails to limit RAD51 chromatin association and shows hyper-recombination in a mouse embryonic stem cell line. These data are consistent with FBH1 restraining RAD51 DNA binding under unperturbed growth conditions to prevent unwanted or unscheduled DNA recombination. FBH1 is also the only known DNA helicase to contain an F-box (i.e., a structural motif of about 50 amino acids that mediates protein-protein interactions), suggesting another function for this protein as a ubiquitin ligase as part of an SCF (S-phase kinase-associated protein 1 (SKP1)/cullin1 (CUL1)/F-box protein1) complex. Accordingly, Chu et al. reported that RAD51 is ubiquitylated by the SCF^{FBH1} complex (Chu et al., 2015). Expression of an ubiquitylation-resistant form of RAD51 in human cells leads to hyperrecombination, as well as several phenotypes indicative of an altered response to DNA replication stress. These data are consistent with FBH1 acting as a negative regulator of RAD51 function in human cells. Interestingly, Ronson et al. reported a requirement for PARP2 in stabilizing replication forks that encounter BER intermediates via the FBH1-dependent RAD51 regulation (Ronson et al., 2018). While PARP2 is dispensable for tolerance of cells to SSBs or HR dysfunctions, they showed that it is redundant with PARP1 in BER. Accordingly, the combined disruption of PARP1 and PARP2 leads to defective BER, resulting in high levels of replication-associated DNA damage owing to the inability to stabilize RAD51 at damaged replication forks and prevent uncontrolled DNA resection.

7.3.11. HELB

HELB (§5.2) is a 5'-3' helicase (Brosh Jr., 2013; van Brabant, Stan, & Ellis, 2000) implicated in chromosomal replication. Under replication stress, HELB is recruited to chromatin in a checkpoint-independent, RPA-dependent manner, and HELB silencing reduces recovery from replication stress (Guler et al., 2012). The role of HELB in DDR has been demonstrated in the work by Liu et al., where the authors reported that HELB silencing results in reduced sister chromatid exchange, impaired HR repair, and delayed RPA late-stage foci formation induced by IR (H. Liu, Yan, & Fanning, 2015). Ectopically expressed HELB colocalizes with RAD51, RAD52, and RPA. Moreover, HELB stimulates RAD51-mediated heteroduplex extension *in vitro* while a helicase-defective mutant fails to do so, thereby establishing a role for HELB in assisting HR.

7.3.12. The S5S1 complex

The fission yeast and the mammalian homologues proteins DNA repair protein SWI5 homolog (SWI5) and SWI5-dependent recombination DNA repair protein 1 homolog (SFR1) are implicated in RAD51-dependent HR and recombination repair of DNA damage (Akamatsu, Dziadkowiec, Ikeguchi, Shinagawa, & Iwasaki, 2003; Akamatsu & Jasin, 2010; Yuan & Chen, 2011). Both the fission yeast and mouse SWI5 and SFR1 proteins form the heterodimeric complex SWI5/SFR1 (S5S1) (Fig. 36, left) that physically interacts with RAD51 and enhances RAD51-mediated DNA strand exchange (Haruta et al., 2006; Kuwabara et al., 2012; Tsai et al., 2012). Working with highly purified mouse proteins, Su et al. showed that the enhancement of RAD51 activity stems from a dual action of S5S1, namely, by stabilizing the presynaptic filament and enhancing the release of ADP from the filament to

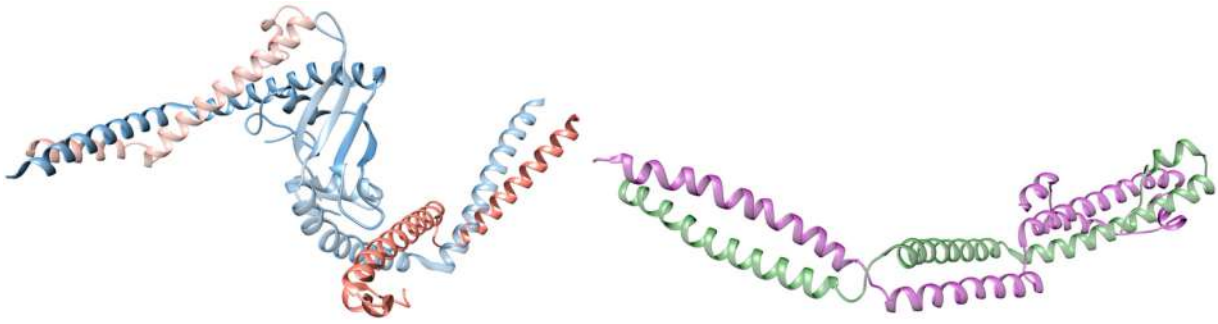

Fig. 36. (Left) Crystal structure of the S5S1 heterodimeric complex. Two SWI5 chains are portrayed as ribbons colored in salmon shades, whereas two SFR1 chains as colored in shades of cornflower blue (PDB: 3VIQ (Kuwabara et al., 2012)). (Right) Crystal structure of the HOP2 (magenta)-MND1 (sea green) heterodimer (PDB: 4Y66 (Kang et al., 2015)).

maintain the presynaptic filament in its active, ATP-bound form (Su et al., 2014; Tsai et al., 2012). In another study, the same group further provided evidence that S5S1 interacts with the oligomeric, but not the monomeric, form of RAD51 (Su et al., 2016). Using a mutant variant of SWI5 that is proficient in complex formation with SFR1 but is strongly affected for the ability to interact with RAD51, they further demonstrated that it is the C-terminal region of SWI5 that makes a major contribution toward complex formation between S5S1 and RAD51. Also, the mutant S5S1 complex is devoid of any ability to stabilize the RAD51 presynaptic filament, to enhance ATP hydrolysis by RAD51, or to stimulate RAD51-mediated DNA strand exchange. Very recently, the same researchers reported that the mammalian S5S1 efficiently stimulated RAD51 nucleus formation and inhibits RAD51 dissociation from filaments, supporting the conserved function of S5S1 by primarily stabilizing the recombinase on DNA, allowing both the formation of the stable nucleus and the maintenance of filament length (Lu et al., 2018).

7.3.13. The HOP2-MND1 complex

As discussed in §7.1, the HOP2-MND1 heterodimeric complex (Fig. 36, right) is required for normal progression of HR, as it stimulates the DNA exchange activity of RAD51. Bugreev et al reported that HOP2-MND1 induces changes in the conformation of RAD51 that profoundly alter the basic properties of the recombinase (Bugreev et al., 2014). In particular, HOP2-MND1 enhances the RAD51 interaction with nucleotide cofactors and modifies its DNA-binding specificity in a manner that stimulates DNA strand exchange. Moreover, its enables RAD51 to perform DNA strand exchange in the absence of divalent cations required for ATP binding and offsets the effect of the ATP-binding-defective K133A RAD51 mutation (§8.2.8). During RAD51 nucleoprotein filament formation, HOP2-MND1 helps recombinase loading onto ssDNA restricting its dsDNA-binding, while during homologous search it promotes dsDNA binding by removing the inhibitory effect of ssDNA. According to these roles, the authors defined HOP2-MND1 as a "molecular trigger" of RAD51 DNA strand exchange.

7.3.14. AGO2

Argonaute proteins are highly specialized binding modules that accommodate small RNA components - such as miRNAs and small interfering RNAs (siRNAs) - and coordinate downstream gene-silencing events by interacting with other protein factors (Hutvagner & Simard, 2008; Meister, 2013). The human protein argonaute-2 (AGO2, Fig. 37, left) is specifically required for RNAi by the RNA-induced silencing complex (RISC). A 'minimal RISC' appears to include AGO2 bound to a short miRNA or siRNA, which direct RISC to complementary mRNAs that are targets for RISC-mediated gene silencing (Janowski et al., 2006). In 2012 Wei et al. reported that a class of DSB-induced siRNAs (diRNAs), produced from sequences in the vicinity of DSB sites, are associated with AGO proteins and are required for DNA repair (Wei et al., 2012). In a successive effort, the same group demonstrated that the role of diRNAs in DSB repair is restricted to HR repair pathway and that, in mammals, it specifically relies on the effector protein AGO2 (Wei et al., 2014). They also showed that AGO2 forms a complex with RAD51, and that this two-protein interaction is enhanced in IR-treated cells. RAD51 accumulation at the DSBs and HR repair depend on catalytic activity and small RNA-binding ability of AGO2. On the contrary, DSB resection and RPA and MRE11 loading is unaffected by AGO2 depletion, suggesting that AGO2 likely functions directly in mediating RAD51 accumulation at DSBs. The authors thus proposed that, guided by diRNAs, AGO2 can promote RAD51 recruitment and/or retention at DSBs to facilitate HR repair.

7.3.15. The SUMO family

Small ubiquitin-like modifier (SUMO) proteins are a family of small proteins that are covalently attached to and detached from other proteins by a pathway involving the SUMO-activating enzyme subunit 1, the SUMO-conjugating enzyme UBC9, and an E3 ligase such as E3 SUMO-protein ligase PIAS1 or PIAS4 (Hendriks & Vertegaal, 2016). SUMOylation is a post-translational modification involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle (Seeler & Dejean, 2017). Mammalian cells possess three SUMO isoforms (SUMO-1/2/3, Fig. 37, right), and several reports suggest the involvement of the SUMO modification system in DNA repair by interacting with RAD51 (Garvin & Morris, 2017; Jackson & Durocher, 2013). In their studies of the regulation of RAD51 dynamics in response to DNA damage, Shima et al. examined the involvement of the SUMOylation system in the recombinase accumulation at the DNA lesions induced by irradiation (Shima et al., 2013). They showed that this process is regulated by UBC9, PIAS1 and PIAS4, and further identified a RAD51 SUMO-interacting domain (SIM) required for the DNA-damage-induced accumulation of RAD51. Accordingly, these authors proposed that the DNA-damage-dependent activation of the SUMOylation system can function in the regulation of the RAD51 localization dynamics through the SUMO-SIM interaction.

7.3.16. The MCM8-MCM9 complex

In 2013 Park and coworkers showed that the minichromosome maintenance protein homologs MCM8 and MCM9 (Fig. 38) form a complex involved in the repair of DNA ICLs by HR (J. Park et al., 2013). This group demonstrated that the depletion of either protein in human cancer cells or a loss of function MCM9 mutation in mouse embryo fibroblasts sensitizes cells to cisplatin, the prototypical DNA ICL-inducing agent. Consistent with a role in repair of ICLs and DSBs by HR, the knockdown of MCM8 or MCM9 significantly reduces the efficacy of the HR

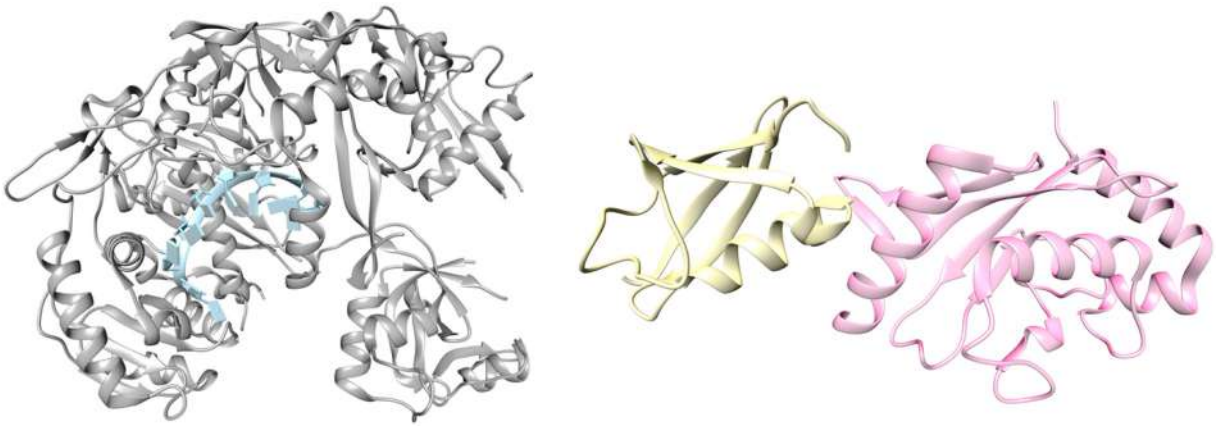

Fig. 37. (Left) Crystal structure of AGO2 (gray) in complex with a ssDNA (light blue) (PDB: 5JS1 (Schirle et al., 2016)). (Right) Crystal structure of SUMO1 (light khaki) in complex with UBC9 (pink) (PDB: 2VRR (Knipscheer et al., 2008)).

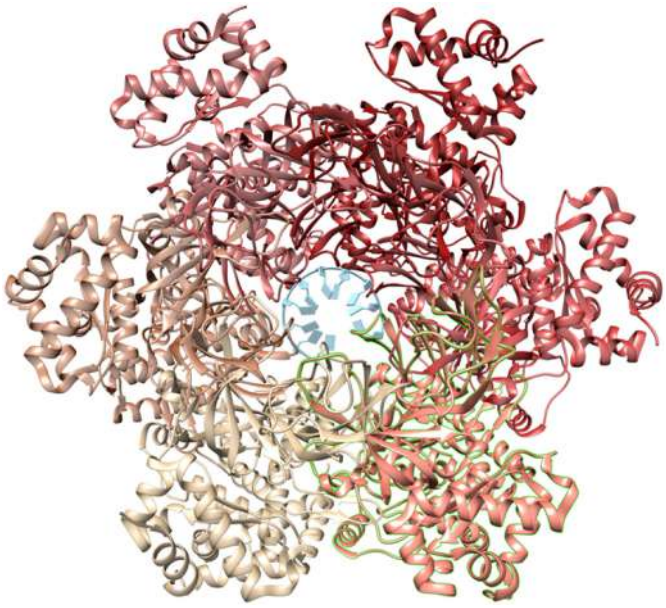

Fig. 38. Crystal structure of an MCM hexamer bound to single-stranded DNA. The different MCM monomers are colored in brown shades, while the nucleic acid is in light blue. (PDB: 6MII (Meagher, Epling, & Enemark, 2019)).

repair. This group also proved that this complex is readily engaged at DNA damage sites and promote RAD51 recruitment. A few years later Lee et al. reported that MCM8-MCM9 is required for DNA resection by the MRN complex (§5) at DSBs to generate ssDNA (Lee et al., 2015). MCM8-MCM9 interacts with MRN and is required for the nuclease activity and stable association of MRN with DSBs, the ATPase motifs of MCM8-MCM9 being indispensable for the recruitment of MRE11 to foci of DNA damage. The authors further showed that a cancer-derived point mutation on MCM8 associated with premature ovarian failure (POF) diminishes the functional activity of MCM8. Therefore, the MCM8-MCM9 complex facilitates DNA resection by the MRN complex during HR repair, genetic or epigenetic inactivation of MCM8 or MCM9 are seen in human cancers, and genetic inactivation of MCM8 may be the basis of the POF syndrome.

7.3.17. CBP and p300 HATs

The histone acetyltransferases (HATs) CBP and p300 (Fig. 39) work as transcriptional activators by producing 'relaxed' chromatin accessible to transcription factors via the acetylation of histones H3 and H4 at gene promoter regions (Roth, Denu, & Allis, 2001). However, the contribution of CBP and p300 to the transcriptional activity of genes involved in DSB repair was not investigated until 2012, when Ogiwara and Kohno demonstrated that these two HATS activate the transcription of the BRCA1 and RAD51 genes in HR (Ogiwara & Kohno, 2012). They showed that the siRNA-mediated depletion of CBP and p300 impairs HR activity and downregulates BRCA1 and RAD51 both at the mRNA and protein levels. Binding of the two HATs to the promoter regions of the BRCA1 and RAD51 genes, and the depletion of CBP and/or p300 reduces H3 and H4 acetylation, inhibits binding of the transcription factor E2F1 to these promoters, and impairs DNA-damage induced phosphorylation and chromatin binding of RPA following BRCA1-mediated DNA end resection. In line with this, subsequent phosphorylation of CHK1 and activation of the G2/M damage checkpoint is also negatively affected.

The E1A-binding protein p400 (p400) is another HAT promoting chromatin remodeling via acetylation of nucleosomal histones H4 and H2A (Fuchs et al., 2001). Courilleau et al. showed that, although this enzyme is not required for DNA damage signaling, DNA DSB repair is defective in the absence of p400 (Courilleau et al., 2012). This group demonstrated that p400 is indeed important for HR-dependent processes, such as the recruitment of RAD51 to DSBs, HR-directed repair, and survival after DNA damage. Remarkably, in this study p400 and RAD51 are both found at DSBs where they favor chromatin remodeling, thereby providing a direct molecular link between RAD51 and a chromatin remodeling enzyme involved in chromatin decompaction around DNA DSBs. All together, these data clearly underlie the multiple roles played by HATs in the activation and function of the DDR in mending these deadly DNA lesions, and highlight the relevant, already broadening landscape for the development of epigenetic modulators as potential cancer therapeutics (Mohammad, Barbash, & Creasy, 2019).

7.3.18. TRF1 and TRF2

Mammalian telomeres consist of 5 to 15 kilobase pairs of TTAGGG repeats that terminate in a 50-500 nucleotide ssDNA 3'-tail. The telomere repeats and the ss-dsDNA junction provide a binding site for shelterin, a six-subunit protein complex (comprising the telomeric repeat-binding factors 1 and 2 (TRF1 and TRF2), the protection of telomeres protein 1 (POT1), the adrenocortical dysplasia protein homolog (TPP1), the TRF1-interacting nuclear protein 2 (TIN2) and the telomeric repeat-binding factor 2-interacting protein 1 (RAP1)) that associates specifically with mammalian telomeres and allows cells to distinguish the natural ends of chromosomes from sites of DNA damage (de Lange, 2018). Literature evidence suggests that the HR pathways cooperates with the components of the sheltering complex - in particular with TRF1 and TRF2, which bind telomeric DNA as homodimers (Fig. 40) to promote both telomere maintenance and nontelomeric HR. This may be due to the ability of both HR and shelterin proteins to

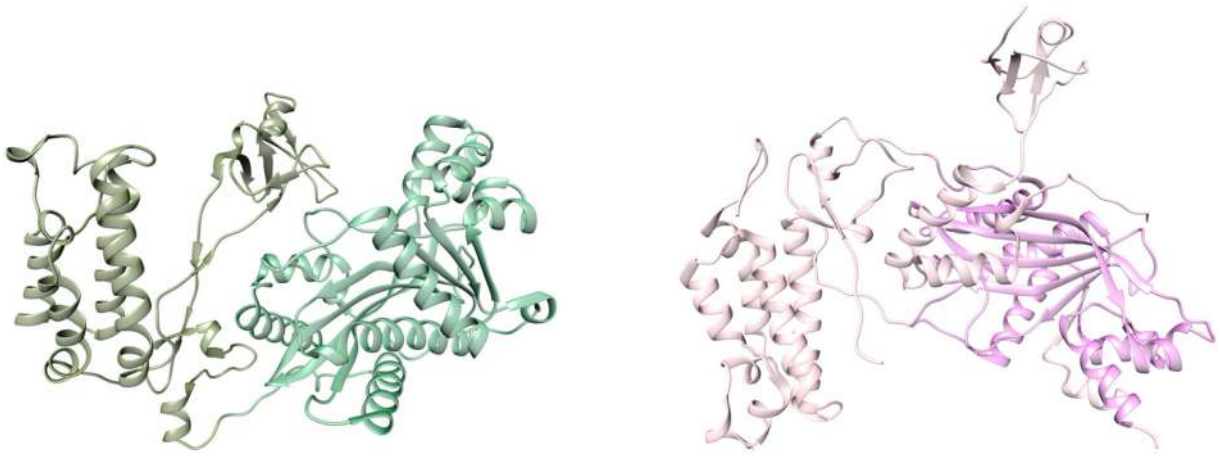

Fig. 39. (Left) Crystal structure of the p300 core protein. The HAT domain is highlighted in sea green (PDB: 4BHW (Delvecchio, Gaucher, Aguilar-Gurrieri, Ortega, & Panne, 2013)). (Right) Crystal structure of the catalytic core of CBP. The HAT domain is highlighted in orchid (PDB: 5U7G (Park et al., 2017)).

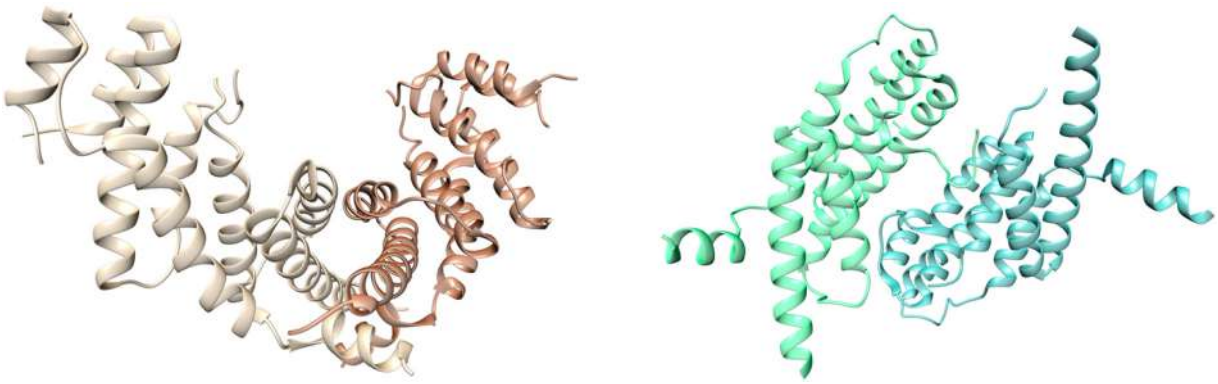

Fig. 40. Crystal structures of the dimerization domains of TFR1 (left, PDB: 1H6O) and TFR2 (right, PDB: 1H6P) (Fairall, Chapman, Moss, de Lange, & Rhodes, 2001).

promote strand invasion, wherein a ssDNA substrate base pair with a homologous dsDNA template displacing a D-loop.

In analogy with the D-loop formation catalyzed by RAD51 during HR (§5.3), TRF2 catalyzes the formation of a telomeric D-loop that stabilizes a looped structure in telomeric DNA, called t-loop, which contributes further telomer protection. In this context, Bower and Griffith reported that preincubation of a telomeric template with TRF2 inhibits the ability to promote telomeric D-loop formation, suggesting that i) RAD51 does not promote t-loop formation and ii) a mechanism in which TRF2 can inhibit HR at telomeres (Bower & Griffith, 2014). The same authors reported that a TRF2 mutant lacking the dsDNA binding domain promotes RAD51-mediated nontelomeric D-loop formation, yielding an explanation on how TRF2 promotes nontelomeric HR. Finally, TRF1 was shown to promote RAD51-mediated telomeric D-loop formation - and hence facilitate HR-mediated replication fork restart - supporting the notion that TRF1 is required for efficient telomere replication.

7.3.19. The SMN-GEMIN2 complex

The survival motor neuron (SMN) complex is essential for the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) and likely functions in the assembly, metabolism, and transport of a diverse number of other ribonucleoproteins (Battle et al., 2006). Specifically, the SMN complex assembles 7 small nuclear proteins - known as Smith or Sm proteins - into a core structure around a highly conserved sequence of RNA found in small nuclear RNAs (snRNAs) (Will & Luhrmann, 2011). Besides Sm proteins, the SMN complex contains 8 additional proteins known as GEMIN2/8 and the Unr-interacting protein (UNRIP), each playing a role in ribonucleoprotein biogenesis (Cauchi, 2010). In 2010, Takizawa et al. initially identified GEMIN2 as a novel RAD51 interacting protein (Takizawa et al., 2010). They found that purified GEMIN2 enhances the RAD51-DNA complex formation by inhibiting RAD51 dissociation from DNA, and thereby stimulates RAD51-mediated homologous pairing. GEMIN2 also enhances the RAD51-mediated strand exchange when RPA is pre-bound to ssDNA before the addition of RAD51. In line, loss of GEMIN2 reduces HR efficiency and results in a significant decrease in the number of RAD51 subnuclear foci, supporting a role for GEMIN2 in regulating HR as a novel RAD51 mediator. One year later the same group successfully purified the SMN-GEMIN2 complex as a fusion protein and verified that it enhances the RAD51-mediated homologous pairing much more efficiently than GEMIN2 alone (Takaku et al., 2011). According to their data, SMN-GEMIN2 possesses DNA-binding activity (not observed for GEMIN2), and significantly stimulates the secondary duplex DNA capture by the RAD51-single stranded DNA complex during homologous pairing. These results provide the first evidence that the SMN-GEMIN2 complex plays a role in HR, in addition to spliceosomal snRNP assembly.

7.3.20. RUVBL1

The RuvB-like 1 protein (RUVBL1, aka Pontin, Pontin52, TP49 or NMP238), is expressed virtually ubiquitously, is evolutionarily conserved, and localizes to the cell nucleus and the cytoplasm. It is an ATPase that is part of the AAA+ superfamily (i.e., ATPases associated with diverse cellular activities), which encompasses a large group of ring-shaped complexes (Fig. 41, left) involved in diverse cellular processes, including gene transcription regulation (although RUVBL1 is not a transcription factor), chromatin remodeling, sensing of DNA damage and repair, and the assembly of protein and ribonucleoprotein complexes (Mao & Houry, 2017). In the field of DDR, Gospodinov and coworkers studied the role of RUVBL1 in the recruitment of RAD51 to DNA damage sites (Gospodinov, Tsaneva, & Anachkova, 2009). To the purpose, they followed RAD51 redistribution to chromatin and recombinase nuclear foci formation induced by DNA DSBs and ICLs under conditions of RUVBL1 depletion via RNAi. According to their data, RUVBL1 silencing reduces both RAD51 recruitment to chromatin and nuclear foci formation to about 50% with respect to control, but this is not the result of defective DNA damage checkpoint signaling, as judged by the normal H2AX phosphorylation and cell cycle distribution. These results led the authors to suggest that RUVBL1 may have a role in facilitating the access of of the repair machinery to the site of DNA damage and in modulating RAD51 foci formation in response to DSBs.

7.3.21. CTCF

The CCCTC-binding factor (CTCF) is a highly conserved zinc finger protein and is best known as a transcription factor (Fig. 41, right). It can function as a transcriptional activator, a repressor or an insulator protein, blocking the communication between enhancers and promoters. CTCF can also recruit other transcription factors while bound to chromatin domain boundaries (Kim, Yu, & Kaang, 2015). The role of CTCF in genome stability has been explored by Lang et al., who showed that this protein is recruited to DNA damage sites, and promotes HR of DSBs (Lang et al., 2017). CTCF depletion increases chromosomal instability, marked by chromosomal breakage and end fusion, elevated genotoxic stress-induced genomic DNA fragmentation, and activates ATM. Knockdown of CTCF impairs HR by reducing IR-induced RAD51 foci, as well as the recruitment of the recombinase to laser-irradiated sites of DNA lesions. Moreover, CTCF is associated with MDC1 (§5) and AGO2 (§7.3.14), and directly interacts with RAD51 via its C-terminus, thereby establishing a direct, functional role in DNA repair.

7.3.22. c-MET

The hepatocyte growth factor receptor (HGFR, aka c-MET) is a tyrosine kinase receptor often overexpressed or constitutively activated in many cancer types, and its inhibition induces the decrease of HR (Zhang et al., 2018). This year Chabot et al. demonstrated *in vitro* that c-MET is able to phosphorylate RAD51 at four tyrosine residues mainly

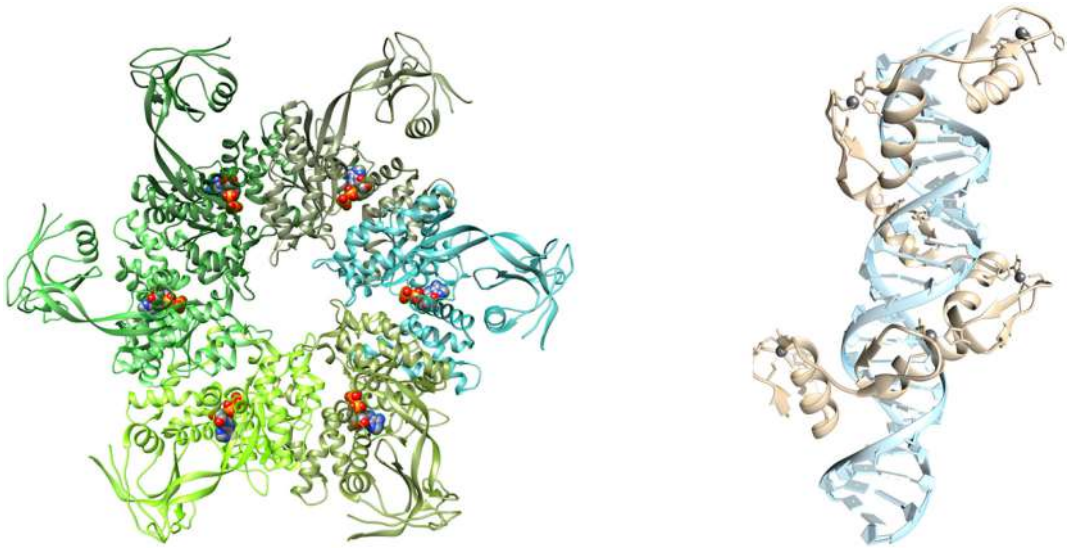

Fig. 41. (Left) Crystal structure of the hexameric ring formed by the RUVBL1 ATPase bound to ADP. Each protein monomer is colored in a shade of green, while the 6 ADP molecules are shown as atom-colored spheres (C, gray; O, red; N, blue. P, orange. H atoms are omitted for clarity) (PDB: 2C9O (Matias, Gorynia, Donner, & Carrondo, 2006)). (Right) Crystal structure of 4 out of 11 zinc finger domains of human CTCF (light tan) bound to DNA (light blue) (PDB: 5T0U (Hashimoto et al., 2017)).

located in the subunit-subunit interface of the recombinase (Chabot et al., 2019). While these post-translational modifications of RAD51 do not affect the presynaptic filament formation, they strengthen its stability against the inhibitory effect of a BRC peptide obtained from BRCA2, supporting the role of these modification in the regulation of the BRCA2/RAD51 interaction and the importance of c-MET in DDR.

7.3.23. SYCP3

The synaptonemal complex protein 3 (SYCP3) is a component of the synaptonemal complexes formed between homologous chromosomes during meiotic prophase, and has been shown to function in meiotic HR biased to interhomologous chromosomes by regulating the strand invasion activity of RAD51 (Cahoon & Hawley, 2016). In a recent effort, Kobayashi et al. reported that SYCP3 significantly suppresses the RAD51-mediated strand invasion reaction by competing with HOP2-MND1 (§7.3.12) (W. Kobayashi, Hosoya, Machida, Miyagawa, & Kurumizaka, 2017). On the other hand, strand invasion mediated by the meiotic recombination protein DMC1/LIM15 homolog (DMC1, a recombinase that participates in meiotic recombination, specifically in homologous strand assimilation, which is required for the resolution of meiotic double-strand breaks (Neale & Keeney, 2006)), is not affected by SYCP3. Since a SYCP3 mutant defective in RAD51 binding is not able to inhibit RAD51-mediated HR in human cells, the authors suggested that SYCP3 may promote the DMC1-driven HR by attenuating RAD51 activity during meiosis.

7.3.24. The I-D complex

The complex formed by the Fanconi anemia group I protein (FANCI) and FANCD2 (FANCI-FANCD2 or I-D, Fig. 42, left) is central to the DNA ICL repair pathway (§6), and localizes to ICLs dependent on its monoubiquitination (C. C. Liang et al., 2016). Sato and coworkers showed that the I-D directly binds to RAD51, and stabilizes the RAD51-DNA filament (Sato et al., 2016). Interestingly, the DNA binding activity of FANCI, but not that of FANCD2, is required for the I-D complex mediated RAD51-DNA filament stabilization. The I-D-stabilized RAD51 filament protects the DNA from nucleolytic degradation by the Fanconi-associated nuclease 1 (FAN1), while this protective action is not observed in the presence of the FA-associated RAD51 T131P mutant (§8.2.8). In all, these results establish the collaborative action of the recombinase and the I-D complex in preventing genomic instability at the replication forks.

7.3.25. The UAF1-USP1 complex

The ubiquitin carboxyl-terminal hydrolase 1 (USP1) is a negative regulator of DNA damage repair which, in association with its USP1-associated factor 1 (UAF1), specifically deubiquitinate the two critical DNA repair proteins FANCD2 and PCNA (Cohn et al., 2007). Murai and

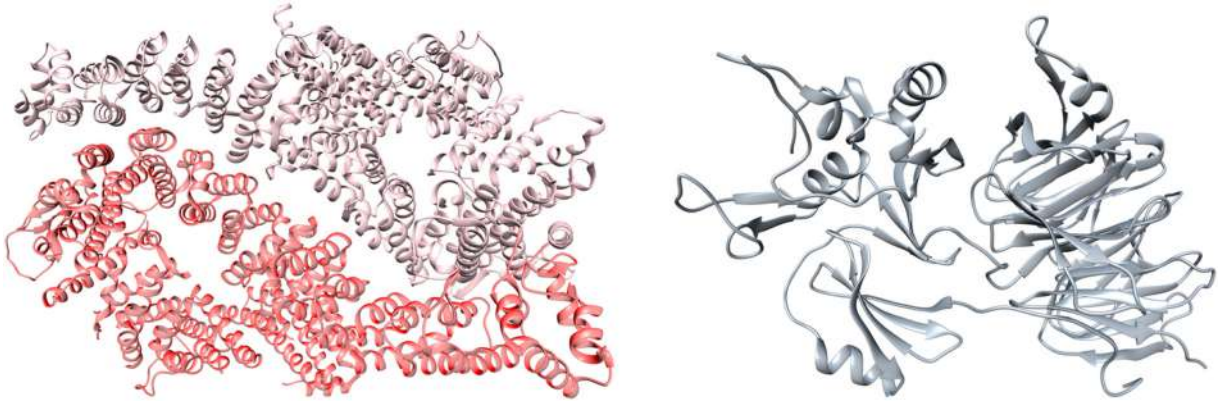

Fig. 42. (Right) Crystal structure of the I-D complex showing FANCI and FANCD2 in pink and red ribbons, respectively (PDB: 3S4W (Joo et al., 2011)). (Right) Crystal structure of UAF1 (PDB: 5L8E (Dharadhar, Clerici, van Dijk, Fish, & Sixma, 2016)).

coworkers showed that the USP1/UAF1 complex is a regulator of the cellular response to DNA damage by promoting HR-mediated DSB repair (Murai et al., 2011). With the purpose of gaining insight into the mechanism of the USP1/UAF1 complex in HR, Liang et al. demonstrated that UAF1 (Fig. 42, right) binds to DNA and forms a dimeric complex with RAD51AP1 (§7.2.9), and a trimeric complex with RAD51 via the recombinase accessory factor (F. Liang, et al., 2016). The formation of these complexes is mediated by two small ubiquitin-like SUMO-like domains in UAF1 and a SUMO-interacting motif in RAD51AP1. Importantly, UAF1 enhances RAD51-mediated homologous DNA pairing in a manner that is dependent on complex formation with RAD51AP1 but independent of USP1. From a mechanistic viewpoint, RAD51AP1-UAF1 cooperates with RAD51 to assemble the synaptic complex, and further cellular experiments reported in this study highlight the biological significance of the RAD51AP1-UAF1 protein complex in HR.

7.3.26. WRNIP1

The WRN interacting protein 1 (WRNIP1) is another AAA+ ATPase, and has been identified as a binding partner of the WRN DNA helicase that plays a crucial role in response to replication stress, significantly contributing to the recovery of stalled replication fork (§5.3). Leuzzi and coworkers recently established that WRNIP1 localizes at stalled replication forks and cooperates with RAD51 to safeguard fork integrity (Leuzzi, Marabitti, Pichierri, & Franchitto, 2016). In particular, these authors showed that WRNIP1 is directly involved in preventing uncontrolled MRE11-mediated degradation of stalled replication forks by promoting RAD51 stabilization of ssDNA, while loss of this ATPase or of its catalytic activity causes extensive DNA damage and chromosomal aberration. Notably, loss of WRNIP1 activity can be compensated by downregulation of the anti-recombinase FBH1 (§7.3.10), as this attenuates replication fork degradation and genomic instability in WRNIP1-deficient cells.

7.3.27. TOPBP1

The mechanistic role of TOPBP1 (§5) in HR has been uncovered by Moudry et al., who reported that this protein depletion abrogates RAD51 loading to chromatin and formation of recombinase foci yet without affecting the upstream HR steps of DNA end resection and RPA loading (Moudry et al., 2016). In detail, TOPBP1 binds to the serine/threonine-protein kinase PLK1 which, in turn, phosphorylates the recombinase at a specific residue (S14), a modification required for RAD51 recruitment to chromatin. In the authors' view, this role of TOPBP1 in HR offers new potential clinical applications in cancer therapy.

7.3.28. TEAD4

The term 'super-enhancer' is used to designate a class of regulatory regions with unusually strong enrichment for the binding of transcriptional coactivators, specifically the mediator of RNA polymerase II transcription subunit 1 (MED1) (Pott & Lieb, 2015). Very recently, Hazan et al. mapped DSBs at high resolution in cancer and non-tumorigenic cells and found a transcription-coupled repair mechanism at oncogenic super-enhancers (Hazan, Monin, Bouwman, Crosetto, & Aqeilan, 2019). At these super-enhancers the transcription factor TEAD4, together with various transcription factors and co-factors, co-localizes with RAD51. Depletion of TEAD4 or RAD51 increases DSBs at RAD51/TEAD4 common binding sites within super-enhancers and decreases expression of related genes, which are mostly oncogenes. Co-localization of RAD51 with transcription factors at super-enhancers occurs in various cell types, suggesting a broad phenomenon. Together, these findings uncover a coupling between transcription and repair mechanisms at oncogenic super-enhancers, to control the hyper-transcription of multiple cancer drivers.

7.4. The RAD51 paralogs

In addition to the RAD51 mediators described above, other recombinase regulators include 5 canonical human paralogs - RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3 (Garcin et al., 2019; J. L. Harris, Rabellino, & Khanna, 2018; Sullivan & Bernstein, 2018; Suwaki, Klare, & Tarsounas, 2011). Although these RAD51 paralogs share only 20-30% amino acid sequence similarity with RAD51), they structurally resemble the recombinase itself, each paralog featuring, among other similarities, both the conserved Walker motifs (§7.1 and §8.2.8) and the BRC repeats (§7.2.2) (Miller, Sawicka, Barsky, & Albala, 2004). However, unlike RAD51, these paralogs are found to exist in cells as two distinct complexes: the heterotetramer BCDX2 (RAD51B/C/D/XRCC2) and the heterodimer CX3 (RAD51C/XRCC) (Masson et al., 2001).

As recently reviewed in details by Sullivan and Bernstein, all these 5 RAD51 paralogs are required for proper RAD51 assembly *in vivo* and are thought to play a role in many facets of RAD51 regulation, including the stabilization, elongation, and remodeling of the nucleoprotein filament; however, the specific function and mechanism each paralog plays in RAD51 assembly remains poorly defined (Sullivan & Bernstein, 2018). Furthermore, in full analogy with the recombinase, RAD51 paralog knockout mice exhibit embryonic lethality (Thacker, 2005), making the *in vivo* assessment of paralog functions rather complicated, as again well reviewed in (Sullivan & Bernstein, 2018). Yet, cells in which the RAD51 paralogs have been knocked down are more sensitive to DNA damaging agents, and display increased chromosome aberration, abnormal centrosome numbers, reduced frequencies of HR-mediated gene targeting and DSB repair, and reduced chromatid exchange, all markers of genomic instability generated by compromised HR repair (Suwaki et al., 2011).

The current view of the role played by the paralog complex CX3 in the early stages of HR is to facilitate the initiation of the recombinase filament assembly on ssDNA overhangs. This is consistent with the evidence that CX3 is characterized by an ATP-independent DNA binding affinity for ssDNA, binds other DNA substrates with reduced affinity, and presents the lowest affinity for dsDNA (Kurumizaka et al., 2001; Masson, Stasiak, Stasiak, Benson, & West, 2001). Since, according to the same studies, CX3 also promotes DNA aggregation, it might also be an actor in annealing complementary DNA during the homology search of RAD51 filaments. In analogy, proofs for the involvement of BCDX2 in RAD51 filament assembly have been directly obtained by EM imaging, which show that this complex binds to both ssDNA and to gaps and nicks in duplexed DNA (Masson, Tarsounas, et al., 2001). The same investigation also verified that BCDX2 also possesses a weak ATPase activity in the presence of ssDNA, but not when in contact with dsDNA or with 5' or 3' tailed DNA. The presynaptic role of the RAD51 paralogs is further supported by research performed on yeasts; in particular, RAD51 paralog-containing complexes in these eukaryotic microorganisms such as RAD55-RAD57 or the Shu complex (a heterotetramer consisting in two yeast RAD51 paralogs, the platinum sensitivity protein 3 (PSY3) and the chromosome segregation in meiosis protein 2 (CSM2), along with the suppressor of HU sensitivity involved in recombination protein 1 and 2 (SHU1/2), (Martino & Bernstein, 2016)) have been shown to promote RAD51 presynaptic filament assembly *in vitro* (Gaines et al., 2015; Godin et al., 2016; Sung, 1997). From a mechanistic standpoint, how the RAD51 paralogs aid in recombinase nucleoprotein filament assembly is still unknown. The actual hypothesis is that they may either intercalate into the filament, or form a co-filament that enables RAD51 elongation after the BRCA2-mediated nucleation (Kurumizaka et al., 2001; Kurumizaka et al., 2002) (§7.1). According to alternative perspectives, these CX3 and BCDX2 could either cap the DNA ends to prevent disassembly of the recombinase filament - in analogy to what observed in yeasts (Sung, 1997) - or they might promote HR depending on the specific nature of the DNA damage (Godin, Zhang, et al., 2016; Martino & Bernstein, 2016). In this respect, again in yeasts it has been proved that Shu

specifically recognize abasic sites at replication intermediates, where it recruits the HR machinery to mediate strand specific damage tolerance (Rosenbaum et al., 2019).

The role of CX3 and BCDX2 in the HR postsynaptic phase is more controversial. For instance, CX3 has been reported both to play and not to play a role in assisting RAD51-mediate D-loop formation (Kurumizaka et al., 2001; Masson, Stasiak, et al., 2001). On the other hand, the DX2 and BC heterodimeric components of BCDX2 were shown to be able to catalyze homologous pairing and enable D-loop formation (Kurumizaka et al., 2002), and to enhance RAD51-mediated strand exchange in the presence of RPA (Sigurdsson et al., 2001), respectively. According to Sullivan and Bernstein, a possible participation of RAD51 paralogs in this phase may involve their incorporation into the recombinase filament with a consequent change in conformation of the latter, to enable increased flexibility required for strand exchange, or to promote filament disassembly to allow the subsequent steps of HR to proceed (Sullivan & Bernstein, 2018).

Investigations of the roles played by the 5 canonical RAD51 paralogs in replication fork protection and restart have started only recently. Accordingly, Somyajit et al. reported that loss of three of these proteins (i.e., RAD51C, XRCC2, and XRCC3) in different cell knockdowns results in increased MRE11-mediated degradation of nascently replicated DNA, thus implicating both CX3 and BCDX2 in replication fork protection (Somyajit, Saxena, Babu, Mishra, & Nagaraju, 2015).

Very recently, Matsuzaki et al, described the role for another RAD51 paralog, SWSAP1, as regulator of the recombinase assembly (Matsuzaki, Kondo, Ishikawa, & Shinohara, 2019). They showed that SWSAP1 interacts with RAD51 through its conserved Phe-XX-Ala BRC-like motif, and its interaction is required for DNA-damage induced RAD51 foci formation, since SWSAP1-depleted cells exhibited defects in DNA damage-induced RAD51 assembly both during mitosis and meiosis. This study also reported that SWSAP1 interacts with the AAA+ ATPase FIGNL1 (§7.3.3); specifically, FIGNL1 depletion suppressed RAD51-focus formation defects in SWSAP1-deficient cells, indicating that the ATPase facilitates RAD51 disassembly in the absence of SWSAP1. Purified FIGNL1 promotes the dissociation of RAD51 from ssDNA in an ATPase activity-independent manner, while SWSAP1 antagonizes the RAD51 filament dismantling activity of FIGNL1 *in vitro*. These results support the idea that the recombinase paralog SWSAP1 protects RAD51 filaments by antagonizing the anti-recombinase FIGNL1.

From these few lines briefly summarizing some of the main features and roles of the mammalian RAD51 paralogs, it is clear that these proteins constitute a thrilling and challenging field of research *per se*. As such, they warrant a wealth of future *in vitro* and *in vivo* studies in order to achieve a clearer picture of their involvement in DNA damage and repair, including their individual purification and/or crystal structure determination.

8. RAD51 and cancer drug resistance

8.1. RAD51 overexpression in cancer

For many years it has been observed that the levels of the RAD51 protein are greatly elevated (~2–7-fold) in many cancer cell lines and in primary tumors (Raderschall et al., 2002). RAD51 overexpression can result in improper and hyper-recombination, namely contributing to genomic instability and genetic diversity (Klein, 2008; Richardson, Stark, Ommundsen, & Jasin, 2004; Son & Hasty, 2018). These, in turn, might drive regular cells towards neoplastic transformation or further contribute to cancer progression, metastasis and anticancer drug resistance, as discussed below.

8.1.1. Brain cancer

As today, the role of RAD51 in gliomas is rather controversial. Large-scale genomic analyses of glioma did not identify any mutation in RAD51, implying it to be a rare event in this human cancer (Cancer Genome Atlas Research, 2008; Parsons et al., 2008). Interestingly, most reports support a suppressive role of RAD51 in these pathologies. Thus, in a screen of 42 human gliomas, a minimal deletion of RAD51 was detected in a subset of tumors (Bredel et al., 2005). Expression of RAD51 mRNA was determined in 40 astrocytomas of grade II–IV and was found not to differ significantly from normal brain samples (Jiang et al., 2006). Also, Westmark et al. described that platelet-derived growth factor subunit B (PDGF-B)-induced gliomas display genomic instability and co-expression of RAD51 can suppress PDGF-B-induced tumorigenesis and prolong survival (Westermark et al., 2011). In line, a study including 68 patients with glioblastoma (GB) showed that elevated RAD51 protein expression at initial diagnosis, as well as at recurrence, correlated with significantly increased survival duration (Welsh et al., 2009).

8.1.2. Head and neck and thyroid cancer

The RAD51 protein levels in tumor samples from 12 head and neck cancer (HNC) patients who received induction chemotherapy (paclitaxel and carboplatin) followed by radiation therapy administered together with additional chemotherapy were quantified by Connell et al. (Connell et al., 2006). In this study, patients with high RAD51 levels in their pre-treatment tumor biopsies had poorer cancer-specific survival rates compared to patients with lower recombinase levels (33% vs. 88.9%. at 2 years), suggesting that RAD51 levels of expression in HNC may influence the outcome in HNC patients treated with a combined chemotherapeutic/radiation regimen. Thyroid gland is the most common site for all endocrine malignancies and thyroid cancer (ThyC) is currently the 5th most prevalent cancer in females (Grimm, 2017). In this field, Sarwar et al. analyzed 102 tissues from ThyC patients and an equal number of healthy thyroid tissue controls for RAD51 expression and its eventual correlation with the cellular proliferation marker Ki-67 (Sarwar et al., 2017). Data showed increased expression of RAD51 in ThyC tissues with respect to control, associated with later stages, poor tissue differentiation, large tumor size, positive lymph node and distant metastasis, and the correlation analysis confirmed a direct relationship between Ki-67 expression and RAD51 expression both at transcriptional and translational level.

8.1.3. Lung cancer

In a study aimed at evaluating RAD51 expression in non-small-cell lung cancer (NSCLC), Qiao et al. analyzed 383 NSCLC tumors, observed high recombinase levels in 100 out of 340 cases (29.4%), reported that the NSCLC patients with high RAD51 levels showed a significant shorted median survival time of 19 vs. 68 months, and suggested that RAD51 expression provides additional prognostic information for surgically treated NSCLC patients (Qiao et al., 2005). In line, a report from the MD Anderson Cancer Center demonstrated that, with reference to 98 NSCLC patients, high levels of cytoplasmic RAD51 were associated to higher percentages of viable tumor cells and, hence, to shorter overall survival (OS) time in patients with NSCLC receiving neoadjuvant chemotherapy (Pataer et al., 2018). Birkelbach et al. studied the clonogenic survival of 16 NSCLC cell lines in response to cisplatin, mitomycin C, and the PARP inhibitor (PARPi) olaparib by assessing foci formation by the HR-associated BRCA1, FANCD2, RAD51, and γH2AX proteins. With respect to untreated cells, four cell lines (25%) showed an impaired RAD51 foci-forming ability in response to cisplatin, which correlated with cellular sensitivity to the anticancer drug. Since baseline foci in untreated cells did not predict drug sensitivity, the authors adapted an *ex vivo* biomarker assay to monitor damage-induced RAD51 foci in NSCLC explants from patients, which identified two tumors (15%) exhibiting compromised RAD51 foci induction upon *ex vivo* cisplatin treatment of the explants (Birkelbach et al., 2013). Data from Gachechiladze obtained from 1109 NSCLC patients further reported loss of nuclear RAD51 to be associated with lymph nodes and distant metastases (Gachechiladze, Skarda, Soltermann, & Joerger, 2017). In a very recent paper, Hu and coworkers analyzed large cohorts of lung adenocarcinomas patients and reported that RAD51 expression is

frequently upregulated in lung cancer tumors compared with normal tissues and is associated with poor survival (Hu et al., 2019). In particular, their systematic investigation of different lung cancer cell lines revealed higher expression of RAD51 in GTPase KRAS (KRAS) mutant cells compared to WT ones. They further showed that mutant KRAS, but not wildtype KRAS, played a critical role in RAD51 overexpression via the MYC proto-oncogene protein (MYC). Moreover, KRAS mutant cells were highly dependent on RAD51 for survival and depletion of RAD51 resulted in enhanced DNA DSBs, defective colony formation and cell death. In all, these results indicate that mutant KRAS promotes RAD51 expression to enhance DDR and lung cancer cell survival, suggesting that RAD51 may be an effective therapeutic target to overcome chemo/radioresistance in KRAS mutant cancers.

8.1.4. Breast cancer

A retrospective analysis of microarray expression data conducted by Martin et al. revealed elevated expression of RAD51 and of its late-acting cofactors (RAD54 (§7.2.6) and RAD51AP1 (§7.2.9)) in BRCA1-deficient vs. sporadic BCs (Martin et al., 2007). According to these authors, these results indicate that upregulation of HR provides a permissive genetic context for cells lacking BRCA1 function by circumventing its requirement in RAD51 subnuclear assembly and support a model in which enhanced HR activity contributes to the etiology of BRCA1-deficient tumors. During the analysis of 20 paired normal tissues and BC cancer specimens, Barbano et al. reported that a BC subgroup – characterized by an estrogen receptor positive/progesterone receptor negative phenotype – had high levels of RAD51 mRNA (Barbano et al., 2011). This finding was confirmed in the same study by the analysis of normal and tumor specimens from an extended cohort (75) of BC patients. Further analysis of microarray expression data from 295 BCs indicated that increased RAD51 mRNA expression was associated with higher risk of tumor relapse, distant metastases and worst overall survival (OS), leading the authors to propose that the determination of RAD51 expression could be of great help not only in a better molecular classification of mammary tumors but also in the evaluation of post-operative adjuvant therapy for BC patients. The expression of RAD51 and its paralog XRCC3 (§7.4) was analyzed in 248 cases of BC tissues and 78 further cases of adjacent non-cancerous samples (Hu, Wang, & Wang, 2013). Data analysis showed that the expression of both proteins was significantly increased in BC samples with respect to control; moreover, while both proteins expression correlated with positive progesterone receptor and receptor tyrosine-protein kinase erbB-2 (HER2) status, XRCC3 high expression was associated with large tumor size and RAD51 expression with axillary lymph node metastasis. In contrast to extensive studies on familial BC, it is currently unclear whether defects in DSB repair genes play a role in sporadic breast cancer development and progression. Accordingly, Wiegmans et al. analyzed an independent cohort of 235 sporadic BCs, and found that RAD51 expression was increased during BC progression and metastasis (Wiegmans et al., 2014). Knockdown of RAD51 repressed cancer cell migration *in vitro* and reduced primary tumor growth in a syngeneic mouse model *in vivo*. Loss of RAD51 also inhibited associated metastasis in human xenografts, and consistently altered the metastatic gene expression profile of cancer cells, highlighting for the first time a new function of RAD51 that may underlie the proclivity of patients with RAD51 overexpression to develop distant metastasis. From a mechanistic standpoint, Parplys et al. described a role for RAD51 overexpression in driving genomic instability caused by impaired replication and intra-S mediated CHK1 signaling by studying an inducible RAD51 overexpression model as well as 10 BC cell lines (Parplys et al., 2015). They demonstrated that cells with high levels of RAD51 display reduced elongation rates and excessive dormant firing during undisturbed growth and after damage, likely caused by impaired CHK1 activation. As a consequence, the authors proposed that the inability of cells with a surplus of RAD51 to properly repair complex DNA damage and to resolve replication stress leads to higher genomic instability and drives tumorigenesis. In another study aimed at assessing the biological and clinical significance of RAD51 expression with relevance to different molecular classes of BC and patient outcome, the expression of RAD51 was evaluated in 1184 cases of early-stage invasive BC with long-term follow-up, while a subset of cases of BC from patients with known BRCA1 germline mutations was included as a control group (Alshareeda et al., 2016). RAD51 was expressed both in the nuclei (RAD51(N+)) and the cytoplasm (RAD51(C+)) of malignant cells, and the subcellular co-localization phenotypes of RAD51 were significantly associated with clinicopathological features and patient outcome, as follows. RAD51(C+) and lack of nuclear expression of RAD51 (RAD(N-)) were associated with features of aggressive behavior, including larger tumor size, high grade, lymph nodal metastasis, and triple-negative phenotypes, together with aberrant expression of BRCA1. RAD51(N+) and RAD51(C+) tumors correlated with longer and shorter breast cancer-specific survival, respectively. Finally, RAD51(N-) was associated with poor prognostic parameters and shorter survival in invasive BC patients. Accordingly, the overall results from this study suggest that the trafficking of RAD51 between nucleus and cytoplasm might play a role in the development and progression of BC.

It is well known that BC frequently metastasizes to the brain (Achrol et al., 2019). A study carried out on 198 BC patients, 96 of which further suffering from brain metastases (BMs) identified a direct correlation between cytoplasmic RAD51 expression and increased risk of brain metastasis (Sosinska-Mielcarek et al., 2013). In order to identify BM-specific genes, the expression profiling of 23 matched sets of human resected BMs and primary BCs was carried out by Woditschka et al. (Woditschka et al., 2014). They discovered that RAD51 and BARD1 (§7.2.5) were overexpressed compared with either matched primary tumors or unlinked systemic metastases. *In vivo* experiments showed that overexpression of either gene increased BMs but not lung metastasis, while RAD51 knockdown via RNAi reduced BMs without affecting lung metastasis development. Finally, the authors detected considerable ROS levels in the metastatic tumor microenvironment and they hypothesized that the brain may require increased DNA repair function in cancer progression because of an endogenous source of genotoxic stress. Accordingly, treatment the affected mice with an oxygen radical scavenger reduced ROS expression in BMs and their microenvironment, and abrogated the brain metastasis stimulatory effects of BARD1 and RAD51 overexpression. The authors hence concluded that nearly the full phenotype of promoting BM initiation by BARD1 and RAD51 overexpression was explained by resisting the effects of ROS in the brain; moreover, since the two proteins overexpression was observed at early points in brain colonization, this suggested that ROS control initial outgrowth of metastases.

8.1.5. Esophageal, pancreatic and colorectal cancer

RAD51 overexpression was significantly associated with poor clinical outcome as a result from a study involving 230 patients affected by squamous-cell esophageal cancer (ESCA) (Li et al., 2011). A multivariate analysis performed in the same study confirmed that RAD51 expression was a significant and independent prognostic factor concerning disease-free and OS. In the same disease context, the levels of RAD51 were analyzed in 89 chemo-naïve ESCA patients undergoing curative surgery (Nakanoko et al., 2014). The results confirmed that disease-specific survival after 5 years was lower in RAD51-positive (59.3%) than in RAD51-negative (79.6%) cases.

Exploiting three-dimensional cell culture models and xenotransplants of human pancreatic cancer cells (PANCCs) Maake et al. reported that, in contrast to conventional monolayer cell systems, RAD51 accumulated to high levels, and this evidence was confirmed in 66% of human pancreatic adenocarcinoma tissue specimens analyzed by these authors (Maacke et al., 2000). Functional tests related RAD51 overexpression to enhanced DNA repair after DSB induction and to the subsequent increased survival of the malignant cells.

Using a tissue microarray analysis of 1213 biopsies taken from colorectal cancers (CRCs), Tennensted et al. investigated the potential

association between the expression of RAD51 and other proteins know to be related to CRC (Tennstedt et al., 2013). The study reported strong recombinase expression in 1% of CRC, moderate in 11% of cases, weak in 34%, and no expression in 44%, and the level of RAD51 expression correlated significantly with OS. Importantly, strong RAD51 expression was found to be associated with loss of two MMR proteins MSH and MLH (§4.1.2) and of β-catenin, thereby establishing a possible link with RAD51 and its role in the epithelial-mesenchymal transition (EMT), as discussed in detail in §8.4.

8.1.6. Prostate, kidney, and cervical cancer

A strong expression of RAD51 in high-grade prostate cancers (PCs), whether sporadic or associated with BRCA germline mutations, was reported by Mitra et al. (Mitra, et al., 2009). According to the authors, a distinct localization of RAD51 between cytoplasm and nucleus reflected distinct levels of recombinase regulatory activity, from transcription to DDR. Renal cell carcinomas (RCCs) show significant histone lysine methylation (Henrique, Luis, & Jeronimo, 2012), this epigenetic change being operated by a class of methyltransferase enzymes that contain the modular protein SET domain (Husmann & Gozani, 2019). In a recent work Liu et al. reported an enhanced expression of these enzymes in all classes of RCCs, metastatic high-grade tumors showing the highest levels (Liu et al., 2016). Although they could not demonstrate a direct correlation, they showed that the histone epigenetic modification by methylation was associated with decreased genomic translation of RAD51. In a proof-of-concept study evaluating the role of prelamin A (a protein that plays an important role in nuclear assembly, chromatin organization, nuclear membrane and telomere dynamics) and RAD51 expression in the clinical outcome of cervical cancer patients, Leonardi et al. analyzed biomarker expression in clinical tumor material from locally advanced cervical cancers (LACCs) and correlated data with clinicopathological parameters and with response to neoadjuvant chemoradiation therapy (CT/RT) (Leonardi et al., 2017). They found that LACC patients subjected to CT/RT and showing high RAD51 expression were less likely to respond to CT/RT treatment than those with low recombinase levels. Remarkably, high RAD51 expression significantly predicted poor outcome, emerging as a potential independent prognostic factor for disease free survival.

8.1.7. Soft tissue sarcomas

In soft tissue sarcomas (STSs), the role played by RAD51 overexpression in chemoresistance and the mechanisms regulating its expression was analyzed by Hannay and coworkers (Hannay et al., 2007). Their study showed high recombinase levels both in a large panel of human STS specimens and in nude rat STS xenografts. Treating STS cells with doxorubicin upregulated RAD51 and arrested them in the S-G2 phase while silencing RAD51 by targeted siRNA increased doxorubicin sensitivity. These authors further verify that, in the studied STS cell lines, p53 participated in regulating RAD51 expression, in that reintroduction of WT p53 in STS cell harboring a p53 mutation resulted in decreased RAD51 mRNA and protein expression by virtue of p53-induced decreased RAD51 promoter activity.

8.2. RAD51 polymorphism in cancer

A large number of genes associated with various cancer types contain single nucleotide polymorphisms (SNPs). SNPs are single-base differences in the DNA sequence that can be observed between individuals and are defined as the least common allele occurring in 1% or more of the population. SNPs can be located in gene promoters, exons, introns as well as 5'- and 3'- untranslated regions (UTRs) and affect gene expression by different mechanisms depending on the role of the genetic elements in which the individual SNPs are located. Accordingly, SNPs can alter the amino acid sequence of the encoded proteins, or alter RNA splicing and gene transcription, resulting in either increased or decreased expression or activity of the encoded proteins, with possible associated susceptibility of cancer development. Moreover, alterations in epigenetic regulation due to gene polymorphisms add to the complexity underlying cancer susceptibility related to SNPs.

Loss of heterozygosity (LOH) is defined as the loss of one parent's contribution to the cell, can be caused by direct deletion, deletion due to unbalanced rearrangements, gene conversion, mitotic recombination, or loss of a chromosome (monosomy). LOH often occurs in cancer, where the second copy of a gene (typically a tumor-suppressor gene) has been inactivated also by other mechanisms, such as point mutation or hypermethylation. When a whole chromosome or a large segment of a chromosome is lost, the remaining chromosome or segment is often duplicated. With complete duplication of the remaining genetic material, the karyotype may appear normal, even though no normal genes are present.

Below, some of the most common SNPs and LOHs found in RAD51 and its main paralogs that may be predictive of increased susceptibility to and/or drug resistance to different cancers are reported. A specific section is devoted to tumor-associated RAD51 SNPs that locate in the recombinase promoter and, either by altering the structural, biochemical or physical properties of the nucleoprotein filament or by interfering with RAD51 interactions with its regulatory partners, may contribute to genome instability and cancer growth.

8.2.1. Brain cancer

The role of RAD51 SNPs as predisposing factors to brain cancer is still a matter of investigation and debate. For example, using a cohort of 309 patients with newly diagnosed glioma and 342 cancer-free controls, Wang et al. did not find statistically significant difference in the distribution of XRCC1 (rs25487, 28152G>A, Q399R), XRCC3 (rs861539, 722C>T, T241M) and RAD51 (rs1801320, 135G>C) SNPs between cases and controls (Wang et al., 2004). Some years later, Franceschi et al. evaluated the association between the same SNPs with the susceptibility to GB (Franceschi et al., 2016). For this study, 85 GB patients and 70 matched controls were recruited and the results indicated that those subjects carrying the RAD51 rs1801320 GC genotype showed an increased risk of GB. The C allele was also significantly associated with GB, in particular when combined with the rs861539 C allele.

8.2.2. Head and neck and oral cancer

A case-control study was conducted to analyze the possible associations between HNC risk and fourteen SNPs and haplotypes in the RAD51 and XRCC3 genes (Gresner et al., 2012). This study involved 81 HNC cases and 111 healthy control subjects. A significant risk-increasing effect of rs3212057 (R94H) SNP in XRCC3 was observed. Interesting, a risk-decreasing effect was reported for rs5030789 (3997A>G) and rs1801321 (60G>T) in 5' near gene and 5'UTR regions of RAD51, respectively. Moreover, these effects were shown to be modulated by tobacco-smoking status and gene-gene interactions. Accordingly, the authors concluded that the genetic variability of XRCC3 and/or RAD51 genes might be of relevance with respect to HNC risk. The effects of SNPs in RAD51 and XRCC3 on susceptibility to oral and oropharyngeal squamous cell carcinomas (SCC) and their clinicopathological significance were recently reported by Santos et al. (Santos et al., 2019). Specifically, SNPs 135G>C (rs1801320) and 172G>T (rs1801321) in RAD51, and T241M (rs861539) in XRCC3 were genotyped in 81 patients presenting oral SCC, 45 presenting oropharyngeal SCC, and 130 healthy controls. In particular, the authors disclosed synergistic effects of the risk alleles of all three SNPs with smoking and alcohol consumption on susceptibility to oral and oropharyngeal SCC. Furthermore, oropharyngeal SCC patients carrying the XRCC3 rs861539 GT/TT genotype (T risk allele) presented a shorter OS than GG genotype carriers. This led the authors to conclude that the combined effects of the analyzed RAD51 and XRCC3 SNPs with environmental carcinogens such as tobacco and alcohol are associated with oral and oropharyngeal SCC development.

8.2.3. Lung cancer

The RAD51 SNP 135G>C was studied by Nogueira et al. in 234 NSCLC patients, and they found that OS was 1 year longer in those carrying the C allele compared to the G-allele carriers (Nogueira et al., 2010). On the other hand, in a cohort of Chinese patients with early stage NSCLC undergoing potentially curative tumor resection followed by adjuvant platinum-based chemotherapy Jiang et al. reported no significant survival difference depending on the RAD51 135 G/C genotype (Y. H. Jiang et al., 2014). Yin and collaborators hypothesized that genetic polymorphisms in RAD51 and its paralogs may affect clinical outcomes among NSCLC patients treated with definitive radio(chemo)therapy. Therefore, they genotyped several potentially functional SNPs, i.e., RAD51 rs1801320 and rs1801321, XRCC2 rs3218384 (4234G>C) and R188H, and XRCC3 T241M and estimated their associations with OS and radiation pneumonitis in 228 NSCLC patients (Yin et al., 2017). These authors found a predictive role of RAD51 135G>C SNP in radiation pneumonitis development; also, they reported that the same RAD51 SNP and the XRCC2 R188H polymorphism were independent prognostic factors for OS and that the SNP-survival association was most pronounced in the presence of radiation pneumonitis. Although larger studies are clearly needed to confirm these findings, the authors suggested that the RAD51 135G>C polymorphism in particular may influence OS and radiation pneumonitis in NSCLC patients treated with definitive CT/RT.

8.2.4. Breast cancer

In 2007 in a major effort Antoniou et al. pooled genotype data for 8512 female mutation carriers from 19 studies for the RAD51 135G>C SNP (Antoniou, et al., 2007). They found evidence of an increased BC risk in CC homozygotes but not in heterozygotes. When BRCA1 and BRCA2 mutation carriers were analyzed separately, the increased risk was statistically significant only among BRCA2 mutation carriers. In addition, they determined that the 135G>C variant affects RAD51 splicing within the 5' UTR e therefore proposed that this recombinase SNP 135G>C may modify the risk of BC in BRCA2 mutation carriers by altering the expression of RAD51. Synowiec et al. reported a strong association between BC occurrence and the genotype C/C of the RAD51 135G>C polymorphism, whereas the G/C genotype correlated with a protective effect against the same pathology (Synowiec, Stefanska, Morawiec, Blasiak, & Wozniak, 2008). Moreover, the same group found that individuals with the RAD51 G/C genotype of the 135G>C SNP displayed a lower extent of basal and oxidative DNA damage, suggesting that this recombinase polymorphism may be linked to BC by the modulation of the cellular response to oxidative stress. In 2013, Wang and collaborators performed a meta-analysis based on 39 case-control studies to investigate the association between BC susceptibility and the RAD51 SNP rs1801320 (Wang et al., 2013). As a result of this study, no overall significant association was observed between this RAD51 polymorphism and cancer susceptibility in any genetic model while, on the other hand, the RAD51 variant 135C homozygote was associated with elevated BC risk among BRCA1 mutation carriers. Besides rs1801320, four other SNPs – one in RAD51 (rs1801321, 172G>T) and three in XRCC3 (rs1799796, 17893A>G (intron 5); rs861539, 722C>T, T241M; and rs1799794, 315A>G) - were studied to examine whether they exhibited an association with BC susceptibility in a Belgian population of BC patients with a known or putative genetic predisposition (Vral et al., 2011). The results showed that low-penetrant variations in both the recombinase and its paralog may modify BC risk in patients already carrying a pathological mutation in the highly-penetrant BC genes BRCA1/2. In particular, a combined risk genotype analysis revealed that RAD51 SNPs enhanced BC risk in BRCA2-patients whilst XRCC3 SNPs significantly enhanced risk in patients carrying BRCA1 mutations and in those with hereditary BCs. Two of the same SNPs just discussed, 135G>T in RAD51 and 722C>T (T241M) in XRCC3, were also analyzed by Krupa et al. for association with BC occurrence and progression in 135 cases and 175 controls (Krupa et al., 2009). While no relation between either SNP was found with tumor size, estrogen and progesterone receptors status, cancer type and grade, according to the results from this Polish group the M241M genotype of the XRCC3 polymorphism slightly increased the risk of local metastasis in BC patients while the combined XRCC3 T241M/RAD51 135G>T genotype decreased the risk of BC occurrence, supporting the hypothesis of a mutual interaction between the recombinase and its paralog in BC onset and progression. The effect of the135G>C SNP was also studied by Söderlund and coworkers in 306 BC patients subjected to radiotherapy and cyclophosphamide, methotrexate, and 5-fluorouracil chemotherapy (Soderlund Leifler, Asklid, Fornander, & Stenmark Askmalm, 2015). According to their results homozygous carriers of the 135G allele had a decreased risk of local recurrence following radiotherapy compared to the individuals carrying the C-allele; contextually, the combination chemotherapy decreased the risk of distant recurrence only in carriers of at least one C-allele. In the same year, Sekhar et al. undertook a meta-analysis on RAD51 135G>C data for 21236 cases and 19407 controls pooled from 28 studies on BC in women (Sekhar, Pooja, Kumar, & Rajender, 2015). The results suggested a significant association of the substitution with BC in the recessive model (GG + GC vs. CC) and in the co-dominant models comparing GG vs. CC and GC vs. CC. Further data analysis suggested that the CC genotype is a significant BC risk factor in comparison to the GG and GC genotypes. Interestingly, the authors also undertook pooled analysis on different ethnic groups and found that CC was a strong risk factor in Caucasians, but not in East-Asian and in populations of mixed ethnicity. In 2016 Al Zoubi et al. proposed that polymorphisms in the 5'-UTR promoter region of the RAD51 gene are prognostic factors for BC development (Al-Zoubi et al., 2016). To the purpose, they performed the direct sequencing of 106 samples from sporadic BC patients and 54 samples from a control group, finding that the homologous variant T172T alone was significantly associated with BC while the heterozygous G135C did not show any meanigful relationship with risk of sporadic BC Moreover, both variants (T172T and G135C) together showed a significant relationship with sporadic BC susceptibility. One year later the same group reported a significant association with BC susceptibility in the Italian population and SNPs in RAD51 (rs1801321, 172G>T) and in its paralog XRCC3 (rs1799794, 315A>G) (Al Zoubi et al., 2017). The SNPs rs1801321 (172G>T) of RAD51 and rs718282 (41657G>T) of XRCC2 were investigated in 70 Polish patients with triple negative breast cancers (TNBCs) and 70 age- and sex matched non-cancer controls (Michalska, Samulak, Romanowicz, & Smolarz, 2015). The results obtained demonstrated a significant positive association between the RAD51 T/T genotype and TNBC. The homozygous T/T genotype was found in 60 % of TNBC cases and in 14 % of the used controls. The variant 172T allele of RAD51 increased cancer risk while no significant association was observed between the 41657G>T genotype of XRCC2 and the incidence of TNBC, implying that the SNP of RAD51, but not of XRCC2, could be positively associated with the incidence of TNBC in the studied cohort.

In terms of somatic RAD51 mutations in tumor tissues, one study reported LOH of this gene - located on chromosome 15q14-15 - in 41 out of 127 BC patients (Gonzalez et al., 1999). Along this line, Nowacka-Zawisza et al. evaluated LOH in RAD51 and its association with BC by analyzing the recombinase polymorphic markers D15S118, D15S214, and D15S1006 (Nowacka-Zawisza, Brys, Romanowicz-Makowska, Kulig, & Krajewska, 2007). Genomic deletion detected by allelic loss ranged from 29 to 46% of informative cases for the RAD51 region, 25% of the analyzed BCs displayed LOH for at least one RAD51 marker, and LOH in RAD51 appeared to correlate with the steroid receptor status, suggesting a role for enhanced risk in BC development.

8.2.5. Esophageal and colorectal cancer

Genotyping of the RAD51 135G>C SNP was performed by Mingzhong et al. on a cohort of 477 volunteers, of whom 219 were esophageal cancer patients (Ming-Zhong, Hui-Xiang, Zhong-Wei, Hao, & Rong, 2015). Individuals carrying the RAD51 C allele (GC + CC) had a

significant increased cancer risk compared to those with the GG genotype, and the risk was drastically exacerbated in the presence of the RAD51 paralog XRCC4 SNP 1394G>T (rs6869366). The association of the RAD51 135G>C and the XRCC3 T241M and R188H (rs3218536) SNPs with CRC risk was considered in the work by Krupa et al. (Krupa et al., 2011). 100 patients with invasive colon adenocarcinoma and an equal number of controls were enrolled in the study, and the results showed that those case positive for R188R/M241M, H188H/T241T, and H188H/G135G genotypes had a significant increased risk of CR occurrence. On the other hand, the RAD51 C135C genotype was associate with a decreased risk of CRC either singly or in combination with the other two SNPs. Following these results, the authors proposed that the RAD51 polymorphism 135G>C could be considered as an independent marker of CC risk, while the T241M and the R188H SNPs in XRCC3 and XRCC2 can modify the CC risk.

8.2.6. Prostate, cervical and ovarian cancer

The relationship between PC risk and presence of SNPs in RAD51 (rs1801320 and rs1801321) and its paralogs RAD51B (rs10483813 and rs3784099), XRCC2 (rs3218536), and XRCC3 (rs861539) was investigated by Nowacka-Zawisza et al. (Nowacka-Zawisza et al., 2015). In particular, this group considered a Polish cohort of 101 patients with prostate adenocarcinoma plus 216 controls and found a significant correlation between the RAD51 rs1801320 polymorphism and increased PC risk, suggesting that this recombinase SNP may contribute to PC susceptibility in Poland. The same group recently analyzed different SNPs in RAD51 (rs2619679, 3879T>A; rs2928140, 7995G>C; and rs5030789) and XRCC3 (rs1799796, 17893A>G (intron 5)) and their relationship to PC (Nowacka-Zawisza et al., 2019). The study group included 99 men diagnosed with prostate cancer and 205 cancer-free controls. A significant association was detected between RAD51 rs5030789 and XRCC3 rs1799796 polymorphisms and an increased susceptibility to PC.

Nogueira et al. analyzed the RAD51 172G>T polymorphism genotypes in cervical cancer patients treated with a combination of platinum-based chemotherapy and radiotherapy and found that the mean survival rates were statistically different according to the patients RAD51 genotypes (Nogueira et al., 2012). Specifically: i) the group of patients carrying the T allele presented a higher mean survival rate than the other patients; ii) an increased OS time for T-carrier patients was noted when compared with GG genotype, with tumor stage, age and presence of lymph nodes as covariates; iii) among the 193 patients considered, RAD51genotype frequency distributions were not under the influence of clinic-pathologic characteristics, i.e., treatment response, recurrence, and tumor stage. This led to the conclusion that the RAD51 172G>T SNP has an influence on OS of cervical cancer and may provide additional prognostic information in cervical cancer patients who underwent cisplatin-based chemotherapy in combination with radiotherapy. In a study aimed at evaluation associations between the risk of ovarian cancer (OC) and the two RAD51 SPNs 135G>C and 172G>T, the distribution of genotypes and frequency of alleles of the recombinase polymorphisms were analyzed in 210 Polish women affected by the pathology and the same number of healthy females (Smolarz et al., 2013). This study showed that the genotype distribution for the RAD51 135G>C SNP in OC patients with respect to control was 20% vs. 30% for G/G, 22% vs. 47% for G/C, and 50% vs. 23% for C/C genotype, respectively, with an increased risk of OC in C/C homozygotes but not in heterozygotes. Also, the meta-analysis performed by Zeng et al. on 4097 cases and 5890 controls supported the notion that the RAD51 135G>C polymorphism increases the risk of 3 common gynecological tumors (endometrial carcinoma, ovarian cancer and cervical cancer), especially for endometrial tumors among hospital-based population (Zeng et al., 2018).

8.2.7. Leukemia

The role of RAD51 polymorphism in modulating the susceptibility to the development of acute myeloid leukemia (AML) was explored by Seedhouse et al. by studying the distribution of SNPs in the recombinase and its paralog XRCC3 in 216 cases of de novo AML, 51 cases of therapy-related AML (t-AML), and 186 control subjects (Seedhouse, Faulkner, Ashraf, Das-Gupta, & Russell, 2004). According to their results, the risk of AML development was found to be significantly increased when both variants rs1801230 (135G>C) in the RAD51 5'-UTR and rs861539 (722C>T, T241M) in the XRCC3 were present. The risk of t-AML development was even higher, presumably because of the large genotoxic insult these patients received after their exposure to radiotherapy or chemotherapy. Further studies from the same group concerning radiation-induced AML and t-AML in mice revealed that the RAD51 SNP 135G>C variant increases t-AML risk, and when analysis combined polymorphisms on both RAD51 and the HLX1 homeobox gene (HLX1, important for hematopoietic development), a synergistic 9.5-fold increase in the risk of t-AML was observed (Jawad, Seedhouse, Russell, & Plumb, 2006). It was suggested that the HLX1 polymorphism has an effect on stem cell numbers, whereas an increased DNA repair capacity via RAD51 suppresses apoptosis, a genetic interaction that may increase the number of genomes at risk during cancer therapy.

8.2.8. Cancer-related mutations in the RAD51 promoter

Because RAD51 nucleoprotein formation and dynamics are essential elements for its DNA strand exchange function (§7.1), even small changes in the recombinase promoter can potentially result in dramatic effects. For instance, single point (or missense) mutations affecting the DNA binding motif, or ATP hydrolysis, or the promoter/promoter docking regions are all expected to influence the RAD51 function during HR. In this respect, three adjacent mutations in the coding sequence of the RAD51 protein - D149N, R150Q, and G151D - were identified in BC patients (Chen et al., 2015; Kato et al., 2000; Marsden et al., 2016). All three variants map to a conserved structural motif on the RAD51 surface that contains the catalytic core of the recombinase (Fig. 43, left), including the Walker A and B consensus sequences for ATP binding and hydrolysis, the loops L1 and L2 of the single- and double-stranded DNA binding interfaces, the promoter-promoter interaction region and recognition sites for other recombinase regulators and mediators like BRCA2, PALB2 (§7.2.4), and p53 (§7.2.10). In particular, these 3 mutated residues occur within the highly conserved sequence 147PIDRGG152 - known as the Schellman loop - which forms a prominent surface feature of both the recombinase monomer and the filament. Yet, their presence has no overall effect on the corresponding recombinase folding and stability (Chen et al., 2015).

From the physical standpoint, all three mutations alter the both the size and shape and the local surface electrostatic properties of RAD51 (Fig. 43, middle and right), the D149N mutation increasing the electropositive character of the Schellman loop surface of +1 unit while R150Q and G151D both increase the electronegativity of the same protein region of -1 unit. The Morrical group studied in details all these three RAD51 mutants in relation to the WT recombinase (J. Chen et al., 2015). Interestingly, although they found that all three variants were folded and stable, they also noted large differences in the ATPase activity for the R150Q and G151D mutants with respect to the WT protein. In particular, under ATP saturating conditions, the catalytic rates and, correspondingly, the catalytic efficiency of the Q150 and D151 variants were similar to each other but substantially lower than the WT and the D149N isoform. Also, after having ruled out the possibility that the observed variations in the kinetic parameters were linked to inefficient binding of the Q150 and D151 variants to ssDNA, they importantly noticed that the ATPase G151D isoform appeared to be independent of ssDNA even at low nucleic acid concentration. This led the authors to speculate that the altered surface electrostatics in the G151D mutation-carrying RAD51 induces it to assume a DNA-independent constitutively active conformation, in agreement with the fact that the same recombinase variant was reported in the same work as a somatic, heterozygous mutation in a patient with advanced BC who had local radiation resistance.

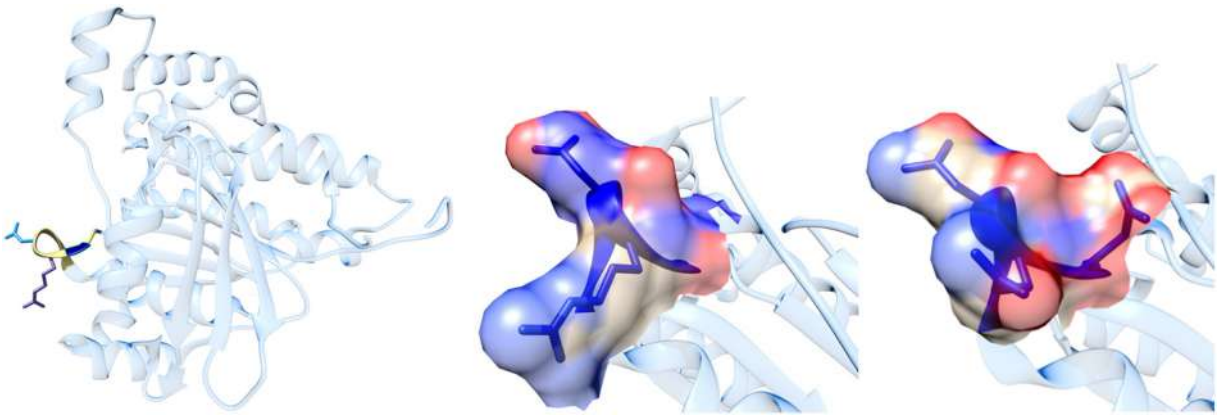

Fig. 43. (Left) The three RAD51 residues D149 (dodger blue), R150 (dark slate blue), and G151 (navy blue) belonging to the Schellman loop (kaki) found mutated in BC. Zoomed views of the variation in the shape, size, and local surface electrostatic properties obtained by replacing the wild type RAD51 D149, R150, and G151 residues (middle) with the three mutants N149, Q150, and D151 (right). Blue regions indicated electropositive character of the surface, while red regions indicate electronegative surface characteristics. For simplicity, all three residues have been changed in the right panel; however, each missense mutation was singularly detected in the clinical samples (PDB: 5JZC (Short et al., 2016)).

From the perspective of DNA strand exchange, the same study demonstrated that the three RAD51 mutants were all equally proficient and endowed with sufficient ssDNA binding affinity in an assay where RPA was used for comparison; yet, with increasing salt concentration (from 100 to 250 mM KCl) in the reaction medium, the G151D variant revealed a slight decrement in both the rate and extent of the corresponding catalyzed strand exchange if compared with the WT recombinase. This led the authors to suggest that the G151D protein could affect HR activity in a cell that is heterozygous for this variant, such as that found in the corresponding BC clinical sample. In terms of nucleoprotein filament formation, although all three forms were able to generate these structures on ssRNA similarly to the WT counterpart, Morrical et al. determined that the changes in the electrostatic surface potentials of the mutants correlated with some of the physical and structural properties of the corresponding filaments on both ssDNA and dsDNA. In particular, the recombinase/nucleic acid complexes involving the more electropositive D149N mutant displayed reduced electrophoretic mobility while, conversely, those containing the other two electronegative variants R150Q and G151D showed enhanced electrophoretic mobility with respect to the WT recombinase nucleofilaments. The authors, supported by electron microscopy imaging showing that the filaments formed by the WT and the D149N proteins were more segmented and stiffer while those formed by the two other mutants appeared as more relaxed and smoother coils, proposed that the combination of the filament mechanical and electrical properties were responsible for the corresponding electrophoretic mobilities, and that these altered properties could have an impact on a variety of biological processes involving RAD51, *in primis* the formation and resolution of the recombinase foci and the turnover of the nucleoprotein filaments in DNA HR. Finally, and perhaps most importantly, the G151D mutation was found to interact with WT RAD51 and to form hybrid nucleofilament on both single and double stranded nucleic acids. Should this *in vitro* behavior be replicated *in vivo*, i.e., in the BC cells where this recombinase variant was identified in the heterozygous state, this could originate hybrid RAD51 foci with likely altered HR behavior.

The location and nature of the three missense mutations D149N, R150Q, and G151D in the Schellman loop motif on the outer RAD51 filament surface (Fig. 44) may exert a considerable influence on the interaction of the recombinase with some of its numerous mediators and regulators.

One candidate in this multitude is the tumor suppressor p53, which interacts via two distinct regions (94-160 and 264-315, Fig. 45, left and middle) with RAD51 residues belonging to the sequence G179-A190, as already discussed in §7.2.10. As seen from Fig. 45, although the RAD51 Schellman loop motif is not directly involved in binding p53, the spatial vicinity of the p53-interacting residues and this loop does not rule out the possibility of an indirect effect of such RAD51 mutations on the contacts of the recombinase with this oncosuppressor.

A survey of 183 lung adenocarcinomas led the same group to identify 1 tumor tissue with an 803A>C SNP in the RAD51 coding region, resulting in the single-point Q268P mutant protein (Silva et al., 2016). The zygosity of this mutation was unknown and its somatic status could not be confirmed. In the same effort, this group also found one sample out of 499 renal clear cell carcinomas characterized by the RAD51 815A>T SNP, corresponding to a protein carrying a the Q272L missense mutation. Although also in this case the zygosity was not known, the somatic status was confirmed. Both sequence variants affect two highly conserved residues and map to the recombinase L2 motif (Fig. 45, right) involved in DNA binding and in the allosteric activation of ATP hydrolysis and DNA strand exchange activities (§7.1). Contrarily to what observed for the three mutations belonging to the Schellman loop discussed above, these two RAD51 variants exhibited thermal profiles quite different from that of the WT. In particular, the relevant thermal stability data indicated that for the WT recombinase the transition from a thermostable to a thermolabile conformation was ATP-dependent whereas for the two variants both conformations seemed to exist in equilibrium in the absence of ATP, their thermolabile forms being characterized by lower melting temperatures with respect to WT RAD51. In line with these findings, the intrinsic ATPase activity of both the Q268P and the Q272L isoforms was practically abrogated, and when stimulated with ssDNA it was still 2.5-fold lower compared to the WT protein, suggesting that the conformational change required to initiate ATP hydrolysis was inefficient in the two variants due to their compromised folding stability. More interestingly, in terms of DNA strand exchange capability both mutants showed severe defects in particular at physiological temperature and in the presence of RPA, the Q268P isoform being completely inactive under these conditions. Accordingly, the authors proposed that this could be related to their incapacity to generate stable or competent presynaptic nucleofilaments on ssDNA in the tested environments. In agreement with this, the Q272L variant was endowed with weak ssDNA and dsDNA affinity, and its low ssDNA binding strength was suggested to prevent this protein from displacing RPA during the presynaptic phase and/or maintaining stable filaments on ssDNA stretches. The alternative variant Q268P, on the other hand, exhibited a modest increment in ssDNA affinity and an unaltered binding ability for dsDNA when compared to RAD51 WT. However, based on electrophoretic mobility data Morrical and coworkers concluded that the presence of the mutation induced an overall change in the nucleofilament morphology which prevented it from adopting the correct extended conformation required to displace RPA and forming the active presynaptic ensemble. Finally, mixtures of WT

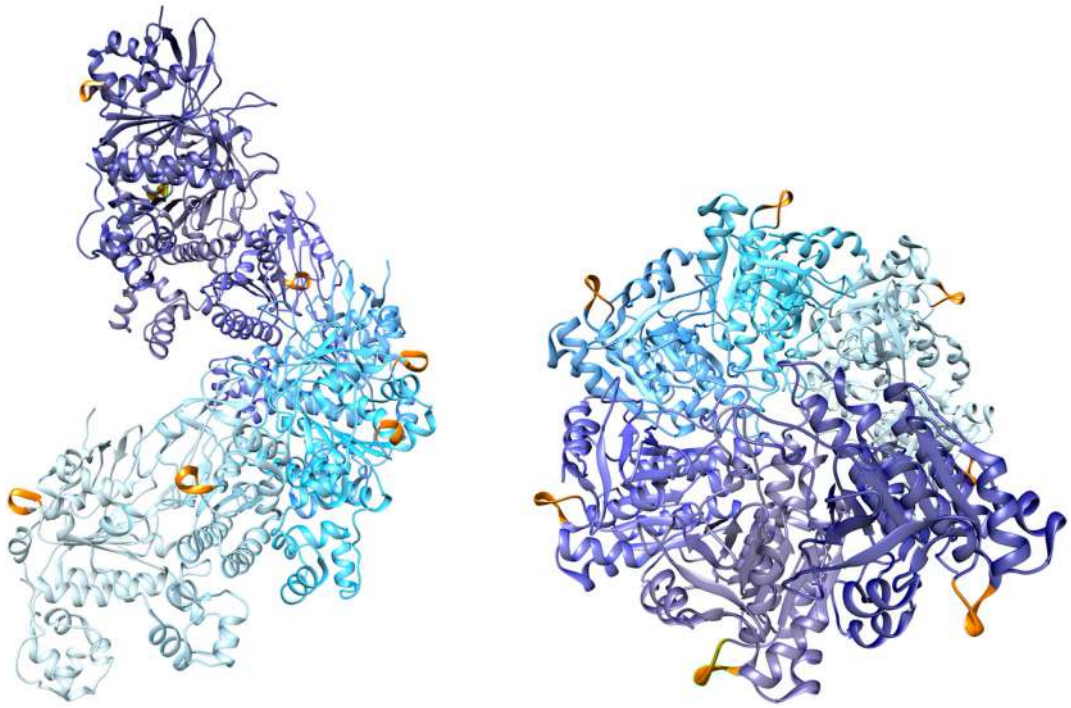

Fig. 44. (Left) The structure of the RAD51 presynaptic filament with 7 protomers colored in different shades of blue and the corresponding 147PIDRGG152 Schellman loop motifs highlighted in orange. (Right) Top-down view of the image in the left panel (PDB: 5JZC (Short et al., 2016)).

and these variants protein also exhibited reduced DNA strand exchange activity, leading to the conclusion that both RAD51 mutants could interfere with HR even if expressed in a heterozygous state with the WT recombinase *in vivo*.

Morrical and collaborators also studied two further cancer-related RAD51 missense variants located in the multimerization/BRCA2 binding region of the recombinase (Silva et al., 2017): the F86L variant, resulting from the 258C>A SNP found during the analysis of 999 invasive BCs and the germline variant E258A (SNP rs191297852), detected in 5 out of 66740 genomes but not yet reported in tumor tissues. Both mutations were found during a survey performed on the cBio cancer genomic portal (Cerami et al., 2012); the former was confirmed somatic with zygosity unknown, while for the second all positive individuals were presumed to be heterozygous for the mutant allele. Notwithstanding the apparent distance of the two mutations sites along the recombinase primary sequence, the three-dimensional structure of the RAD51 promoter shows that residue E258 locates at the RAD51/BRCA2 binding interface (Fig. 46, left) (§7.2.2) while an analogous interaction was predicted by the authors on the basis of the homology of the human RAD51 with the S. cerevisiae recombinase promoter (Fig. 46, right).

As seen for the two missense RAD51 Q268P and Q272L variants discussed above, also these two RAD51 isoforms were found to be less stable than the WT recombinase in the presence of ATP and presented a biphasic thermal profile, suggesting the existence of two or more conformational states in equilibrium. In further analogy, both F68L and E258A mutants were endowed with lower ssDNA and dsDNA affinities, yet with two different consequences. In fact, in the presence of ATP the E258A mutant was still able to form stable nucleoprotein filaments on both ssDNA and dsDNA whilst those filaments formed by the alternative variant appeared to be unstable under the adopted experimental conditions. Interestingly, F86L and E258A were both found partially deficient in their ATPase activity, but for two opposite reasons: the former had little intrinsic ATP hydrolysis but a strong ssDNA response while the latter presented a robust ATPase activity but a weak ssDNA response. In terms of DNA strand exchange activity, the E258A mutant preserved intrinsic DNA exchange activity albeit this was greatly reduced with respect to

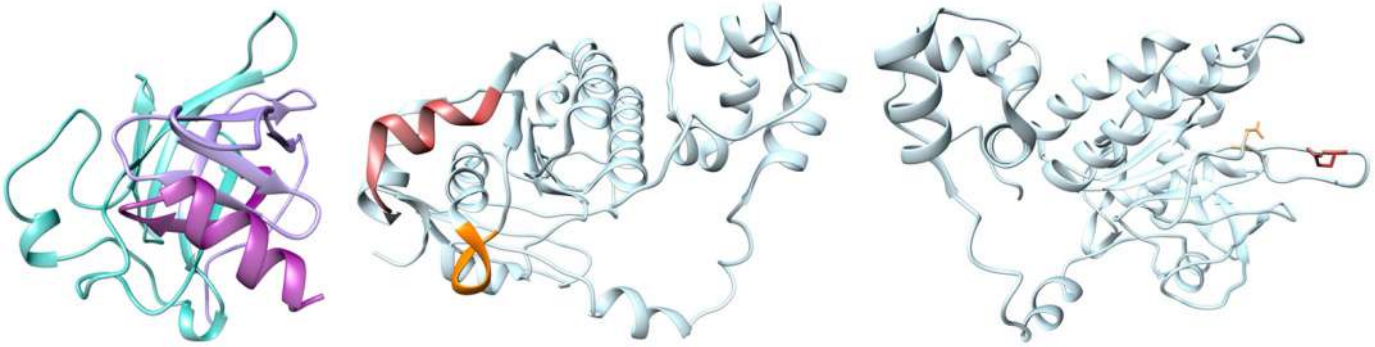

Fig. 45. (Left) Structure of the tumor suppressor p53 (sea green) with the two RAD51 interacting regions highlighted in medium purple (94-160) and dark magenta (264-315), respectively (PDB: 1TUP, (Cho, Gorina, Jeffrey, & Pavletich, 1994)). (Middle) One RAD51 promoter (light blue) showing the special proximity of the p53 binding region (firebrick) and the Schellman loop (orange) found mutated in BC (PDB: 5JZC (Short et al., 2016)). (Right) The same RAD51 promoter showing the two residues Q268 (goldenrod) and Q272 (brown) found mutated into P and L in lung and kidney carcinoma samples, respectively (Silva et al., 2016).

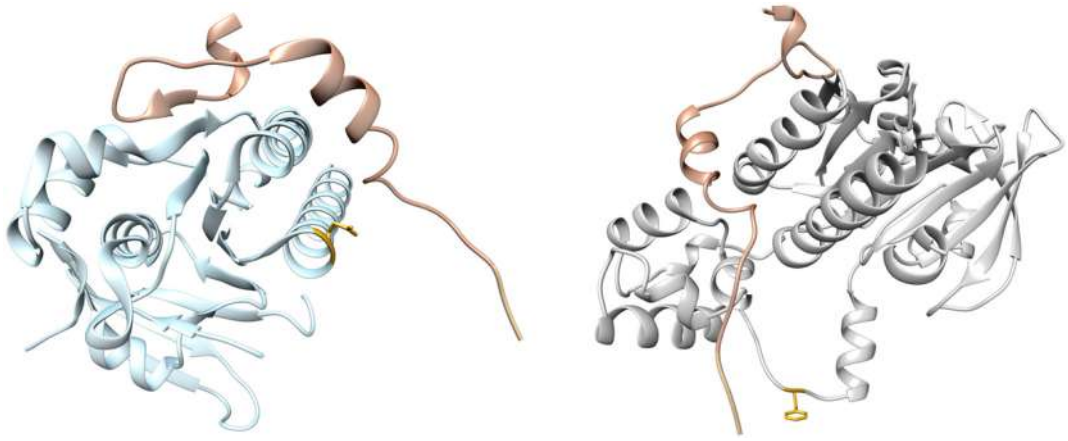

Fig. 46. (Left) Structure of a complex between the core domain of the RAD51 promoter (light blue) and the BRCA BRC4 repeat (sienna) (PDB: 1N0W, (Pellegrini et al., 2002)) showing the E258 residue (goldenrod) found mutated to A in BCs (Silva et al., 2017). (Right) The same BRCA BRC4 repeat in the putative complex formed with the homologous RAD51 core domain of the *Saccharomyces cerevisiae* (light gray, PDB: 3LDA, (Chen, Villanueva, Rould, & Morrical, 2010)), highlighting the relative position of the human F86 residue (goldenrod, F144 in *S. cerevisiae*), found mutated to L in the cBio cancer genomic portal (Silva et al., 2017).

WT and completely abrogated in reactions performed in the presence of RPA. When mined with the WT recombinase, this isoform exerted a dominant negative effect on RAD51 WT through the formation of partially active, hybrid presynaptic filaments, and this data suggested that individual heterozygous for this mutation might have reduced HR activities. Most surprisingly, the biochemical recombination activity of the F68L mutant was found to drastically diverge from that of the WT recombinase, as it exhibited DNA strand exchange hyperactivity that the authors attributed to a likely rapid release of the heteroduplex product and the subsequent fast turnover of the oligonucleotide substrate. Even more interesting was the observation that, when mixed with RAD51 WT, the F86L mutant poisoned the DNA strand exchange activity of both recombinases. This constitutes a brilliant demonstration of the fact that even conservative single-point mutations (like F–>L) in critical position on the RAD51 filament may result in protein interface regions incompatible with presynaptic filament geometry and impaired HR.

The dominant effects of two further RAD51 missense mutations defective for ATP binding (K133A) or ATP hydrolysis (K133R) expressed in cells that also expressed normal RAD51 from the other allele were reported by Kim and coworkers (Kim et al., 2012). These cells were defective for restarting stalled replication forks and repairing DSBs and exhibited a wide range of structural chromosomal changes that included multiple breakpoints within the same chromosome. Compared to normal protein very low levels of mutant RAD51 were visualized at sites of replication and repair, suggesting that low levels of mutant recombinase isoforms were sufficient for disruption of RAD51 activity and induction of chromosomal rearrangements.

Fanconi anemia is a genetic disorder that is characterized by bone marrow failure, developmental abnormalities and predisposition to cancer linked to defects in DNA ICL repair (§6) and chromosomal instability (Nalepa & Clapp, 2018). Recently, a recombination-independent role of RAD51 in ICL repair was reported (Wang et al., 2015). In this study, cells derived from a FA patient carrying the RAD51 missense mutation T131P were found to be hypersensitive to DNA crosslinking agents, yet remained HR proficient. Contextually, another FA-related RAD51 point mutation, A293T, was also reported to be sensitive to the same type of DNA damaging agents and to camptothecin (Ameziane et al., 2015). However, how these FA-related RAD51 mutations affect DNA replication beyond ILC repair and the exact molecular mechanism by which they alter RAD51 filaments was not investigated. In 2017, Zadorozhny et al. showed that, upon replication fork stalling, both FA-associated RAD51 mutants T131P and A293T failed to protect nascent DNA from aberrant nucleolytic cleavage by MRE11 (Zadorozhny et al., 2017). Reconstitution of DNA protection *in vitro* using synthetic DNA substrates confirmed that the defect was due to the properties of the relevant RAD51 filaments. EM with subsequent 3D reconstructions showed pronounced structural changes within the corresponding RAD51 nucleoprotein filaments, directly resulting in their destabilization that was not rescued by prevention of ATP hydrolysis due to aberrant ATP binding. This works thus uncovered a general mechanism by which these two FA-related RAD51 mutations affect protection of replication forks independently of ICL repair and highlights the importance of the delicate cooperation between the FA and HR factors during DNA replications.

8.2.9. RAD51 polymorphisms in relation to toxicity following anticancer therapy

Although most of the research efforts in RAD51 SNPs characterization were and are focused on the related risk of cancer development and/or patient outcome, interesting studies reported that the RAD51 135G>C polymorphism can be associated to toxicity following anticancer radio- and chemotherapy. For instance, among other DNA repair genes Osti et al. investigated the association between the RAD51 315G>C polymorphism with acute adverse effects in 67 locally advanced rectal cancer patients treated with neoadjuvant radiochemotherapy (Osti et al., 2017). Specifically, RAD51 correlated with acute severe gastrointestinal toxicity in heterozygosity (GC) and homozygosity (CC), in that severe abdominal/pelvis pain toxicity was higher in the GC group and in the GC+CC compared with the GG patients. Acute skin toxicity occurred in 55.6% of the mutated patients versus 22.8% in the WT group for RAD51, suggesting that this RAD51 polymorphism may be a predictive factor for radiation-induced acute toxicity in rectal cancer patients treated with preoperative combined therapy. Pratesi and coworkers investigated the association between the occurrence of acute reactions in 101 patients with head and neck squamous cell carcinoma after radiotherapy and two different RAD51 SNPs, 135G>C and 172G>T (Pratesi et al., 2011). Their results showed that the likelihood of developing moderate to severe dysphagia was higher in carriers of RAD51 135CC/GC genotypes and that the presence of at least one RAD51 SNP or the co-presence of the RAD51 SNP 135G>C and the Q399R polymorphism in its paralog XRCC1 were associated to higher likelihood of occurrence of acute toxicities.

8.3. RAD51 and drug resistance in leukemia - a peculiar relationship

The liaison between the etiological cause of CML - the aberrant, constitutively active cytoplasmic tyrosine kinase BCR-ABL – and RAD51 has been already presented in §7.2.12, where it was reported that elevated levels of RAD51 contributed to drug resistance in BCR-ABL transformed cells (Slupianek et al., 2002), and that the expression of the recombinase

and several of its paralogs is directly regulated by STAT5 (Ferbeyre & Moriggl, 2011). B-cell chronic lymphocytic leukemia (B-CLL) cells are resistant to ICL-inducing agents, such as nitrogen mustards, mitomycin C, cisplatin; yet, the mechanisms governing this drug resistance are still unknown (Sampath & Plunkett, 2007). In this respect, Christodoulopoulos et al. demonstrated that increased chlorambucil-induced RAD51 nuclear foci formation correlated with a drug-resistant phenotype in B-CLL lymphocytes (Christodoulopoulos et al., 1999). In a further study aimed at defining the role of HR in B-CLL resistance to nitrogen mustards, the same group measured the protein levels of RAD51 and its paralog XRCC3 in lymphocytes from seventeen B-CLL patients (Bello, Aloyz, Christodoulopoulos, & Panasci, 2002). They found a significant correlation between both protein levels and drug lethal dose (LD_{50}), suggesting that both proteins influence the cytotoxicity of this aromatic nitrogen mustard. Moreover, since RAD51 expression varies in cell lines during the cell cycle, these authors determined the levels of PCNA to assess possible differences in cell cycle progression. As no correlation between PCNA levels and chlorambucil LD_{50} or RAD51/XRCC3 expression was found, they proposed that the levels of expression of the recombinase and its paralog could be predictive of the response in B-CLL patients treated with this type of ICL-inducing agents. The presence of internal tandem duplications (ITD) mutations in the FMS-like tyrosine kinase 3 (FLT3) receptor influences the risk of relapse in AML (Daver, Schlenk, Russell, & Levis, 2019). Accordingly, Seedhouse and coworkers investigated DNA repair in FLT3-ITD and WT cells and showed that the FLT3 inhibitor PKC412 (Weisberg et al., 2002) significantly inhibited repair of DNA damage in the MV4-11-FLT3-ITD cell line and FLT3-ITD patient samples but not in the HL-60-FLT3-WT cell line or FLT3-WT patient samples (C. H. Seedhouse et al., 2006). Following the discovery that transcript levels of RAD51 were significantly correlated with FLT3 transcript levels in FLT3-ITD patients, they further investigated the role of RAD51 in FLT3-ITD-AML. The reduction in DNA repair in PKC412-treated FLT3-ITD cells was shown to be associated with downregulation of RAD51 mRNA and protein expression and correlated with the maintenance of phosphorylated γH2AX levels, implying that PKC412 inhibits the DSB HR pathway in FLT3-ITD cells. Furthermore, using FLT3-targeted siRNA this group also proved that genetic silencing of FLT3 resulted in RAD51 downregulation in FLT3-ITD cells but not in FLT3-WT cells, and suggested that the use of FLT3 inhibitors such as PKC412 could reverse the drug-resistant phenotype of FLT3-ITD-AML cells by inhibiting repair of chemotherapy-induced genotoxic damage, thereby reducing the risk of disease relapse (Seedhouse et al., 2006).

8.4. RAD51 and the epithelial-mesenchymal transition-associated drug resistance

The epithelial-mesenchymal transition (EMT) is the process by which polarized epithelial cells that are connected via adhesion lose their characteristics and acquire migratory and invasive properties distinctive of mesenchymal cells. Particularly, during EMT actin cytoskeleton reorganization results in a reduction of the expression of proteins that promote epithelial cell-cell junctions (e.g., E-cadherin and β-catenin, Fig. 47) and stimulates the expression of mesenchymal markers including vimentin, fibronectin, α-smooth muscle actin and N-cadherin (Lamouille, Xu, & Derynck, 2014).

Although EMT is crucial for the development and differentiation of multiple tissues and organs, it also contributes to the tumorigenic properties of the cell and cancer cell metastasis (Brabletz, Kalluri, Nieto, & Weinberg, 2018; Dongre & Weinberg, 2019; Mittal, 2018). Furthermore, the process of EMT confers drug resistance characteristics to cancer cells against a variety of drugs (Shibue & Weinberg, 2017). In this respect, the role of RAD51 in the complex mechanisms leading to the acquisition of EMT-associated drug resistance in cancer cells has not been identified yet. However, while investigating gemcitabine-resistant PANCCs that acquired EMT phenotype Nagathihalli and Nagaraju discovered that

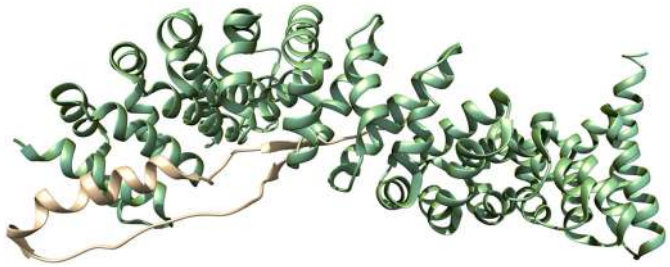

Fig. 47. Crystal structure of the E-cadherin cytoplasmic domain (light tan) complexed with the so-called armadillo repeat region of β-catenin (olive drab) (PDB: 1I7X (Huber & Weis, 2001)).

siRNA-mediated downregulation of RAD51 in highly chemoresistant PANCC lines resulted in a partial EMT reversal (i.e., mesenchymal-epithelial transition or MET), associated with a decreased expression of the mesenchymal markers vimentin and N-cadherin (Nagathihalli & Nagaraju, 2011). However, whether silencing of RAD51 and the related observed MET effectively restore chemosensitivity in drug-resistant cancer cell lines still remains to be determined.

Most, if not all, invasive BCs have a pre-invasive stage defined as ductal carcinoma in situ (DCIS). There is increasing evidence that progression from early ductal hyperplasia to the onset of invasive ductal carcinoma (IDC) is a result of escalating levels of genomic instability, which culminate in the accrual of detrimental mutations in tumor suppressing genes (Duijf et al., 2019). The singleminded homolog 2s (SIM2s) transcription factor is a key regulator of mammary epithelial cell differentiation (Wellberg, Metz, Parker, & Porter, 2010), and loss of SIM2s expression is associated with EMT both in normal breast and BC cell lines (Laffin et al., 2008). In 2013, Scriber et al. demonstrated that i) SIM2s is lost with progression from DCIS to IDC, ii) SIM2s re-expression inhibited growth and metastasis and promoted a more luminal-like phenotype, and iii) down-regulation of SIM2s led to an increase in invasive potential (Scribner, Behbod, & Porter, 2013). In a more recent effort, Pearson and coworkers described a novel role for SIM2s activation in response to DNA damage. Specifically, they found that SIM2s interacts with ATM and is stabilized through ATM-dependent phosphorylation in response to IR. Once stabilized, SIM2s interacts with BRCA1 and supports RAD51 recruitment to the site of DNA damage. Silencing of SIM2s via RNAi or by mutating SIM2s at one of the predicted ATM phosphorylation sites (S115) reduces HR efficiency through disruption of RAD51 recruitment, resulting in genomic instability EMT induction. In particular, the EMT induced by the S115A mutation is characterized by a decrease in E-cadherin and an induction of the basal marker, KER-14, resulting in increased invasion and metastasis. Together, these results identify this transcription factor as a novel player in the DDR pathway and provides a link in DCIS progression to IDC through loss of SIM2s, increased genomic instability via negative interference with RAD51, EMT, and metastasis (Pearson et al., 2019).

Although this complex field undoubtedly awaits further research, the results discussed above provide mechanistic evidence of a relationship between RAD51 and the acquisition of EMT phenotype by different cancer cells, further suggesting that inactivation/inhibition of RAD51 could constitute a potential alternative approach for overcoming drug resistance in cancer therapeutics.

8.5. RAD51 and drug resistance in cancer stem cells

A critical aspect underlying the transition from benign to malignant lesions is the progressive acquisition of the undifferentiated state. In general, benign tumors are often more differentiated while malignant cancers are poorly differentiated (anaplastic) implying a reversal of the differentiation signals during development. Many of the signals that drive the undifferentiated state play also a major role in conferring a stem cell fate – that is, the ability of a cell to perpetuate itself through

self-renewal and to generate mature cells of a particular tissue through differentiation. Stem cells (SCs) are relatively rare among other cell types, can remain dormant during most of their life time, are resistant to toxin and chemicals, and are endowed with enhanced DDR (Reya, Morrison, Clarke, & Weissman, 2001). In addition, SCs are also integrally linked to cancer initiation; in fact, driver mutations that cannot transform differentiated cells can transform undifferentiated ones, suggesting that the SC or progenitor cell state provides a more permissive context for transformation (Lytle, Barber, & Reya, 2018). Even after cancer establishment, perpetuation of a SC state in a small population of cells creates cancer stem cells (CSCs) (Nassar & Blanpain, 2016), a niche of driver cells that are preferentially aggressive and contribute a substantial risk of therapy resistance and disease relapse (Phi et al., 2018).

The peculiar resistance of CSCs to radiation and/or chemotherapy appears to be related to the associated overexpression of drug efflux pumps and the fact that, being SCs, the DNA repair machineries in these cells are distinct and more efficient with respect to normal cells, as mentioned above (Dean, Fojo, & Bates, 2005; Schulz, Meyer, Dubrovska, & Borgmann, 2019). SCs undergo asynchronous DNA synthesis (ADS) and asymmetric self-renewal (ASR). During ADS, the parental 'immortal' DNA strand always segregates with the parental SC rather than with the differentiating progeny, thus contributing to protect the SC niche from DNA damage. In addition, SCs also undergo immortal DNA strand co-segregation, which prevents accumulation of mutations associated with replication errors or DNA lesions arising from damaging agents (Merok, Lansita, Tunstead, & Sherley, 2002). Under this perspective, it is tempting to speculate that RAD51, as a key player in HR of damaged DNA, might have a role in the efficient DDR characterizing both SCs and CSCs. This hypothesis is supported by the fact that several genes operative in the alternative DDR pathways of MMR (§3.l.2) and NHEJ (§5.2.1) (e.g., Ku80, XPG, XRCC1, XPD, RAD23B and MSH2, just to name a few) are overexpressed in SCs (Vitale, Manic, De Maria, Kroemer, & Galluzzi, 2017).

To date, whether RAD51 is overexpressed and/or modulated differently by HR mediators to increase DNA repair efficiency in SCs/CSCs remains to be definitively ascertain. Concerning cancer, both patient-derived and cultured CSCs exhibit a robust DDR as compared to relatively more differentiated malignant cells, which underlies their elevated resistance to antineoplastic agents with genotoxic activity. In 2006, Bao et al. firstly reported that human glioblastoma stem cells (GSCs) activate DNA damage checkpoint in response to IR, and that they repair IR-induced DNA damage more efficiently than normal cells (Bao et al., 2006). The role of RAD51-dependent DNA repair on sensitivity to radiation and the alkylating agent temozolomide (TMZ) as single agent or in combination was investigated by Short et al. using established glioma cell lines, early passage glioblastoma multiforme (GBM) cell lines, and normal human astrocytes (NHAs) (S. C. Short et al., 2011). Their data revealed that in established glioma cell lines RAD51 was upregulated compared to NHAs, with a dose-dependent increase in RAD51 foci formation after IR and TMZ. Also, in these cells RAD51 levels inversely correlated with radiosensitivity, and recombinase downregulation with RNAi markedly increased TMZ cytotoxicity and promoted more residual γH2AX foci 24h after combined treatment. Newly established GBM cell lines were also characterized by high levels of RAD51 and high sensitivity to recombinase knockdown. Successive efforts confirmed that patient-derived GSCs display both increased HR efficiency (Lim et al., 2012) and superior NHEJ activity (Yuan, Eberhart, & Kai, 2014) as compared to neural progenitors. In particular, GSCs obtained from patients or xenografts contain high levels of RAD51 (and of cell cycle checkpoint protein RAD17 (RAD17)) involved in ATR signaling, §5) at baseline, together with signs of replication stress (Bao et al., 2006). Along this line, very recently the study by King et al. performed again using clinical samples and patient-derived GSCs confirmed that RAD51 is highly expressed in these cells, which are reliant on RAD51-dependent DSB repair after radiation (King et al., 2017). RAD51 expression and RAD51 foci numbers fall when these cells move toward astrocytic differentiation. Interestingly, treating GSCs with RAD51 specific inhibitors prevented RAD51 focus formation, reduced DNA DSB repair, and caused significant radiosensitization. All these results led the authors to conclude that RAD51-dependent DNA repair represents an effective and specific target in GSCs. In the same setting, Tachon and coworkers examined the consequence of IR and the potential effect of RAD51 inhibition on cell cycle progression in GSCs (Tachon et al., 2018). Five radiosensitive and five radioresistant GSC lines were exposed to IR (4Gy) and analyzed at different times after exposure with or without the presence of RI-1, a RAD51 inhibitor (Budke et al., 2012). Upon irradiation only, all GSC lines showed a significant increase in the G2 phase at 24h, which was maintained up to 72h; however, when subjected to IR in the presence of RI-1, radioresistant GSCs showed delayed G2 arrest post-irradiation up to 48h. These results demonstrate that all GSCs can promote G2 arrest in response to IR-induced DNA damage, but inhibition of RAD51 leads to different cell cycle response, and support the rationale of targeting RAD51-dependent HR in view of radiosensitizing GSCs.

In the field of BC, Al-Assar et al. found that the MDA-MB231 BC cells were more resistant to IR when sorted for the two SC markers - the signal transducer CD24 (CD24) and the epithelial specific antigen (ESA), and correlated this evidence with a statistically significant increase in RAD51 expression and decrease of γH2AX foci compared to the unsorted population as a direct consequence of a larger S-G2 fraction (Al-Assar et al., 2011). Liu and coworkers investigated the role of RAD51 in mediating CSCs resistance to PARPis using both *in vitro* and *in vivo* models (Y. Liu et al., 2017). These authors demonstrated that the BCSCs in BRCA1-mutant TNBCs are resistant to PARPi and have elevated RAD51 protein levels and activity; moreover, downregulation of RAD51 via RNAi sensitizes CSCs to PARP inhibition and reduces tumor growth. Notwithstanding they found that BRCA1-WT cells were relatively resistant to PARP inhibition alone, the reduction of RAD51 sensitized both CSCs and bulk cells in these tumors to treatment with PARPi. Accordingly, these authors suggested that strategies aimed at targeting RAD51 may increase the therapeutic efficacy of PARPi for the treatment of both BRCA1-mutant and BRCA1-WT TNBCs (Liu et al., 2017). Using pancreatic CSCs isolated on the basis of their ability to form tumor spheres, Mathews et al. found that these structures showed increased expression of the DNA repair proteins – especially BRCA1 and RAD51 and were able to repair gemcitabine-induced DNA damage while their control pancreatic cancer cells failed to do so (Mathews et al., 2011). Finally, Ruiz et al. found that CSCs derived from cervical cancer cell lines overexpressed RAD51 and were less sensitive to the topoisomerase II inhibitor etoposide (VP16) (Ruiz et al., 2018). By inhibiting RAD51 in CSC-enriched cultures either using the natural compound resveratrol or via RNAi, they observed a decrease in cell viability and induction of apoptosis when cells were treated simultaneously with VP16, and reported that resveratrol-mediated inhibition of RAD51 expression also sensitized CSCs to VP16 treatment.

Concomitantly, however, a number of studies reported no difference or even lower DDR in CSCs (Lundholm et al., 2013; Magee, Piskounova, & Morrison, 2012; McCord, Jamal, Williams, Camphausen, & Tofilon, 2009; Ropolo et al., 2009). These contradictory observations suggest that an improved DNA damage response may not be a common feature of CSCs. More obvious is that CSCs and non-CSCs are transient populations and that, in addition to intertumoral heterogeneity, intratumoral heterogeneity must also be considered in DNA damage reaction functionality (Magee et al., 2012).

8.6. RAD51 and hypoxia chemoresistance in cancer cells

During tumor development and progression, cancer and stromal cells often have restricted access to nutrients and oxygen. Most solid tumors indeed have regions permanently or transiently subjected to hypoxia because of aberrant vascularization and poor blood supply (Harris,

2002; Hockel & Vaupel, 2001). Hypoxic cancer cells are usually aggressive, therapy resistant, and have the ability to metastasize. Also, cancer cells exposed to hypoxia undergo replication stress, resulting in the activation of the DDR pathways (Eales, Hollinshead, & Tennant, 2016; Petrova, Annicchiarico-Petruzzelli, Melino, & Amelio, 2018). In this context, already in 2004 Bindra et al. reported that hypoxia specifically down-regulates the expression of RAD51 (Bindra et al., 2004), as decreased recombinase levels were observed in multiple cancer cell types during hypoxic exposure and were not associated with the cell cycle profile. Their analyses of RAD51 gene promoter activity, as well as mRNA and protein stability, indicated that the hypoxia-mediated regulation of this gene occurs via transcriptional repression and independently of the expression of the hypoxia-inducible factor 1-α (HIF1-α). In the same study, the decreased expression of RAD51 was also observed to persist in post-hypoxic cells for as long as 48 h following reoxygenation. Correspondingly, reduced levels of HR were found in both hypoxic and post-hypoxic cells, and the hypoxia-mediated downregulation of RAD51 was further confirmed by these authors *in vivo*. Three years later the same group confirmed that the downregulation of RAD51 by hypoxia is transcriptionally controlled and specifically mediated by the repressive complex formed by the transcription factor E2F4 and the retinoblastoma-like protein 2 (p130) (Bindra & Glazer, 2007). Mechanistically, these authors found that hypoxia induces substantial p130 dephosphorylation and nuclear accumulation, leading to the formation of the E2F4/p130 complexes and their subsequent binding to a single E2F site in the proximal promoter of the RAD51 gene. In close connection, they also reported the unexpected evidence that the clinical targeting of PARP, either via PARPi or siRNAs targeted to PARP1, can inhibit HR by suppressing expression RAD51 (and of BRCA1) via the same mechanism in play under hypoxia (Hegan et al., 2010). Indeed, PARP inhibition was found to cause increased occupancy of the RAD51 (and BRCA1) promoter(s) by the repressive E2F4/p130 complex, and the disruption of p130 by e.g., siRNA knockdown, reversed the cytotoxicity and radio-sensitivity associated with PARP inhibition, suggesting that the down-regulation of RAD51 and BRCA1 is central to these effects.

Under a similar perspective, Wu and coworkers reported that PC cells with mutant p53 i) were resistant to PARPi or DNA-damaging agents under hypoxia, ii) upregulation of RAD51 by the transcription factor E2F1 upon DNA damage under oxygen deprivation contributed to such resistance, and iii) resistance was reversed by inhibiting RAD51 transcription via RNAi (Wu, Wang, McGregor, Pienta, & Zhang, 2014). Contextually, combination therapies based on PARPi and DNA-damaging agents significantly enhanced DNA damage and apoptosis via RAD51 upregulation under both hypoxic and normoxic conditions *in vitro* and *vivo*. Overall, these data illustrate a dynamic regulation of RAD51 by E2F1 and p53 in the response of PC cells to hypoxia and DNA damage. In a BC context, Lu et al. demonstrated that hypoxia induces epigenetic modifications of the RAD51 and BRCA1 promoters, with the HIF1-α-independent histone H3 lysine 4 (H3K4) demethylation as a key repressive modification produced by the lysine-specific histone demethylase 1A (LSD1, Fig. 48, left) resulting in BRCA1 epigenetic silencing (Y. Lu, Chu, Turker, & Glazer, 2011).

Interestingly, although RAD51 can be downregulated by hypoxia in a manner utterly similar to that for BRCA1, no evidence for RAD51 silencing was observed. The authors speculated that this reflects the severe growth disadvantage that the absence of RAD51 would place on human cells, consistent with reports that full RAD51 knockout is lethal to cells while BRCA1 silencing is not (Sonoda et al., 1998). In fact, cancer cells in which the BRCA1 promoter is silenced would lack the genome maintenance and tumor suppressor functions of the gene and so could in theory develop a growth advantage that would lead to expansion during tumor progression. Accordingly, this could explain the frequent observation of silenced BRCA1 genes in human cancers.

In primary CRCs, and particularly in the aggressive Consensus Molecular Subtype 4 (CMS4) (Guinney et al., 2015), gene expression signatures reflecting hypoxia and a stem-like phenotype are highly expressed; conversely, the expression of DNA repair genes is strongly suppressed in CMS4 and inversely correlated with both HIF1-α and HIF2-α co-expression signatures (Jongen et al., 2017). In particular, Jongen et al. verified that in human CRCs the expression of the repair proteins RAD51, Ku70 (§5) and RIF1 (§5.2) was strongly suppressed in hypoxic peri-necrotic tumor areas. Also, in this study experimentally induced hypoxia in patient derived colonospheres *in vitro* or *in vivo* was sufficient to downregulate repair protein expression and to cause DNA damage, while hypoxia-induced DNA damage could be prevented by expressing the hydroperoxide-scavenging enzyme glutathione peroxidase-2 (GPx2, Fig. 48, right), suggesting that ROS play a role in hypoxia-induced DNA damage (Jongen et al., 2017).

The von Hippel-Lindau tumor suppressor gene (VHL) is inactivated in the vast majority of human clear cell renal carcinomas. The pathogenesis of VHL loss is currently best understood to occur through stabilization of the hypoxia-inducible factors, activation of hypoxia-induced signaling pathways, and transcriptional reprogramming towards a pro-angiogenic and pro-growth state. However, hypoxia also drives other pro-tumorigenic processes, including the development of genomic instability via down- regulation of DNA repair gene expression. In this context, the Glazer group investigated the possibility that VHL mutations, through induction of hypoxia-like signaling pathways, may lead to down-regulation of DDR pathways and sensitivity to DNA damage (Scanlon, Hegan, Sulkowski, & Glazer, 2018). They found that VHL-deficient human renal carcinoma cells have reduced protein and mRNA expression of key HR and MMR genes down-regulated by hypoxia, including BRCA1, RAD51, FANCD2, and MLH1. Using siRNA

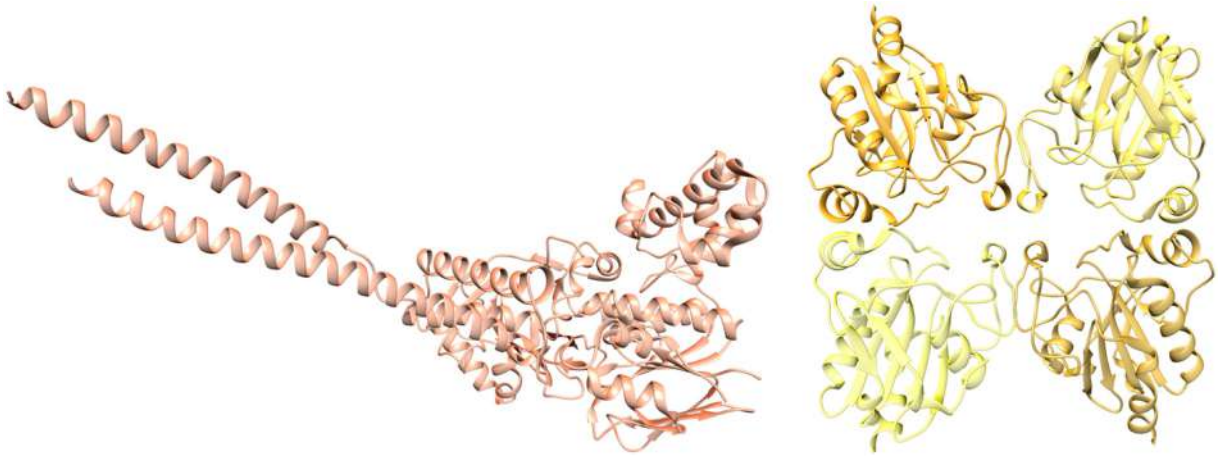

Fig. 48. (Left) Crystal structure of LSD1 (PDB: 6NQM (Tan et al., 2019)). (Right) Crystal structure of biomolecular assembly of GPx2 (PDB: 2HE3, unpublished).

depletion, they demonstrated that this reduced gene expression is directly linked to loss of the VHL protein, and have further established that the decrease in HR gene expression is associated with reduced repair of DNA DSBs by HR and consequent sensitivity to PARP inhibitors in VHL-deficient renal carcinoma cells.

8.7. miRNAs and long non-coding RNAs as mediators of RAD51 drug resistance

miRNAs are small, non-coding endogenous RNAs that function in regulation of gene expression (Gebert & MacRae, 2019). Compelling evidences have demonstrated that miRNA expression is dysregulated in cancer through various mechanisms, including amplification or deletion of miRNA genes, abnormal transcription control of miRNAs, and dysregulated epigenetic changes and defects in the miRNA biogenesis machinery. miRNAs may function as either oncogenes or oncosuppressors under certain conditions, and dysregulated miRNAs have been shown to affect the hallmarks of cancer, including sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, promotion of EMT, activation of invasion and metastasis, and induction of angiogenesis (Peng & Croce, 2016).

The contribution of miRNAs in the regulation of HR is currently an issue of active investigation (Natarajan, 2016; Thapar, 2018). In 2012, Wang et al. reported a study showing that overexpression of miR-96 in human cancer cells reduced the levels of both RAD51 and the TLS polymerase REV1, and impacted the cellular response to agents that cause DNA damage (Wang, Huang, Calses, Kemp, & Taniguchi, 2012). Specifically, miR-96 directly targeted the coding region of RAD51 and the 3'-untranslated region of REV1, and that its overexpression decreased the efficiency of HR and enhanced sensitivity to the PARPi AZD2281 *in vitro* and to cisplatin both *in vitro* and *in vivo*. Thus, miR-96 was indicated as a regulator of DNA repair and chemosensitivity via repression of RAD51 and REV. One year later, the same group performed a systematic screening of a library of human miRNA mimics to identify several miRNAs that significantly reduce RAD51 foci formation in response to IR in human osteosarcoma cells (Huang et al., 2013). Consistent with the inhibition of RAD51 foci formation, they found that other two miRNAs - miR-103 and miR-107 - reduced HR and sensitized cells to various DNA-damaging agents, including cisplatin and a PARPi. Mechanistic analyses revealed that both miRNAs directly target and regulate RAD51 and its paralog RAD51D, which is critical for miR-103/107-mediated chemosensitization. Furthermore, endogenous regulation of RAD51D by miR-103/107 was observed in several tumor subtypes, confirming the role for these two miRNAs in regulating DDR and identifying new players in the progression of cancer and response to chemotherapy (Huang & Li, 2013).

The miR-34s family consists of miR-34a, and mir-34b/34c, which are located in two disparate loci but share the same seed sequence. Concerning the specific role of miR-34s in HR, recently Chen et al. reported that miR-34s overexpression results in suppression of RAD51 and upregulation of γH2AX, supporting the involvement of this microRNA in the process of HR (Chen et al., 2019). In another effort, the same group demonstrated that miR-34s directly targets the RAD51 mRNA 3'-UTR or indirectly inhibits RAD51 expression via the p53-signaling, highlighting a novel mechanism of HR pathway via the miR-34s/p53/RAD51 axis (Shen et al., 2018). Cortez et al. also verified that miR-34a binds to the 3'-UTR of RAD51 and regulates homologous recombination by inhibiting DSB repair in NSCLC cells (Cortez et al., 2015). Further, they demonstrated the therapeutic potential of miR-34a delivery in combination with radiotherapy in a mouse models of lung cancer. miR-506 is a potent inhibitor of the EMT and, according to the study by Liu et al., it was associated with better response to therapy and longer progression-free survival in two independent epithelial ovarian cancer (EOC) patient cohorts (Liu et al., 2015). In the same work, using an orthotopic OC mouse model the authors found that miR-506 sensitized cells to DNA damage through directly targeting RAD51. Furthermore, the systemic delivery of miR-506 in 8 to 12-week-old female athymic nude mice statistically augmented cisplatin and the PARPi olaparib response, in line with the clinical observations. Croce and coworkers showed that overexpression of miR-155 in human BC cells reduced the levels of RAD51 by directly targeting its 3'-UTR region and, in so doing, enhanced the cellular response to IR (Gasparini et al., 2014). Most importantly, high miR-155 levels were also associated with decreased HR efficiency, enhanced sensitivity to IR and better OS of patients in a large series of TNBCs. Accordingly, the authors proposed that testing for expression levels of miR-155 could be useful in the identification of BC patients who might benefit from an IR-based therapeutic approach. Choi et al. conducted a gain-of-function screen to identify miRNAs that regulate HR-mediated DSB repair, and found that miR-1255b, miR-148b*, and miR-193b* specifically suppress the HR pathway by targeting the transcripts of HR factors BRCA1, BRCA2 and RAD51 in the G1 phase (Choi et al., 2014). Inhibition of these miRNAs increased the expression of BRCA1/2 and RAD51 leading to impaired DSB repair, while depletion of CtIP rescued this phenotype. Also, according to this study, the deletion of miR-1255b, miR-148b*, and miR-193b* in independent cohorts of OCs correlated with significant increase of LOH events/chromosomal aberrations and BRCA1 expression. Piotto and coworkers examined a pool of HR and NHEJ genes including RAD51, BRCA2, XRCC5 and LIG1 to determine whether they could be real targets of selected miRNAs by functional and biological studies (Piotto, Biscontin, Millino, & Mognato, 2018). In aggregate, their results showed that miR-96-5p and miR-874-3p directly regulated the expression these target genes; moreover, these miRNAs synergized with IR in decreasing the survival of NSCLC cells to an extent comparable to that achieved upon combination of IR and specific HR or NHEJ inhibitors.

Besides miRNAs, also long non-coding RNA sequences (lncRNA) – usually molecules longer than 200 nucleotides – are found to be involved in mRNA translation, transcription processes, cell development, proliferation and apoptosis (Yao, Wang, & Chen, 2019). It is therefore not surprising to find a direct implication of lncRNA in human diseases. Specifically, in cancer, increasing evidence has strengthened the notion that lncRNA exert cooperative functions to tumor suppression or tumorigenesis (Sanchez Calle, Kawamura, Yamamoto, Takeshita, & Ochiya, 2018). In a study performed by Shen et al., the knockdown of the ionizing radiation-inducible lncRNA (lnc-RI) resulted in a significant increase of spontaneous DNA DSBs, confirmed by the associated decrease efficiency of the HR pathway and the drastic decline in the related expression of RAD51 (L. Shen et al., 2018). In the same effort, the authors verified that the miRNA miR-193a-3p could bind both lnc-RI and the RAD51 mRNA, thereby repressing their expression. In particular, lnc-RI was shown to act as a competitive endogenous RNA (ceRNA) by stabilizing RAD51 mRNA via competitive binding with miR-193a-3p and release of its inhibition of RAD51 expression. These results support a critical role of lnc-RI un regulating DSB repair and, hence, in the maintenance of genomic integrity.

9. RAD51 as a target in cancer therapeutics

In the light of what reported above it is evident that RAD51 plays a role in the progression of malignancy on addition to its pivotal function in DNA HR. Specifically, the recombinase can promote cancer progression by two distinct mechanisms: indirectly, via increased/aberrant HR, and directly, by upregulating pro-metastatic gene expression. Accordingly, RAD51 can be considered a clinically relevant cancer target, and a number of strategies are currently being developed to exploit this protein either directly (by inhibiting its recombinase functions and activity or interfering with its interaction with other proteins), or indirectly (e.g., by downregulating its expression) in oncology therapeutics. However, so far only 1 clinical trial directly targeting RAD51 is currently active (https://clinicaltrials.gov/ct2/show/NCT03997968), despite the huge efforts devoted to the design, synthesis and activity evaluation of small molecule RAD51 inhibitors (Budke, Lv, Kozikowski, &

Connell, 2016). On the other hand, synthetic lethality (SL) (O'Neil, Bailey, & Hieter, 2017) targeting the DDR pathways and HR deficiencies has shown some clinical success, and is currently a hot topic of research (Ward, Khanna, & Wiegmans, 2015). SL is a term introduced by geneticists and refers to the death of cells caused by concomitant perturbations of two genes (loss-of-function mutations, RNA interference, drug treatment, etc.), each of which is nonlethal alone. Accordingly, synthetic lethal interactions can expand the repertoire of anticancer therapeutic targets, as they facilitate the indirect targeting of e.g., non-druggable oncogenes through the identification of a second-site synthetic lethal target that may be druggable.

Given the fact that RAD51 is an essential gene, and that the currently available RAD51 direct inhibitors have not yet entered their translational phase, the last part of this review will examine the recent development in indirect RAD51 targeting, highlighting the most promising SL studies endowed with exploitation potential as anticancer therapeutics. The reader interested in the drug discovery and medicinal chemistry aspects related to the development of small molecules directly targeting RAD51 and/or other DDR proteins are referred to the last, excellent review works in the field (Brown, O'Carrigan, Jackson, & Yap, 2017; Budke et al., 2016; Carvalho & Kanaar, 2014; Desai, Yan, & Gerson, 2018; Fujii, 2017; Gavande et al., 2016; Hengel, Spies, & Spies, 2017; F. Huang & Mazin, 2014; Kopa, Macieja, Galita, Witczak, & Poplawski, 2019; Minchom, Aversa, & Lopez, 2018; O'Connor, 2015; Pearl, Schierz, Ward, Al-Lazikani, & Pearl, 2015; Srivastava & Raghavan, 2015; Velic et al., 2015; Ward et al., 2015).

Before beginning any discussion about SL and RAD51, it is important to observe that there is a considerable crosstalk among SSBs, DSBs, and stalled replication fork repair systems, reflecting several mechanistic commonalities in these pathways, i.e., lesion recognition, ssDNA binding, structure-specific endo- and exonuclease cleavage, strand annealing, polymerase gap filling, and ligation. Repair pathways display several types of crosstalk, like i) signaling crosstalk (e.g, between HR and cNHEJ pathways through ATR, ATM, and DNA-PK), ii) functional crosstalk (in which the overexpression of a DNA repair component in one pathway compensates for a repair deficit in another, conferring therapeutic resistance), and iii) direct crosstalk (where specific components are shared among pathways, e.g., PARP1 in BER and aNHEJ).

If on the one hand a defect in any of these DDR pathways can result in malignant transformation and any pathway can be subverted to assist cancer cell proliferation and survival, on the other hand the crosstalk network in the DDR can be advantageously exploited from the therapeutic perspective like in e.g., SL. A prototypical example is the inherited mutations in the HR components BRCA1 and BRCA2 in breast and ovarian cancers (H. Kobayashi, Ohno, Sasaki, & Matsuura, 2013). All cells in these patients have one mutated allele of BRCA1 (or 2), but the cancer has both alleles mutated, and the cancer cell has an unstable genome because it has lost HR capability. Since HR is the most important pathway for repairing and restarting stalled replication forks, these cancers become dependent on PARP1-mediated SSB repair and aNHEJ to repair and restart their replication forks (Farmer et al., 2005). When PARP1 is inhibited by a drug, then these cancers cannot repair and restart replication forks, the stalled forks collapse and can aberrantly ligate together, and distinct chromosomes can fuse resulting in mitotic catastrophe and subsequent apoptosis (Nickoloff, Jones, Lee, Williamson, & Hromas, 2017).

Unfortunately, the molecular rationale to induce SL by targeting defective HR in TNBCs has also shown several shortcomings. Not meeting the expected minimal outcomes in clinical trials has highlighted common clinical resistance mechanisms including increased expression of PARP1, increased expression or reversion mutation of BRCA1, or upregulation of RAD51 (Ashworth & Lord, 2018). To overcome selective pressure on DDR pathways, the Wiegmans group examined new potential targets within TNBC that demonstrate SL in association with RAD51 depletion (Wiegmans, Miranda, Wen, Al-Ejeh, & Moller, 2016). They confirmed complementary targets of PARP1/2 and DNA-PK as well as a new SL combination with p38 mitogen-activated protein kinase (MAPK) signaling pathway, a relevant target in BC as implicated in resistance to several chemotherapeutics including tamoxifen (Kruger et al., 2018). The combination of targeting RAD51 and MAPK inhibited cell proliferation both *in vitro* and *in vivo*, which was further enhanced by targeting of PARP1. Analysis of the molecular mechanisms revealed that depletion of RAD51 increased both extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and MAPK signaling, highlighting a potential compensatory mechanism via MAPK that limits DNA targeted therapy.

Insulin and insulin-like growth factors (IGFs) are well known as key regulators of energy metabolism and growth. Yet, there is now considerable evidence that these hormones and the signal transduction networks they regulate have important roles in neoplasia (Pollak, 2008). In particular, BRCA1 has been shown to directly affect the IGF type 1 receptor (IGF-1R) (Werner & Bruchim, 2012), and studies have suggested that BRCA1/2 deficient BC and ovarian cells are associated with elevated expression of this receptor (Kang et al., 2012). Accordingly, using BC and OC cell lines with known BRCA1 status Amin and coworkers showed that those cells with mutated/methylated BRCA1 exhibited impaired HR function and overactivation of the IGF-1R pathway (Amin et al., 2015). These cells were more sensitive to IGF-1R inhibition compared to HR-proficient cells, and the reasons for this was ascribed to the reduced RAD51 expression at mRNA and protein levels induced by IFG-1R inhibition. Along a similar line, experiments performed by Lodhia et al. showed a time-dependent accumulation of γH2AX foci in IGF-1R-inhibited or depleted PC cells, and that RAD51 depletion enhanced cell sensitivity to IGF-1R inhibitors in phosphatase and tensin homolog (PTEN) WT PC cells but not in cells lacking functional PTEN (Lodhia, Gao, Aleksic, Esashi, & Macaulay, 2015).

The Hippo pathway is the major regulator of organ growth and proliferation, and the mammalian transcriptional activator yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ) are two core kinases in this pathway (Ma, Meng, Chen, & Guan, 2019). In cancer, the Hippo signaling is inactivated, and YAP and TAZ are activated and free to translocate into the nucleus to promote cell proliferation. Furthermore, nuclear YAP/TAZ activate or suppress transcription factors that regulate target genes involved in cell proliferation, tissue growth, control of organ size and shape or metastasis (Zanconato, Cordenonsi, & Piccolo, 2019). Independently of its role in angiogenesis and vascular permeability, the vascular endothelial growth factor (VEGF) signaling mediated by neuropilins (NRPs, another family of VEGF receptors) contributes in enhancing the aggressive and drug-resistant characters of several cancer cells. TNBCs, that manifest VEGF-NRP2 signaling (Napolitano & Tamagnone, 2019) and are resistant to standard therapy (Bianchini, Balko, Mayer, Sanders, & Gianni, 2016), are prototypical examples. Very recently, Elaimy and coworkers demonstrated that the autocrine VEGF-NRP2 signaling contributes to DNA HR and therapy resistance in TNBC cells by promoting YAP/TAZ-dependent RAD51 transcription (Elaimy et al., 2019). Specifically, these authors showed that RAD51 is a YAP/TAZ target gene, and that VEGF-NRP2-YAP/TAZ-mediated cisplatin resistance occurs through downstream RAD51 expression. These observations provided the first evidence of an integrated mechanism that governs RAD51 expression and HR in TNBC.

PTEN deletions in prostate cancer are associated with tumor aggression and poor outcome; yet, similarly to BRCA1/2-defective tumor cells, PTEN-null prostate and other cancers have been reported to be sensitive to PARPi (Jamaspishvili et al., 2018). To investigate whether PTEN is implicated as a determinant of HR through RAD51, and to determine the eventual liaison between PTEN and RAD51, Fraser et al. analyzed the expression of both PTEN and RAD51 in primary PCs of known PTEN status (Fraser et al., 2012). They found that PTEN status was not associated with reduced RAD51 mRNA or protein expression in these cancers, and that PTEN-deficient calls had only mild PARPi sensitivity and no loss of HR or RAD51 recruitment, suggesting an indirect and more

complex relationship between PTEN status and DNA repair. STAT5 is overexpressed in PC compared with normal prostate epithelium (Igelmann, Neubauer, & Ferbeyre, 2019), and the levels of this protein positively correlate with the aggressiveness of this malignancy (Gu et al., 2010; Mirtti et al., 2013). Also, STAT5 undergoes gene amplification during PC progression to castrate-resistant metastatic disease (Haddad et al., 2019). Since STAT5 has been linked to DNA repair in CML (§8.3), Maranto et al. investigate whether this signal transducer and activator of transcription could have a role in the regulation of DNA DSB repair in PC (Maranto et al., 2018). Their results clearly showed that STAT5 is critical for RAD51 expression in PC via a tyrosine-protein kinase JAK2 (JAK2)-dependent mechanism by reducing RAD51 mRNA levels. Consistently with this, silencing of STAT5 suppressed HR while it did not affect NHEJ. Also, the pharmacological inhibition of STAT5 potently sensitized PC to IR *in vitro* and *in vivo*, with the remarkable absence of radiation-induced sensitivity in neighboring tissues in the last case.

In EOC, Ceccaldi and coworkers have demonstrated an inverse correlation between HR activity and Polθ expression by virtue of the crosstalk between the aNHEJ and HR pathways (Ceccaldi et al., 2015). Accordingly, while the knockdown of Polθ in HR-proficient cells upregulated HR activity and RAD51 nucleofilament assembly, the same process in HR-deficient EOCs, which overexpress the polymerase, enhanced cell death. Therefore, this study revealed a SL relationship between Polθ-mediated repair and RAD51 in EOCs, identifying Polθ as a novel druggable target in cancer therapy. Another crosstalk between NHEJ and HR was assessed by Mueck et al. via the RAC-alpha serine/threonine-protein kinase (AKT1) (Mueck, Rebholz, Harati, Rodemann, & Toulany, 2017). AKT1 is one of 3 closely related serine/threonine-protein kinases (AKT1/2/3) called the AKT kinases, which regulate many processes including metabolism, proliferation, cell survival, growth and angiogenesis (Nitulescu et al., 2018). AKT1 is known to promote NHEJ-mediated DNA DSB repair via stimulation of DNA-PKcs (Q. Liu, Turner, Alfred Yung, Chen, & Zhang, 2014). However, in NSCLC cell lines (A549 and H460) the authors found that AKT1 knockdown significantly reduced RAD51 protein levels, foci formation and its co-localization with γH2AX foci after irradiation. Although further analyses are necessary to investigate the functional interaction between AKT1 and RAD51 in stimulating DSB repair, this study offers new aspects for the development of novel strategies for selective targeting of NSCLC cells. Actually, other authors previously reported suppression of RAD51 as a consequence of AKT inhibition. For instance, Pal et al. documented that the inhibition of PI3K in Barrett carcinoma cell lines expressing high levels of its downstream effector AKT resulted in RAD51 suppression, cell growth arrest, and apoptosis (Pal et al., 2012). In another interesting study, inhibition of the epidermal growth factor receptor (EGFR) with the targeted tyrosine kinase inhibitor (TKI) erlotinib in NSCLC cell lines reduced AKT phosphorylation. This, in turn, suppressed RAD51 expression by enhancing both RAD51 mRNA and protein instability, and led to apoptotic cell death (Ko et al., 2009). Interestingly, the enforced expression of a constitutively active AKT vector restored RAD51 protein levels and decreased erlotinib-induced cytotoxicity. Furthermore, endogenous RAD51 knockdown by RNAi significantly enhanced erlotinib cytotoxic effects while induced overexpression of RAD51 protected the cells from the TKI cytotoxic activity. Finally, in line with the study of Pal et al. reported above, inhibition of PI3K with wortmannin inhibited the activation of AKT and, concomitantly, suppressed the expression of RAD51, enhancing the erlotinib-induced cell death even in erlotinib-resistant NSCLC cell lines.

In the same cancer context, Ko et al. reported that the inhibition of heat shock protein 90 (Hsp90) by 17-allylamino-17-demethoxygeldanamycin (17-AGG) decreased cellular RAD51 both at the protein and mRNA level, and that the disruption of the Hsp90/RAD51 interaction by the small molecule promoted RAD51 degradation via the 26S proteasome pathway (Ko et al., 2012). Treating NSCLC cells with 17-AAG also decreased cell's HR capacity, which was recovered by the forced expression of the Flag-RAD51 vector. Also, silencing RAD51 expression via RNAi further enhanced 17-AAG-induced cytotoxicity. The authors hence concluded that Hsp90 inhibition can exert cytotoxicity in NSCLC cells via RAD51 downregulation and impaired HR. On the other hand, Hansen et a. demonstrated the in small cell lung cancer (SCLC) the repair of DNA DSBs induced by etoposide - a highly potent drug yielding remission in 70% of SCLC patients in single drug regimens (Alvarado-Luna & Morales-Espinosa, 2016) - is mediated by both RAD51-dependent HR and DNA-PKcs-dependent NHEJ, and suggested that the levels of these two proteins (and hence the relative contribution of each of these two DDR pathways) could be a determinant of the variation in clinical treatment effects observed in human SCLC tumors of identical histologic type (Hansen, Lundin, Spang-Thomsen, Petersen, & Helleday, 2003).

Radiotherapy has long been considered as the mainstay of treatment for nasopharyngeal carcinoma (NPC). However, locoregional recurrence or distant metastasis may occur in some patients due to the radiation resistance of cancer cells. Autophagy plays a vital role in protecting cells against radiation. However, the mechanism of autophagy in radiation therapy remains obscure. In their study, Mo and coworkers demonstrated that suppression of autophagy related 5 (ATG5) by RNAi aggravated IR-induced DNA damage and apoptosis in human NPC cells without accelerating the cell cycle, whereas regulation of the cell cycle has been widely regarded as the most important determinant of IR sensitivity (Mo et al., 2014). Further experiments showed that inhibition of autophagy suppressed the mRNA expression of RAD51; moreover, suppression of ATG5 had no impact on the radiosensitivity when cells were pre-treated by a RAD51 inhibitor, and the enhanced radiosensitivity by ATG5 suppression was reversed by overexpression of RAD51 in human NPC cells. Thus, these results suggest that inhibition of autophagy enhances the susceptibility of NPC cells to radiation by reducing RAD51 expression.

In the field of brain cancer, IR combined with TMZ represents the standard therapy of the still poorly curable high-grade gliomas (Aldape et al., 2019). Contextually, integrins have been suggested as possible targets in anticancer therapy (Hamidi & Ivaska, 2018). Christmann and colleagues showed that knockdown of integrins αVβ3, αVβ5, α3β1 and α4β1 and pharmacological inhibition using a cyclo-RGD integrin $\alpha\nu\beta3/\alpha\nu\beta5$ antagonist sensitized multiple high-grade glioma cell lines to TMZ-induced cytotoxicity (Christmann et al., 2017). In particular, integrin β3 knockdown led to the proteasomal degradation of RAD51, reduction of RAD51 foci, and reduced repair of TMZ-induced DNA DSBs by impairing HR efficiency. The downregulation of integrin β3 in RAD51 knockdown cells neither further sensitized them to TMZ nor increased the number of γH2AX foci, confirming causality between this integrin silencing and RAD51 reduction. Increased H2AX phosphorylation, caspase-3 cleavage, reduced expression of RAD51 and of the receptor-interacting serine/threonine-protein kinase 1 (RIP-1, a kinase which transduces inflammatory and cell-death signals, activation of pathogen recognition receptors, and DNA damage (Christofferson, Li, & Yuan, 2014)) were also observed in mouse glioma xenografts treated with the cyclo-RGD inhibitor and TMZ, confirming the molecular mechanism *in vivo*, indicating that β3 silencing in glioma cells represents a promising strategy to sensitize high-grade gliomas to TMZ therapy. Quiros and coworkers assessed whether inhibiting DSB repair by HR was a feasible strategy for sensitizing glioma cells to alkylating agents (Quiros, Roos, & Kaina, 2011). The siRNA-mediated knockdown of RAD51 or BRCA2 greatly increased cell death following treatment with TMZ or nimustine (a nitrosourea), while the induced expression of O^6-methylguanine-DNA methyltransferase (MGMT) abolished these effects, indicating that O6-alkylguanine induced by these drugs was the primary lesion responsible for DSBs and glioma cell increased sensitivity following RAD51/BRCA2 silencing. Finally, a triple strategy based on RAD51 silencing, MGMT depletion and PARP1 inhibition by olaparib greatly enhanced the therapeutic effect of TMZ.

Microarray analysis focused on DDR genes performed with the aim of searching for correlations between expression patterns and survival prognosis in astrocytomas revealed that 19 genes were significantly altered (de Sousa et al., 2017). Combining these genes in all possible arrangements, the authors found 421 expression signatures strongly associated with poor survival. Among others, EXO1, BRCA2, and NEIL3 were independently correlated with worse prognosis, revealing single-gene signatures. Silencing of EXO1, which was remarkably overexpressed, promoted faster restoration of DNA DSBs, while NEIL3 knockdown, also highly overexpressed, caused an increment in DNA damage and cell death after irradiation of GB cells. These results disclosed the importance of DNA repair pathways for the maintenance of genomic stability of high-grade astrocytomas and suggest that EXO1 and NEIL3 overexpression confers more efficiency for DSBs repair and resistance to ROS, respectively. In this context, Klattehnoff and coworkers reported that NEIL3 localized at the DSB sites during oxidative DNA damage and replication stress (Klattenhoff et al., 2017). Loss of NEIL3 significantly increased spontaneous replication-associated DSBs and recruitment of RPA. In contrast, the authors reported a marked decrease in RAD51 on nascent DNA strands at the replication fork, suggesting that HR-dependent DDR is compromised in NEIL3-deficient cells. At the same time, NEIL3-deficient cells were sensitive to ATR inhibitors alone or in combination with PARPis, suggesting possible clinical implications in the utilization of ATR and PARPi inhibitors to enhance cytotoxicity in brain and other tumors carrying altered levels of NEIL3.

Krumm et al. reported that malignant melanomas *in situ* contain a high level of histone deacetylases 1 and 2 (HDAC1/2) and malignant melanoma cells overexpress HDAC1/2/3 compared to noncancer cells (Krumm et al., 2016). Accordingly, these authors inhibited HDAC1/2/3 and observed sensitization of melanoma cells on TMZ *in vitro* and in melanoma xenografts *in vivo*. In particular, HDAC1/2/3 inhibition resulted in suppression of DNA DSBs repair by HR because of downregulation of RAD51 (together with FANCD2). Furthermore, knockdown experiments identified HDAC2 as being responsible for the regulations of RAD51, leading to the idea that class I HDAC inhibitors could be used to counteract RAD51/FANCD2-mediated melanoma cell drug resistance. Suberoyl anilide hydroxamic acid (SAHA, aka vorinostat) is another HDAC inhibitor with promising anticancer activity against several malignancies (Behera, Jayaprakash, & Sinha, 2015). In 2017 Wu et al. showed that SAHA enhanced the radiosensitivity of pancreatic cancer cells by downregulating the HR proteins Ku70, RAD51 and RAD54, thereby inducing G2-M cell phase arrest and apoptosis (Wu et al., 2017). SAHA was also found to sensitize HR-proficient OC cells to the PARPi olaparib (Konstantinopoulos, Wilson, Saskowski, Wass, & Khabele, 2014). According to the microarray analysis performed in this study, SAHA induced coordinated down-regulation of HR-pathway genes including RAD51 and BRCA1. In particular, the nuclear co-expression of RAD51 and γH2AX was reduced by nearly 40% by the combined SAHA/olaparib treatment; also, SAHA enhanced olaparib-mediated cell viability reduction in 4 different OC cell lines and in SKOV-3 xenografts *in vivo*.

In the field of AML, Zhao and coworkers employed class- and isoform-specific HDAC inhibitors and siRNA-mediated silencing of individual HDACs to determine which of these enzymes was responsible for the observed decreased expression of BRCA1, CHK1 and RAD51 following AML cell treatment with the pan-HDAC inhibitor panobinostat (Zhao et al., 2017). They found that inhibition of both HDAC1 and HDAC2 was required not only to decrease the expression of the 3 HR key proteins but also to enhance DNA damage and apoptosis, and to abrogate the cell cycle checkpoint activation induced by two AML gold standard treatment, cytarabine- and daunorubicin. Interestingly, Lai et al identified the role of HDACs in silencing miR-182 in AML (Lai et al., 2016). Most importantly, they discovered that this microRNA directly targets RAD51. Specifically, both overexpression of miR-182 or HDAC inhibition-mediated induction of miR-182 were linked to time- and dose-dependent decreases in RAD51 expression, increased levels of residual DNA damage and decreased AML cell survival after exposure to DSB-inducing agents, highlighting a potential new therapeutic strategy in AML.

While investigating melanoma resistance to cisplatin, Song et al. identified and characterized a novel DNA damage response mechanism according to which, instead of increasing levels of RAD51 on encountering cisplatin-induced ICLs during replication, melanoma cells shut down RAD51 synthesis and instead boost levels of the TLS Polθ (§4.2.2) to allow replication to proceed (Song et al., 2017). However, this response resulted in SL to olaparib, suggesting that, of the one side this approach of DNA damage tolerance rather than immediate repair leads to aggressive and rapidly growing tumors, on the other it also results in melanoma exposing an 'Achille's heel' more susceptible to PARPi-based therapies.

MMR-deficient colon cancer cells are sensitive to TOP1 inhibitors such as irinotecan and camptothecin, presumably due to microsatellite instabilities of the MRE11 locus (Fallik et al., 2003). Accordingly, Tahara et al. investigated the synergistic effect of SN-38 (an active metabolite of the TOP1 inhibitor irinotecan) in combination with the PARPi olaparib in colon cancer cells (Tahara et al., 2014). They found that olaparib potentiated S-phase-specific DNA DSBs induced by SN-38, followed by RAD51 recruitment. The RAD51 knockdown via siRNA increased cancer cell sensitivity to olaparib and/or SN-38 treatment, and *in vivo* study using mouse xenografts demonstrated that olaparib was effective in potentiate the antitumor effect of irinotecan. The authors hence concluded that the triple synthetic lethality comprising topoisomerase I-mediated DNA breakage-reunion, PARP inhibition and RAD51-mediated HR pathway may contribute as a potential target for future chemotherapy.

Esophageal cancer progression and chemoresistance are critical factors that impact the survival of patients with ESCA, the sixth leading cause of mortality that accounts for 6.6% of all cancer-related deaths (Bray et al., 2018). In a recent effort, while investigating the role of the important cell cycle regulator cyclin dependent kinase inhibitor 3 (CDKN3) in ESCA progression and chemoresistance *in vitro* and *in vivo*, Wang et al. discovered that this protein was highly expressed in this cancer and served as an independent prognostic factor of this disease (Wang et al., 2019). In detail, their bioinformatic analysis showed CDKN3 involvement in DNA replication, cell cycle G2/M phase transition, and DDR signaling pathways. Functional *in vitro*/*in vivo* experiments demonstrated that CDKN3 promoted ESCA progression and enhanced cisplatin resistance. Importantly, CDKN3 inhibition resulted in reduced expression of RAD51, while recombinase overexpression reversed cisplatin-induced DNA damage and chemosensitivity in CDKN3 inhibited ESCA cell lines.

Chemotherapy is the only choice for the treatment of advanced hepatocellular carcinoma (HCC). (Kumari, Sahu, Tripathy, Uthansingh, & Behera, 2018). HCC is known to frequently overexpress EGFR, which is associated with more aggressive diseases and a poor prognosis (Komposch & Sibilia, 2015). In this respect Shao and coworkers reported a synergistic action between gefitinib (an EGFR inhibitors aka Iressa) and the irinotecan metabolite SN-38 in inducing caspase-mediated apoptosis in HCC cells (Shao et al., 2016). Mechanistically, the authors verified that gefitinib dramatically promoted the ubiquitin-proteasome-dependent degradation of RAD51, thereby suppressing DNA HR and generating more DNA damage in tandem with SN-38. The increased antitumor efficacy of the combined gefitinib/irinotecan treatment was further validated in a HepG2 xenograft mice model, providing a rationale for clinical trials investigating the efficacy of the doubly synthetically-lethal strategy involving the inhibition of topoisomerase I and EGFR.

The SL between EGFR and RAD51 was also exploited by Ko et al. in targeting NSCLC (Ko et al., 2008). Using both human adenocarcinoma (H1650) and bronchoalveolar carcinoma (A549) cell lines, they found that gefitinib decreased the cellular levels of phosphorylated ERK1/2 and, contextually, also the levels of RAD51 by enhancing its instability via 26S proteasome-mediated degradation. Inhibition of endogenous

RAD51 by RNAi significantly enhanced gefitinib-induced toxicity, whereas cell transfection with a constitutively active mitogen-activated protein kinase kinase 1 vector (MAP2K1 or MEK1, an ERK1/2 activator) restored both RAD51 expression levels and cell survival. This demonstrates that the MAP2K1-ERK1/2 signaling pathway constitutes an upstream pathway involved in maintaining high levels of RAD51 expression and, hence, protecting the NSCLC cells against the cytotoxic effects of gefitinib. Thus, inhibition of this pathway leading to RAD51 suppression could constitute a potential therapeutic option for overcoming EGFR-targeted therapy in NSCLC.

As mentioned in §8.1.3, over 90% of pancreatic adenocarcinomas express oncogenic mutant KRAS that constitutively activates the RAF proto-oncogene serine/threonine-protein kinase (RAF)-MAP2K1/ERK pathway conferring resistance to both radiation and chemotherapy. In this field, Estrada-Bernal et al. evaluated whether the MEK1 inhibitor trametinib (GSK212) could alter DNA repair mechanisms in PANCC lines (Estrada-Bernal et al., 2015). They found that the combined IR/trametinib treatment resulted in delayed resolution of DNA damage, with the suppressed expression and activation of a number of DSB repair pathway intermediates, including BRCA1, DNA-PKcs, and RAD51.

Bromodomain and extraterminal domain (BET) proteins are epigenetic readers that regulate gene expression by recruiting and activating the positive transcription elongation factor b (p-TEFb), and are involved in cancer pathogenesis; as such, this family of 4 protein members (BRD2, BRD3, BRD4 and BRDT) represents interesting, emerging targets in cancer therapeutics (Stathis & Bertoni, 2018). This year Mio and collaborators assessed the specific role of BRD4 regulation in HR-mediated DNA repair in TNBC (Mio et al., 2019). By performing a dual approach, based on chromatin immunoprecipitation and RNAi, they confirmed the direct relationship between BRD4 and BRCA1/RAD51 expression in TNBC cells. According to their data, the pharmacological inhibition of BRD4 using two BET inhibitors induced a dose-dependent reduction in both BRCA1 and RAD51 levels, was able to hinder HR DDR, and triggered SL when combined with a PARPi, opening new potentials for BET proteins as targets in TNBCs.

Numerous genetic and environmental insults impede the ability of cells to properly fold and post-translationally modify secretory and transmembrane proteins in the endoplasmic reticulum (ER), leading to a buildup of misfolded proteins in this organelle - a condition called ER stress (Oakes & Papa, 2015). ER stress triggers a signaling reaction known as the unfolded protein response (UPR), which induces adaptive programs that improve protein folding and promote quality control mechanisms and degradative pathways or can activate apoptosis when damage is irreversible (Almanza et al., 2019; Hetz, 2012). In their study, Yamamori and coworkers showed that ER stress suppressed DNA DSB repair and increased radiosensitivity of tumor cells by altering RAD51 levels (Yamamori, Meike, Nagane, Yasui, & Inanami, 2013). They further proved that the ER stress induced either by tunicamycin (a natural antibiotic) or glucose deprivation stimulated selective degradation of RAD51 via the 26S proteasome, thereby impairing HR and enhancing radiosensitivity in human lung cancer A549 cells. As such, the authors proposed that ER stress caused by the intratumoral environment can affect cancer cell radiosensitivity, and this could be exploited a a strategy to improve cancer radiotherapy.

10. Conclusions

In this effort we have reviewed the complexity of the DNA damage repair system, the different mechanisms available to cells to preserve genome integrity, the main role played by one of them - homologous repair and its main molecular actor, the RAD51 recombinase protein - and the impact of a range of defects in the HR pathway relevant to cancer inception, progression, and therapeutics. Yet RAD51 continues to offer new, exciting and challenging perspectives. For instance, at the time of finalizing this review Mason et al. reported a new, non-enzymatic roles for this human recombinase at stalled replication forks (Mason, Chan, Weichselbaum, & Bishop, 2019). Using a separation-of-function allele of RAD51 that retains DNA binding, but not D-loop activity, these authors revealed mechanistic aspects of the roles of RAD51 roles in the response to replication stress. Specifically, they found that cells lacking RAD51 enzymatic activity protect replication forks from MRE11-dependent degradation, as expected from previous studies. Unexpectedly, however, they verified that RAD51 strand exchange activity is not required to convert stalled forks to a form that can be degraded by DNA2. Such conversion was shown previously to require replication fork regression, supporting a model in which fork regression depends on a non-enzymatic function of RAD51. They also showed that RAD51 promotes replication restart by both strand exchange-dependent and strand exchange-independent mechanisms. Thus, what we offered here only represent the tip of an iceberg, which will undoubtedly catalyze the efforts of scientists active in different fields in the near future.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We wish to acknowledge the generous financial support from the Italian Association for Cancer Research (AIRC – grant IG17413) and the Regione Friuli Venezia-Giulia (REFVG, Strategic planning 2016-2018).

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