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The fellowship of the RING: BRCA1, its partner BARD1 and their liaison in DNA repair and cancer

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ABSTRACT

The breast cancer type 1 susceptibility protein (BRCA1) and its partner – the BRCA1-associated RING domain protein 1 (BARD1) – are key players in a plethora of fundamental biological functions including, among others, DNA repair, replication fork protection, cell cycle progression, telomere maintenance, chromatin remodeling, apoptosis and tumor suppression. However, mutations in their encoding genes transform them into dangerous threats, and substantially increase the risk of developing cancer and other malignancies during the lifetime of the affected individuals. Understanding how BRCA1 and BARD1 perform their biological activities therefore not only provides a powerful mean to prevent such fatal occurrences but can also pave the way to the development of new targeted therapeutics. Thus, through this review work we aim at presenting the major efforts focused on the functional characterization and structural insights of BRCA1 and BARD1, *per se* and in combination with all their principal mediators and regulators, and on the multifaceted roles these proteins play in the maintenance of human genome integrity.

Abbreviations: 3D, three-dimensional; 3'UTR, 3′ untranslated region; 6,4PP, pyrimidine-6,4-pyrimidone photoproduct; 7SK snRNP, 7SK small nuclear ribonucleoprotein; 9–1-1, RAD9/RAD1/HUS1 complex; 53BP1, TP53-binding protein; AD, acquired degron; AF-2, carboxyl-terminal activation function; AhR, aryl hydrocarbon receptor; AKT1, RAC-alpha serine/threonine-protein kinase; ALT, recombination-based alternative lengthening of telomeres; AMPK, adenosine monophosphate–activated protein kinase; aNHEJ, alternative non-homologous end joining; ANK, ankyrin; AP1/JUN, transcription factor AP-1; APC/C, anaphase promoting complex; APE1/REF1, DNA-(apurinic or apyrimidinic site) endonuclease; ARD, ankyrin repeat domain; ARM, armadillo repeats domain; ATF, activating transcription factor; ATM, ataxia telangiectasia mutated protein; ATR, ataxia telangiectasia and Rad3-related protein; ATRIP, ATR-interacting protein; AURKA, aurora kinase A; AURKB, aurora kinase B; BACH1/BRIP1/FANCJ, BRCA1-associated carboxyl-terminal helicase; BAF, breast adipose fibroblast; BAP1, BRCA1 associated protein 1; BARD1, breast cancer type 1 protein-associated RING domain protein 1; BARD1α, BARD1 α isoform; BARD1β, BARD1 β isoform; BARD1γ, BARD1 γ isoform; BARD1δ, BARD1 δ isoform; BARD1ε, BARD1 ε isoform; BARD1η, BARD1 η isoform; BARD1κ, BARD1 κ isoform; BARD1ω, BARD1 ω isoform; BAX, apoptosis regulator BAX; BCL2, apoptosis regulator Bcl-2; BCL-3, B-cell lymphoma 3 protein; BER, base excision repair; bHLHZip, basic-helix-loop-helix-leucine zipper; BIP, Bcl-3 interacting protein; BIR, break-induced DNA replication; BRCA1-, associated carboxyl-terminal helicase; BIME, bacterial interspersed mosaic element; BLBC, basal-like breast cancer; bp, base pair; BOD1L, biorientation of chromosomes in cell division protein 1-like 1; BRAP2, BRCA1-binding protein 2; BRCA1, breast cancer type 1 protein; BRCA2/FANCD1, breast cancer type 2 protein; BRCC36, Lys-63-specific deubiquitinase BRCC36; BRCC45, BRISC and BRCA1-A complex members 2; BRCT, BRCA1 C-terminal tandem repeat; BRD4, bromodomain-containing protein 4; BRD7, bromodomain-containing protein 7; BRG1/SMARCA4, transcription activator BRG1; BUBR1, mitotic spindle checkpoint protein; CBP, CREB binding protein; CBX/HP1, chromobox protein homolog 5; CC, coiled-coil; CCDC98/ABRAXAS, coiled-coil domain-containing protein 98 or BRCA1-A complex subunit Abraxas 1; CCR4-NOT, carbon catabolite repression-negative on TATA-less; CCP, cell cycle checkpoint; CDK, cell cycle dependent kinase; CDKI1/p21, cyclin-dependent kinase inhibitor; ceRNA, competing endogenous RNA; CHK1, serine/threonine-protein kinase CHK1; CHK2, serine/threonine-protein kinase CHK2; ChIP, chromatin immunoprecipitation; ch-TOG, cytoskeleton-associated protein 5; c-MYC, MYC proto-oncogene protein; cNHEJ, classical non-homologous end joining; COSA-1, pro-crossover factors crossover site associated; CPC, chromosomal passenger complex; CPD, cyclobutane pyrimidine dimer; CPSF, cleavage and polyadenylation specificity factor; CRC, colorectal cancer; CREB, cAMP-responsive element-binding; CRM1, chromosome region maintenance 1; cryo-EM, cryogenic electron microscopy; CSR, class-switch; CstF, cleavage stimulation factor; CSTF1/CSTF-50, cleavage stimulator factor subunit 1; CSTF2/CSTF-64, cleavage stimulator factor subunit 2; CSTF3/CSTF-77, cleavage stimulator factor subunit 3; CtBP1, C-terminal binding protein 1; CTCF, transcriptional repressor CTCF or CCCTC-binding factor; CTSD, cathepsin D; CTSS, cathepsin S; CUL1, cullin1; DBS, double-strand break; DDB2, DNA damage-binding protein 2; DDR, DNA damage response; DDS, DNA damage site; DDSR1, DNA damage-sensitive RNA1; DDT, DNA damage tolerance; DDX1, ATP-dependent RNA helicase DDX1; dHJ, double Holliday junctions; DNA2, DNA replication ATP-dependent helicase/nuclease DNA2; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; dsDNA, double-strand DNA; DUB, deubiquitylating enzymes; DSS1, deleted in split hand/split foot syndrome protein; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; E6, protein 6; E2F, E2 factor; E2FA, E2F binding site A; E2FB, E2F binding site; EME1, crossover junction complex endonuclease EME1; EOC, epithelial ovarian cancer; EP300, histone acetyltransferase p300; ER, estrogen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERCC1, DNA excision repair protein ERCC-1; ERCC4, DNA repair endonuclease XPF; ES, 17β-estradiol/estrogen; ESC, embryonic stem cells; ESCC, esophageal squamous cell carcinoma; ESE, exonic splicing enhancers; ESFT, Ewing sarcoma family of tumors; ESR1, gene encoding ERα; EU, Europe; EWSR1, RNA-binding protein EWS; EXO1, exonuclease 1; FA, Fanconi anemia; FACT, facilitates chromatin transactions;

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1. Introduction

Any organism survival and trait inheritance by offspring critically depend on genome integrity preservation. Thus, cells have evolved a sophisticated DNA repair machinery (Ciccia & Elledge, 2010; Giglia-Mari, Zotter, & Vermeulen, 2011; Hakem, 2008; Jackson & Bartek, 2009; Roos, Thomas, & Kaina, 2016; Sancar, Lindsey-Boltz, Unsal-Kaçmaz, & Linn, 2004; Sirbu & Cortez, 2013) to protect their genetic information from damage caused by both endogenous and external threats (Chatterjee & Walker, 2017; Tubbs & Nussenzweig, 2017). As a result of the many types of DNA lesions that might occur, different repair pathways may be triggered, each of which may be successful on its own or in conjunction with others to complete the whole DNA repair process. Deficiencies in DNA healing – with incorrect repair and/or extended DNA damage as outcomes – may have catastrophic consequences, including gene mutations, chromosomal rearrangements, genomic instability, and, ultimately, carcinogenesis and/or tumor growth (Carusillo & Mussolino, 2020).

The DNA damage response (DDR), a complex network of cellular pathways that coordinates the whole DNA repair process – from injured DNA detection, recognition and repair, to the eventual commitment of unrepairable cells to senescence or death – is the key strategy implemented by eukaryotes to deal with DNA damage and to preserve somatic cell homeostasis. Among the ~10^5 spontaneous DNA lesions human cells may experience daily (Hoeijmakers, 2009), DNA double-strand breaks (DSBs) are likely the most dangerous threat to genomic stability (Katsuki, Jeggo, Uchihara, Takata, & Shibata, 2020), and even a single unrepaired DSB may elicit cell death (Rich, Allen, & Wyllie, 2000). Ultra-violet (UV) light is the most common exogenous factor inducing DNA DSBs (Mullenders, 2018); however ionizing radiations (IRs), molecules that imitate the action of IRs (Tiwari & Wilson 3rd., 2019), topoisomerase inhibitors (Pommier, 2013), and compounds produced by tobacco smoke (Weng et al., 2018) and in overcooked foods (Bedard & Massey, 2006) also contribute to DNA DSB formation. DSBs in DNA are produced during cellular metabolism as well. Endogenous reactive oxygen species (ROS), for example, can cause DSBs upon DNA base oxidation (Woodbine, Brunton, Goodarzi, Shibata, & Jeggo, 2011), while mechanical stress on the chromosomes (Gelot, Magdalou, & Lopez, 2015) and defective telomere metabolism (Aksenova & Mirkin, 2019) can also elicit these dangerous nucleic acid lesions. Because DNA intermediates at replication forks are unstable and prone to breaking, DNA replication is assumed to be the primary source of DSBs in growing cells. Notably, DNA breaks can arise as a result of polymerase stalling, leading to the formation of persistent single-strand DNA (ssDNA) intermediates. Broken or collapsed replication forks containing ssDNA resemble DSBs at various stages of processing and can cause genomic instability if not promptly and effectively repaired (Ait Saada, Lambert, & Carr, 2018). In other cases, DNA DSBs are purposefully created by the cell for a specific biological function, such as initiating recombination between homologous chromosomes during meiosis (Murakami & Keeney, 2008). Furthermore, DNA DSBs occur spontaneously as intermediates during developmentally controlled rearrangements such as V(D)J (variable (V), diversity (D), and joining (J)) recombination – a process that requires the creation and resolution of programmed DSBs to effect the gene rearrangements necessary for immunoglobulin (Ig) and T cell receptor formation (D. B. Roth, 2014) – and class-switch recombination (CSR), a DNA recombination process that replaces the Ig constant region for the isotype that can best protect against a given pathogen (Nicolas, Cols, Choi, Chaudhuri, & Vuong, 2018). Although the cell exerts tight control over such activities, they can occasionally go astray, with potentially disastrous repercussions for the cell and/or the entire organism.

Classical non-homologous end joining (cNHEJ) and homologous recombination (HR) are the two main pathways that preside over the repair of DNA DSBs (Fig. 1) (Scully, Panday, Elango, & Willis, 2019).

Notably, the choice between cNHEJ and HR is determined by the phase of the cell cycle (CCy) in which the DNA DSB is detected (Her & Bunting, 2018). In fact, although most common in mitotic cells during the G0-, G1-, and early S-phases, cNHEJ can occur throughout the cell cycle since it does not require a sister chromatic (SC) with a homologous sequence (HS); as such, it is a fast, high-capacity yet error-prone DSB repair. Diversely, HR is an error-free route that entails HSs to align DSB ends prior to ligation. Therefore, HR takes place predominantly during the S-phase of the vertebrate cell cycle, in which a replicated SC is available as a homologous template to copy and restore the DNA sequence missing on the damaged chromatid. Furthermore, HR necessitates a 5'end excision at the break (*vide infr*a), an activity elicited during the S/G2 phases of the cell cycle but repressed during G1 by the TP53-binding protein 1 (53BP1)/telomere-associated protein RIF1 (RIF1, Fig. 2, top left)/mitotic spindle assembly checkpoint protein MAD2 (MAD2, Fig. 2, top right) pathway, which targets the DDR to cNHEJ (Escribano-Diaz et al., 2013).

In cNHEJ, the heterodimeric KU protein sensor complex (composed by the ATP-dependent DNA helicase 2 subunits Ku70 – aka X-ray repair cross-complementing protein 6 or XRCC6 – and Ku80, the X-ray repair cross-complementing protein 5 or XRCC5, respectively (Shibata, Jeggo, & Lobrich, 2018)) locates and binds to the DNA free ends at the DSBs. Once bound, KU promotes the recruitment and activation of DNA-PKcs (the DNA-dependent protein kinase catalytic subunit, Fig. 2, bottom); this event, in turn, initiates a complex signaling cascade that coordinates repair, including end resection processing, gap filling, and break sealing (H. H. Y. Chang, Pannunzio, Adachi, & Lieber, 2017).

The nucleolytic degradation of the 5'strand of a DNA DSB (aka *end resection*) and the subsequent generation of a 3'ssDNA tail shuttle the DSB damage to the HR pathway (Fig. 1). Here, the replication protein A complex (RPA, Fig. 3, top left) swiftly coats the 3'ssDNA overhang to prevent its degradation/self-annealing; then, various protein partners, including the breast cancer type 1 and 2 susceptibility proteins (BRCA1 and BRCA2), the DNA repair protein RAD52 homolog (RAD52, Fig. 3, top right), the partner and localizer of BRCA2 (PALB2, Fig. 3, middle), and the breast cancer type 1 protein-associated RING domain protein 1 (BARD1) promote RPA replacement with the DNA repair protein RAD51 homolog 1 (RAD51) recombinase (Laurini et al., 2020). The RAD51-ssDNA nucleoprotein filament thus formed (Fig. 3, bottom) initially performs a search for DNA homology in either the SC or the homologous chromosome and, once found, catalyzes the invasion of

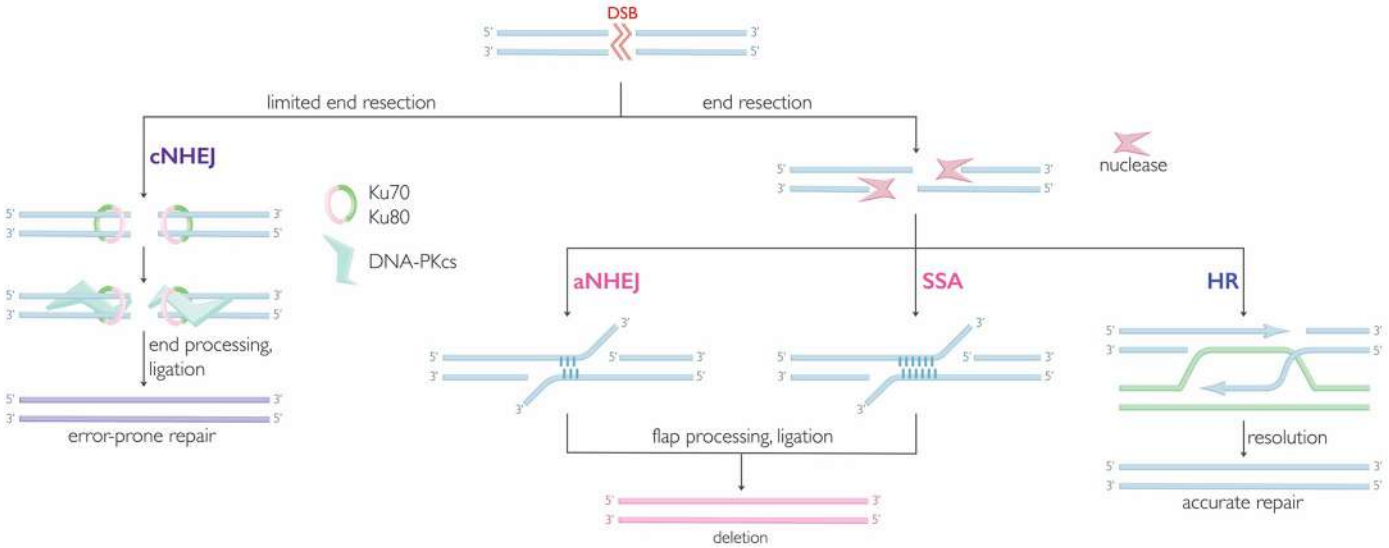

Fig. 1. Schematic view of the main pathways governing DNA DSBs in human cells.

the ssDNA end into the homologous double-strand DNA (dsDNA) to create a *D-loop*. Finally, next, the extended structure is resolved via one of several paths to produce various types of DNA products (San Filippo, Sung, & Klein, 2008; Scully et al., 2019).

In the absence of cNHEJ proteins, another direct end joining repair process – alternative non-homologous end joining (aNHEJ, Fig. 1) – operates on 3'ssDNA ends during the S- and G2 stages of the cell cycle (H. H. Y. Chang et al., 2017; Nagaria & Rassool, 2018). In aNHEJ, the primary nick DNA sensor poly(ADP-ribose) polymerase 1 (PARP1) identifies and tethers either ssDNA nicks or blunt DS ends (Fig. 4, top left), and resection follows immediately after the heterotrimeric complex MRN – composed by the double-strand break repair protein MRE11 (MRE11, Fig. 4, top right), the DNA repair protein RAD50 (RAD50, Fig. 4, bottom left), and the Nijmegen breakage syndrome protein 1 (NBS1/nibrin) (Syed & Tainer, 2018) – binds the DSB ends. Through endonucleolytic cleavage/3′-5'exonucleolytic processing 3'ssDNA overhangs are then formed, and the 3'flaps produced from the remaining non-complementary DNA segments are removed to assist in DNA end stable association and to provide a suitable substrate from which DNA synthesis can by filling the gap (likely by DNA polymerase θ (Polθ), Fig. 4 bottom right), and sealing the nick via DNA ligase 1 (LIG1) or DNA ligase IIIα (LIGIIIα). Whether aNHEJ is a true DSB repair

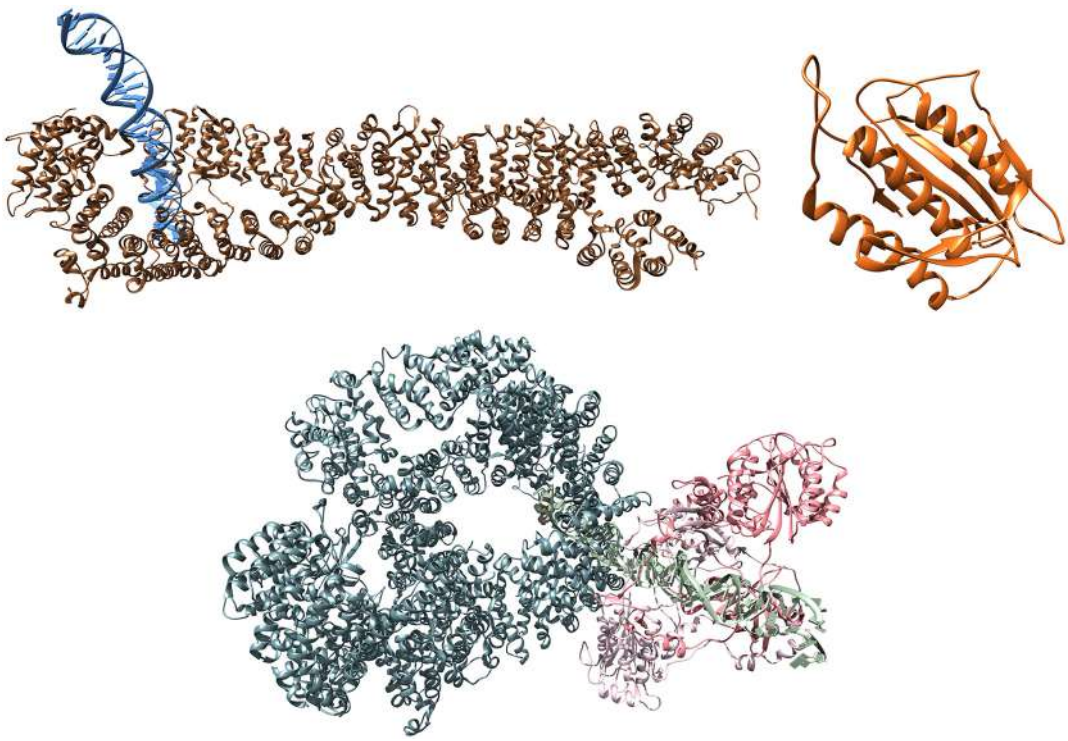

Fig. 2. (Top left) Crystal structure of the RIF1 N-terminal domain (mule fawn) in complex with DNA (marina) (Protein Data Bank (PDB): 5NW5 (Mattarocci et al., 2017)). (Top right) Solution structure of MAD2 as solved by nuclear magnetic resonance (NMR) (pumpkin orange, PDB: 1DUJ (X. Luo et al., 2000)). (Bottom) Cryogenic electron microscopy (cryo-EM)-derived structures of a KU heterodimer bound to DNA and in complex with DNA-PKcs (Ku70/Ku80: cherry blossom/conch shell; DNA: smoke green; DNA-PKcs: arctic, PDB: 5Y3R (Yin, Liu, Tian, Wang, & Xu, 2017)). In this and all remaining Figures, reported color names refer to Pantone® colors (www.pantone.com).

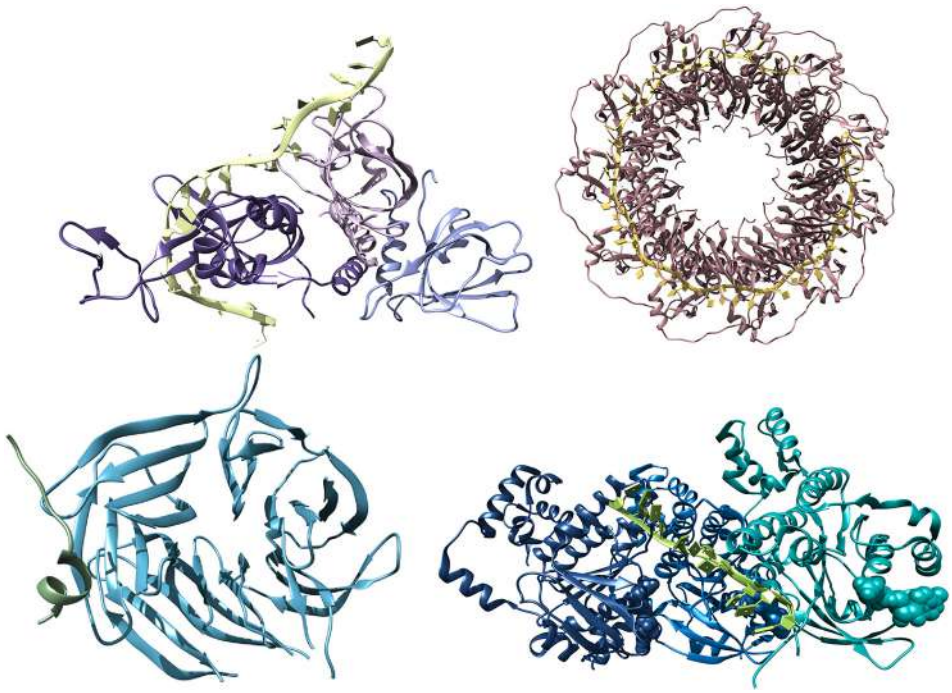

Fig. 3. Crystal structures of the trimeric RPA/ssDNA complex (top left, violet shades/luminary green, PDB: 6I52 (Yates et al., 2018)), the RAD52-ssDNA complex (top right, ssDNA in cream gold and the 10 RAD52 subunits in nostalgia rose, PDB: 5XRZ (Saotome et al., 2018)), and the PALB2 C-terminal domain (heavenly) bound to a BRCA2 peptide (bottom left, green glimmer, PDB: 3EU7 (Oliver, Swift, Lord, Ashworth, & Pearl, 2009)). (Bottom right) Cryo-EM-derived structure of the RAD51 presynaptic filament (PDB: 5H1B (Xu et al., 2017)). The three RAD51 protomers are colored in true blue, directoire blue and viridian green, respectively; the ssDNA is in greenery, while the three adenosine monophosphate molecules, each linked to a RAD51 chain, are portrayed as matching-color spheres.

mechanism is still unclear, but it may serve as a plan-B strategy for both cNHEJ and HR, whenever both pathways must deal with a large number of DSBs. Finally, single-strand annealing (SSA) is a RAD51-independent, non-conservative DSB repair pathway that also does not require the presence of a SC. Activated by end resection during late S- and G2 phases, SSA connects direct repeat sequences (like *tandem repeats*) at 3'ssDNA ends via RAD52 annealing, removal of the non-homologous 3'ssDNA tails performed by the DNA excision repair protein ERCC-1 (ERCC1)/DNA repair endonuclease XPF (ERCC4) complex, and gap filling and ligation by polymerase/ligase enzymes still non uniquely identified (Bhargava, Onyango, & Stark, 2016). During SSA, substantial DNA end resection and RPA displacement are required in order to expose complementary homologous sequences; furthermore, sequence information may be lost or altered if DNA ends are not properly linked together. As a result, SSA is regarded as a *de rigueur* error-prone pathway.

The main focus of this review is on two key protein players in human DDR pathways of DNA DSBs and a plethora of other vital cellular functions: BRCA1 and its obligatory partner BARD1. It has been over 25 years since mutations in the tumor suppressor genes BRCA1 and BRCA2 were found associated with a small number (~10%) of hereditary breast cancers (HBCs) and/or ovarian cancers (HOCs) (Ford & Easton, 1995). Since then, genetic or hereditary factors including BRCA1/2 mutations have been found to be responsible for between 5% to 10% of BC cases overall (Gorodetska, Kozeretska, & Dubrovska, 2019; Valencia et al., 2017), and the most recent data report BRCA1 mutations as responsible for approximately 35% of HBCs, with an increased risk of developing BC by age 70 between 44% to 78% in females (and between 0.22 and 2.8% in males), and an increased risk of developing OC comprised between 18% to 54% by women at the same age (Casaubon, Kashyap, & Regan, 2021). Additionally, mutations in BRCA1 lead not only to hereditary breast and ovarian cancer syndrome (HBOC) but are also the likely drivers for a variety of other cancers including prostate, pancreatic, and stomach malignancies among others (Mersch et al., 2015). Because BRCA1 is a large polypeptide that interacts with many other effector proteins that perform a variety of functions, determining how defects in its function could lead to cancer has proven to be a challenging task. Nevertheless, it has been demonstrated that one of these specific proteins – BARD1 – is the master regulator of BRCA1 activity, in addition to being a tumor-suppressor itself (Cimmino, Formicola, & Capasso, 2017; Irminger-Finger & Jefford, 2006; Irminger-Finger, Ratajska, & Pilyugin, 2016; Tarsounas & Sung, 2020). Accordingly, in what follows, we will first briefly review the initial studies that identified BRCA1 as an HBC suppressor, as well as how BARD1 was isolated as its interactor and regulator. We will next focus on these proteins structures and functions in combination and proceed by analyzing the roles that the BRCA1/BARD1 system plays in cell biology and DDR – and in HR in particular – and their liaison with carcinogenesis and cancer progression. Our intention is to provide all readers with most of the basic aspects of the topics just mentioned above along with an updated literature references on these subjects. Nonetheless, given the wealth of data now available, we were forced to make decisions for which we are solely responsible. This is particularly true for BRCA1 (and its cancer-related mutations), for which a quick survey of the literature databases (*e.g.*, PubMed) using BRCA1 as entry returned more than 18,800 items. As a result, we sincerely apologize to those colleagues whose excellent work was not directly cited due to space constraints. As a last note, our own nature of computational/experimental structural biologists has led us to make the choice of representing the structures of the proteins involved in BRCA1 and BARD1 function and control – when available – for at least two main reasons. The first is that we wanted to give a comprehensive and original view of BRCA1/BARD1 and their partners also from a molecular structure viewpoint. Second, but not less important, reporting the structure of a given protein has itself a twofold meaning: a) if a protein can be crystallized or seen by *e.g.*, electron microscopy, this means it can be expressed (at full length or at least some of its domains) with high purity and in relevant quantities; alternatively, it can be modeled using *in silico*-based techniques which are

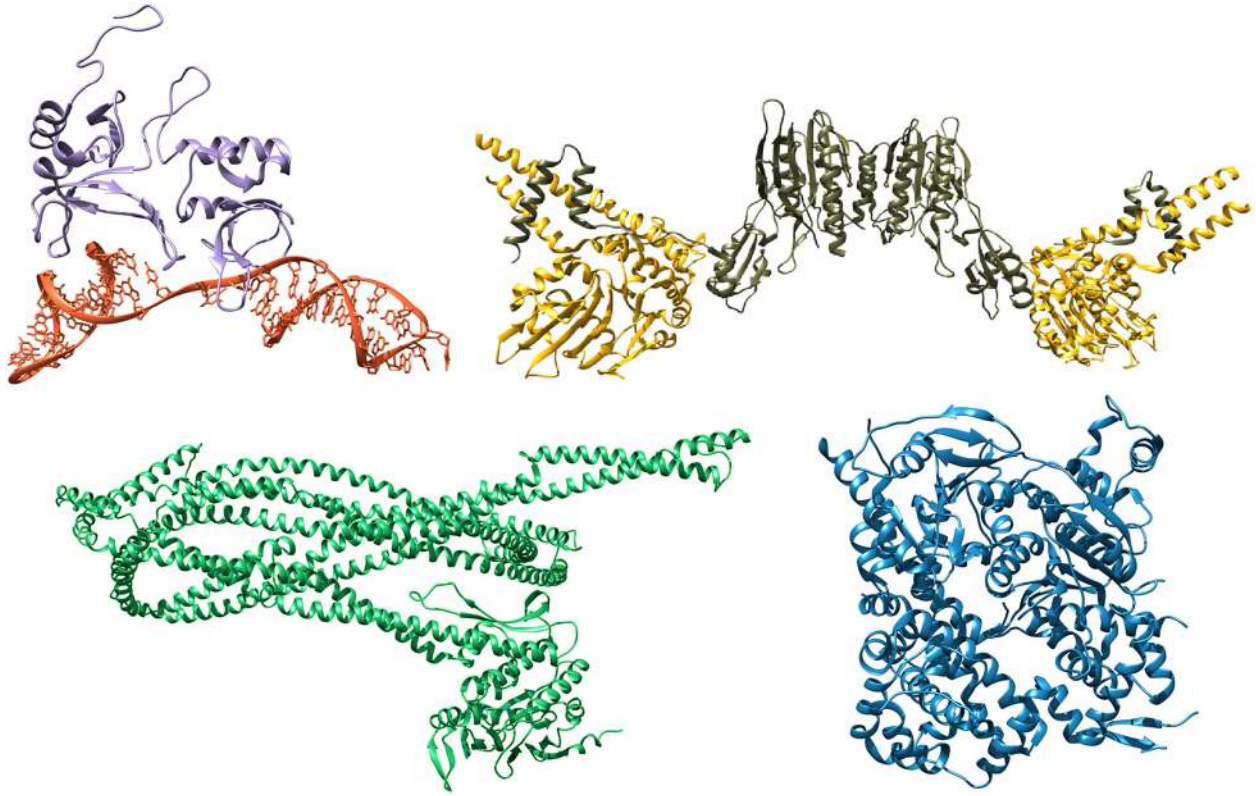

Fig. 4. (Top left) Structure of the N-terminal zinc finger domain of PARP1 (Bougainvillea) detecting a DNA SSB as determined by a combination of X-rays and NMR (vermillion orange, PDB: 2N8A (Eustermann et al., 2015)). (Top right) Crystal structure of the dimeric form of MRE11 (taxi cab yellow) in complex with two Rad50 nucleotide-binding domains (mayfly green, PDB: 3QG5 (Lammens et al., 2011)). (Bottom left) Three-dimensional (3D) structure of RAD50 as predicted by AlphaFold2 (AlphaFold2 is an artificial intelligence (AI) system developed by Google's DeepMind (https://deepmind.com/research/case-studies/alphafold) able to predict a protein 3D structure from its amino acid sequence. AlphaFold2 is the top-ranked, high accuracy protein structure prediction method, and most of structures protein models predicted by this AI-based technology are competitive with experimentally-determined measurements (Jumper et al., 2021). The large database of protein structures currently predicted by AlphaFold2 is freely available at https://alphafold.ebi.ac.uk/ and actually covers the human proteome and the proteomes of several other key organisms. In the coming months, the AlphaFold2 team plans to expand the database to cover a large proportion of all catalogued proteins (the over 100 million in UniProt Reference Cluster 90 (UniRef90), https://www.uniprot.org/help/uniref).) (island green, AlphaFold2 PDB: Q92878, (Jumper et al., 2021)). In this and all other Figures showing proteins structures predicted by AlphaFold2, only those structures characterized by an average per residue confidence score > 80 (in a scale 0–100) will be reported. (Bottom right) Crystal structure of the helicase domain of Polθ (French blue, PDB: 59AJ (Newman, Cooper, Aitkenhead, & Gileadi, 2015)).

now easily accessible due to the widespread availability of (super)computer facilities; and b) as such, these proteins – especially those less studied – can be produced and be the subject of further, fundamental investigations. In other words, we reasoned that showing the structures of these BRCA1 and BARD1 mediators, most of which indeed deserve a lot of additional studies, can be a source of research inspiration to a plethora of scientists working in this specific discipline as well as in other related research fields.

2. Overview of the BRCA1 and BARD1 proteins

2.1. BRCA1 as a HBOC gene

The first convincing genetic linkage-based localization of a BC gene (later named BRCA1) dates back to the early 90's (Hall et al., 1990; Miki et al., 1994). In a study of 23 families, Hall and coworkers found a two-point logarithm of the odds (LOD)[1] score of 3.28 at a recombination fraction[†] of 0.14 from D17S74, a marker on chromosome 17q21, with an estimated 40% of families linked (Hall et al., 1990). To corroborate this finding and to investigate whether OC was also linked to this locus, the year after Narod and collaborators studied five large families with a hereditary predisposition to BC and OC (Narod et al., 1991). The link to D17S74 was confirmed in three out of five families with HBOC, with the LOD score for the largest family equal to 2.72. These findings therefore provided compelling evidence that the chromosomal region 17q12-q23, previously shown to contain a gene for early-onset BC, was also associated with a proportion of HOC, and a plethora of successive studies reported confirming data in this respect (Hodgson & Turashvili, 2020; Kuchenbaecker et al., 2017; Nielsen, van Overeem Hansen, & Sørensen, 2016a; Yoshida, 2020). Cancer onset in BRCA1-mutated individuals typically involves loss of heterozygosity (LOH) either by simple deletion of the wild-type (WT) allele (copy-loss LOH), or by deletion of the normal allele accompanied by duplication of the faulty one (copy-neutral LOH) (Maxwell et al., 2017). However, BRCA1-deficient BOCs can develop also with no familial linkage, and the role for this tumor suppressor gene in sporadic primary BOC tumorigenesis is ascribed to its epigenetic silencing by promoter hypermethylation, especially in the presence of LOH and in specific histopathologic subgroups (Esteller et al., 2000). Lastly, triple negative breast cancer (TNBC) – an aggressive disease subtype representing ~10% of all BCs and defined by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) – is also linked to germline or somatic mutations in BRCA1 (and other genes involved in DNA DSB repair), ultimately resulting in an increased

[1] In genetics, the LOD score estimates the probability that two or more genes are located on the same chromosome and that they are thus likely inherited together. A LOD score ≥ 3 is usually taken as an indication that two genes are located near each other on the same chromosome and, in terms of significance, LOD = 3 indicates that the chances of the two genes being linked and thus inherited together are 1000:1. [†]The recombination fraction is the number of offspring that inherit different alleles of a trait from each parent, rather than inheriting all alleles from the same parent. The recombinant fraction is an important mean of determining genetic distance.

BC lifetime risk to 60–70% (Marra, Trapani, Viale, Criscitiello, & Curigliano, 2020).

2.2. BARD1: the major partner of BRCA1 yet a tumor suppressor and an oncogene per se

In the quest of isolating proteins that associate with BRCA1 *in vivo*, Wu and collaborators used the BRCA1 N-terminal region as a bait in a yeast 2-hybrid interaction screen; as a result, BARD1 emerged as the major BRCA1 interacting protein partner (L. C. Wu et al., 1996). Later evidences that i) BARD1 and BRCA1 genes are co-expressed in almost all tissues, ii) BARD1 and BRCA1 knock-out mice exhibit nearly the same phenotype, and iii) the fact that these two proteins form a stable heterodimeric complex (§2.3.1) all confirmed the BARD1 accessory role to BRCA1. However, the latest data demonstrate that these two proteins have both common and diverse functions. In particular, not only BARD1 works as a controller of BRCA1, but it is a tumor suppressor itself, with dominant negative BC, OC and other tumor promoting activities associated with a number of its mutated isoforms (Cimmino et al., 2017; Irminger-Finger et al., 2016; Irminger-Finger & Jefford, 2006). For example, in a recent comprehensive case-control association study comprising ~14,000 unselected BC patients and ~ 5900 controls from Polish and Belarusian populations, Suszynska and coworkers detected the highest number of BARD1 variants in BC cases in any individual BARD1-specific study, including a recurring nonsense mutation c.1690C > T/p.Gln564* (M. Suszynska et al., 2019). Among all analyzed cases, only the p.Gnl564* BARD1 isoform was associated with a moderately increased risk of BC (odd ratio OR[2] = 2.30), leading the authors to classify it as a low/moderate risk BC allele. Given its prevalence in the Central European population, the authors further suggested BARD1 p.Gln564* as a mutation of potential relevance for future genetic testing. A number of BARD1 single nucleotide polymorphisms (SNPs) are also associated with protein overexpression, HBOC cases in different populations, and with few cases of spontaneous BCs and OCs not linked to BRCA1/2 mutations (Irminger-Finger et al., 2016). Additional research has discovered a number of SNPs, genetic modifications, and epigenetic alterations within or around the BARD1 gene that were shown to have significant impacts on carcinogenesis in a range of malignancies other than BCs and OCs (Watters, et al., 2020). Interestingly, BARD1 mutations in HBC are less common than BRCA1 variations for yet unknown reasons, and BARD1 mutations identified in non-BRCA mutant BC families (Alenezi, Fierheller, Recio, & Tonin, 2020; Weber-Lassalle et al., 2019), are likely to account for more instances of non-BRCA1/2 hereditary BC (Cimmino et al., 2017; Keeney, Couch, Visscher, & Lindor, 2017; Klonowska et al., 2015; Malwina Suszynska & Kozlowski, 2020). Remarkably, germline pathogenic variants in BARD1 were also associated with high TNBC risk (OR = 5.92), and an overall lifetime risk for BC > 20% in Caucasian individuals in the recent work by Shimelis and colleagues (Shimelis et al., 2018). On the other hand, a liaison between BARD1 as an oncogene and OC has been recently proposed by Norquist and coworkers based on a study comprising 1915 women with OC and accessible germline DNA (Norquist et al., 2016). For BARD1, these authors found a mutation frequency of 0.2% and an OR of 4.2, in line with similar findings reported by *e.g.*, Couch and colleagues upon searching for multigene pathogenic variants in hereditary BC patients (mutation frequency 0.18% and OR = 2.16) (Couch et al., 2017). The latter group also evaluated the mutation frequency in 17 genes linked to BC predisposition in a large cohort of patients with TNBC (Couch et al., 2015). Concerning BARD1, they reported 6 BARD1 mutations in these patients, four frameshift variations (Pro300fs, Val510fs, Glu652fs, and Val767fs), and two missense alterations (Leu275* and Cys645*). In summary, most of the reported literature currently support the view of BARD1 as a gene linked to increased OC and BC predisposition (da Costa et al., 2020; De Brakeleer et al., 2010; Ghimenti et al., 2002; Ishitobi et al., 2003; Karppinen et al., 2006; W. Li et al., 2021; Ratajska et al., 2012; Rofes et al., 2021; Thai et al., 1998; J. Y. Wu et al., 2006); as such, BARD1 is now routinely included in the clinical gene panel testing for these two malignancies (Irminger-Finger, 2010; Śniadecki et al., 2020; Malwina Suszynska & Kozlowski, 2020). Of note, the BARD1 pathogenic nonsense germline mutation p.Gln564* was confirmed in a case of synchronous endometrioid endometrial and ovarian carcinoma (SEOC) (Hájková et al., 2019), a malignancy that constitutes ~5% of endometrial carcinomas and from 10 to 20% of OCs, respectively (Matias-Guiu & Stewart, 2018). The search of putative BC susceptibility genes within a familiar BC/OC multiple-case large cohort led Li and coworkers to the identification of 4 BARD1 mutations: the frameshift c.627_628delAA/p.Lys209Asnfs*, the nonsense c.1652C > G/p.Ser551*, and the two missense c.2317C > T/p.Leu773Phe, and c.1915 T > C/p.Cys639Arg, respectively, all of which were described as potentially deleterious according to 5 different *in silico* prediction tools (Li et al., 2016).

Down-regulation of BARD1 (§2.4.2) also may lead to tumorigenesis. For example, a partial repression of BARD1 in TAC-2 cells (a mammary epithelial cell (MEC) line of murine origin that preserves normal breast epithelium morphogenetic properties) resulted in significant phenotypic alterations (*i.e.*, different cell shape/size, increased presence of multinucleated cells, and abnormal CCy progression), suggestive of a premalignant phenotype (Irminger-Finger, Soriano, Vaudan, Montesano, & Sappino, 1998). *In vivo*, BARD1-null mice were reported to show symptoms of mental retardation and to die between embryonic day 7.5 and 8.5 (McCarthy, Celebi, Baer, & Ludwig, 2003). McCharty and colleagues also proved that this embryonic lethality is a result of a severe cell proliferation in the absence of increased apoptosis and this, together with the observed augmented chromosomal aneuploidy of BARD1 mutant cells, supported the role of BARD1 as a key factor in preserving genome stability (McCarthy et al., 2003).

Lastly, some BARD1 splice variants (α, β, κ, γ, δ, φ, ε, η and ω) have been reported to have lost tumor suppressor functions while acquiring oncogenic potential. As a notable exemplar, the BARD1 β isoform (BARD1β), which lacks exons 2 and 3, codifies for a protein product lacking the really interesting new gene (RING) domain (§2.3.1), and this *RING-less* BARD1 is the oncogenic driver associated with neuroblastoma (Bosse et al., 2012a; Capasso et al., 2013), nephroblastoma (Fu et al., 2017a), and with poor survival in patients with lung (Zhang et al., 2012) or colorectal cancer (CRC) (Gautier, Irminger-Finger, Grégoire, Meflah, & Harb, 2000b; Ozden et al., 2016; Sporn, Hothorn, & Jung, 2011; Zhang et al., 2012) (§2.7.6). The BARD1 α isoform (BARD1α) removes exon 2, while the γ isoform (BARD1γ) loses either exon 4 or exons 4–11, as found during investigations of gynecological and CRCs (Lepore et al., 2013; L. Li et al., 2007; Zhang, Pilyugin, et al., 2012). In the former set of malignancies, using cell lines derived both from hormone-dependent and independent tumors Li and colleagues discovered a unique combination of BARD1 isoforms, in the majority of which the BRCA1 interaction domain was missing; interestingly, these BARD1 variants were absent in hematologic cancer cell lines (L. Li et al., 2007). In the same study, the authors reported that the expression of BARD1 isoforms truncated at their N-terminal was associated with advanced OC stages, and that spliced isoform BARD1 variants were characteristic of clear cell carcinoma, the form of OC currently with the highest fatal *exitus*. Silencing of BARD1 isoforms in OC cells that lacked WT BARD1 led to a complete proliferation arrest, confirming that i) malignant cell survival requires BARD1 isoform expression and ii) BARD1 isoforms act as cancer maintenance genes. Zhang et al. found that CRCs characterized by the expression of BARD1 isoforms (but not of full length (FL) BARD1), and this affects the progression and clinical outcome of this tumor (Zhang, Pilyugin, et al., 2012). In particular,

[2] OR is defined as the chance of an event occurring in one group compared to the chance of the same event occurring in another group. ORs are most often employed in cancer research in case-control (typically retrospective) studies to decide whether being exposed/subjected to a particular factor increases the risk of cancer (for example, many people in each group have a specific gene mutation by considering a group of people with cancer (cases) and a group of healthy people (controls)).

they suggested that BARD1 isoforms κ (BARD1κ, lacking exon 3 and the exon 4 N-terminal region), β, and π (BARD1π, missing the C-terminal portion of exon 4) play a role in promoting colon cancer insurgence and progression; as such, the authors suggested that these BARD1 isoforms could be considered as specific prognostic markers for CRC. Also, all these three BARD1 isoforms might also be involved in non-small cell lung cancer (NSCLC) initiation and invasive progression, and again could represent novel prognostic markers for this tumor (Zhang, Bianco, et al., 2012). The δ isoform (BARD1δ) lacks exons 2–6 (that encode most of the RING finger domain and the full ankyrin repeat motif (§2.3.4.3)) (Feki et al., 2005; Tsuzuki et al., 2006); as such, it was reported not to interact with BRCA1, although it was still found to interact and colocalize to cytoplasmic dots with another partner, the mRNA polyadenylation factor cleavage stimulator factor subunit 1 (CSTF1/CSTF-50, §2.3.4.3). Exons 4 to 9 are missing in the ε isoform (BARD1ε), exons from 2 to 9 are removed in the η isoform (BARD1η), while the absence of exons 3–6 characterizes the ϕ isoform (BARD1ϕ) (L. Li et al., 2007). Finally, the ω isoform (BARD1ω, in which exons from 1 to 3 are missing) codifies for proteins of various lengths as a result of diverse translation starting sites located on exons 4 and 5; given their specificity, these BARD1ω products could be adopted as tumor diagnostic/prognostic biomarkers in acute myeloid leukemia, as reported by Lepore and colleagues (Lepore et al., 2013). A more detailed discussion on the role of BARD1 mutations and isoforms in cancer (and other diseases) will be dealt with in section §2.7.6.

2.3. Structure and functions of the BRCA1 and BARD1 protein domains

The BRCA1 gene, located on chromosome 17 (17q21), comprises 24 exons and encodes for a large (1863) amino acid protein (Albertsen et al., 1994; Koonin, Altschul, & Bork, 1996; Miki et al., 1994; T. M. Smith et al., 1996), whereas the human BARD1 gene is mapped close to the telomers of chromosome 2 (2q34-2q35), is composed by 11 exons, and codifies for a protein of 777 residues (L. C. Wu et al., 1996). Both proteins feature a RING domain (instrumental for their heterodimerization and the related E3 ubiquitin ligase activity (§3.1), two BRCA1 tandem repeats (BRCTs), which bestow the ability to cooperate with numerous proteins involved in the DDR, nuclear export sequences (NESs) and nuclear localization signals (NLSs) for cytoplasm-nucleus two-ways shuttling, and unstructured regions that mediate contacts with DNA and the RAD51 recombinase during DNA HR. At variance, BRCA1 features a coiled-coil domain (CC) that directs the formation of a larger supramolecular unit involving BRCA2 which, in turn, binds to and coordinates the activity of several other mediators – including the deleted in split hand/split foot syndrome protein (DSS1) and PALB2 (§1) – to further assist in RAD51 loading and function. BRCA1 also includes a domain known as the serine-glutamine cluster domain (SQCD), with SQCDs being protein motifs that are recognized targets for DDR kinases. Diversely, BARD1 is characterized by four ankyrin repeat domains (ARD), responsible for targeting the BRAC1/BARD1 complex to DNA lesions by interacting with unmethylated form of histone H4, a constituent of newly duplicated DNA nucleosomes (see Fig. 5, top).

2.3.1. The BRCA1 and BARD1 RING domains

Residues 24–65 in BRCA1 and 46–90 in BARD1 constitute the C3HC4-type RING domains of the two proteins (Brzovic, Rajagopal, et al., 2001). A conserved pattern of eight cysteine and histidine residues arranged in pairs in the primary sequence characterizes the RING motif (Cassandri et al., 2017); these residues bind Zn^{2+} ions in an interleaved fashion, resulting in the formation of two distinct Zn^{2+}-binding sites in the corresponding proteins (termed site I (Cys24, Cys27, Cys44, and Cys47) and site II (Cys39, His41, Cys61, and Cys64), respectively). Concomitantly, the regions immediately adjacent to the central RING motifs are critical for the correct assembly of the two proteins to take place. In detail, the α-helices formed by the amino acids 8–22 and 81–96 of BRCA1 flank its central RING motif (residues 23–76) in an anti-parallel fashion. In utter analogy, the α-helices formed by BARD1 residues 36–48 and 101–116 are adjacent to the protein central RING structure (amino acids 49–100). These helices globally combine in a four-helix bundle that constitutes the protein/protein heterodimerization interface (Fig. 5, middle left). RING-mediated BRCA1/BARD1 heterodimerization both increases the ubiquitin ligase activity of BRCA1 (§2.5.1) and causes the NESs located on the N-terminals of both proteins (Rodriguez, Schüchner, Au, Fabbro, & Henderson, 2004) to be buried within the four-helix bundle, resulting in the nuclear retention of the two proteins (§2.3.3). Moreover, as it will be discussed in detail later on, the BRCA1/BARD1 heterodimer promotes genome integrity by performing a variety of tasks, and this structural ensemble is needed for mutual stability, HR function, and tumor suppression. The upstream signaling mechanisms that control BRCA1/BARD1 heterodimerization still remain unknown but it has been shown recently by Minter and coworkers that the NAD-dependent protein deacetylase sirtuin-2 (SIRT2, a deacetylase that performs the removal of acetyl groups from specific lysine residues on histones, α-tubulin and other proteins including some transcription factors, and is also a BC suppressor itself (Y. Wang, Yang, Hong, Chen, & Cui, 2019)), enhances BRCA1/BARD1 heterodimerization via deacetylation (Minten et al., 2021). In particular, their data suggest a scenario in which BARD1 RING acetylation (specifically at lysines 46, 96, and/or 110) results in the elimination of interface-neutralizing salt bridges involving the corresponding BRCA1 negatively charged residues, thereby preventing the formation of the BRCA1/BARD1 heterodimer. This promotes BRCA1 ubiquitination and proteasomal degradation which, consequently, destabilizes BARD1. Furthermore, inefficient BRCA1/BARD1 heterodimerization exposes both BRCA1 and BARD1 NESs; thus, both proteins tend to accumulate in the cytoplasm, with their consequent reduced localization to DNA damage sites (DDSs) and inefficient HR. Intuitively, SIRT2 deacetylation of the BARD1 RING motif has exactly opposite effects, that is BRCA1/BARD1 binding interface stabilization, heterodimer facilitated formation and increased stability, NES masking with subsequent nuclear retention, accumulation at DDSs and effective HR activity of the two proteins.

Although the homozygous deletion of BRCA1 exon 2 generally leads to embryonic lethality in mice, Li and coworkers successfully showed that the product of such gene is a protein mutant isoform lacking the N-terminal RING domain (Li et al., 2016), in complete analogy with BARD1 (§2.2). This stable *RING-less* BRCA1 protein was proficiently recruited to the DDSs; moreover, in cells expressing *RING-less* BRCA1 the authors observed formation of RAD51 foci in response to DNA-induced damage although the same cells displayed substantial genomic instability. The latter could be rescued by silencing 53BP1 (§1), while animals expressing *RING-less* BRCA1 did not demonstrate an enhanced susceptibility to malignancies in the absence of 53BP1. Notably, the group found that genomic instability related to *RING-less* BRCA1 *in vitro* correlated with loss of BARD1 and a deficiency in replication fork restart, suggesting a RAD51-independent function for BRCA1/BARD1 in genomic integrity preservation (as discussed in detail later in §3.3.2.3).

2.3.2. The BRCA1 and BARD1 BRCT domains

Many DDR and cell cycle checkpoint (CCP) proteins contain BRCT repeats (Gerloff, Woods, Farago, & Monteiro, 2012). BRCT domains may be found as isolated BRCT motifs, tandem repetitions (as in BRCA1 and BARD1), multiple repeats, or two-domain fusions (C. C. Leung & Glover, 2011); moreover, BRCTs can generate homo/hetero BRCT multi-dimers, and BRCT-non-BRCT dimers (Huyton, Bates, Zhang, Sternberg, & Freemont, 2000). BRCA1 residues 1642–1855 create two tandem BRCT repeats – BRCT1 (amino acids 1642–1736) and BRCT2 (amino acids 1756–1855), respectively – connected by a 20 amino acid linker (Fig. 5, bottom left). Each BRCT repeat is composed of three α-helices organized around a four-stranded β-sheet, and the two BRCT repeats interact in a head-to-tail orientation mostly through hydrophobic contacts between

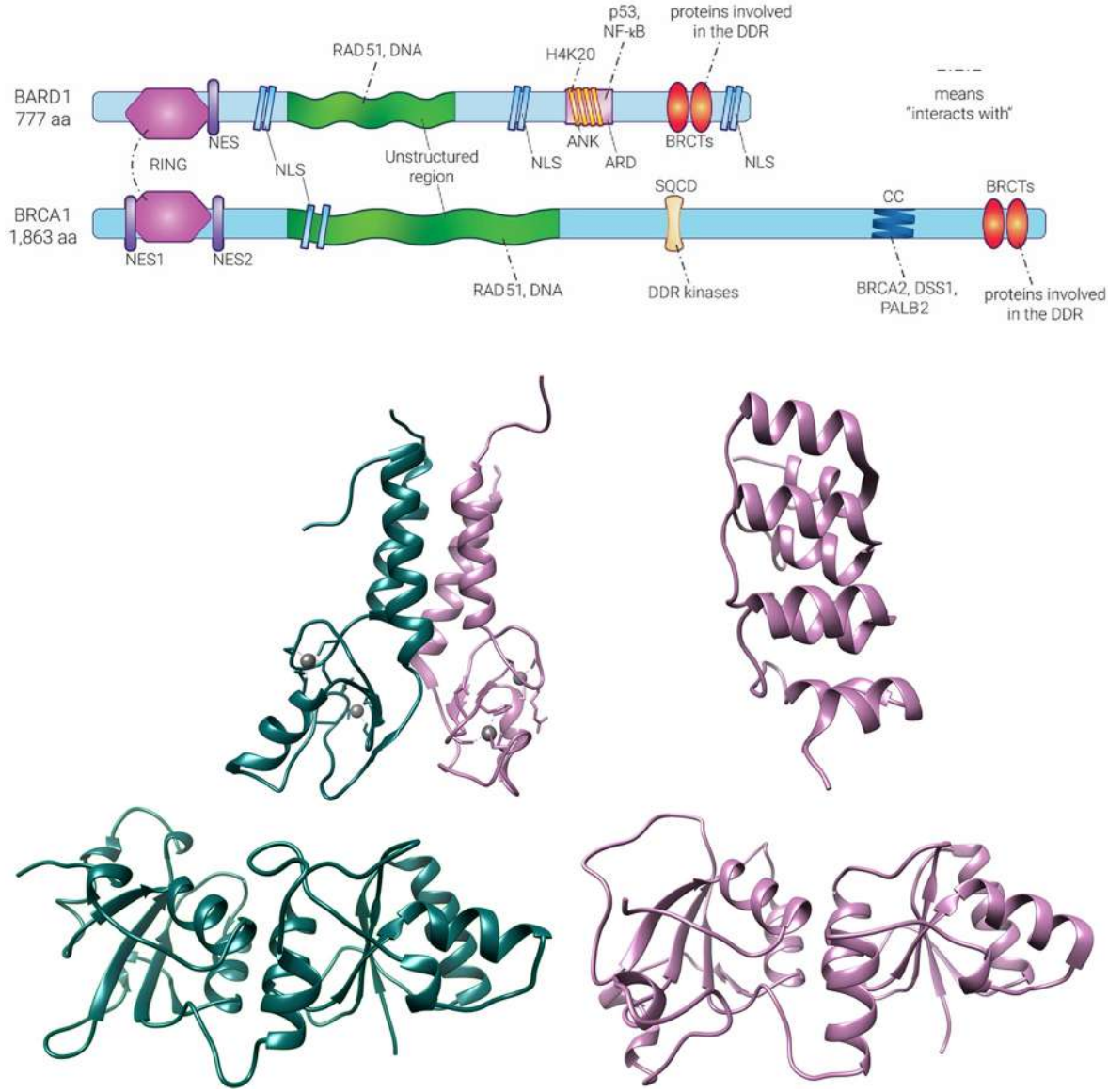

Fig. 5. (Top) Comparison of the functional domains of BARD1 and BRCA1. (Middle, left) Structure of the BRCA1 (teal)/BARD1 (mulberry) heterodimeric RING-RING complex obtained from NMR in solution. Zinc atoms are shown as small gray spheres (PDB: 1JM7 (Brzovic, Rajagopal, Hoyt, King, & Klevit, 2001)). (Middle, right) Crystal structure of the BARD1 ARD (mulberry, PDB: 3C5R (Fox 3rd et al., 2008)). (Bottom) Crystal structures of the BRCT domains of BRCA1 (left, teal, PDB: 1JNX (R. S. Williams, Green, & Glover, 2001)) and BARD1 (right, mulberry, PDB: 2NTE (Birrane, Varma, Soni, & Ladias, 2007)).

the α-helix 2 of BRCT1 and the BRCT2 α-helices 1 and 3. BRCT domains are classified into two categories based on their capacity to recognize phosphoproteins; accordingly, phosphoserine (pSer) residues are recognized by Class-I BRCT domains, while Class-II BRCT domains additionally recognize phosphothreonine (pThr) residues. The BRCA1 BRCT domain specifically senses the sequence pSer-X-X-Phe in its phosphorylated binding partners; accordingly, it is classified as a Class-I BRCT domain. As a phospho-epitope binding domain, BRCA1 BRCT specifically interacts with a plethora of phosphoproteins, including the retinoblastoma binding protein 8 (RBBP8/CtIP) (Fig. 6, top left) (Varma, Brown, Birrane, & Ladias, 2005) – a DNA endonuclease that, after being phosphorylated by the cell cycle dependent kinase (CDK), detects the CCy phase and eventually convert this information to start DNA resection (Ira et al., 2004) – the BRCA1-associated carboxyl-terminal helicase (BACH1/BRIP1) (Fig. 6, top right) (Clapperton et al., 2004a; Litman et al., 2005; Peng, Litman, Jin, Fong, & Cantor, 2006), and the BRCA1-A complex subunit Abraxas 1 (Q. Wu et al., 2016) (aka coiled-coil domain-containing protein 98 or CCDC98/ABRAXAS, Fig. 6, bottom) (Castillo et al., 2014), which both act as mediators of BRCA1 DNA repair, tumor suppression and genome stability functions. While the BRCA1 BRCT domain primary function is to modulate BRCA1-phosphoprotein interactions (Glover, Williams, & Lee, 2004; Manke, Lowery, Nguyen, & Yaffe, 2003; R. S. Williams, Lee, Hau, & Glover, 2004; Yu, Chini, He, Mer, & Chen, 2003), BRCT domains can also mediate DNA binding and non-phosphoprotein interactions, as discussed in detail later on (*e.g.*, §3.3.2.3).

Similarly, the BRCT domain of BARD1 is also composed by two tandem repeats, made up of residues 560–653 (BRCT1) and 667–777 (BRCT2), joined by a 14 residue-long connector (Fig. 5, bottom right) (Koonin et al., 1996; R. S. Williams et al., 2005; R. S. Williams et al., 2001). However, at variance with its partner, BARD1 BRCT recognizes a pSer-Asp/Glu-Asp/Glu-Glu motif, which indicates a different selectivity of this domain with respect to its counterpart on BRCA1 (Birrane et al., 2007). BARD1 tandem BRCT domains have been discovered to be a poly(ADP-ribose) (PAR) binding module, and binding of the BARD1 BRCTs to PAR directs the BRCA1/BARD1 heterodimer to the DDSs (§2.6.1.4).

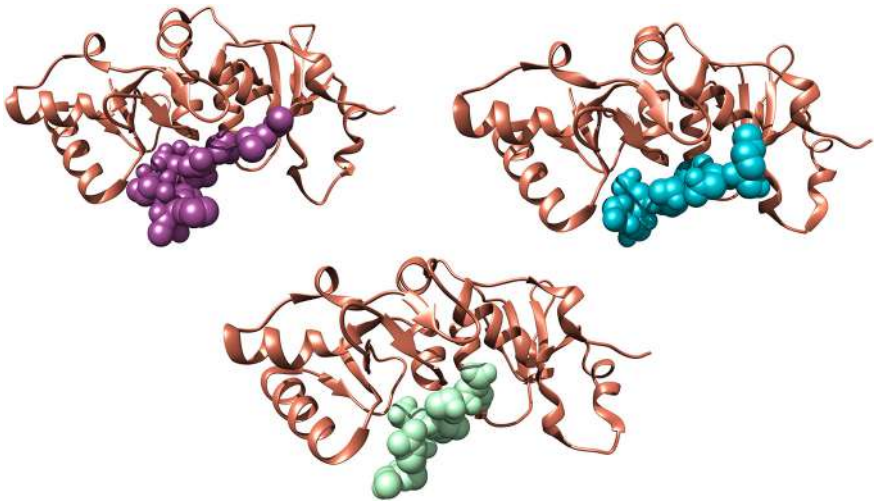

Fig. 6. X-ray structure of the BRCA1 BRCT repeats (spice route ribbon) in complex with a CtIP phosphopeptide (willowherb spheres, top left, PDB: 1Y98 (Varma et al., 2005)), to the phosphorylated interacting region from the BACH1 helicase (peacock blue spheres, top right, PDB: 1T15 (Clapperton et al., 2004b)), and to a single phosphorylated ABRAXAS peptide (green ash spheres, bottom, PDB: 4Y2G (Q. Wu et al., 2016)).

2.3.3. The BRCA1 and BARD1 NLS and NES domains

Although BRCA1 is prevalently a nuclear protein, it shuttles between nucleus and cytoplasm. The active import of BRCA1 into the cell nucleus is governed by two pathways (Henderson, 2005). The former is based on the interaction of the two nuclear localization signals (NLS1 (residues 503–508) and NLS2 (606–615), both encoded in exon 1 and situated in the middle of the protein, Fig. 5, top (Chen et al., 1996; S. Thakur et al., 1997)), with importin-α/β receptors (Fig. 7, top) being in charge of translocating the NLS-cargo into the nucleus through the nuclear pore complex (NPC) (Beck & Hurt, 2017). Of the two sequences, NLS1 has been identified to be the most critical, as mutations of NSL1 completely impaired the ability of BRCA1 to interact with importin-α, while mutations of NSL2 did not fully abolish nuclear localization (Chen, Li, et al., 1996). BRCA1 variants that lack NLSs due to exon 11 splicing enter the nucleus via a second import mechanism that entails BARD1 as a binding partner (Qin et al., 2011a). This alternative mechanism also relies on importins, but it is mediated by a *piggy-back* process according to which BRCA1 binds BARD1 and then the latter uses its own NLSs (*vide infra*) to escort BRCA1 into the nucleus (Fabbro, Rodriguez, Baer, & Henderson, 2002). Such a reciprocal regulatory mechanism of nuclear shuttling could also explain why BRCA1/BARD1 heterodimers are nearly exclusively detected in the nucleus in both fixed (Fabbro et al., 2002) and living cells (Mok & Henderson, 2012). Qin and colleagues proposed that binding of the SUMO-dependent E3 ubiquitin ligase (UBC9/UBE2I, Fig. 7, bottom left) to the N-terminus of BRCA1 could also elicit its nuclear entry and localization (Qin et al., 2011b). Changes in various signaling pathways or the expression of binding partners may therefore affect BRCA1 nuclear import, and protein import targeting has

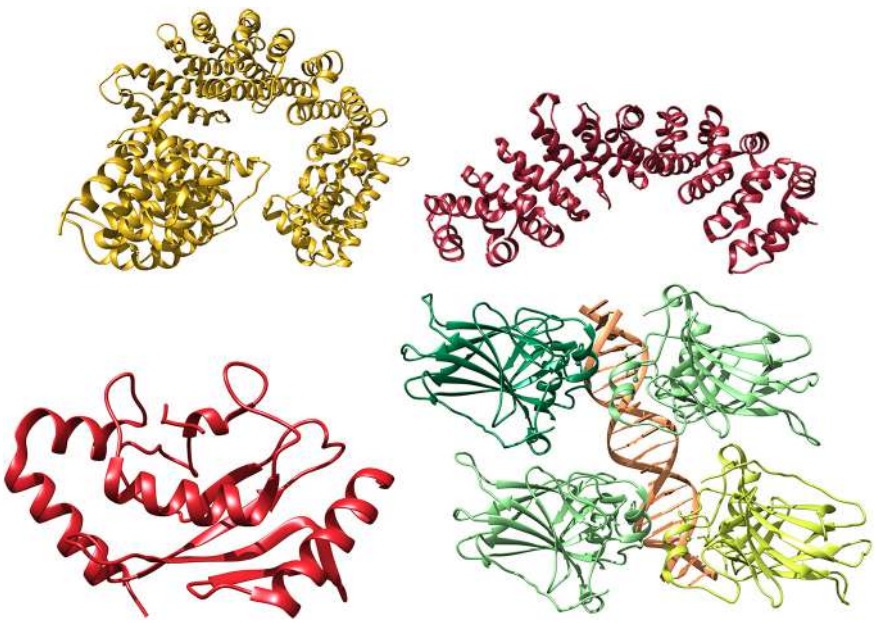

Fig. 7. Crystal structure of importin-β (top left, lemon curry, PDB: 1QGK (Cingolani, Petosa, Weis, & Müller, 1999)) and the so-called armadillo repeats domain (ARM) of a cargo-free importing-α (top right, chili pepper, PDB: 6BVT (K. M. Smith et al., 2018)). Both proteins use they ARM repeats to bind NLSs. (Bottom) Crystal structure of UBC9 (left, flame scarlet, PDB: 6SYF (Hofmann, Akimoto, Wucherpfennig, Zeymer, & Bode, 2020)) and the p53 core domain bound to DNA as a self-assembled tetramer (right, DNA in muskmelon and P53 protomers in green shades, PDB: 3KMD (Y. Chen, Dey, & Chen, 2010)).

been related to different diseases and pathological conditions, including cancer (Kosyna & Depping, 2018).

In this context, the amino and carboxy terminal ends of BRCA1 are the two most functionally sensitive regions of the gene that have been targeted by mutations, and therefore substitution/termination variations within these BRCA1 regions could cause the protein to be imported into the nucleus less efficiently. Indeed, a variety of BRCT mutations inserted into FL BRCA1 have been verified to prevent nuclear localization (Henderson, 2005; Rodriguez, Au, & Henderson, 2004), most likely by virtue of significant changes in the global conformation of the protein (Liang et al., 2017). Within its N-terminal domain BRCA1 also contains two NESs located from residues 22 to 30 (NES1, 22LECPICLEL30) (Thompson, Robinson-Benion, & Holt, 2005) and from positions 81 to 99 (NES2, 81QLVEELL KIICAFQLDTGL99) (Rodríguez & Henderson, 2000). NES2 in particular has been shown to facilitate the export of BRCA1 from the nucleus via the chromosome region maintenance 1 (CRM1)/exportin pathway (Ishizawa, Kojima, Hail, Tabe, & Andreeff, 2015). Unusually for this type of proteins, one of the two α-helices flanking the RING domain (§2.3.1) corresponds exactly to NES1. Intriguingly, studies focused on point mutations in the BRCA1 RING domain (*e.g.*, C61G) revealed no effects in the protein nuclear localization (Fabbro et al., 2002). However, Qin et al. reported that the C61G mutation increased cytoplasmic accumulation of BRCA1, likely as a result of reduced binding of UBC9 (Qin et al., 2011b). The nucleus-cytoplasm shuttling of BRCA1 is also mediated by the tumor suppressor protein p53 (p53, Fig. 7, bottom right),[3] as originally shown by Feng and coworkers (Z. Feng, Kachnic, Zhang, Powell, & Xia, 2004). Jiang and collaborators also investigated the mechanisms of p53-dependent BRCA1 subcellular distribution and DNA damage-induced nuclear export (J. Jiang et al., 2011). This group showed that i) rather than modulating its transcription, p53 mediates BRCA1 nuclear export through protein-protein binding; ii) the BRCA1 BRCT region is essential for this BRCA1/p53 interaction and iii)) p53 may foster BRCA1 nuclear export by interfering with BRCA1/BARD1 heterodimerization. Importantly, using sporadic BC samples these authors demonstrated that WT BRCA1 is retained in the cell nucleus in the presence of dysfunctional p53; accordingly, this p53-dependent BRCA1 shuttling defines the susceptibility of cells to DNA damage as an increase of BRCA1 in the cytosol drastically enhances the susceptibility of cancer cells to IRs. The findings of Jiang et al. than indicate that p53 failure impairs nuclear export of WT BRCA1, which may serve as a mechanism for increasing cellular resilience to DNA damage in BC.

In BARD1, one single NES has also been identified within the heterodimerization region (residues 102–120), coherently with the fact that both proteins are subjected to nuclear entrapment when they dimerize (Rodriguez, Schüchner, et al., 2004). In addition, BARD1 contains six NLS (NLS1-NLS6), which allow the protein to backtrack into the nucleus (Schüchner, Tembe, Rodriguez, & Henderson, 2005); these NLS sequences are located at residues 127–130, 139–155, 321–337, 365–371, 657–663, and 706–709, respectively (Watters, et al., 2020). Deletion of amino acids 292–338 (comprising NLS3) within FL BARD1 resulted in an almost complete protein loss from the nucleus, whilst a deletion of residues 342–379 (encompassing BARD1 NLS4) elicited a consistent yet substantially less drastic impairment of the protein nuclear import (Rodriguez, Schüchner, et al., 2004; Schüchner et al., 2005).

[3] P53, also known as the *guardian of the genome*, is a transcription factor that, upon receiving/modifying/relaying information, masters a number of different cellular functions, including the regulation of cellular senescence, cell metabolism, inflammation, autophagy, and other biological pathways that determine the survival and death of aberrant cells. P53 is also essential in controlling cell response to various biological stressors (*e.g.*, DNA damage or hypoxia) by increasing the transcription of genes that govern cell cycle and death (Levine, 2019).

2.3.4. Unique structural features of BRCA1 and BARD1

2.3.4.1. The BRCA1 CC domain. Exons 11–13 of BRCA1 also code for a protein coiled-coil domain, encompassing residues 1397–1424, which serves as the molecular scaffold for the formation of a higher-order complex composed of BRCA1, BRCA2 and PALB2 (§1), an important player in the assembly of the recombinational DDR machinery at the DDSs (Sy, Huen, Zhu, & Chen, 2009). The group of Sy also demonstrated that the BRCA1/PALB2 association is mainly mediated by apolar interactions between the CC domains of the two proteins (Fig. 3, middle). Through modeling and mutagenesis studies, these authors finally identified amino acids Lys14, Leu21, Tyr28, Leu35, and Glu42 on PALB2 and Met1400, Leu1407, and Met1411 on BRCA1 as the main residues making up the relevant protein/protein binding interface (Sy, Huen, & Chen, 2009). More importantly, cells carrying mutations that disrupted the BRCA1/PALB2 connection were endowed with poor HR repair, in agreement with the converging roles of BRCA proteins in DDR. Thus, the authors proposed that BRCA1 fine-tunes HR through its direct interaction with PALB2, partly *via* its modulatory role in the PALB2-dependent loading of the BRCA2/RAD51 repair machinery at DNA DSBs, and that defective HR repair is one of the central reasons of genomic instability and carcinogenesis in patients with mutations in the BRCA1, BRCA2, or PALB2 genes.

2.3.4.2. The BRCA1 SQCD. BRCA1 includes a SQCD, a serine- and threonine-rich region comprising residues from 1280 to 1524 (also encoded by the BRCA1 gene exons 11–13) which is phosphorylated by DNA damage checkpoint kinase ataxia telangiectasia mutated (ATM, Fig. 8) (Cortez, Wang, Qin, & Elledge, 1999; Gatei et al., 2000; Gatei et al., 2001; Shiloh & Ziv, 2013) and the ataxia telangiectasia and Rad3-related protein (ATR) (Lecona & Fernandez-Capetillo, 2018; Saldivar, Cortez, & Cimprich, 2017; Tibbetts et al., 2000) kinases. Ser1423 and Ser1524 are redundantly phosphorylated by ATM and ATR, whilst Ser1387 and Ser1457 are specific targets for ATM and ATR, respectively (Cortez et al., 1999; Gatei et al., 2001). Besides being key players in the early stages of DSB and single-strand break (SSB) DDR, ATM is involved as an activator of some mediators at later time points along the HR pathway (Ahlskog, Larsen, Achanta, & Sorensen, 2016; Bakr et al., 2015) and, although ATR mainly presides over SSBs and replication stress repair, ATM/ATR cross-talks do occur during DSB repair (J. Smith, Tho, Xu, & Gillespie, 2010), for example, at 5′-end resection during repair of IR-induced DSBs (Jazayeri et al., 2006; Laurini et al., 2020).

The group of Beckta reported that serine-to-alanine changes at key SQCD sites (known targets of ATM and ATR phosphorylation) resulted in decreased HR and abnormal mitosis (Beckta et al., 2015). In particular, the authors showed that while the Ser1387Ala BRCA1 mutant reflected only in a slight decrease in HR efficiency, the Ser1387Ala/Ser1423Ala BRCA1 double mutant ($BRCA1^{2P}$) reduced HR to the level of the empty transfection vector control. Interestingly, the effects described for $BRCA1^{2P}$ were comparable to those observed in the presence of a quadruple mutant also including the S1457A and S1524A variations ($BRCA1^{4P}$), and all these effects did not seem to be reliant on PALB2. Furthermore, the same group discovered that $BRCA1^{4P}$ promoted a protracted and struggling HR late in the cell cycle and switched DSB repair from HR to NHEJ, resulting in mitotic catastrophe in the face of irreversible chromosomal damage. These results demonstrate that BRCA1 SQCD phosphorylation is essential for providing sufficient time for normal HR completion prior to mitosis and preventing cells from entering G1 prematurely, thus avoiding severe chromosomal abnormalities.

2.3.4.3. The BARD1 ARD. BARD1 residues 425–555 comprise the protein ARD domain (Fig. 5, middle right), a structure located upstream of the BRCT domains that includes four ankyrin (ANK) repeats with a non-canonical C-terminal capping ankyrin repeat and a well-ordered extended loop preceding the first repeat (Fox 3rd et al., 2008). Conserved surface features show an acidic patch (Arg427, Glu429, Asp458, Ala460,

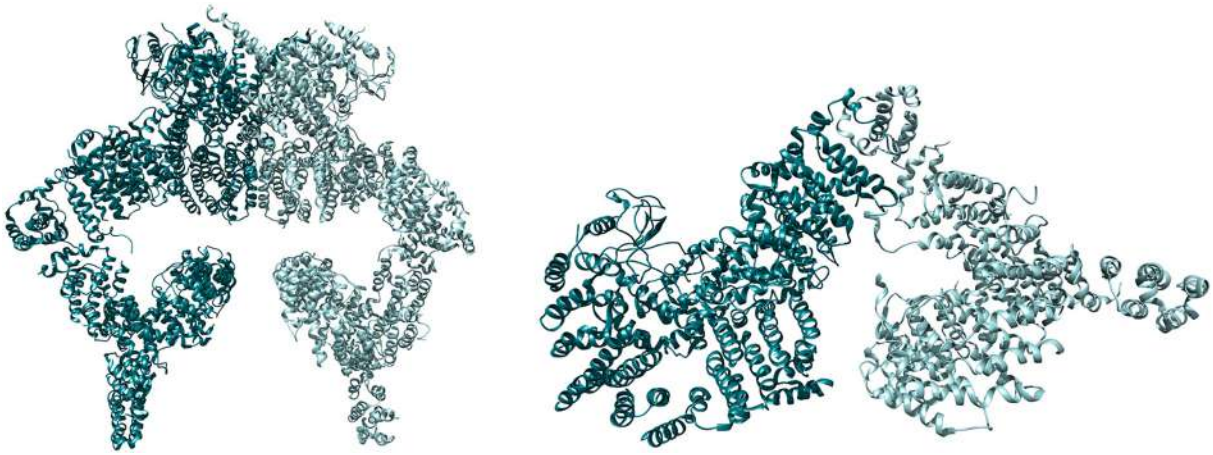

Fig. 8. Structures of the closed (left) and open (right) ATM in its dimeric form (PDB: 5NP0 and 5NP1 (Baretic et al., 2017)), as reconstructed from cryo-EM imaging. The two ATM units are colored in harbor blue and aqua haze, respectively.

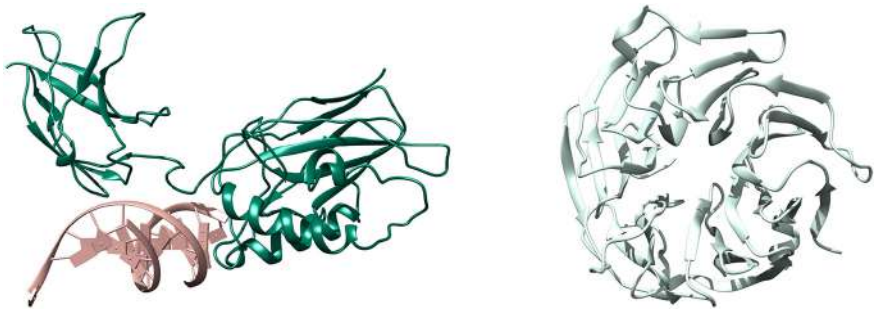

Fig. 9. Crystal structures of NF-κB p50 subunit/DNA complex (left, ultramarine green/rose tan, PDB: 1NFK (G. Ghosh, van Duyne, Ghosh, & Sigler, 1995)) and of CSTF1 (right, opal blue, PDB: 6B3X, (Yang, Hsu, Yang, Song, & Varani, 2018)).

Trp462, Glu467, and Tyr492) and an acidic pocket (Trp462, His466, Gly491, Tyr492, Asp495, Asp500, and Lys503) along the surface typically used by ankyrin repeat domains for binding cognate proteins (J. Li, Mahajan, & Tsai, 2006). Several proteins have been reported to interact with the BARD1 ARD, including p53 (§2.3.3) and the p50 subunit of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB, Fig. 9, left) (Irminger-Finger & Leung, 2002) (§2.6.2.4 and §2.7.5). Interestingly, the ARD and BRCT domains appear to be functionally linked in BARD1 as both motifs are required for BARD1-p53-dependent apoptosis (Feki et al., 2005) (§2.7.2). Furthermore, the ARD-BRCT connector is also important for BARD1 interactions with p53 and CSTF1 (§2.2, Fig. 9, right), and contains two BARD1 BOC-predisposing mutations, Cys557Ser and Gln564His. The former increases BC risk in patients also harboring a BRCA2 mutation (Karppinen et al., 2006; Stacey et al., 2006), whereas the latter is associated with augmented OC risk (Thai et al., 1998) and abrogation of p53-dependent apoptosis (Irminger-Finger et al., 2001).

Choudhary and collaborators studied the dynamics of the ARD domain present in the BARD1 WT and Gln564His mutant proteins in association with CSTF1 using biophysical, biochemical and computer-based molecular dynamics (MD) simulations (Choudhary et al., 2017). They observed that i) the ARD domain is relatively more flexible than the BRCT domain; ii) the relative orientation of these two domains varies in time due to the highly flexible nature of their connector, and iii) the Gln564His mutant ARD is more dynamic in nature compared to the corresponding WT motif. MD studies showed that the motion of the BARD1 ARD was stabilized when complexed with CSTF1 and contextually predicted loss of interaction between Gln564His mutant BARD1 and the cleavage stimulation factor. The group of Fox also showed that two further reported BARD1 ARD mutations (N470S and V507M) – putatively linked to cancer predisposition (Ishitobi et al., 2003; Vahteristo et al., 2006) – do not result in observable structural defects, and that in the absence of any interacting protein, the BARD1-ARD and tandem BRCT domains behave independently in solution (Fox 3rd et al., 2008). Notably, two independent studies on cohorts from South America (Gonzalez-Hormazabal et al., 2012) and Iceland (Stacey et al., 2006) proposed the low-frequency BARD1 mutation Cys557Ser as a BC risk-conferring variant; however, other investigations did not confirm this association in different populations, including Australian (Johnatty et al., 2009), Chinese (Ding et al., 2011), Polish (Jakubowska et al., 2008) and a multiethnic group (Spurdle et al., 2011).

2.4. Transcriptional regulation of BRCA1 and BARD1

2.4.1. Regulation of transcription of the BRCA1 gene

The BRCA1 gene is a component of an intricate pathway network that regulates its expression at both the transcriptional and translational levels, and which is also influenced by the cell cycle and other factors (P. B. Mullan, Quinn, & Harkin, 2006; Xu, Chambers, & Solomon, 1997). Two different promoters – promoter α and β – master the human BRCA1 gene (Santana Dos Santos et al., 2018). Promoter α (aka the minimum or proximal BRCA1 promoter) in particular is a TATA-less bidirectional promoter (Y. Jin, Eser, Struhl, & Churchman, 2017) that regulates transcription of both the BRCA1 and next to BRCA1 gene 2 (NBR2)[4] genes. BRCA1 and NBR2 start sites are separated by a 218 base pair intergenic region (Xu et al., 1997), within which the segment from −202 to −166 (relative to the BRCA1 1a exon start site) has been verified to be

[4] NBR2 is a non–protein coding gene that encodes a long non-coding RNA (lncRNA) (§2.4.2) and suppresses tumor development through regulation of adenosine monophosphate–activated protein kinase (AMPK) activation (Xiao, Liu, Zhuang, & Gan, 2016).

indispensable for the activity of the BRCA1 promoters (S. Thakur & Croce, 1999; S. Thakur et al., 2003). Two sub-elements compose the BRCA1/NBR2 intergenic region: the RIB elements[5] – which span 22 base pairs (from −204 to −182) and are constitutively activated by the GA-binding protein α/β transcription factor (GABPα/β, Fig. 10, top left) (E. Atlas, Stramwasser, Whiskin, & Mueller, 2000) – and the CRB/ATF1 element, which spans 7 base pairs (−174 to −167) and is constitutively activated upon binding to another transcription factor, the cAMP-response element binding protein (CREB) (Ella Atlas, Stramwasser, & Mueller, 2001; Mancini et al., 1998).

Several other transcription factors or activating assemblies participate in the complex transcriptional regulation of BRCA1. Wang and coworkers demonstrated that the retinoblastoma susceptibility gene product (RB, Fig. 10, top right) – a nuclear phosphoprotein that is inactivated in retinoblastoma and other tumors (W.-H. Lee et al., 1987) – regulates the expression of the BRCA1 gene (Wang, Schneider-Broussard, Kumar, MacLeod, & Johnson, 2000) through its ability to modulate the activity of the E2 factor (E2F) transcriptional network (Bindra & Glazer, 2006; Dimova & Dyson, 2005; Li et al., 2018; Oberley, Inman, & Farnham, 2003). Two E2F binding sites have been identified, located between base pairs −39 to −32 (E2FA) and − 18 to −11 (EF2B) upstream of the BRCA1 promoter, and the reported evidence that mutations in either E2FA or E2FB reduce BRCA1 promoter activity in OCs suggests that both binding sites are required for optimal transcriptional activity (Kent & Leone, 2019). Kanakkanthara et al. showed that, upon interaction with an E2FA site on the BRCA1 promoter, the zinc finger CCCH-type containing 18 (ZC3H18) promotes BRCA1 transcription by recruiting E2F4 to a nearby E2F site. (Kanakkanthara et al., 2019). The same group also discovered that the levels of BRCA1 mRNA positively correlate with both ZC3H18 and E2F4 mRNA levels in patient-derived xenografts and primary high-grade serous ovarian cancers (HGSOCs), confirming the role of ZC3H18 in regulating BRCA1.

The tumor suppressor p53 (§2.3.3) is central to genome integrity preservation (S. Armstrong, 2014; D. P. Lane, 1992; F. Mantovani, Collavin, & Del Sal, 2019). Its oncosuppressor function has been principally linked to the decision of cell faith in response to damage, with the ability to eliminate malignant cells while preserving the integrity of the organism (Aubrey, Kelly, Janic, Herold, & Strasser, 2018). Following DNA damage, p53 swiftly masters temporary delays in cell cycle progression, allowing cells more time to activate the DDR system prior to critical cell cycle transitions, particularly the transition from G1- to S-phase. In the event of significant DNA damage, p53 may irreversibly prevent further proliferation of the affected cells by activating either senescent or apoptotic processes (Valente et al., 2013). Different efforts testify the role of p53 in modulating BRCA1 expression at the transcription stage, and in particular how BRCA1 expression levels are down-regulated in response to p53 induction in cells that undergo either growth arrest, senescence, or apoptosis (Arizti et al., 2000; MacLachlan, Dash, Dicker, & El-Deiry, 2000). Remarkably, 53BP1 (§1) – another tumor suppressor and a crucial component of DNA DSB signaling and repair in mammalian cells that include two carboxy-terminal BRCT repeats (Panier & Boulton, 2014) – is a positive regulator of the BRCA1 promoter α *via* binding to an element that overlaps E2FA between base pairs −40 and − 25 (Moureau, Luessing, Harte, Voisin, & Lowndes, 2016; Rauch, Zhong, Pfeifer, & Xu, 2005). BRCA1 promoter activity is also inhibited upon interaction of its region including base pairs from −209 to −169 with the splice variant 1b of the high mobility group 1 protein (HMGA1b, (Baldassarre et al., 2003)), a non-histone chromatin protein involved in many cellular processes related to *e.g.*, regulation of gene transcription, organization of heterochromatin, DNA replication and cancer cell metastatic progression. Actually, two protein isoforms (HMGA1a and HMGA1b) are generated *via* an alternate transcriptional splicing process of the structural gene HMGA1 (Friedmann, Holth, Zoghbi, & Reeves, 1993; Reeves & Beckerbauer, 2001).

The MYC proto-oncogene protein (c-MYC) is a transcription factor member of the bHLHZip (basic-helix-loop-helix-leucine zipper) family. It is located in the cell nucleus – where it is involved in the regulation of cell growth, differentiation, metabolism, and death – and is found to be frequently dysregulated in a wide range of human cancers (Madden, de Araujo, Gerhardt, Fairlie, & Mason, 2021; Pelengaris, Khan, & Evan, 2002). In the context of BRCA1 gene regulation, c-MYC activates the BRCA1 promoter upon binding two E-box motifs[6] located in the regions from −1292 to −1286 and from −912 to −907, respectively, upstream of the BRCA1 exon 1a transcription starting site (Y. Chen et al., 2011). Using an *inverse genomics* approach based on a randomized ribozyme gene library, Beger and coworkers identified inhibitor of differentiation 4 (ID4, a member of the helix-loop-helix (HLH) family of proteins, which are dominant negative regulators of transcription (R. Benezra, Davis, Lockshon, Turner, & Weintraub, 1990; Norton, Deed, Craggs, & Sablitzky, 1998)), as a negative regulator of BRCA1 (Beger et al., 2001). More recent data based on ID4 overexpression and amplification support a possible role for this protein as a protooncogene in a subgroup of basal-like breast cancers (BLBCs), where it suppresses *BRCA1* function conferring poor prognosis (Baker, Holliday, & Swarbrick, 2016). Finally, yet not less importantly, De Siervi and coworkers discovered a remarkable connection between the functions of BRCA1 as a coregulator member of the transcriptional machinery and the modulation of its own expression through an autoregulatory transcriptional loop (De Siervi et al., 2010). They found that BRCA1-depleted cells respond by upregulating BRCA1 transcripts, while BRCA1-overexpressing cells react in the opposite way, and thus suggested an intriguing model according to which BRCA1 is involved in environmental-sensitive, auto-regulatory assembly at its own promoter. Along a similar line Horwitz et al. identified an ubiquitin-dependent mechanism by which BRCA1 inhibits transcription (Horwitz, Affarel, Heine, Shi, & Parvin, 2007). According to their work, BRCA1 can elicit transcription repression by ubiquitinating some components of the pre-initiation complex, implying that environmental factors (*e.g.*, genotoxic stress) may stimulate dynamical changes in the ubiquitylation of proteins at the BRCA1 promoter *via* the BRCA1/BARD1 heterodimer E3-ligase activity (§3.1). These findings imply that the roles of BRCA1 as a transcriptional repressor areas important as its functions as a transcriptional activator; however, since these evidences were obtained *in vitro*, further research will be required to determine if and how such changes occur at the BRCA1 promoter *in vivo*.

It has also been reported that other environmental variables – including, hypoxia, genotoxic agents and mitogenic hormone stimulation – may alter BRCA1 expression *via* the regulation of BRCA1 transcription factors. For example, the work of Bindra et al. revealed that hypoxic conditions induce a dynamic redistribution of promoter occupancy by EF2, resulting in the transcriptional repression of BRCA1 expression (Bindra et al., 2005). In the absence of genetic mutation, hypoxia-induced repression of BRCA1 expression therefore offers an interesting mechanism of functional BRCA1 inactivation which, in turn, may result in genomic instability by changing the balance between the high-fidelity HR and the error-prone NHEJ pathways of the DDR system, as suggested by these authors. Two research groups also found that the C-terminal binding protein 1 (CtBP1, Fig. 10, bottom) represses BRCA1 transcription by binding to the E2F site of the BRCA1 promoter (Deng et al., 2010; Di, Fernandez, De Siervi, Longo, & Gardner, 2010). Additionally, Deng et al. discovered that i) the recruitment of CtBP1 to the BRCA1 promoter

[5] The term RIBs - reiterative IHF BIMEs - refers to a group of bacterial repetitive DNA elements located at the 3'end of transcription units. RIBs were discovered as a class of integration host factors (IHFs), where IHF is a histone-like heterodimeric protein in the bacterium *Escherichia coli* (Freundlich, Ramani, Mathew, Sirko, & Tsui, 1992). The term BIMEs (bacterial interspersed mosaic elements) refers to prokaryotic repetitive extragenic palindrome (REP) or palindromic unit (PU) sequences (Boccard & Prentki, 1993).

[6] The name *E-box* refers to DNA motifs characterized by the consensus sequence CANNTG, which serve as protein binding sites in a wide range of promoters and enhancers.

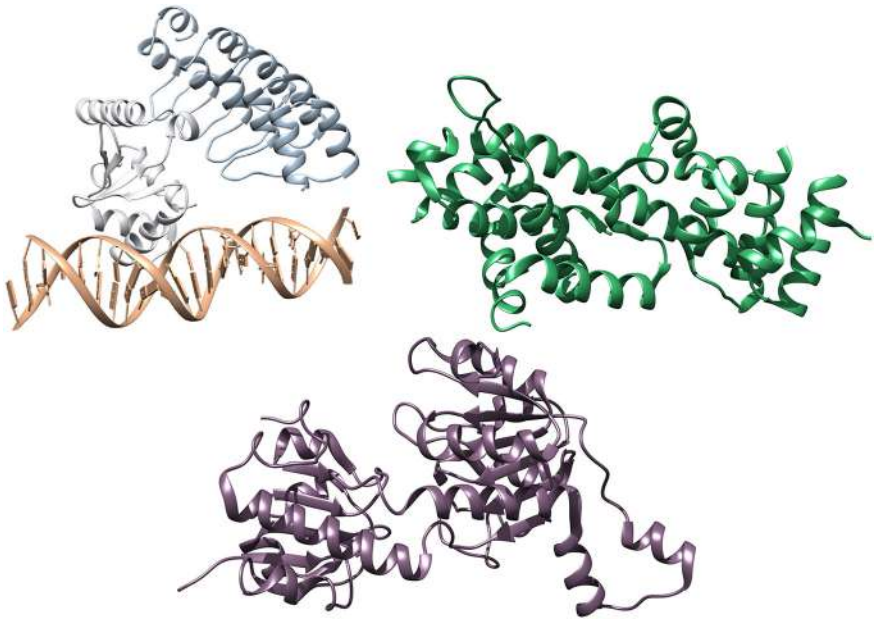

Fig. 10. (Top left) X-ray structure of the GABPα/β transcription factor (microchip gray/blue fog) bound to DNA (tan, PDB: 1AWC (Batchelor, Piper, de la Brousse, McKnight, & Wolberger, 1998)). The α-subunit features a DNA-binding domain that belongs to the ETS family (§2.7.2), whereas the β-subunit is constituted by a series of ANK repeats (§2.3.4.3). (Top right) Crystal structure of the RB N-terminal domain (Kelly green, PDB: 2QDJ (Hassler et al., 2007)). (Bottom) Crystal structure of CtBIP1 (grape jam, PDB: 6CDR (Bellesis, Jecrois, Hayes, Schiffer, & Royer Jr., 2018)).

is redox-dependent, that is, it increases at the high levels of reduced nicotinamide adenine dinucleotide (NADH) characterizing hypoxic conditions, and ii) the disruption of the CtBP1 interaction at the BRCA1 promoter induced by the presence of the antioxidant Tempol (which reduces NADH levels) removed the CtBP1-mediated repression of BRCA1 and restored DNA repair in cancer cells. Considering that tumor cells commonly become hypoxic (and hence have high NADH levels), the dynamic regulation of BRCA1 by this mechanism – that the authors termed a *metabolic switch* – offers an essential connection between tumor metabolism and tumor suppressor expression. DNA damaging agents and other cytotoxic substances also play a role in regulating BRCA1 expression, and already in 1998 Andres and collaborators found that a number of pharmacological treatments, including DNA intercalators and inhibitors of topoisomerase II (*e.g.*, adriamycin), topoisomerase I inhibitors (*e.g.*, camptothecin), and UV radiation significantly lowered the levels of BRCA1 and BRCA2 mRNA (Andres et al., 1998). Likely the best-characterized stimulant of BRCA1 expression is estrogen, which elicit the highest increase in BRCA1 mRNA levels that routinely peak just before the onset of DNA synthesis (Marks et al., 1997; P. B. Mullan et al., 2006). In this manner, BRCA1 acts as a feedback regulator, monitoring and inhibiting the growth and pro-proliferative effects of the most potent estrogen 17β-estradiol (ES)[7] in hormone-responsive tissues (Gorski, Kennedy, Hosey, & Harkin, 2009; T. F. Lane et al., 1995; Marks et al., 1997). Further efforts focusing on the mechanisms underlying the modulation of BRCA1 by estrogen showed that this sex hormone stimulates BRCA1 promoter activity in transfected cells and that, upon ES stimulation, the estrogen receptor α (ERα, Fig. 11, left) with its cofactor, the nuclear protein p300 (p300), is recruited to the AP-1[8] site located in the region from −27 to −31 upstream of BRCA1 exon 1b transcription starting site (Jeffy et al., 2005). Contextually, the authors documented that p53 overexpression prevents the recruitment of ERα to the AP-1 site and represses BRCA1 promoter activity. Accordingly, these findings support a model in which an ERα/AP-1 complex modulates BRCA1 transcription under conditions of ES stimulation, while the formation of this transcription complex is abrogated in cells overexpressing p53. In a follow-up study, the same group reported that the activation of BRCA1 transcription by ES requires occupancy of the BRCA1 promoter by the unliganded aryl hydrocarbon receptor (AhR, Fig. 11, right), and proposed that the ligand status of the AhR modulates the E2-dependent activation of the BRCA-1 promoter (Hockings et al., 2006).

Epigenetic modifications and the action of enhancers/repressors also modulate the expression of the BRCA1 gene. When Mancini and colleagues published their findings in 1998, they were the first to report a difference in the pattern of DNA methylation within the regulatory region of the BRCA1 gene. Specifically, they found no evidence of methylation at CpG islands within the BRCA1 promoter in a variety of normal human tissues, while the presence of CpG methylation adjacent to the BRCA1 transcription start site were detected in a screening of a series of randomly sampled BCs (Mancini et al., 1998). One such methylated CpG occurs within the CREB transcription factor binding site in the BRCA1 promoter (position −173 relative to exon 1a), and inhibits CREB binding, thereby suppressing BRCA1 expression (DiNardo, Butcher, Robinson, Archer, & Rodenhiser, 2001). To investigate the preservation of methylation-free zone (MFZ) in the BRCA1 promoter regions observed in normal breast tissues, Butcher et al. provided data implicating two proteins – the specific protein 1 (SP1) (Vizcaíno, Mansilla, & Portugal, 2015) and the transcriptional repressor CTCF (aka CCCTC-binding factor, CTCF) (Somi Kim, Yu, & Kaang, 2015) – in the maintenance of this MFZ which, in healthy tissues, starts 650 base pairs (bps) upstream of the transcription start site and extends for 1.4 kilobases (kbs) through most of the BRCA1 CpG island (Butcher, Mancini-DiNardo, Archer, & Rodenhiser, 2004).

2.4.2. Regulation of transcription of the BARD1 gene

Compared to its partner, substantially less information is available on the regulation of the BARD1 gene both at a transcriptional and

[7] Although the commonly used abbreviation for 17β-estradiol is E2, in this work we adopted the acronym ES to indicate estrogen in order to avoid confusion with the ubiquitin-conjugating enzyme E2, for which we maintained the canonical abbreviation E2.

[8] AP-1 is a collective term referring to dimeric transcription factors composed of the transcription factor AP-1 (AP1/JUN), the protooncogene c-FOS (FOS) or the activating transcription factor (ATF) subunits (Hai & Hartman, 2001; van Dam & Castellazzi, 2001) that bind to a common DNA site termed the AP-1-binding site (Karin, Liu, & Zandi, 1997).

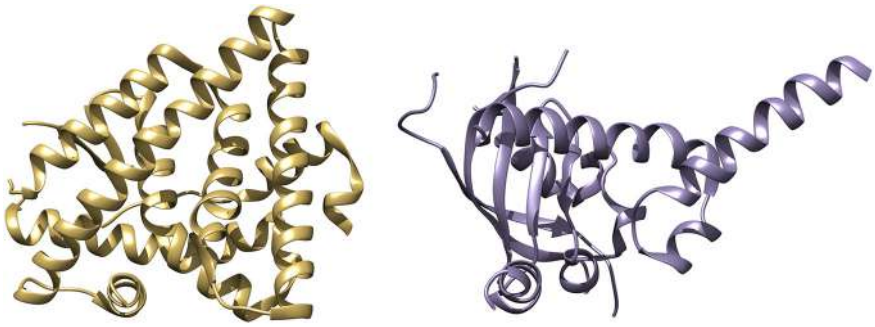

Fig. 11. Crystal structures of ERα (left, rich gold, PDB: 2QSE (Nettles et al., 2008)) and AhR (right, purple haze, PDB: (Schulte, Green, Wilz, Platten, & Daumke, 2017)).

molecular level. In 2002, Ren et al. reported that the promoter region of BARD1 was occupied by E2F4 (Ren et al., 2002). Five years later, in the quest of isolating and identifying genes associated with ERα (§2.4.1) in MCF-7 human breast cancer cells – a widely studied epithelial cancer cell line derived from breast adenocarcinoma (Comşa, Cîmpean, & Raica, 2015) – the group of Creekmore reported one of such gene regions as a 288 bp fragment from the 9th intron of the BARD1 gene (Creekmore, Ziegler, Bonéy, & Nardulli, 2007). Using MCF-7 cells, they demonstrated that i) ERα associated with the 288 bp BARD1 gene region *via* binding to three out of five estrogen response elements (EREs) located in this zone of BARD1 9th exon, and ii) this 288 bp BARD1 region conferred ES responsiveness to a heterologous promoter. Importantly, treatment of MCF-7 cells with ES increased BARD1 mRNA and protein levels, thereby providing a likely mechanism through which the fate of mammary cells might be influenced by the ES-induced regulation of the BARD1 gene.

lncRNAs are RNA molecules of more than 200 nucleotides in length that are ubiquitously expressed yet do not include significant open reading frames (ORFs); they have the potential to serve as epigenetic regulators of gene expression, influencing transcription, messenger RNA (mRNA) stability/transport, and translation, despite the fact that exact functions have been ascribed only to a few of these molecules (Yao, Wang, & Chen, 2019). In a suitable environment, both coding and non-coding RNAs may be the target of the same microRNAs (miRNAs)[9] and they can indirectly control each other by vying for them. Such RNAs, also known as competing endogenous RNAs (ceRNAs), contribute to the formation of an extra post-transcriptional regulatory layer, ncRNAs may take on new importance and functions (Ala, 2020). During their investigation focused on the epigenetic regulation of the BARD1 gene, Pilyugin and Irminger-Finger found that the BARD1 3'untranslated region (3'UTR) is almost 3000 nucleotide (nt) long and harbors a large number of miRNA binding elements (M. Pilyugin & Irminger-Finger, 2014). In addition, they reported a newly discovered lncRNA – BARD1 9′L – which results from the transcription of an alternative BARD1 promoter on intron 9 and shares a portion of the 3'UTR with the protein-coding BARD1 mRNAs. Using two well-known miRNAs (*i.e.*, miR-203 and miR-101), the authors demonstrated that they down-regulate the expression of the FL BARD1 and cancer-associated BARD1 mRNAs, while BARD1 9′L counteracts the effect of both miR-203 and miR-101, highlighting BARD1 9′L as a possible ceRNA regulating the expression of BARD1 mRNA. Further, they also observed another alternative promoter in intron 9 governing production of the putative isoform BARD1 9′S, that may encode a 148-residue long protein construct. Of these, amino acids 1–6 are exclusive for BARD1 9′S, while amino acids 7–148 correspond to FL BARD1 residues 636–777.

As seen for BRCA1 (§2.4.1) and discussed above for BARD1, the hormone-induced expression of both proteins or of BARD1 isoforms may serve as a connection that partly explains the elevated BC/OC risk linked with BARD1 or BRCA1 deficits and ES exposure. Moreover, since FL BARD1 is expressed at very low level or is completely absent in tumor tissues while, on the contrary, different BARD1 spliced isoforms are overexpressed and linked to tumorigenesis (§2.7.6), a better knowledge and a deeper understanding about the mechanisms that master the expression of FL BARD1 and its isoforms constitutes an actual, fundamental issue that imperatively warrants further studies.

2.5. Regulation and stability of the BRCA1 and BARD1 proteins

2.5.1. Regulation and stability of the BRCA1 protein

Since the BRCA1 protein was shown to exhibit several important nuclear functions, and is most frequently detected in that compartment, it is usually defined as a predominantly nuclear phosphoprotein (Wilson et al., 1999). Yet, BRCA1 is ubiquitously expressed – albeit at different levels – in human tissues and, in line with what discussed above about the BRCA1 gene transcription regulation, the post-translational control of the BRCA1 protein levels and compartment location are also very complex (Henderson, 2005). In terms of protein life-time, this has been verified to be rather short in asynchronous cells: for example, studies performed with human embryonic kidney (HEK 293 T), prostate cancer (DU145) and cervical cancer (HeLa) cell lines reported half-life values of 2 to 4 h following treatment with cycloheximide[10] (Blagosklonny et al., 1999; Choudhury, Xu, & Baer, 2004). BRCA1 protein expression varies in a cell cycle-dependent manner, with low steady-state levels in resting cells (G0) and early G1 cycling cells, levels rising as the cells move into late G1-phase (where the protein is mostly hypophosphorylated), peaking during the S- and M phases and remaining high in the G2-phase (in which the protein is in a hyperphosphorylated state) before declining as the cells move back into G1 (corresponding again to an hypophosphorylated protein state) (Chen et al., 1996; Gudas et al., 1996; Ruffner & Verma, 1997). This decrease in BRCA1 levels corresponds with the emergence of proteasome-sensitive BRCA1 ubiquitin conjugates at the beginning of G1, which seems to be different from autoubiquitination products and are most likely mediated by the activity of other cellular E3 ligases. BARD1 co-expression prevents the production of these conjugates, suggesting that BARD1 aids in the stabilization of BRCA1 expression by limiting the proteasome-sensitive ubiquitination of BRCA1 polypeptides (Choudhury et al., 2004). BRCA1 ubiquitination, which results in its subsequent proteasomal degradation, occurs in its N-terminal domain,

[9] miRNAs are a subtype of small (20–22 nucleotides) ncRNAs (Gebert & MacRae, 2019) synthesized via a complex biogenesis process (Ha & Kim, 2014; Treiber, Treiber, & Meister, 2019), starting from nuclear transcription to cytoplasmic processing by the RNase III enzyme Dicer (M. S. Song & Rossi, 2017). Mature miRNAs are incorporated within the multiprotein complex RISC (RNA-induced silencing complex) (Paroo, Liu, & Wang, 2007), and direct it to target transcripts, where they often interfere with their translation and, in certain instances, promote their destruction (Catalanotto, Cogoni, & Zardo, 2016; Khraiwesh et al., 2010).

[10] Cycloheximide is a protein synthesis inhibitor as it prevents translational elongation. It is often used in cell biology to estimate the half-life of a particular protein. (Kao et al., 2015).

which is located inside the degron[11] region of the protein comprised between residues 1 and 167 (Y. Lu et al., 2007). There are a total of 14 lysine residues within the BRCA1 degron, all of which are conserved among vertebrate orthologs of BRCA1 and can serve as sites for polyubiquitination and proteasome-mediated protein degradation. Remarkably, Lu et al. reported that, although this region is also populated by highly conserved serines and threonines, mutating each of these residues did not abolish the ubiquitin ladder or resulted in a stabilized protein fragment. Vice versa, the concomitant mutation of all degron lysines prevented ubiquitination and, thus, protein degradation (Y. Lu et al., 2007). Only a few proteins that ubiquitinate BRCA1 within its degron region have been discovered to date. Among these, Wu and collaborators reported that the HECT and RLD domain containing E3 ubiquitin protein ligase 2 (HERC2),[12] a protein recently implicated in diverse cellular functions which include DDR, cell proliferation and neurodevelopment (García-Cano et al., 2019), targets BARD1-uncoupled BRCA1 for degradation by interacting with the BRCA1 degron *via* its C-terminal HECT-containing domain (W. Wu et al., 2010). HERC2-BRCA1 interaction depends on the cell cycle, being maximal during the S-phase and then quickly decreasing as cells progress into G2-M, a behavior inversely correlated with the steady-state level of BRCA1 discussed above. Notably, a depletion in HERC2 counteracts the effects of a depletion in BARD1 depletion via restoration of BRCA1 expression and G2-M checkpoint functions, while BARD1 exerts as protective role on BRCA1 by preventing its HERC2-mediated ubiquitination.

HERC2-BRCA1 interaction is maximal during the S-phase of the cell cycle and rapidly weakens as cells enter G2-M, a behavior inversely correlated with the steady-state level of BRCA1 discussed above. Notably, HERC2 depletion antagonizes the effects of BARD1 depletion by restoring BRCA1 expression and G2-M checkpoint activity while BARD1 protects BRCA1 from HERC2-mediated ubiquitination. Using mass spectrometry-based investigations, in 2012 the group of Lu found that the SKP1-CUL1-F-box-protein 44 (SCF^{FBXO44}) complex[13] (Fig. 12, top and bottom left) ubiquitinates FL BRCA1 *in vitro*. Furthermore, they showed that the N-terminal of BRCA1 mediates the interaction between BRCA1 and FBXO44, and that overexpression of SCF^{FBXO44} reduces BRCA1 protein levels, confirming the role of SCF^{FBXO44} as an E3 ubiquitin ligase responsible for BRCA1 degradation. Two years later Wang et al. identified the HECT, UBA and WWE[14] domain containing E3 ubiquitin-protein ligase 1 (HUWE1, Fig. 12, bottom right), also known as Mule or ARF-BP1 (Gong et al., 2020) as a novel BRCA1-interacting protein involved in the control of BRCA1 protein level by binding to its degron domain (X. Wang et al., 2014). According to this study, silencing of the HUWE1 gene *via* RNA interference (RNAi) – a natural mechanism in which the cell promotes suppression of gene expression by using sequence-specific dsRNAs (aka small interfering RNAs or siRNAs) (Setten, Rossi, & Han, 2019) – increased both the half-life and the levels of the BRCA1 protein, whereas HUWEI exogenous expression elicited the BRCA1 degradation *via* the ubiquitin-proteasome route, emphasizing HUWE1 as a critical negative regulator of BRCA1.

Using again RNAi-based experiments Kim et al. showed that the depletion of cathepsin S (CTSS (Fig. 13, top left), a lysosomal protease member of the cysteine cathepsin protease family which can promote degradation of damaged/unwanted proteins in the endo-lysosomal pathway (Wilkinson, Williams, Scott, & Burden, 2015)) enhances the protein stability of BRCA1 by inhibiting its ubiquitination (SeoYoung Kim, Jin, Seo, Lee, & Lee, 2019). At variance with HERC2, however, CTSS promotes ubiquitin-mediated proteolytic degradation of BRCA1 by interacting with its BRCT domain (§2.3.2), indicating that RING domain-independent BRCA1 degradation may also play a key role in determining BRCA1 stability. Interestingly, *in vivo* ubiquitination assay carried out by Ueki and coworkers indicated BRCA1 to be polyubiquitinated by the E2 ligase ubiquitin-conjugating enzyme E2T (UBE2T, Fig. 13, top right) (Ueki et al., 2009). Knocking down of UBE2T protein by siRNAs induced upregulation of BRCA1 protein in BC cells, whereas its overexpression caused the decrease of the BRCA1 protein, thereby implying a critical role of UBE2T in BRCA1 regulation.

Ubiquitylation, like other PTMs, is a reversible process, and specific enzymes called deubiquitinases (DUBs) indeed not only cleave ubiquitin from specific substrate proteins but also modify ubiquitin chains and/or process ubiquitin precursors. (Harrigan, Jacq, Martin, & Jackson, 2018). DUBs which remove ubiquitination from BRCA1 in order to counteract its turnover, however, are less well understood and only a few of them have been identified to date. Besides its cognate partner BARD1, one of such DUBs is the probable ubiquitin carboxyl-terminal hydrolase FAF-X (USP9X, Fig. 13, bottom), very recently described by Lu et al. as a *bona fide* deubiquitinase for BRCA1 in human cancer cells (Q. Lu, Zhang, Lu, Shao, & Li, 2019). USP9X depletion by RNAi or USP9X inhibition by the small compound WP1130 (Harrigan et al., 2018) drastically reduced BRCA1 protein abundance and half-life, although its mRNA levels were not affected. On the other hand, overexpression of WT USP9X let to BRCA1 upregulation whereas a USP9X mutant (C1566S) defective in deubiquitinase activity had no effect, thus establishing USP9X as a BRCA1 deubiquitinase.

Recently, Kim et al. found that high levels of the transforming acidic coiled-coil protein 3 (TACC3)[15] can cause genomic instability possibly in part through destabilizing BRCA1 *in vitro* (J. L. Kim, Ha, Campo, & Breuer, 2018). They also discovered that high amounts of TACC3 hindered the connection between BRCA1 and BARD1, thereby flagging the BARD1-free BRCA1 for ubiquitin-mediated proteosomal degradation. Moreover, in BC tissues this research group discovered an inverse relationship between TACC3 and BRCA1 expression, providing fresh insight on TACC3's involvement in BRCA1 regulation, genomic instability, and insurgence of BC.

Despite the fact that BRCA1 has mostly been identified as a nuclear protein, it is known to shuttle back and forth between the cytoplasm and the nucleus. Within the former, BRCA1 has been discovered to bind γ-tubulin at centrosomes during the process of mitosis (Chen, Li, et al., 1996; Hsu, Doan, & White, 2001; Hsu & White, 1998) (§3.2). A number of additional cell compartments, including the mitochondria and the endoplasmic reticulum, have been discovered to contain BRCA1 (Coene et al., 2005; Laulier et al., 2011). Two distinct pathways regulate the nuclear import of BRCA1. The former transfers BRCA1 into

[11] Based on the properties of the signal that causes protein degradation, degrons may be classified into two main categories: inherent degrons (IDs) and acquired degrons (AD). IDs - which are invariably present in proteins - may be particular aminoacidic sequences (*e.g.*, the protein N- and C-termini, corresponding to the N- and C-degron routes, respectively (Varshavsky, 2019)) or hydrophobic sequences, which are typically buried in the protein core or on contact surfaces between different components of protein complexes. ADs, are transient elements generated by post-translational modifications (PTMs), which include phosphorylation, ligation of small ubiquitin-like modifier (SUMO) and hydroxylation (Ella, Reiss, & Ravid, 2019).

[12] HERC2 is a highly mutable gene of 93 exons found at a deletion breakpoint hotspot on human chromosome 15q11-q13, which encodes a giant (4834 amino acids) protein (García-Cano, Martinez-Martinez, Sala-Gaston, Pedrazza, & Rosa, 2019). The HERC2 protein is evolutionarily highly conserved, and possesses multiple functional domains, including a homologous to the E6-AP carboxyl terminus (HECT) domain, a motif endowed with E3 ubiquitin ligase activity (Scheffner & Kumar, 2014).

[13] The S-phase kinase-associated protein 1 (SKP1, Figure 12, left), cullin-1 (CUL1, Figure 12, top right), and the F-box only protein 44 (FBXO44, Figure 12, bottom left) are all members of multiple cullin-RING-based SCF (SKP1-CUL1-F-box protein) E3 ubiquitin-protein ligase complexes, which preside over the ubiquitination of several proteins involved in different biological processes (*e.g.*, signal transduction, transcription and cell cycle progression (Cardozo & Pagano, 2004).

[14] Ubiquitin-associated (UBA) domains are protein motifs which interact with ubiquitin *via* non-covalent protein-protein interactions (Dikic, Wakatsuki, & Walters, 2009), whereas WWE (*i.e.*, Trp-Trp-Glu) domains are characteristic of two classes of functional proteins: one associated with ubiquitination and the other with ADP-ribosylation (Aravind, 2001).

[15] The protein TAAC3 is involved in the microtubule-dependent coupling of the nucleus and the centrosome. As component of the TACC3/cytoskeleton-associated protein 5 (ch-TOG)/chlatrin complex, it has a role in the stabilization of the mitotic spindle kinetochore fibers by acting as an inter-microtubule bridge (Cheeseman, Harry, McAinsh, Prior, & Royle, 2013).

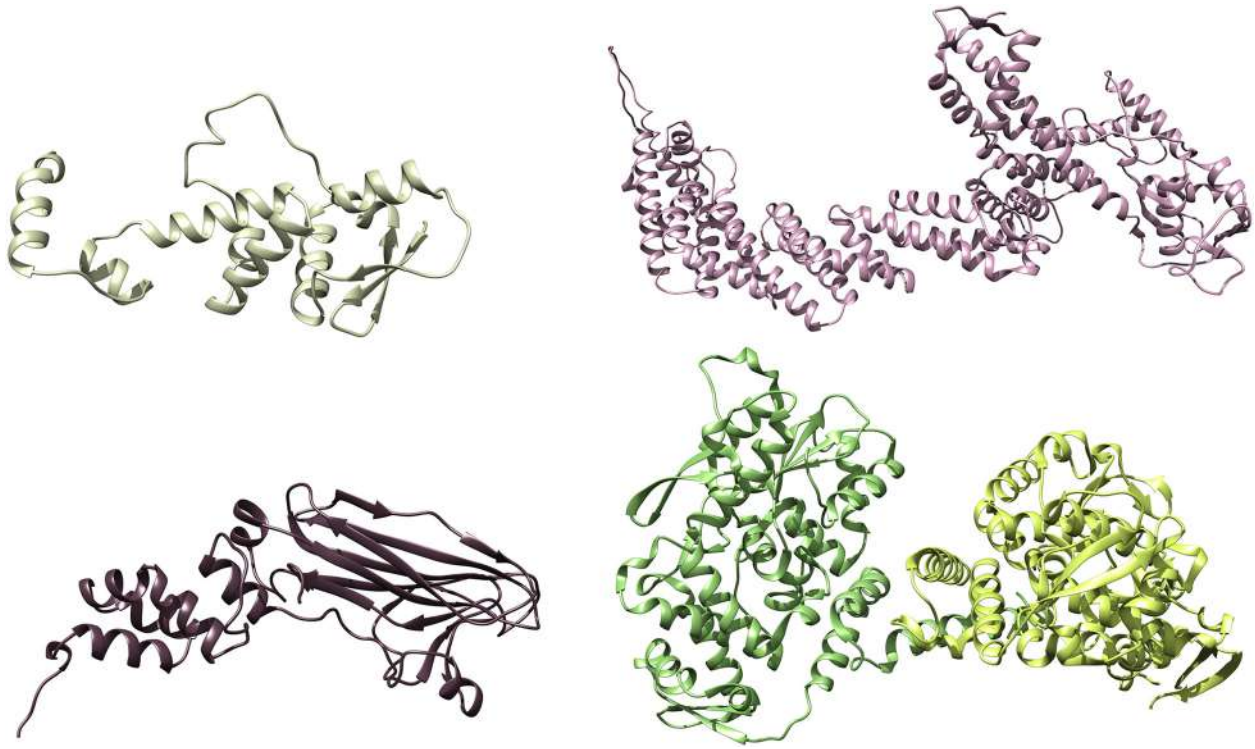

Fig. 12. 3D structures of SKP1 (top left, lint, AlphaFold2 PDB: P63208), CUL1 (top right, orchid haze, AlphaFold2 PDB: Q13616) and FBXO44 (bottom left, fig, AlphaFold2 PDB: Q9H4M3) as predicted by AlphaFold2 (Jumper et al., 2021)). (Bottom right) Crystal structure of a C-terminal part of HUWE1, including the catalytic domain, in its asymmetric auto-inhibited dimeric form (protomers in green shades, PDB: 5LP8, (Sander, Xu, Eilers, Popov, & Lorenz, 2017)).

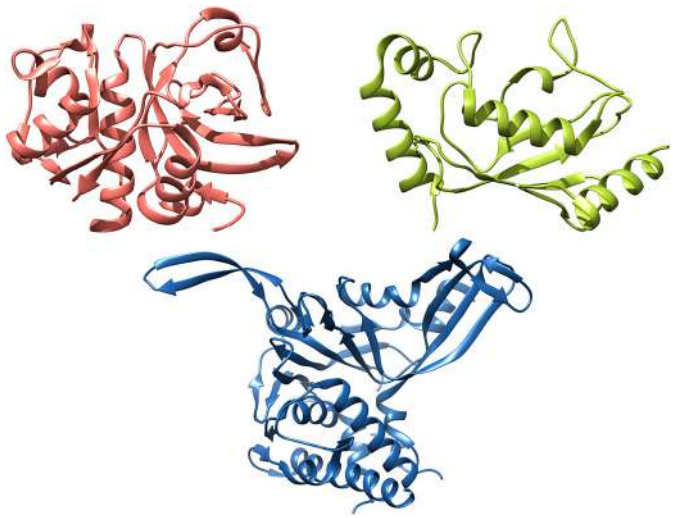

Fig. 13. Crystal structures of CTSS (top left, living coral, PDB: 2OP3 (Inagaki et al., 2007)), UBE2T (top right, lime green, PDB: 5NGZ, (Morreale et al., 2017)), and the catalytic domain of USP9X (bottom, strong blue, PDB: 5WCH (Paudel et al., 2019)).

the nucleus *via* the interaction of BRCA1 NLS1 and NLS2 sequences (§2.3.3), while the second, NLS-independent mechanism involves BARD1, and will be discussed in more detail later on (§2.6.2.4). Other proteins as well as BRCA1 mutations (§2.6.2.6) also influence the cellular localization of this protein. For instance, the BRCA1-binding protein 2 (BRAP2) has been shown by two different groups to bind the BRCA1 NLS sequences (potentially only when these are flanked by phosphorylation), thereby abrogating BRCA1/importin α interaction and sequestering BRCA1 into the cytoplasm (Fulcher, Roth, Fatima, Alvisi, & Jans, 2010; S. Li et al., 1998). Another protein active in retaining BRCA1 in the cytoplasm is the RAC-alpha serine/threonine-protein kinase (AKT1, Fig. 14, left) (Plo et al., 2008), a member of the AKT family that regulates many processes including metabolism, proliferation, cell survival, growth and angiogenesis (Hoxhaj & Manning, 2020). On the other hand, the apoptosis regulator Bcl-2 (BCL2, Fig. 14, right) fosters the localization of BRCA1 within mitochondria and the endoplasmic reticulum (Laulier et al., 2011), likely in relation with the apoptotic role of BRCA1, whilst Qin et al. reported that UBC9 (§2.3.3) also mediates BRCA1 nuclear localization and growth suppression (Qin et al., 2011b).

2.5.2. Regulation and stability of the BARD1 protein

BARD1 protein stability is regulated by phosphorylation and by the action of degrading and/or cleaving enzymes. BARD1 stability is increased by phosphorylation during the cell cycle; in detail, BARD1 expression follows three distinct patterns throughout the cell cycle: the protein is moderately expressed (and mainly unphosphorylated) in early G1, it is found at low levels (both phosphorylated and unphosphorylated) during late G1- and S-phases, while it is highly expressed (and phosphorylated) during mitosis, concomitantly with

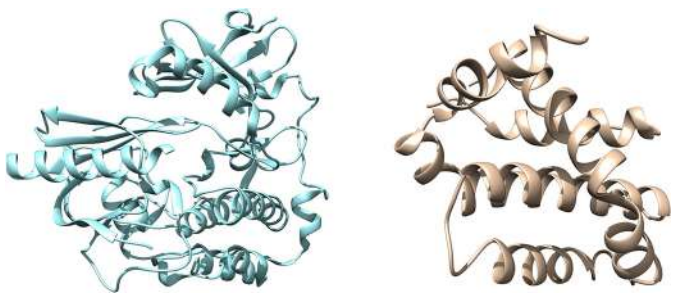

Fig. 14. X-ray-derived structures of AKT1 (left, island paradise, PDB: 6S9W (Quambusch et al., 2019)) and BCL2 (right, hazelnut, PDB: 5JSN (Berger et al., 2016)).

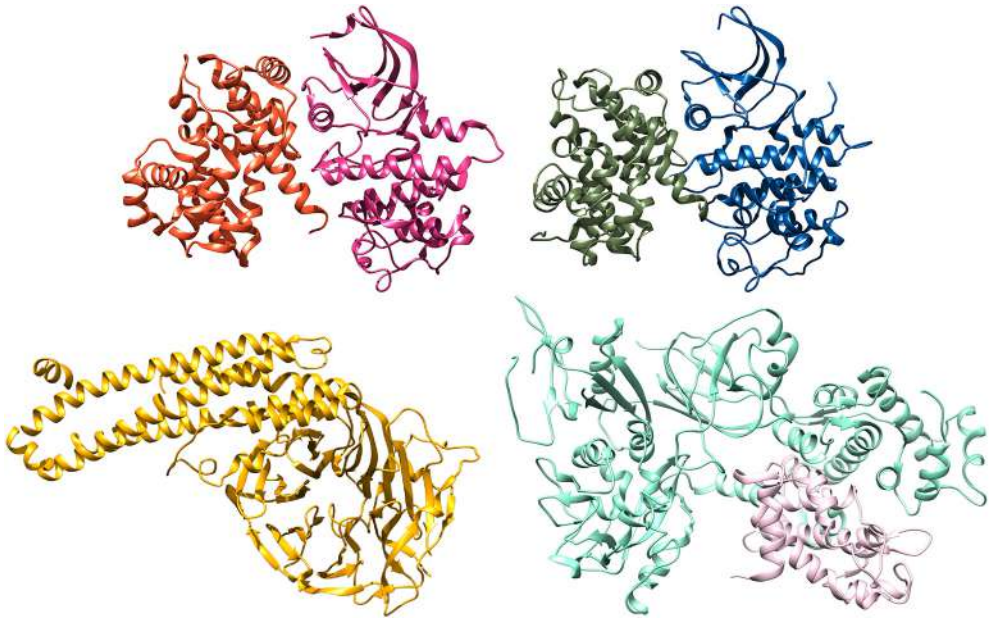

Fig. 15. Crystal structures of the CDK1/cyclin B1 complex (top left, pink yarrow/flame, PDB: 5HQ0 (N. R. Brown et al., 2015)), the CDK2/cyclin A1 complex (top right, lapis blue/kale, PDB: 1FIN (Jeffrey et al., 1995)), the APC/C subunits APC4 and the APC5 N-terminal domain (APC5(N)) (APC4 comprises a WD40 domain (lower right) divided by a long α-helical domain, whereas APC5(N) is characterized by α-helical fold (upper left). WD40 domains are made up of multiple repetitions, the first of which is a variable area of approximately 20 residues, followed by a more frequent repeating set of residues. These repetitions usually result in a four-stranded anti-parallel β-sheet or blade. These blades combine to create a propeller, the most common of which is a 7-bladed β-propeller.) (bottom left, saffron, PDB: 5BPT (Cronin et al., 2015)) and the full-length, heterodimeric human calpain (bottom right, large subunit in carnival grass and small subunit in fairy tale, PDB: 1KFX, (Strobl et al., 2000)).

BRCA1 expression (Choudhury et al., 2005). By mobility shift analysis and mass spectrometry Choudhury and coworkers identified seven sites of mitotic phosphorylation within BARD1, and showed that i) all sites exist within either a SerPro (SP) or ThrPro (TP) sequence (suggesting the involvement of proline-directed kinases), ii) two sites (Ser148 and Thr299) are reminiscent of the consensus sequence (Ser/Thr) ProX1-2(Arg/Lys) recognized by different CDKs and could therefore be substrates for the mitotic cyclic dependent kinase 1 (CDK1)-cyclin B1 complex (Fig. 15, top left) (Nigg, 1993), and iii) Thr299 is a BARD1 confirmed phosphorylation site during mitosis (Choudhury et al., 2005). In the same year, Hayami et al. reported that, besides CDK1-cyclin B1, CDK2-cyclin A1/E1 (Fig. 15, top right) also phosphorylate BARD1 on its N-terminal both *in vivo* and *in vitro*, thereby affecting its stability and heterodimeric functions with BRCA1 (Hayami et al., 2005).

The anaphase promoting complex (APC/C, Fig. 15 bottom left) – another E3 ubiquitin ligase that marks target cell cycle proteins for degradation by the 26S proteasome (Peters, 2006) – is also responsible for the degradation of BARD1 to mediate mitotic spindle-pole assembly (L. Song & Rape, 2010), a function that depends upon BRCA1/BARD1 E3 ubiquitin ligase activity (§3.1) (Joukov et al., 2006). Another proteolytic cleavage of BARD1 occurs during apoptosis, and is promoted by calpain (Fig. 15, bottom right, an intracellular Ca^{2+}-dependent cysteine protease that localizes to the cytosol and is involved in various proteolytic physiological events (Ono & Sorimachi, 2012)) acting at a cleavage site of BARD1 located again at the protein N-terminal domain but downstream of the RING motif (Gautier, Irminger-Finger, Grégoire, Meflah, & Harb, 2000a).

2.6. Cellular functions of the BRCA1 protein

2.6.1. BRCA1 and the DDR

2.6.1.1. Recruitment of BRCA1 at the DNA damage site. When considered in the framework of the DDR, early indications to BRCA1 gene function came from investigations of BRCA1-deficient fibroblasts and malignancies showing signs of widespread genomic instability including *e.g.*, chromosomal abnormalities, centrosomal amplification and aneuploidy patterns (Tirkkonen et al., 1997; Z. Weaver et al., 2002; B. Xu, Kim, & Kastan, 2001). Yet, the most direct and obvious evidence supporting the role of BRCA1 in DDR is that this protein relocates to DDSs along with RAD51 (§1), where it forms nuclear foci (NFs) upon DNA DSB formations (Scully et al., 1997). The signaling cascade that triggers this BRCA1 translocation is extremely complex (Kolas et al., 2007) and begins with the phosphorylation of the chromatin-associated histone H2AX[16] that locates close to the damaged DNA site by ATM and ATR (§2.3.4.2) (Burma, Chen, Murphy, Kurimasa, & Chen, 2001). This step is followed by the on-site recruitment of the mediator of DNA damage checkpoint 1 (MDC1) – a 2080 amino acid long protein required for cell cycle arrest promoted by a check point in response to DNA injury during both S- and G2/M phases (G. S. Stewart, Wang, Bignell, Taylor, & Elledge, 2003) – and the E3 ubiquitin ligase RING finger protein 8 (RNF8) (T. Zhou et al., 2019) that, along with the E3 ubiquitin conjugase Ubc13 (UBC13, Fig. 16, top left) (Hodge, Spyracopoulos, & Glover, 2016), ubiquitinates histone H2A (Fig. 16, top right) and H2B at chromatin lesions,[17] which in turn regulates the translocation of BRCA1 to the DNA damage site (J. Wu et al., 2009).

The so-called BRCA1-A complex, including BRCA1, the receptor-associated protein 80 (RAP80), CCDC98/ABRAXAS (§2.3.2), the Lys-63-specific deubiquitinase BRCC36 (BRCC36), and the BRISC and BRCA1-A complex members 1 (aka mediator of RAP80 interactions and targeting subunit of 40 kDa or MERIT40/BABAM1) and 2 (aka BRCA1/BRCA2-

[16] The histone variant H2AX constitutes about 2.5–25% of total H2A in the mammalian genome. After a DNA DSB, a serine at position 139 becomes phosphorylated (γH2AX), and renders H2AX an important player in preserving genome integrity (Turinetto & Giachino, 2015).

[17] More recently, Mattiroli et al. reported that the E3 ubiquitin ligase RING finger protein 168 (RNF168), instead of RNF8, is the priming ligase for histones during DNA DSB repair (Mattiroli et al., 2012). According to their data, the action of RNF8 on a non-nucleosomal target recruits RNF168 to the DSBs. Once there, RNF168 monoubiquitinates Lys 13–15 on the H2A-type histones and this, in turn, leads to the RNF8-catalyzed formation of the Lys63 ubiquitin chains required for proper DSB signaling. These data indicate that the collaboration between the two ligases likely takes place on H2A/H2AX, with RNF168 catalyzing the priming reaction and RNF8 efficiently extending the Lys63 chains.

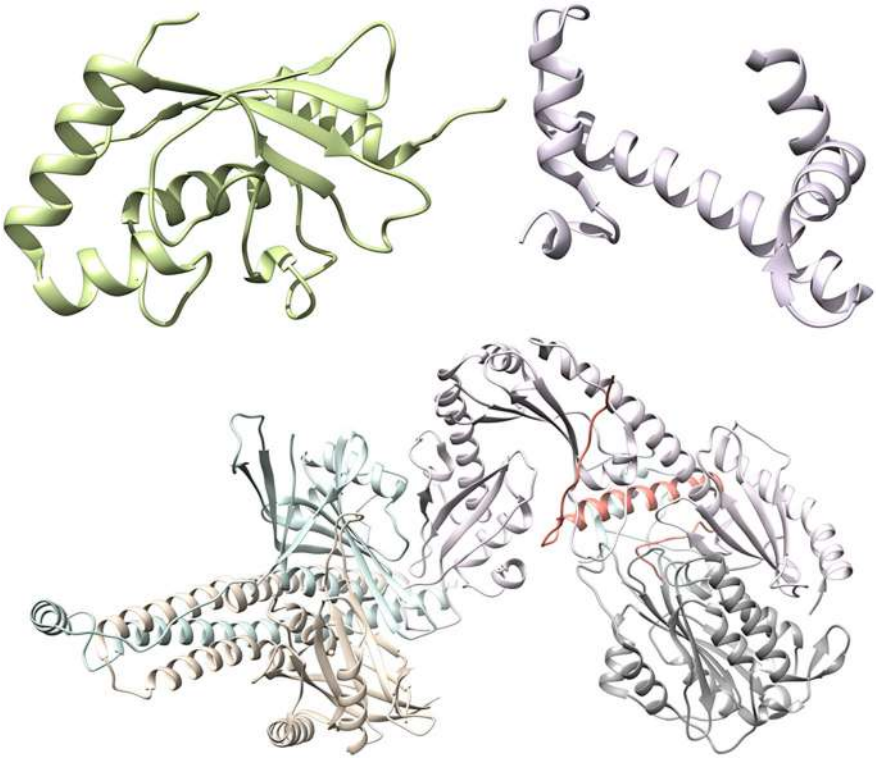

Fig. 16. 3D structure of UBC13 (top left, lettuce green, AlphaFold2 PDB: P61088) as predicted by AlphaFold2 (Jumper et al., 2021) and crystal structure of H2A (top right, lavender fog, PDB: 6N1Z (Padavannil et al., 2019)). (Bottom) X-ray structure of the BRCA1-A complex: ABRAXAS (glacier), BRCC36 (mother of pearl), BRCC45/BRE (orchid ice), MERIT40 (silver) and RAP80 (fusion coral) (PDB: 6GVW (Rabl et al., 2019)).

containing complex subunit 45/BRE or BRCC45/BRE) (Fig. 16, bottom) is also known to target BRCA1 in its heterodimeric form with BARD1 to DDSs (Savage & Harkin, 2015). Specifically, RAP80 includes two tandem ubiquitin-interacting motifs that promote the migration of BRCA1 to DDSs through contacts with ubiquitinated histones. ? (a ubiquitin-binding protein) contains two tandem ubiquitin-interacting motifs that facilitate the relocation of BRCA1 to DNA damage sites *via* the interaction with ubiquitinated histones (Sobhian et al., 2007). By directly interacting with BRCA1 between its C-terminal phospho-Ser motif and the BRCT domain, CCDC98/ABRAXAS (B. Wang et al., 2007) was reported to mediate the association of BRCA1 with RAP80 and to control both DNA damage-induced formation of BRCA1 foci and BRCA1-dependent G2/M checkpoint activation (H. Kim, Huang, & Chen, 2007; Z. Liu, Wu, & Yu, 2007). BRCC36 is a DUB that specifically digests Lys63-based polyubiquitin chains (L. Feng, Wang, & Chen, 2010), while both BRCC45 and MERIT40 are adaptors in the BRCA1-A complex (G. Shao et al., 2009; B. Wang, Hurov, Hofmann, & Elledge, 2009). In detail, MERIT40is assembled into the RAP80/CCDC98-containing complex *via* direct contacts with BRCC45/BRE. Importantly, MERIT40 controls BRCA1 DSB retention and checkpoint function (§2.6.1.2) mainly by preserving the stability of the whole BRCA-A complex at the DDSs (L. Feng, Huang, & Chen, 2009). Leung and coworkers identified ZMYM3 (zinc finger, myeloproliferative, and mental retardation-type 3) as a chromatin-interacting protein that stimulates DDR by HR (J. W. Leung et al., 2017). They found that, by interacting with DNA, histones and other components of the nucleosome, ZMYM3 is recruited to DNA DSBs where links BRCA1 to damaged chromatin *via* specific contacts with the BRCA1-A subcomplex components (§2.3.2). ZMYM3 further fine-tunes the contacts of BRCA1 with DDSs and chromatin by controlling ABRAXAS recruitment to damaged chromatin, and ZMYM3 loss leads to poor HR repair and genomic instability, consistent with a role in regulating BRCA1 function.

2.6.1.2. BRCA1 and DNA damage-induced cell cycle checkpoints. CCPs are critical to prevent the cell from progressing to the next phase of the cell cycle before the prior phase has been completed. Premature entry into the next phase of the cell cycle can result in catastrophic consequences for the cell and cell death (Barnum & O'Connell, 2014). In the face of DNA damage and other stresses that affect DNA replication, the cell has developed a number of sophisticated checkpoint pathways to prevent cell cycle progression, promote DDR or, in case of irreparable damage, activate apoptosis with the ultimate goal of avoiding transmission of an altered genome to daughter cells (Kastan & Bartek, 2004). As a natural consequence, developmental abnormalities, genetic instability, and cancer are all often associated with dysfunction of proteins involved in CCPs (Negrini, Gorgoulis, & Halazonetis, 2010; Visconti, Della Monica, & Grieco, 2016; Wenzel & Singh, 2018). There are four CCPs that can be activated upon DNA damage and in which BRCA1 was found to be involved with: the G1/S checkpoint, the G2/M checkpoint, the S-phase checkpoint and the spindle assembly checkpoint (SAC). Both G1/S and G2/M checkpoints are initiated to avoid transmission of damaged or incomplete chromosomes to daughter cells, the former by preventing cells from replicating damaged DNA, the latter by precluding cells from dividing with defective DNA (Swift & Golsteyn, 2016). The activation of the S-phase checkpoint upon DNA damage prevents S-phase advancement and stops DNA replication immediately after injury (Larner, Lee, & Hamlin, 1997). Cells also arrest at the SAC when they enter mitosis with damaged DNA (Nitta et al., 2004a).

Concerning the role of BRCA1 in G1/S checkpoint, Fabbro and coworkers adopted RNAi techniques to deplete BRCA1 and BARD1 with the purpose of verifying that the BRCA1-BARD1 complex is required for ATM/ATR-mediated phosphorylation of p53 following induced DNA damage (Fabbro et al., 2004). p53 is a key player in DNA damage-induced G1/S checkpoint, and its phosphorylation (specifically at Ser15) is necessary to elicit G1/S arrest *via* transcriptional induction

of the cyclin-dependent kinase inhibitor p21 (CDKI1/p21) (Senturk & Manfredi, 2013). They found that i) following UV- and IR-induced DNA damage the phosphorylation of p53 at its Set15 requires BRCA1; ii) for BRCA1 to work as an adaptor of p53, the ATM/ATR-mediated phosphorylation at BRCA1 Ser1423 or Ser1524 is also a *sine qua non*, and iii) BRCA1 depletion not only abrogates p53 Ser15 phosphorylation but also compromises p21 induction and G1/S checkpoint arrest following IR. Conversely, cell depleted in BRCA1 were still able to arrest at the G1/S checkpoint in response to UV radiation, notwithstanding the reduced levels of both p53 Ser15 phosphorylation and p21. Interestingly, in the same year the group of Shorrocks found that primary fibroblasts from individuals carrying heterozygous mutations in BRCA1 displayed defective G1/S checkpoint compared to WT cells in response to UV exposition, although this was not accompanied by genomic instability as measured by micronuclei induction after oxidative stress or treatment with mitomycin C, a DNA synthesis inhibitor (Shorrocks et al., 2004).

The crucial role of BRCA1 in the activation of the S-phase checkpoint in response to DNA damage has been established by the work of Xu and colleagues, who showed that ATM and BRCA1 are required for both the S- and G2-phase arrests induced by IR, while NBS1 (§1) is essential only for the S-phase arrest (B. Xu et al., 2001). They also reported that the phosphorylation of BRCA1 at Ser1387 as mediated by ATM is a prerequisite for S-phase checkpoint activation following IR-induced DNA damage; on the contrary, mutation of BRCA1 Ser1423 – the other target of phosphorylation by ATM – resulted in the abrogation of BRCA1 ability to master the G2/M checkpoint although it did not affect its S-phase function. Further works suggest that, following DNA damage during S-phase, BRCA1 may also act as an ATM regulator, as it colocalizes and interacts with the MRN complex (§1), a DNA DSB sensor and an activator of ATM (Bian, Meng, Zhang, & Li, 2019).

ATM and ATR are also both involved in the initiation of the G2/M checkpoint, although ATR is the main effector kinase associated with G2/M arrest (Saldivar et al., 2017). When a ssDNA is produced as a result of DSBs or in the presence of broken, stalled or collapsed replication forks (§1), the heterotrimeric protein RPA coats it quickly to i) protect it from destruction and ii) coordinate excision and repair processes (Dueva & Iliakis, 2020). RPA-ssDNA interactions acts as a hub for the binding of many other polypeptides, including the ATR-interacting protein (ATRIP) (Namiki & Zou, 2006) and the DNA repair protein RAD9 (RAD9)-cell cycle checkpoint protein RAD1 (RAD1)-checkpoint protein HUS1 (HUS1) (or 9–1-1) complex (Parrilla-Castellar, Arlander, & Karnitz, 2004), which facilitate the recruitment of ATR to the lesion. Localization of the ATR-ATRIP complex (Fig. 17, top) to the DDS is however insufficient for the activation of the ATR kinase, which further requires a conformational change of ATR itself promoted by the DNA topoisomerase 2-binding protein 1 (TOPBP1, Fig. 17, bottom left), (Bagge, Oestergaard, & Lisby, 2021)) as recruited by 9–1-1. Finally, the active ATR prompts the CCP by phosphorylating the serine/threonine-protein kinase CHK1 (CHK1, Fig. 17, bottom right).[18] This causes cell cycle arrest in the G2/M phase *via* suppression of the activity of CDK1/cyclin B (§2.5.2), thereby providing the injured cells with time to proceed with DDR (Cuddihy & O'Connell, 2003).

In this setting, BRCA1 was also found to be essential for CHK1 expression, phosphorylation and cellular localization by Yarden and coworkers (Ronit I. Yarden, Pardo-Reoyo, Sgagias, Cowan, & Brody, 2002). They also verified that BRCA1 affects the expression of the Wee1-like protein kinase (WEE1, Fig. 18, top left, an inhibitor of CDK1/cyclin B (§2.5.2) and, thus, a negative regulator of the G2-to-M transition (Ghelli Luserna di Rorà, Cerchione, Martinelli, & Simonetti, 2020)) and of the 14–3-3 family of proteins (a family of structurally related phospho-binding proteins that control almost every important cellular function (Pennington, Chan, Torres, & Andersen, 2018)) that sequesters phosphorylated CDK1/cyclin B in the cytoplasm (K. Liu et al., 2020). These findings show that BRCA1 masters critical effectors that govern the G2/M checkpoint and, as a result, is involved in governing the onset of mitosis. In 2000, Lee et al. reported that the serine/threonine-protein kinase CHK2 (CHK2, Fig. 18, top right) regulates BRCA1 function after DNA damage by phosphorylating BRCA1 at S988 (J.-S. Lee, Collins, Brown, Lee, & Chung, 2000). In particular, they showed that BRCA1 pSer988 is essential for BRCA1 release from CHK2, as well as for the BRCA1 capacity to reinstate cell survival after DNA injury. Accordingly, this study indicates that CHK2 phosphorylation, in addition to other factors (*e.g.*, ATM and CHK1, as discussed above), modulates BRCA1 G2/M checkpoint regulation.

BRCA1 and ATR were also shown to be involved in the so-called decatenation checkpoint, another G2 CCP that checks the state of chromatid unwinding and delays cell entrance into mitosis until the chromatids are adequately unwound (decatenated), preventing chromosomal stress that may result in aneuploidy or polyploidy (Damelin & Bestor, 2007). Deming and coworkers showed that the mitotic delay observed in human fibroblasts in the presence of ICRF-193 (a topoisomerase II catalytic inhibitor that precludes chromatid decatenation without generating DNA DSBs (Kaufmann & Kies, 1998)) was abrogated when an ATR kinase-inactive allele was expressed in these cells (Deming et al., 2001). The same group additionally reported that HCC1937, a tumor cell line derived from a primary BC characterized by a BRCA1 germline mutation (Tomlinson et al., 1998), also presented defects in mitotic delay induced by ICRF-193 that was rectified upon WT BRCA1 expression, supporting the notion that ATR and BRCA1 enforce the decatenation G2 checkpoint.

The spindle assembly checkpoint maintains proper chromosomal segregation and is triggered in mitosis during the metaphase-to-anaphase transition in response to microtubule (MT) abnormalities (Schwartz & Shah, 2005), erroneous kinetochore[19] attachment (Tauchman, Boehm, & DeLuca, 2015), or to prevent the cell from entering mitosis with damaged DNA (Nitta et al., 2004b). Accordingly, SAC inactivation may in chromosome mis-segregation and aneuploidy (Holland & Cleveland, 2009). By using a mouse model deficient for BRCA1 FL isoform ($BRCA1^{\Delta11/\Delta11}$) Wang and colleagues showed that cells expressing $BRCA1^{\Delta11/\Delta11}$ displayed decreased expression of a number of genes that are involved in SAC (Wang, Yu, & Deng, 2004), including MAD2 (§1), a key SAC component that inhibits APC/C (§2.5.2) (Ciliberto & Shah, 2013). They further showed that BRCA1 binds to the POU domain, class 2, transcription factor 1 (OCT-1 or NF-A1, Fig. 18, bottom left)[20] and upregulates MAD2 transcription. Finally, they proved that BRCA1 induction to endogenous MAD2 or transfected MAD2 luciferase reporter *in vitro* was completely inhibited by BRCA1 suppression *via* RNAi, confirming the role of BRCA1 in maintaining genome integrity by interplaying with SAC genes.

BRCA1 also controls SAC by fine-tuning the expression of genes associated with orderly mitosis progression (Baer & Ludwig, 2002; Wang, Yu, & Deng, 2004). In this case, BRCA1 depletion resulted in defective SAC and faulty cytokinesis, leading to multinucleated cells accumulation. Recent studies shed more light on the role of BRCA1 in SAC. For instance, Stolz et al. suggested that BRCA1 phosphorylation mediated by

[18] Actually, CHK1 activation also requires the cell cycle checkpoint protein RAD17 (RAD17)-replication factor C (RFC) complex (Zou, Liu, & Elledge, 2003), the 9–1-1 complex, and the adaptor protein claspin (Chini & Chen, 2003) are also required for CHK1 activation. The RAD17-RFC assembly functions as a 9–1-1 clamp loader while claspin connects ATR and CHK1, enabling phosphorylation of CHK1 Ser317 and Ser345 (Q. Liu et al., 2000). In particular, Ser345 is required for CHK1 activation, while Ser317 has a contributory role (Walker, Black, Oehler, Gillespie, & Scott, 2009).

[19] Kinetochores are multifunctional macromolecular super-complexes that establish the attachment of spindle microtubules to chromosomes and are thus essential for faithful chromosome segregation (Hinshaw & Harrison, 2018).

[20] OCT-1 belongs to the octamer-binding proteins (Octs), a family of transcription factors that are highly conserved and endowed with specific affinity for the octamer motif (ATGC AAAT) and/or closely related sequences, which populate both promoters and enhancers of many ubiquitously expressed or cell type-specific genes (F. Q. Zhao, 2013). The POU domain is a binary DNA binding domain consisting of two subunits (the N-terminal or POU-specific (POUs) domain, and the C-terminal or homeobox domain) separated by a non-conserved stretch of 15 to 55 residues (Verrijzer et al., 1992).

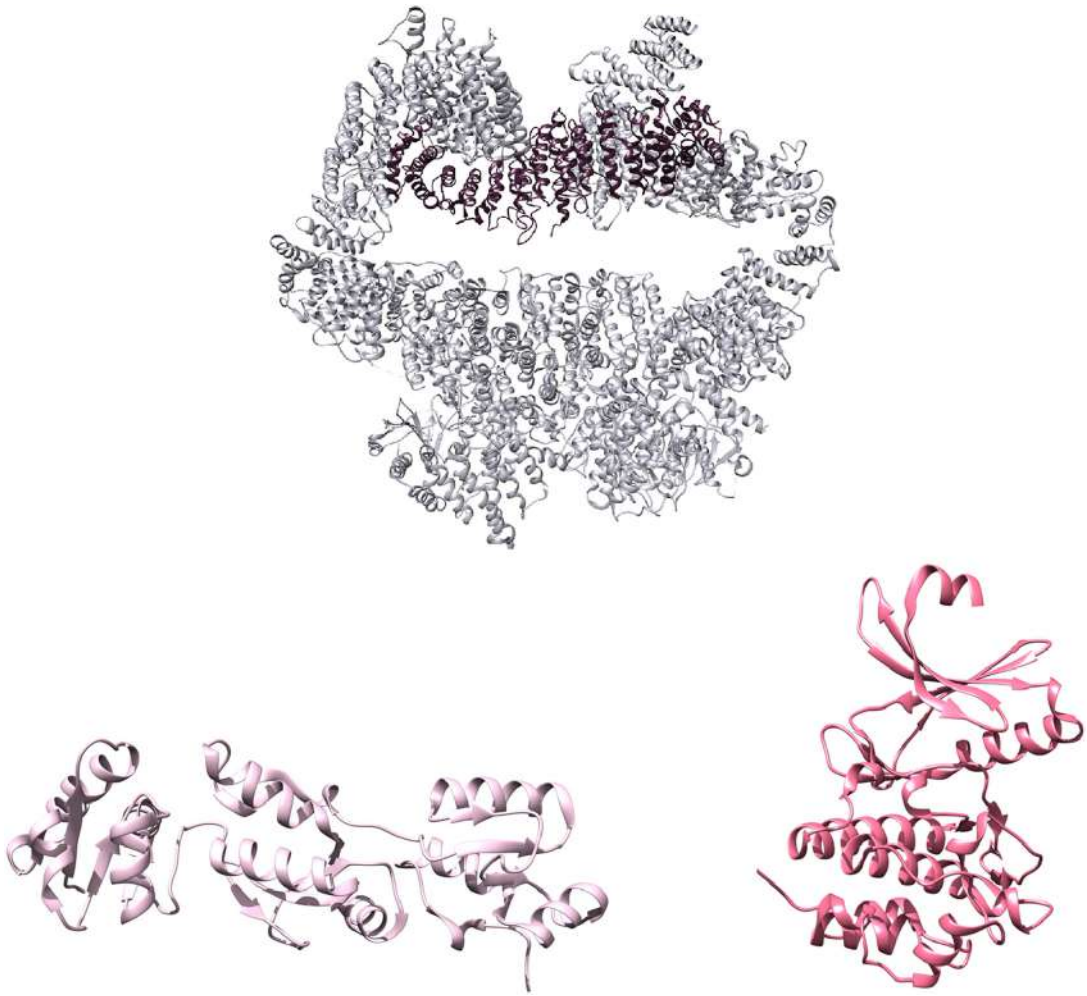

Fig. 17. (Top) Cryo-EM-derived structure of the dimeric ATR/ATRIP complex (lilac gray/winter bloom, PDB: 5YZ0 (Rao et al., 2018)). (Bottom) X-ray structures of the RAD9-binding region of TOPBP1 (left, pink lady, PDB: 2XNH (Rappas, Oliver, & Pearl, 2011)) and CHK1 (right, pink lemonade, PDB: 6FCK (Yang et al., 2018)).

CHK2 is needed for correct mitotic spindle assembly and proper chromosome segregation (Stolz et al., 2010; Stolz, Ertych, & Bastians, 2010). In this year, using mesothelioma cells Busacca and coworkers showed that *BRCA1* silencing abrogated recruitment of the mitotic spindle checkpoint protein (BUBR1, Fig. 18, bottom right, an essential component of the mitotic checkpoint required for normal mitosis progression) to kinetochores and apoptosis in mesothelioma cells (Busacca et al., 2021). *BRCA1* silencing also led to co-depletion of *MAD2* at the mRNA and protein levels, consistent with its status as a transcriptional target of *BRCA1 described above.* In BRCA1-transfected MCF-7 cells, Chabalier et al. also demonstrated that siRNA downregulation of BRCA1 reduced the mitotic index, and triggered premature cyclin B1 degradation and a decrease in CDK1 activity following MT stabilization with paclitaxel treatment, implying that BRCA1 downregulation results in premature inactivation of the SAC (Chabalier et al., 2006). These results were further supported by the discovery that BRCA1 deficiency causes early sister chromatid separation in MCF-7 cells following spindle injury (Chabalier et al., 2006).

2.6.1.3. BRCA1 in HR of DNA damage. Because the homologous strand of the matching sister chromatid is needed as a template for repair-related DNA synthesis, HR can only occur during the S- and G2-phase of the cell cycle. This mechanism of DNA DSB repair is often classified as error-free (Scully et al., 2019), and therefore a method for maintaining genomic integrity, although an aberrant error-prone form of HR termed hyper homologous recombination (HHR) has been described, as discussed later in this section. Already in 1999 Moynahan et al. discovered that a BRCA1-deficient reporter mouse embryonic stem cell (ESC) line failed to repair a chromosomal DSB caused by the I-*Sce*I endonuclease (SCEI, Fig. 19, top left)[21] indicating that BRCA1 plays an important role in HR (Moynahan, Chiu, Koller, & Jasin, 1999). The same group also showed that HR defects in BRCA1-deficient cells could be repaired by either expressing a WT BRCA1 transgene or correcting one mutant BRCA1 allele *via* gene targeting (Moynahan, Cui, & Jasin, 2001). As discussed in §1, HR is activated by the DDR and relies on ATM and MRN complex-mediated 5′-3′ end resection of DNA starting at the site of the DSB into ssDNA. Here, BRCA1 in complex with CtIP (§2.3.2) facilitates end resection by allowing the recruitment of RPA (§1). After end resection and binding of RPA to the newly created ssDNA, RAD51 can promote the invasion of ssDNA into the sister chromatid and its pairing with the complimentary DNA strand, allowing repair initiation. Both BRCA2 and PALB2 are further functional partners of RAD51 in

[21] As a mitochondrial DNA endonuclease, SCEI has an active role a role in intron homing. It inserts a particular DBS specifically into the DNA of the 21S rRNA gene, allowing an intron with its own coding sequence (group I intron) to be inserted into an intronless gene. It precisely identifies and cleaves the nucleotidic sequence 5′-TAGGGATAACAGGGTAAT-3′.

SCEI is a mitochondrial DNA endonuclease involved in intron homing. It introduces a specific DBS in the DNA of the 21S rRNA gene and thus mediates the insertion of an intron, containing its own coding sequence (group I intron), into an intronless gene. It specifically recognizes and cleaves the sequence 5′-TAGGGATAACAGGGTAAT-3′.

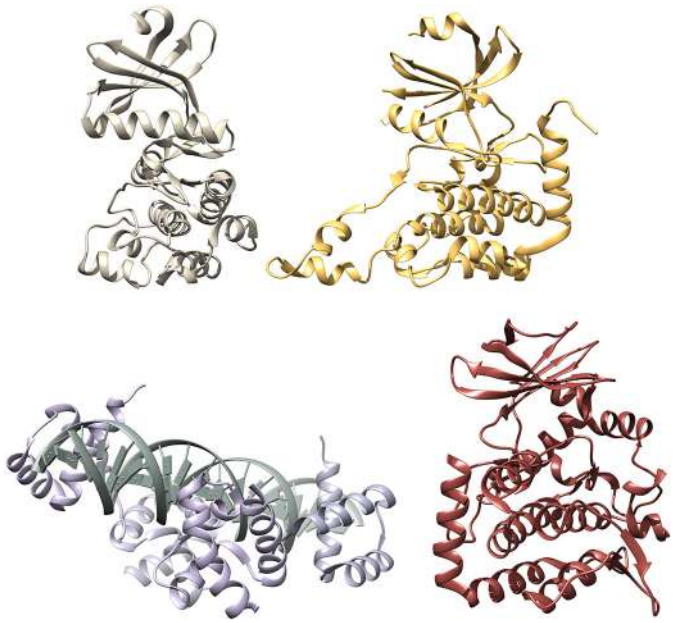

Fig. 18. Crystal structures of WEE1 (top left, oyster gray, PDB: 5VC3 (J. Y. Zhu et al., 2017)) and CHK2 (top right, sunset gold, PDB: 2W7X (Jobson et al., 2009)). (Bottom left) Crystal structure of the dimeric OCT-1 POU domain (protomers in lilac shades) bound to DNA (slate gray, PDB: 1HF0 (Reményi et al., 2001)). (Bottom right) Crystal structure of the kinase domain of BUBR1 (Tandoori spice, PDB: 6JKK (Y. Huang et al., 2019)).

generating the RAD51-ssDNA filament (Laurini et al., 2020; B. Xia et al., 2006) and, at this stage, BRCA1 in complex with PALB2 is required for the localization of BRCA2 at DNA damage sites. Indeed, Zhang et al. have shown that loss of BRCA1 disrupts the stability of BRCA2 and PALB2 at the lesion, with the consequent abrogation of the DNA HR process (Zhang et al., 2009; Zhang, Fan, Ren, & Andreassen, 2009). The last step in DSB repair by HR – in which BRCA1 has not been reported to play any major role so far – is the creation of Holliday junctions (*i.e.*, branched nucleic acid structures containing four double-stranded arms joined together), which are subsequently resolved without crossing, returning the DNA to its original state usually with no sequence aberrations. However, the abnormal, error-prone HR variant HHR has been discovered ten years ago in the early 2010s, which is directed by BRCA1 (Dever, White, Hartman, & Valerie, 2012). Specifically, HHR was reported in the presence of BRCA1 mutants (*e.g.*, M1775R) that impair the BRCT domain interaction with phosphopeptide sequences or when BRCA1-A complex components (ABRAXAS, RAP80, or BRCC36) (§2.6.1.1) were knocked down. In this context, it has been proposed that the BRCA1-A acts as a de-ubiquitinating complex in the early stages of HR to restrict end resection and avoid excessive buildup of RAD51 and RPA on the invading DNA strand (B. Wang, 2012).

2.6.1.4. BRCA1 in NHEJ repair of DNA damage. NHEJ is a mechanism of DDR which involves a completely different collection of proteins with respect to HR and, again at variance with HR, occurs mostly during G1- and – although to a lesser extent – during S- or G2 phases of the cell cycle as it does not require a homologous region of DNA as a template for repair synthesis (H. H. Y. Chang et al., 2017). In NHEJ, DNA damaged ends are directly ligated after limited processing, and this results in the removal or additions of bases at the broken ends. This, along with the absence of a template strand, implies that NHEJ can be an error-prone DDR process. The role of BRCA1 in cNHEJ is still somewhat controversial, in that initial works reported conflicting evidence using both *in vitro* and *in vivo* experiments (Baldeyron et al., 2002; Bau et al., 2004; Mérel, Prieur, Pfeiffer, & Delattre, 2002; Moynahan et al., 1999; Wang et al., 2001; Zhong, Boyer, Chen, & Lee, 2002; Zhong, Chen, Chen, & Lee, 2002). Subsequent studies aimed at determining how BRCA1 mediates/influences cNHEJ showed that phosphorylation of BRCA1 at Ser988 by CDK2, and at Ser1423 and 1524 (§2.6.1.2) are critical for precise end-joining activity *via* cNHEJ (Kato et al., 2009; Löbrich et al., 2005). The BRCA1 N-terminal containing the RING domain (§2.3.1) has similarly been shown to bind to IR-induced DNA DSBs and to dissociate from them quickly. This rapid interaction with DSBs is reliant on the c-NHEJ factor Ku80 (§1) as cancer-causing missense mutations in the BRCA1 RING finger domain resulted in the elimination of

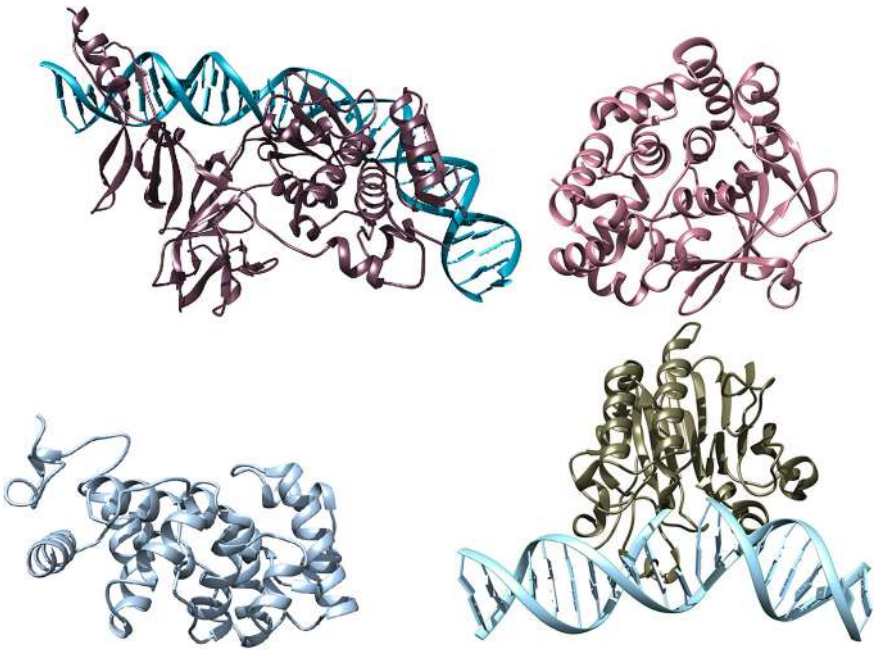

Fig. 19. (Top left) Crystal structure of SCEI (mauve wine) bound to its DNA recognition site (Caribbean blue, PDB: 1LWS (Moure, Gimble, & Quiocho, 2002)). (Top right) Crystal structure of OGG1 (heather rose, PDB: 6RLW (Visnes et al., 2020)). (Bottom left) 3D structure of NTH1 (powder blue, AlphaFold2 PDB: P78549) as predicted by AlphaFold2 (Jumper et al., 2021). (Bottom right) Crystal structure of APE1 (Capulet olive) captured while processing an AP site (polar wind, PDB: 5DFF (Freudenthal, Beard, Cuneo, Dyrkheeva, & Wilson, 2015)).

the BRCA1-KU interaction as well as the fast recruitment of BRCA1 to DSBs (Wei et al., 2008). Interestingly, the paper by Jiang et al. found that the amino acids 262–803 of BRCA1, rather than the RING domain, are responsible for mediating the interaction between BRCA1 and Ku80 and in maintaining Ku80 binding to chromosomal breaks throughout the G1-phase of the cell cycle (G. Jiang et al., 2013). In addition, these authors reported that the suppression of BRCA1 also caused a substantial decrease in cNHEJ in G1-phase cells, with no impact on cNHEJ in G2/S phase cells. In aggregate, these evidences indicate that the interaction of BRCA1 with the cNHEJ component Ku80 stabilizes the KU heterodimer at DNA damage sites and that this is necessary for accurate end-joining repair *via* cNHEJ during the G1-phase of the cell cycle.

The role of BRCA1 in aNHEJ (§1) is also controversial, as BRCA1 has been reported both to favorably affect the aNHEJ pathway and to inhibit it. For example, BRCA1-null mouse embryonic fibroblasts had a 50-100-fold reduction in aNHEJ of a specified chromosomal DSBs with respect to WT animals (Rothkamm & Löbrich, 2003). Furthermore, BRCA1 and CtIP are also needed for telomeric fusions in telomeric repeat-binding factor 2 (TRF2)-depleted cells (uncapped telomeres), a process independent of Ku80 (cNHEJ), but reliant on PARP1/LIGIIIα (§1), two well-known aNHEJ components (Badie et al., 2015). The rapid recruitment of the BRCA1/BARD1 complex to DNA lesions is also governed by a PAR-dependent mechanism, according to which binding of the BARD1 BRCTs to PAR *via* its BRCT (PAR)-binding module targets the BRCA1/BARD1 heterodimer to damage sites (M. Li & Yu, 2013). Also, PAR polymerases (PARPs) connect PARs to proteins, with PARP1 being a factor needed for aNHEJ (Redon, Dickey, Bonner, & Sedelnikova, 2009), and PARP1 suppression prevents the BRCA1/BARD1 complex from being recruited to DNA lesions early, suggesting that the aNHEJ pathway influences BRCA1 recruitment to DSBs.

In contrast, a substantial body of evidence indicates that BRCA1 inhibits aNHEJ. For instance, Rothkamm and collaborators showed that, following DSB formation, BRCA1 protein knockdown or deletion enhanced the incidence of total plasmid DNA mutagenesis and aNHEJ (Rothkamm, Balroop, Shekhdar, Fernie, & Goh, 2007). The same authors further reported that inhibiting the exonuclease activity of the DNA end processing and aNHEJ factor MRE11 (§1) substantially reduced the incidence of aNHEJ repair but had little effect on the overall mutagenic frequency of plasmid DSB repair, suggesting that BRCA1 shields DNA against mutagenesis during non-homologous DSB repair. Along the same line, disruption of the BRCA1/BACH1 (§2.3.2) complex *via* BACH1 mutation impaired cNHEJ and accelerated aNHEJ, as shown by Burdak-Rothkamm et al. (Burdak-Rothkamm, Short, Folkard, Rothkamm, & Prise, 2007). In conclusion, following Saha and Davies (J. Saha & Davis, 2016), it could be proposed that BRCA1 usually inhibits aNHEJ, but may enhance this more error-prone repair when cNHEJ is absent or hindered, such as in clustered or complex lesions and telomeric DSBs, where repair of broken DNA ends takes priority over restoring sequence correctness.

2.6.2. Other cellular functions of BRCA1

2.6.2.1. Other DDR-related BRCA1 functions. Although the best characterized DDR-related function of BRCA1 refers to its role in DSB repair *via* HR or NHEJ, BRCA1 has been described as an active player in other DNA lesion repair processes. For instance, base excision repair (BER) is the prime (usually error free) process the DDR activates for repairing oxidized DNA. Here, BRCA1 has been used by Saha et al. (Saha, Rih, Roy, Ballal, & Rosen, 2010; Saha, Smulson, & Rosen, 2010) as a reporter to increase the activity of the BER pathway through a transcriptional process involving the stimulation of the production of several important BER enzymes (*e.g.*, the N-glycosylase/DNA lyase (OGG1), Fig. 19, top right (Ba & Boldogh, 2018), the endonuclease III-like protein 1 (NTH1), Fig. 19, bottom left, (S. C. Williams & Parsons, 2018), and the DNA-(apurinic or apyrimidinic site) endonuclease (APE1/REF1), Fig. 19, bottom right (Whitaker & Freudenthal, 2018)). Bae and colleagues (Bae et al., 2004) also showed that BRCA1, like ATM, promotes a cytoprotective antioxidant response, which is characterized by activation of nuclear factor erythroid 2-related factor 2 (NFE2L2), a transcription factor that plays a key role in the response to oxidative stress (He, Ru, & Wen, 2020). Further, the group of Saha also showed that BRCA1 RCA1 downregulates cellular levels of ROS by working in tandem with APE1 (Tapas Saha, Rih, & Rosen, 2009).

DNA interstrand covalent crosslinks (ICLs), like DSBs, are also very hazardous DNA lesions as they completely obstruct DNA replication. Both replication-dependent and independent mechanisms can preside over ICLs repair. During the G1/0 phase, ICLs repair basically consists in three steps: 1) dual incisions flanking the ICL, 2) DNA repair by NER (nucleotide excision repair) – a versatile mechanism that eliminates a plethora of lesions resulting in DNA helix distortions and/or anomalous structures produced mainly by exogenous threats like UV radiation and bulky chemical adducts (Spivak, 2015) – and 3) gap filling by DNA synthesis. Although ICL repair in the S-phase is comparable, it requires HR to guarantee a precise template for repair synthesis over the excised lesion. When a replication fork converges on an ICL, both BRCA1 and RAD51 safeguard the stalled fork from MRE11-mediated degradation, and the Fanconi anemia (FA)[22] repair pathway (Niraj, Farkkila, & D'Andrea, 2019) repairs the faulty crosslink. In brief, upon its ubiquitination the Fanconi anemia group D2 protein (FANCD2, Fig. 20, top left (Liang et al., 2016)) recruits the endonuclease pool composed of ERCC4, the crossover junction complex endonucleases MUS81 (MUS81) and EME1 (Fig. 20, top right), and the structure-specific endonuclease subunit SLX1 (SLX1) for crosslink incision followed by translesion DNA synthesis (TLS)[23] across the lesion. Next NER is activated for lesion removal, and finally HR is started to repair the replication fork (Taniguchi, et al., 2002). Both are error-free mechanisms, with the exception of the TLS step, which can be subjected to nucleotide mutations depending on fidelity of the specific operative DNA polymerase. Other studies have uncovered further distinct roles of BRCA1 in ICL repair. Accordingly, Cheng et al. demonstrates that the Werner syndrome ATP-dependent helicase (WRN, Fig. 20, bottom (Rossi, Ghosh, & Bohr, 2010)) is essential for the cellular processing of DNA ICLs, and that WRN collaborates with BRCA1 during the cell response to this type of DNA lesions (Cheng et al., 2006). Specifically, the direct interaction between BRCA1 and WRN promotes both WRN helicase and exonuclease activities *in vitro* and, again according to data by Cheng et al., the contact between WRN and BRCA1 increases in cells treated with DNA ICL-inducing agents. The binding site of WRN on BRCA1 was identified by the authors to the BRCA1 region spanning residues 452–1079. Another significant finding by this research team was that the BRCA1/BARD1 heterodimer interacts with WRN *in vivo* and this elicits WRN helicase activity on forked and Holliday junctions, indicating that WRN and BRCA1 work in concert to enable DNA ICLs repair. Bunting and coworkers also reported that depletion of both BRCA1 and 53BP1 (§1) renders cells hypersensitive to DNA ICLs (Bunting et al., 2010). They further verified that disruption of KU in BRCA1-deficient cells fosters DNA repair; yet, the alternative deletion of 53BP1 or KU in cells deprived of FANCD2 exacerbated genomic instability. According to their findings, BRCA1 has two distinct functions in ICL repair, both of which may be regulated by modulating NHEJ, while FANCD2 has a critical activity that cannot be circumvented by 53BP1 or KU ablation. Of note, several BRCA1-interacting proteins already described above, also belong

[22] Fanconi anemia is a hereditary disease marked by bone marrow failure, developmental abnormalities, and a susceptibility to malignancy caused by faulty DNA ICL repair and chromosomal instability (Nalepa & Clapp, 2018).

[23] DNA damage tolerance (DDT) pathways (also known as post replication repair pathways) – such as TLS – have been developed by cells to cope with the difficulty of replicating damaged DNA. These pathways let the replication machinery to bypass the DDS by starting DNA synthesis downstream of the injury and allowing for its repair once DNA replication has terminated. (D. J. Chang & Cimprich, 2009; Marians, 2018).

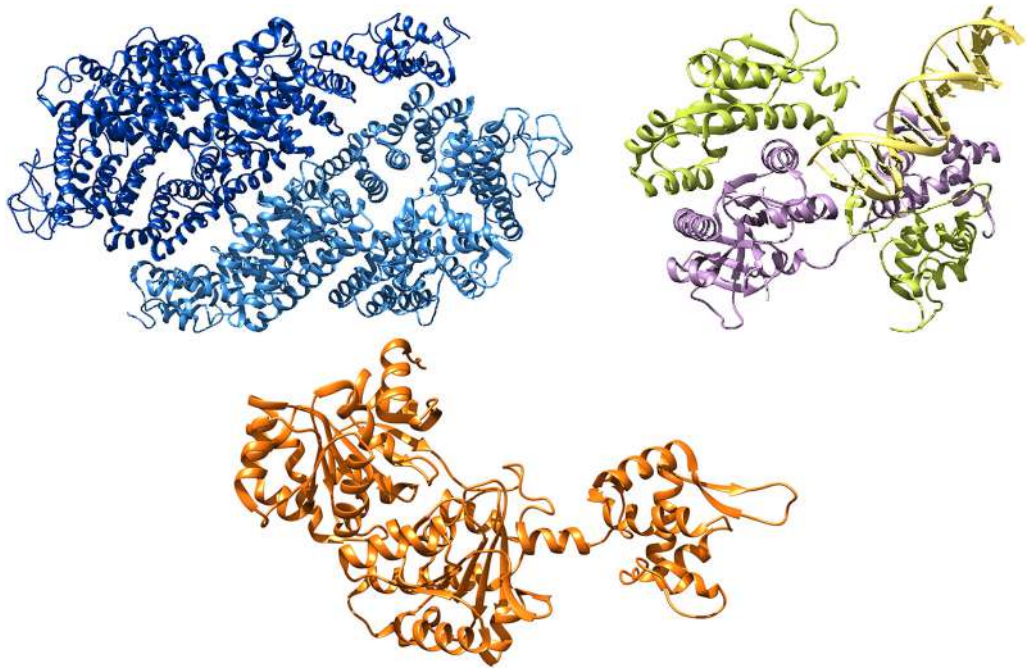

Fig. 20. (Top left) Cryo-EM structure of a FANCD2 dimer (protomers in blue shades, PDB: 6TNI (Alcón et al., 2020)). (Top right) Crystal structure of the MUS81/EME1 complex (African violet/tender shoots) bound to a DNA substrate (lemon zest, PDB: 4P0P (Gwon et al., 2014)). (Bottom left) X-ray structure of WRN (orange popsicle, PDB: 6YHR (Newman et al., 2021)).

to the FA pathway, and they include BACH1 (aka FANCJ), PALB2 (aka FANCN), and BRCA2 (aka FANCD1).

As mentioned in §1, ATM controls overall BRCA1 phosphorylation during DDR. However, Tibbetts and coworkers showed that, in response to DNA caused by IR, BRCA1 phosphorylation only partially depends on ATM while the process is ATM-independent in response to UV light- induced lesions, and provided evidence that ATR (§2.3.4.2) is the responsible for BRCA1 phosphorylation under these circumstances both *in vitro* and *in vivo* (Gatei et al., 2001; Tibbetts et al., 2000). They also found that ATR reacts to DNA damage and obstacles to replication by creating NFs at stalled replication fork sites, and that these NFs produced by ATR overlay with those generated by. Together, these results demonstrate that ATR and BRCA1 are both members of the same genotoxic stress-responsive route, and that BRCA1 is a direct phosphorylation substrate for ATR in response to DNA damaging or halted DNA replication. In 2011 Pathania and coworkers discovered that BRCA1 contributes to the response to UV irradiation. They proved that during the S—/G2-phase of the cell cycle BRCA1 is recruited to UV-damaged sites *via* its BRCT motifs, and this process is DNA replication-dependent but independent of NER. More specifically, at UV-stalled replication forks, BRCA1 was found to promote photoproduct excision, suppression of TLS, and the localization and activation of RFC complex subunits (note 20), a complex comprising 5 subunits which acts as a primer recognition factor for DNA polymerases (Li et al., 2018). The last function, in turn, triggers post-UV checkpoint activation and post-replicative repair. Contextually, Zhang and coworkers showed that BRCA1 rapidly binds to DNA UV-damaged sites when cells are undergoing DNA synthesis (L. Zhang, Chen, Gong, & Gong, 2013). In contrast, two phosphorylated forms of BRCA1 were not seen to accumulate at the same sites. Most importantly, they also proved that depletion of the transcription activator BRG1 (BRG1/SMARCA4, Fig. 21) – a core subunit of the human switch/sucrose non-fermentable-BRM-associated factors (SWI/SNF-BAF)[24] complex (Tang, Nogales, & Ciferri, 2010) – impaired the recruitment of BRCA1 to the DDSs and attenuated DNA damage-induced BRCA1 phosphorylation. Since stalled replication forks at UV lesions the activation of the ATM/ATR kinases was also found to be attenuated when BRG1 was depleted, the authors also proposed that BRG1 controls the response of BRCA1 to UV irradiation by mastering ATM/ATR activation.

Sunlight is a powerful and inevitable genotoxic stressor that cannot be avoided. A high dose of residual UVA and UVB under bright sunlight may cause up to 10^5 lesions per exposed cell every hour, despite the fact that the ozone layer filters the most harmful portion of the solar UV spectrum (UVC) (Mullenders, 2018). As is well known, this natural hazard may result in a variety of diseases ranging from minor sunburns to the development of skin cancer (Cadet & Douki, 2018). UVB in particular damages DNA directly by promoting the formation of covalent bonds between neighboring pyrimidine bases, resulting primarily in the production of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-6,4-pyrimidone photoproducts (6,4PPs). In 2002, Takimoto et al. already reported that the DNA damage-binding protein 2 (DDB2, Fig. 22, top left) gene was upregulated by BRCA1 in a p53-dependent manner following

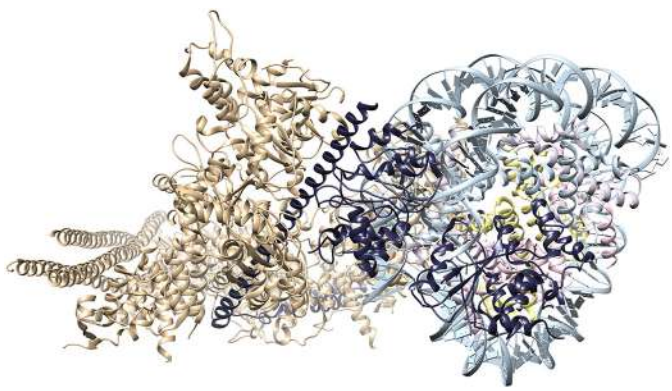

Fig. 21. Cryo-EM-derived structure of the human BAF complex consisting of the catalytic subunit BTG1/SMARCA4 (astral aura) and nine auxiliary subunits (in fading-out tan) bound to the nucleosome (DNA in baby blue, histone H3 in cherry blossom, histone H4 in yellow iris, histone H2A in orchid rush, and histone H2B in peach bud, PDB: 6LTJ (He et al., 2020)).

[24] SWI/SNF-BAF is one of the ATP-dependent chromatin remodeling complexes. These are specialized protein machineries capable of reshaping the nucleosome to allow for DNA accessibility during the processes of transcription, replication and DDR (Clapier, Iwasa, Cairns, & Peterson, 2017).

exposure to UVC or DNA damaging agents (Takimoto et al., 2002). Their *in vivo* data showed that BRCA1 improved the binding of p53 to the DDB2 promoter along with the p53-dependent transactivation of DDB2 promoter-reporter constructs via a typical p53 DNA responsive element. BRCA1 silencing prevented DDB2 overexpression following UVC or exposure to drugs, and BRCA1 insertion into WT cells substantially restored DDR activity relative to DDB2-deficient cells. Furthermore, abrogation of BRCA1 expression in UV-exposed human cells slowed the elimination of CPDs and 6,4PPs. Thus, they concluded that BRCA1 DDR functions could be ascribed (at least in part) to the induction of DDB2 transcription by p53, and that the abrogation of BRCA1-dependent DDB2 repair function could lead to cancer susceptibility and cell sensitivity to DNA damage provoked by UV and/or chemical agents. In a successive effort from the same group, the authors investigated the effects of BRCA1 and p53 on the repair of various forms of UV-induced DNA damage using damage-specific antibodies and different cell types, including human BC cells expressing p53- and BRCA1-mutated isoforms (Navaraj, Mori, & El-Deiry, 2005). They found a cooperative interaction between BRCA1 and p53 in the efficient repair of CPDs with respect to 6,4PPs. Interestingly, besides DDB2 the effects of BRCA1 and p53 on repair were also associated with the transcriptional induction of the DNA repair protein complementing XP-C cells (XPC, Fig. 22, top middle) gene, a component of the XPC complex that detects a broad range of damaged DNA that is characterized by helix deformities such as single-stranded loops/overhangs and mismatched bubbles (Emmert, Kobayashi, Khan, & Kraemer, 2000).

NER is made up of two separated but eventually converging pathways: transcription-coupled repair (TC-NER) and global genome repair (GGR) (GG-NER). The former efficiently repairs DNA damage that selectively impedes the advancement of RNA polymerase II (RNAPII) along the DNA strand, while GG-NER is a slow, transcription-independent route that examines the whole genome for damage. Different evidences support a role for BRCA1 in the transcriptional upregulation of the genes involved in both NER sub-pathways (Gudmundsdottir & Ashworth, 2006; Hartman & Ford, 2002). In particular, BRCA1 is also thought to be involved in DDR *via* the TC-NER pathway through the BRCA1-associated genome surveillance (BASC) complex (Wang et al., 2000), which is composed of the MRN complex (§1), ATM (§2.3.4.2), four key proteins of the DNA mismatch repair pathway (MMR)[25] – mutL homologue 1 (MLH1, Fig. 22, top right), mutS homologue 2 (MSH2), mutS homologue 6 (MSH6) (Fig. 22, bottom left) and postmeiotic segregation increased 2 (PMS2, Fig. 22, bottom middle) – the Bloom syndrome protein RecQ helicase (BLM, Fig. 22, bottom right), and RFC. Further, in tandem with BARD1 BRCA1 drives RNAPII through degradation *via* E3 ubiquitin ligase activity of the BRCA1/BARD1 heterodimer (§3.1) to enable the repair machinery to access the DDSs, and possibly prevent transcription of damaged genes (Kleiman et al., 2005; Starita et al., 2005).

2.6.2.2. BRCA1 functions at the telomeres. Mammalian telomeres (chromosome ends) entail 5 to 15 kb pairs of TTAGGG repeats that terminate with a ssDNA 3'tail of 50–500 nucleotides in length. Together, the telomere repeats and the ss-dsDNA connection constitute the binding site for shelterin, a protein complex of six elements (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), Fig. 23, top to bottom left)[26] that, by selectively associating with mammalian telomeres, endows cells with the ability to discriminate chromosomal natural ends from DDSs (de Lange, 2018). Replication of telomeres is a multistage mechanism cells have developed to avoid the shortening of telomeres that would otherwise occur when a DNA polymerase is unable to duplicate the 5'end of DNA (R. A. Wu, Upton, Vogan, & Collins, 2017). During carcinogenesis, telomere shortening has two opposing effects: on the positive side, by activating ATM and ATR at unprotected chromosome ends it can elicit an effect of tumor suppression. However, a failure in telomere protection can result in a telomeric crisis – an extensive genome instability situation that can stimulate cancer development (Maciejowski & de Lange, 2017). BRCA1 has been implicated in many studies as a regulator of telomere length and stability. Accordingly, overexpression of BRCA1 was discovered by Xiong et al. to decrease telomerase enzymatic activity by transcriptionally suppressing the expression of the telomerase catalytic component (*i.e.*, the telomerase reverse transcriptase or TERT, that promotes the extension of 3′-termini of chromosomes with the specific telomeric repeat unit 5′-TTAGGG-3′ in an RNA-dependent mode, Fig. 23, bottom right, (Xiong et al., 2003)), and the authors postulated that the capacity of the c-MYC oncoprotein (§2.4.1) to boost TERT expression through the c-MYC E-box inside the TERT proximal promoter was the mechanism underlying this repression. In the same study, the overexpression of WT BRCA1 but not a cancer-associated BRCA1 RING mutation (Cys61Gly) induced telomere shortening in numerous tumor cell lines as a result of TERT suppression, leading to the conclusion that an intact BRCA1 RING domain is required for inhibition of TERT. Notably, while inducing telomere shortening to extremely tiny sizes (under 2 kb), BRCA1 did not affect cellular growth, cell cycle arrest, senescence or death (Xiong et al., 2003). In aggregate, these results support the view that BRCA1 promotes telomere erosion while also protecting against telomeric malfunction. BRCA1 overexpression was also shown by the Rosen group to cause inhibition of telomerase activity and telomere shortening in BC and prostate cancer (PC) cells. (Xiong et al., 2003). A follow-up work of the same group (Ballal, Saha, Fan, Haddad, & Rosen, 2009) reported that BRCA1 knockdown caused increased TERT expression, telomerase activity, and telomere length; yet, results obtained by silencing both BRCA1 and TERT via RNAi suggested that BRCA1 was also involved in telomere length regulation irrespective of telomerase activity. Using telomeric chromatin immunoprecipitation (ChIP) assays, they were able to locate BRCA1 at the telomere and reported that BRCA1 was progressively removed from the telomeres in a time-dependent manner after DNA damage. Further data from this study also revealed i) BRCA1 interaction and nuclear colocalization with TRF1 and TRF2 in a DNA-dependent manner, ii) that RAD50 (a component of the MRN complex, §1) was essential for BRCA1 localization to the telomeres, and iii) that the length of the 3′G-rich overhang was controlled by BRCA1 in a RAD50-dependent mechanism.

To maintain telomere length, approximately 15% of tumors exploit recombination-based alternative lengthening of telomeres (ALT) (Nabetani & Ishikawa, 2011), the underlying mechanisms of which are still not completely clear but certainly involve different components of the DDR system, including BRCA1 and BLM (§2.6.2.1). In order to clarify their roles in telomere maintenance, Acharya et al. studied the association of these two proteins in telomere metabolism in cells exploiting ALT (Acharya et al., 2014). Their work showed that, contrarily to cells that use telomerases for telomere maintenance, BLM and BRCA1 colocalized with RAD50 at telomeres in immortalized human cells that make use of ALT during S/G2-phases of the cell cycle; moreover, BRCA1 and BLM co-immunoprecipitation of BRCA1 and BLM was higher in ALT cells at G2. Further data demonstrated that BRCA1 and BLM interact with RAD50 predominantly in S- and G2-phases, respectively, suggesting that BRCA1 participates in ALT through its interactions with RAD50 and BLM. Another, more recent work showed that BRCA1 and BLM cooperate with the Fanconi anemia group M protein (FANCM), an ATP-dependent RNA helicase of the FA network, in alleviating the replication stress at ALT telomeres (Pan et al., 2017). In addition, the BRCA1 knockdown in two MEC lines performed by Cabuy and

[25] MMR eliminates base mismatches and minor insertions/deletions (indels) caused by replication mistakes, as well as spontaneous/induced base changes (*e.g.*, methylation or oxidation), and fixes DNA adducts like those caused, for instance, by chemotherapeutics based on platinum complexes (Jiricny, 2006; Kunkel & Erie, 2015). It is a conserved cellular mechanism that has further roles in DNA DSB repair, recombination and apoptosis.

[26] Within the shelterin complex, TRF1, TRF2, and POT1 directly recognize TTAGGG repeats in dsDNA (TRF1/TRF2) and ssDNA (POT1). TIN2 acts as a hub by promoting TRF1/TRF2 dimerization and recruiting POT1 to the complex via TPP1. Finally, RAP1 contacts the telomere protein complex by making connections with TRF2 (Lim & Cech, 2021).

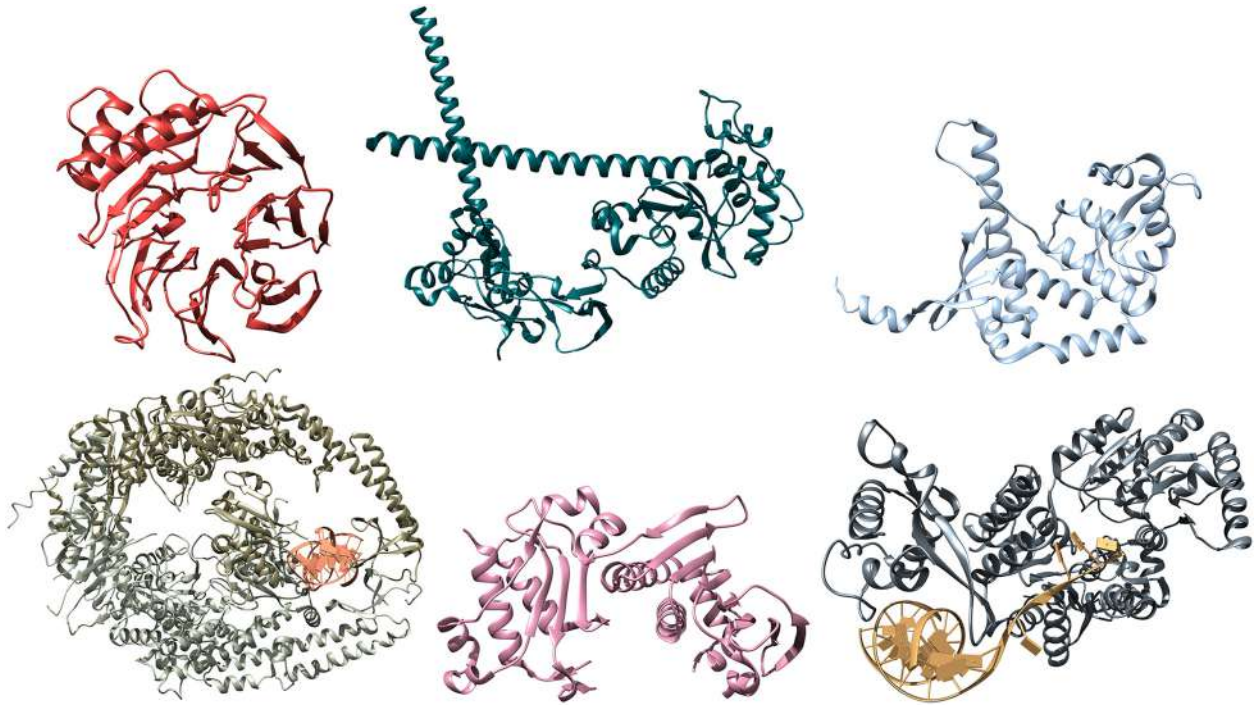

Fig. 22. (Top left) Crystal structure of DDB2 (grenadine, PDB: 3EI4 (Scrima et al., 2008)). (Top middle) 3D structure of XPC (shaded spruce, AphaFold2 PDB: Q01831) as predicted with AlphaFold2 (Jumper et al., 2021). (Top right) Crystal structure of MLH1 (cerulean, PDB: 6RMN (J. Dai, Chervy, Legrand, Ropars, & Charbonnier, 2019)). (Bottom left) Crystal structures of (bottom left) MSH2/MSH6 heterodimer (aka MutSα, dried herbs/desert sage) sensing a DNA lesion (cadmium orange, PDB: 2O8B (Warren et al., 2007)), (bottom middle) PMS2 (cashmere rose, PDB: 6MFQ (D'Arcy, Blount, & Prakash, 2019)), and (bottom right) BLM (stormy weather) in complex with DNA (oak buff, PDB: 4O3M (Swan et al., 2014)).

collaborators *via* RNAi had no effect on both telomerase activity and telomere length, although in anaphase cells it showed a correlation with the increase in chromatin bridges, in line with telomere dysfunction (Cabuy, Newton, & Slijepcevic, 2008). In the light of these findings, it seems that BRCA1 has the capacity to control telomere length and stability, and that it may be involved in mediating some of the detrimental

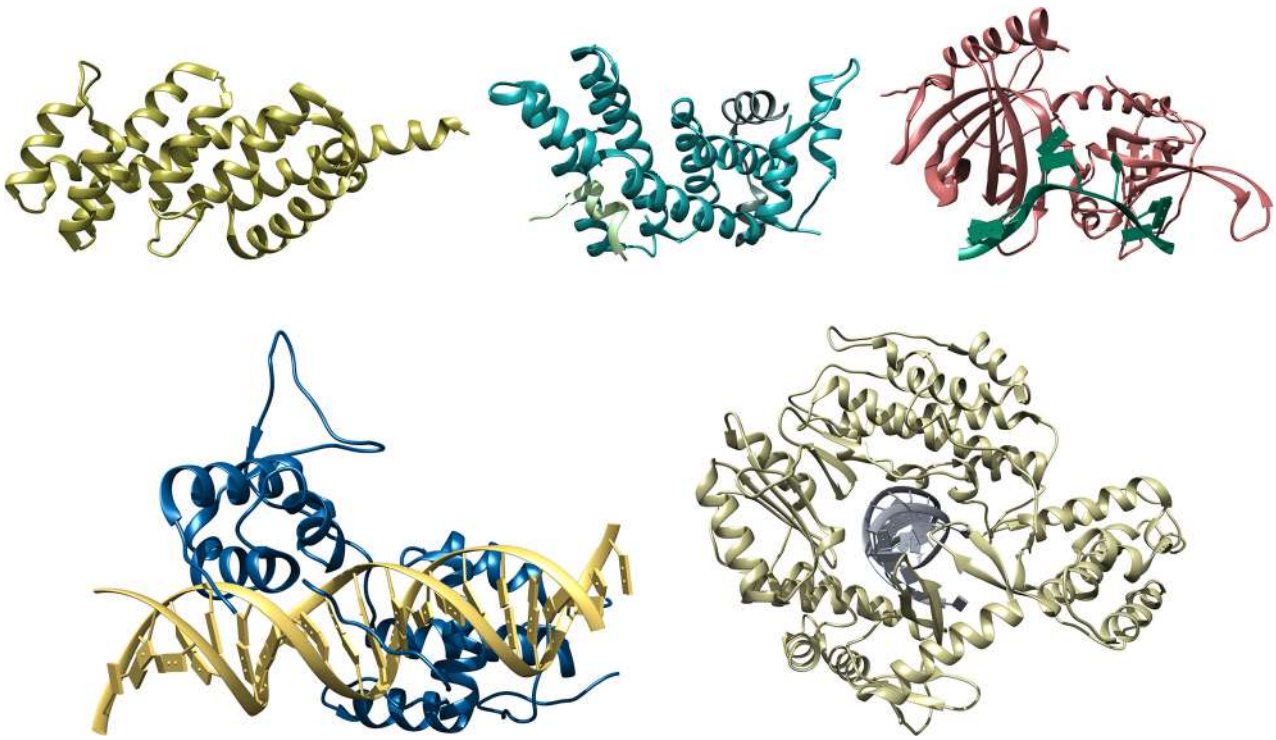

Fig. 23. Crystal structures of (top left) TRF1 (golden lime, PDB: 3L82 (Z. Zeng et al., 2010)), (top middle) the N-terminal domain of TIN2 (Baltic) in complex with the TIN2-binding motifs from TRF2 (pistachio green) and TPP1 (sagebrush green, PDB: 5XYF (C. Hu et al., 2017)), (top right) POT1 (dusty cedar) bound to telomeric ssDNA (lush meadow, PDB: 1XJV (Lei, Podell, & Cech, 2004)), (bottom left) RAP1 (snorkel blue) in complex with DNA (cream gold, PDB: 4GFB (Le Bihan et al., 2013)), and (bottom right) TERT (weeping willow) bound to an RNA-DNA hairpin designed to resemble the putative RNA-templating region and telomeric DNA (Tradewinds, 3KYL: (Mitchell, Gillis, Futahashi, Fujiwara, & Skordalakes, 2010)).

effects of the MRN complex on telomeres (*e.g.*, overhang length). These results are also consistent with the fact that cells lacking functional BRCA1 show signs of telomere dysfunction, extremely short telomeres, and the development of chromosomal aberrations associated with telomere disorder (Al-Wahiby & Slijepcevic, 2005; McPherson et al., 2006; Sedic et al., 2015; Uziel et al., 2015; Vohhodina et al., 2021; Yerushalmi et al., 2015).

2.6.2.3. BRCA1 functions in the estrogen response signaling pathway. In the formation and maintenance of normal sexual and reproductive function, estrogens play a critical role. Furthermore, they have a wide variety of biological effects on both men and women, affecting the cardiovascular, musculoskeletal, immunological, and central nervous systems (Heldring et al., 2007). Estrogen signaling is a balance between two opposing pathways in the form of two distinct receptors (ERα and ERβ) (§2.4.1) and their splice variants; whatever the pathway, ligand-dependent estrogen signaling begins with ES binding to and stimulation of ERs, which in turn reflects into receptor activation upon dimerization and successive binding to the promoters of estrogen responsive genes which include those associated with cell proliferation (*e.g.*, cyclins D and E, the epithelial growth factor (EGF) and the vascular endothelial growth factor (VEGF)). Back in 2001 Zheng and collaborators showed that BRCA1 mediates ligand-independent transcriptional repression of ERα (Lei Zheng, Annab, Afshari, Lee, & Boyer, 2001), the master of the growth, differentiation, and normal functional status of breasts and ovaries. They reported that ERα exhibited ligand-independent transcriptional activity in BRCA1-null mouse embryo fibroblasts and BRCA1-deficient human OC cells that was not observed in BRCA1-proficient cells. Ectopic expression of WT BRCA1 but not clinically validated BRCA1 missense mutants (*i.e.*, Cys64Gly, Gln536Arg, Ala1780Glu and Gln536Arg/Ala1780Glu) in BRCA1-deficient cells restored ligand-independent repression of ERα, and BRCA1 was found to be associated with ERα at endogenous estrogen-response elements before but not after estrogen stimulation in estrogen-dependent human BC cells. Collectively, these results supported the notion that BRCA1 functions as a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα and advocated a possible mechanism according to which BRCA1 functional inactivation could promote cancer insurgence by altering the hormonal control of mammary and ovarian epithelial cell growth. In the same year, Fan et al. found that BC-associated mutations of BRCA1 abolished or reduced its ability to inhibit ERα activity and that domains within both the N- and C-terminal of BRCA1 were required for this inhibition (Fan et al., 2001). In addition, BRCA1 was seen to block the expression of two endogenous estrogen-regulated gene products in human BC cells: the trefoil factor 1 (TFF1, aka breast cancer estrogen-inducible protein or pS2, a stabilizer of the mucous gel that protects the gastrointestinal mucosa, which acts as a physical barrier against the presence of different harmful substances, Fig. 24, left) and cathepsin D (CTSD), an acid protease active in intracellular protein breakdown (Fig. 24, middle). *In vitro* and *in vivo*, the BRCA1 protein was found to interact with ERα *via* an ES-independent binding that was localized within the N-terminal domain of BRCA1 (residues 1–300) and the conserved C-terminal activation function domain (AF-2) of ERα. A further finding was that several truncated BRCA1 isoforms, which nonetheless maintained the N-terminal ERα binding motif, abrogated the ERα-inhibitory activity of FL BRCA1. These data led the authors to propose that the BRCA1 N-terminal is the ERα interacting region while its C-terminal may function as a transcriptional repression domain. This suggestion was confirmed one year later by Kawai et al. who reassessed ERα/BRCA1 interaction *in vitro* and *in vivo*, and identified the two BRCA1 regions involving residues 1–306 and 428–683 as mediators of BRCA1 interaction with the AF-2 domain of ERα (Kawai, Li, Chun, Avraham, & Avraham, 2002). In normal MCF-10A breast epithelial cells and in BC cells (MCF-7 and T47D), these authors observed an endogenous interaction of ERα with BRCA1, and reported this interaction to be significantly negatively affected by presence of ES. Most importantly, however, these authors demonstrated i) the ERα-induced activation of VEGF, ii) that the presence of BRCA1 significantly inhibited VEGF gene transcription activation and VEGF protein secretion in a dose-dependent manner, and iii) that the BRCA1 region composed of residues of 1–683 was indispensable for the inhibition of VEGF gene transcription activation. In line with the results of Zheng et al. discussed above (Lei Zheng et al., 2001) three BC-related BRCA1 mutants (A1708E, M1775R and Y1853*) failed to associate with ERα and to suppress VEGF promoter activity and VEGF protein secretion; contextually, overexpression of WT BRCA1 in the HCC-1937 BC cell line that lacks endogenous functional BRCA1 significantly reduced VEGF secretion in these cells. In aggregate, these results revealed a new mechanism through which, by interacting with ERα, BRCA1 mutated proteins could support both oncogenesis and angiogenesis *via* both hormone-mediated MEC proliferation and impaired VEGF function.

An extensive number of coactivators/corepressors are known to mediate the role of ERα in gene regulation, the majority of which essentially alter chromatin structure and/or impact the assembly of regulatory complexes active at the beginning of the transcriptional process. In this field, Aiyar and colleagues described a novel mechanism of attenuating the ERα activity, according to which the native elongation factor B/cofactor of BRCA1 (NELF-B/COBRA1) – an integral subunit of the human negative elongation factor (NELF), a complex that negatively regulates the elongation of transcription by RNAPII (Narita et al., 2003) – directly binds to ERα and represses ERα-mediated transcription (Aiyar et al., 2004; Aiyar, Blair, Hopkinson, Bekiranov, & Li, 2007). The authors also showed that reduction of the endogenous NELF proteins in BC cells using RNAi resulted in enhanced ERα-mediated transcription and cell proliferation. ChIP assays reported that COBRA1 and the other NELF subunits recruitment to endogenous ERα-responsive promoters was greatly stimulated upon ES treatment. Intriguingly, COBRA1 did not affect the ES-dependent assembly of transcription regulatory complexes at the ERα-regulated promoters but it halted RNAPII at the promoter-proximal region, leading to the identification of COBRA1 as the first corepressor of nuclear receptors that modulates ERα-dependent gene expression by stalling RNAPII. The authors also proposed that this additional level of regulation may be essential in

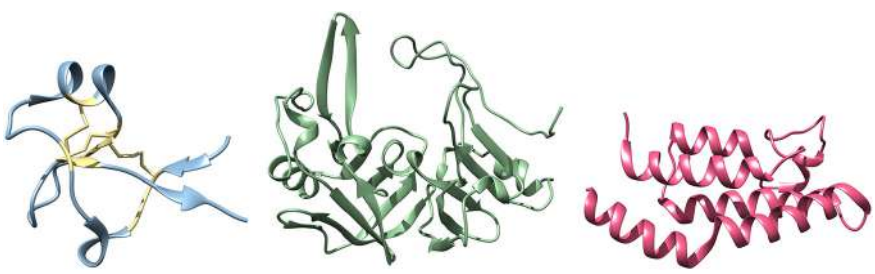

Fig. 24. Crystal structures of TFF1 (left), comprising three loops (foils, dusk blue) formed by three disulfide bonds (highlighted in custard, PDB: 6V1D (Järvå et al., 2020)), CTSD (middle, peapod, PDB: 1LYW (A. Y. Lee, Gulnik, & Erickson, 1998)), and the bromodomain of BRD7 (left, honeysuckle, PDB: 6PPA (R. M. Karim, Chan, Zhu, & Schönbrunn, 2020)).

controlling the length and amplitude of a fast and reversible hormonal response.

The bromodomain-containing protein 7 (BRD7, Fig. 24, right) is a subunit of the SWI/SNF-BAF chromatin remodeling complex (note 26, §2.6.2.1) that was identified by Harte et al. as a novel binding partner of BRCA1 (Harte et al., 2010). These authors investigated the role of BRD7 in BRCA1-dependent transcription, and found that several targets were coordinately regulated by BRCA1 and BRD7, including ERα. *In vitro* depletion of BRCA1 or BRD7 led to suppression of ERα expression at mRNA/protein level which, in turn, was associated with resistance fulvestrant, an antiestrogen drug. Interestingly, BRD7 was found to be present, along with BRCA1 and OCT-1 (note 22, §2.6.1.2), on the promoter of ESR1 (the gene which encodes ERα), while the depletion of BRD7 prevented the recruitment of BRCA1 and OCT-1 to the same gene promoter. According to the authors, these results supported a model wherein BRD7 regulates ERα transcription by recruiting BRCA1 and OCT-1 to the ESR1 promoter. The 300ArgXLysLys and 266LysXLys motifs have been identified previously as sites for acetylation of ERα (Kim, Woo, Chong, Homenko, & Kraus, 2006; Wang et al., 2001), and Lys302 was also found to be a site for BRCA1-mediated mono-ubiquitination of ERα *in vitro* by Ma and coworkers (Y. Ma et al., 2010). This group also showed that ERα proteins with single or double lysine mutations of these motifs (including Lys303Arg, a cancer-associated mutant) were resistant to inhibition by BRCA1, even though the mutant ERα isoforms retained the ability to bind BRCA1. BRCA1 overexpression reduced while its knockdown increased the level of acetylated WT ERα, without changing the total ERα protein level. Interestingly, increased acetylation of ERα by BRCA1 silencing *via* RNAi was reported to be dependent upon phosphatidylinositol 3-kinase (PI3K)/AKT signaling and on upregulation of the coactivator p300 (§2.4.1). In addition, the Ma group found that WT BRCA1 (but not its pathogenic mutation C61G (§2.3.3)) inhibited ERα acetylation, a process mediated by p300. Furthermore, they reported that i) mono-ubiquitinated ER-α protein levels were increased by BRCA1 overexpression ii) the BRCA1 Ile126Ala mutant, that is deficient in ubiquitin ligase activity but preserves other BRCA1 functions (§3.3.1), was not able to ubiquitinate ERα or to suppress its *in vivo* activity, and iii) ERα proteins bearing mutations within their 300ArgXLysLys and 266LysXLys motifs exhibited limited or no BRCA1-induced ubiquitination. In conclusions, the authors proposed a rationale according to which BRCA1 controls ERα activity, in part, by modulating its relative degree of acetylation *vs.* ubiquitination.

2.6.2.4. BRCA1 transcriptional activities and apoptosis. As a tumor suppressor, BRCA1 has been linked to the apoptotic pathway by Shao et al., who suggested that the lack/decreased levels of functional BRCA1 proteins could be the cause of the declined ability of a wide variety of human malignancies, including BC and OC, to undergo apoptosis (N. Shao, Chai, Shyam, Reddy, & Rao, 1996). Actually, BRCA1 seems to have opposing functions in apoptosis, as it both inhibits and promotes the process; the current view of BRCA1 indicates that it serves as a gatekeeper for deciding the destiny of a cell (*i.e.*, repair or death) based on the amount of DNA damage that has occurred. For example, in 2000 Ouchi et al. showed that BRCA1 (residues 502–802) interacts with the C-terminus of the signal transducer and activator of transcription 1-alpha/beta (STAT1, Fig. 25, top left) (Ouchi, Lee, Ouchi, Aaronson, & Horvath, 2000). The authors demonstrated that, through the activation of CDKI1/p21 (§2.6.1.2), which includes an interferon gamma (IFNγ)-responsive region in its promoter, this relationship was essential for the growth arrest after IFNγ therapy. Also, they reported that WT BRCA1 was required for its interaction with and regulation of the transcriptional activity of STAT1, since IFNγ stimulation of p21 was abolished in BRCA1 mutant HCC1937 cells. In a subsequent effort, using microarray analysis the Harkin group identified a number of additional BRCA1 transcriptional targets – many of which had previously been demonstrated to be activated by interferons (Andrews et al., 2002). BRCA1 activation of several of these targets, including *e.g.*, the interferon regulatory factor 7 (IRF7, the crucial regulator of type I interferons (IFNs) against pathogenic infections (Ning, Pagano, & Barber, 2011)), the 2′-5′-oligoadenylate synthase 2 (OAS2, an interferon-induced, dsRNA-activated antiviral enzyme which plays a critical role in cellular innate antiviral response, Fig. 25, top right (Choi, Kang, Hwang, & Kim, 2015)), and the interferon-induced protein with tetratricopeptide repeats 2 (IFIT-2, an IFN-induced antiviral protein which

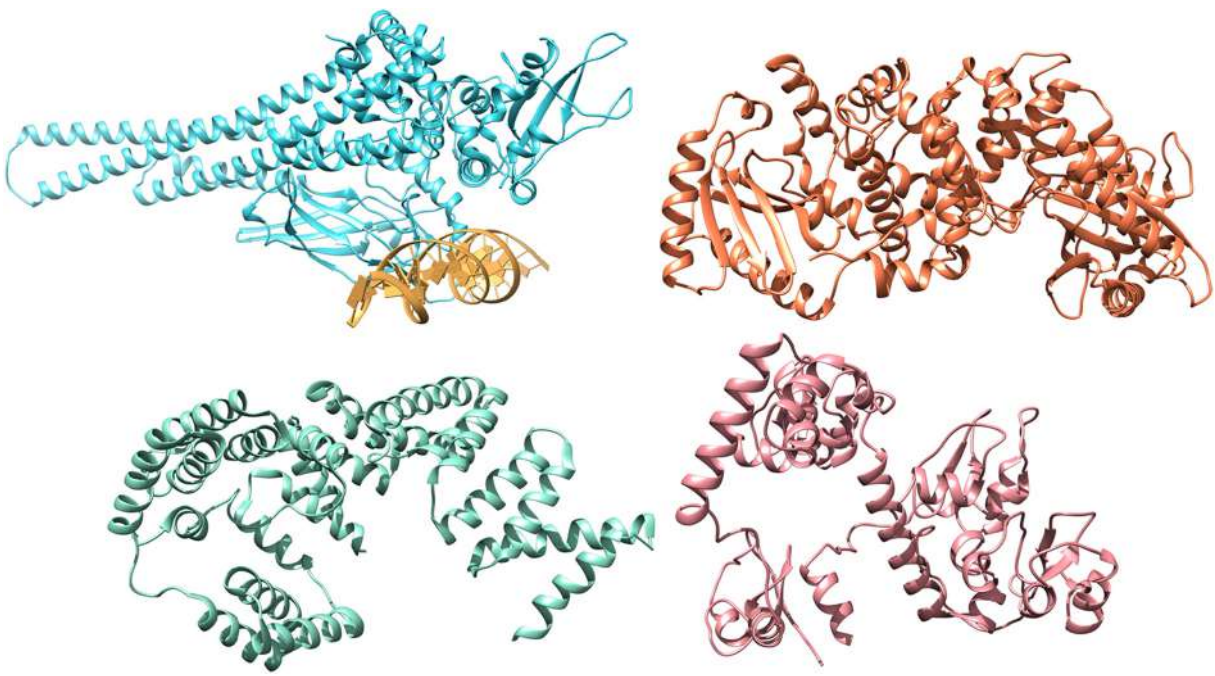

Fig. 25. (Top left) Crystal structure of STAT1 (blue Curacao) bound to DNA (beeswax, PDB: 1BF5 (X. Chen et al., 1998)). (Top right) 3D structure of OAS2 (coral rose, AlphaFold2 PDB: P29728) as predicted by AlphaFold2 (Jumper et al., 2021). (Bottom left) Crystal structure of IFIT2 (Lucite green, PDB: 4G1T, (Z. Yang et al., 2012)). (Bottom right) 3D structure of XIAP (strawberry ice, AlphaFold2 PDB: P98170) as predicted by AlphaFold2 (Jumper et al., 2021).

inhibits expression of viral messenger RNAs lacking 2'-O-methylation of the 5'cap (Sen & Fensterl, 2012), Fig. 25, bottom left), was synergistically increased with IFNγ (but not with type I interferons) and was associated by apoptotic induction (P. B. Mullan et al., 2006). In particular, Buckley et al. identified IRF7 as a BRCA1 transcriptional target, and demonstrated that it was synergistically upregulated by BRCA1 specifically in the presence of IFNγ, coincident with the synergistic induction of apoptosis (Buckley et al., 2007). These authors also showed that BRCA1, STAT1, and STAT2 (signal transducer and activator of transcription 2) were all required for the induction of IRF7 following stimulation with IFNγ, while Welcsh and coworkers showed that STAT1 levels increased in a BRCA1-dependent manner after inducible expression of BRCA1 (Welcsh et al., 2002). Of note, since IRF7 is a critical molecule in the amplification of the interferon cascade in response to viral infection, Marié et al. originally suggested that BRCA1 may have a transcriptional function in the innate immunological response to viral infection (Marié, Durbin, & Levy, 1998). OAS2, on the other hand, was also found by Mullan and collaborators to function as an apoptotic mediator in a BRCA1 and IFNγ -dependent way (Paul B. Mullan et al., 2005). As a result, the interaction of BRCA1 with STAT1 and the need for BRCA1 in the increase of transcription after IFNγ indicate yet another function for BRCA1, namely sensitization of BC and other cell types to the immunosurveillance and antiproliferative effects of IFNγ as confirmed, for instance, by the recent work of Cardenas et al., who determined that IFNγ signaling is associated with BRCA1 loss-of-function mutations in high grade serous ovarian cancer (Cardenas et al., 2019).

In the quest of identifying BRCA1 downstream target genes, Harkin and colleagues established cell lines with tightly regulated inducible expression of BRCA1 and found that the DNA damage-responsive gene GADD45A (GADD45A) is a major BRCA1 target (Harkin et al., 1999). GADD45A is best known as a p53-regulated growth arrest and DNA-damage-inducible gene, although it is also regulated in a p53-independent manner (Tamura et al., 2012) which involves BRCA1 (S. Jin et al., 2000). Specifically, BRCA1 is a robust GADD4A5 inducer, and its activity relies on at least three essential motifs in the GADD45A gene sequence: i) the BRCA1 binding site, mapped to the −121 to −75 region of the GADD45A promoter (Harkin et al., 1999), (ii) a specific binding sequence (5'-GGGxxxCAGxxxTTT-3′) within GADD45A intron 3 for the zinc finger protein 350 (ZNF350, aka zinc finger and BRCA1-interacting protein with a KRAB domain 1 (ZBRK1) (L. Zheng et al., 2000)), and iii) OCT-1 (§2.6.1.2) and CCAAT-box[27] elements in the proximal promoter region of the GADD45A gene between −121 to −75 (S. Jin et al., 2000). Both OCT-1 and NF-YA – the nuclear transcription factor Y subunit alpha, a member of the sequence-specific heterotrimeric transcription factor (NF-Y) that identifies a 5'-CCAAT-3′ box motif within their target gene promoters (Dolfini, Gatta, & Mantovani, 2012) – were found to be essential in the activation of the GADD45 promoter upon different DNA damage (Hirose et al., 2003; S. Jin et al., 2001; Takahashi, Saito, Ohtani, & Sakai, 2001). On the contrary, ZNF350/ZBRK1 is a transcriptional repressor that regulates the GADD45A 3rd intron activation driven by BRCA1. ZBRK1 encodes a 60-kD protein comprising a BRCA1 binding site, a zinc finger domain, and a KRAB (Krüppel-associated box) domain[28] which, upon BRCA1 binding, inhibits GADD45 transcriptional activity (L. Zheng et al., 2000). Finally, like ZBRK1 also BARD1 inhibits BRCA1 transcriptional activity by repressing BRCA1-mediated trans-activation of the GADD45A promoter, while increasing BRCA1 accumulation in the nucleus (Fabbro & Henderson, 2008). Most importantly, Harkin et al. identified a pathway that is likely to contribute to the function of BRCA1 as a tumor suppressor. According to their evidences, the induction of BRCA1 expression in different cancer cells (*e.g.*, osteosarcoma and BC) triggered apoptosis, and this effect involved induction of GADD45A and was associated with activation of the mitogen-activated protein kinase 8 (MAPK8/JNK1)/mitogen-activated protein kinase 11 (MAPK11/SAPK2b) stress response pathway (Johnson & Nakamura, 2007). Inhibition of JNK1/SAPK2b signaling abrogated BRCA1-mediated cell death and, since the induction of GADD45 expression and JNK/SAPK2b-dependent apoptosis by BRCA1 are independent of p53, these results support a functional pathway for BRCA1-mediated apoptosis, consistent with a role in the p53-independent cellular response to DNA damage. To examine the apoptotic function of BRCA1 in more detail, Thangaraju and coworkers investigated the impact of WT and C-terminal-truncated dominant negative BRCA1 on BC and OC cell lines that had been exposed to a variety of pro-apoptotic stimuli that included deprivation of growth factors and exposition to IR and anticancer therapeutics, among others (Thangaraju, Kaufmann, & Couch, 2000). Interestingly, all of these were shown to trigger significant amounts of apoptosis in the presence of WT BRCA1, while the BRCA1 truncated isoform had a suppressive effect on the apoptosis response. This study was significant because it showed that BRCA1 increased signaling *via* a route that included the GTPase HRas (HRAS, a member of the Ras proteins that activate signaling networks controlling cell proliferation, differentiation, and survival (Simanshu, Nissley, & McCormick, 2017)), the mitogen-activated protein kinase kinase kinase 4 (MAP3K4/MEKK, a component of a protein kinase signal transduction cascade activated by GADD45A), JNK1, the tumor necrosis factor receptor superfamily member 6 receptor/ligand system (FAS/FASLG (Pinkoski & Green, 1999)) and caspase-9 activation. Fabbro and coworkers also demonstrated that the apoptosis induced by BRCA1 was not dependent on p53 while it was promoted by BRCA1 nuclear export; contrarywise, BARD1 repressed BRCA1-dependent apoptosis via a mechanism that included BRCA1 nuclear sequestration (Fabbro, Schuechner, Au, & Henderson, 2004). They also showed that BARD1-mediated apoptosis was less efficient in the presence of BRCA1 mutations that impair its ubiquitin ligase activity. By transfecting cells with BRCA1 N-terminal peptides that prevented the formation of the BRCA1/BARD1 heterodimer, the authors reported a substantial decrease in BRCA1 nucelar localization, which was associated with increased apoptosis but did not affect both endogenous BARD1 localization or expression. Contextually, lowering BARD1 expression by RNAi reflected only in a small increase in apoptosis. Accordingly, these data revealed an original role of BARD1 in apoptosis inhibition and indicated that retention of BRCA1/BARD1 heterodimers in the cell nucleus promotes both DNA repair and cell survival.

The programmed cell death machinery involves the caspases, a family of fate-determining cysteine proteases that are best known for driving cell death, either *via* apoptosis or pyroptosis, under the condition of a variety of stresses (Julien & Wells, 2017). In this context, Martin and Ouchi found that activation of caspase-3 by UV was abrogated in cell lines expressing mutated BRCA1 (Martin & Ouchi, 2005). Capsase-3 was restored by re-expressing WT BRCA1, but not the phosphorylation-deficient BRCA1 Ser1423Ala/Ser1524Ala double mutant isoform. *In vitro*, the authors also found that the X-linked inhibitor of apoptosis protein (XIAP, Fig. 25, bottom right), which binds to and prevents caspase-9 from activating caspase-3 during apoptosis, also interacts with BRCA1; this XIAP/BRCA1 complex is disrupted upon UV-induce phosphorylation of BRCA1 on the two serine residues 1423 and 1524, and this allows for caspase-3 activation and apoptosis induction. These findings provide credence to a mechanism in which suppression of BRCA1 phosphorylation results in the abrogation of caspase-3-mediated apoptosis. On the other hand, Harte et al. showed that, following treatment with DNA-damaging agents (*i.e.*, etoposide or camptothecin), BRCA1 is required for the activation of NF-κB (§2.3.4.3), and that BRCA1 and NF-κB cooperate to regulate the expression of the NF-κB antiapoptotic targets BCL2 (§2.5.1) and XIAP (Harte et al., 2014). The substantially decreased survival of WT BRCA1 cells after NF-κB inhibition supported the

[27] The CCAAT box (and its ATTGG complementary sequence within the opposite strand) is one of the most important and widely distributed cis-elements in eukaryotic promoters, including those implicated in embryogenesis and development.

[28] The KRAB domain, located in the N-terminal region of a wide family of zinc-finger transcription factors, acts as a repressor of transcription by binding to corepressor proteins, whilst the C2H2 zinc-finger motifs bind DNA (Urrutia, 2003).

functional significance of BRCA1 activation of NF-κB in response to DNA damage. In essence, this study discovered a novel BRCA1/NF-κB complex and proved for the first time that NF-κB is needed for cell resistance to DNA damage mediated by BRCA1. In addition, it highlighted a functional dependency between BRCA1 and NF-κB, further clarifying the role performed by NF-κB in regulating the cellular resistance of WT BRCA1 wild-type cancer to DNA-damaging agents.

2.6.2.5. BRCA1 functions in chromatin remodeling. Chen et al. reported that the histone-binding protein RBBP7 (RBBP7, Fig. 26, top left), a histone modifying and remodeling complex component which is also a growth suppressor, interacts with and alters the transcriptional activity of BRCA1 (G.-C. Chen et al., 2001). These authors found that RBBP7 interacted specifically with the BRCA1 BRCT domain, and that the RBBP7/BRCA1 interaction required the first two of the four Trp-Asp (WD)-repeats of RBBP7. They also showed that expression of RBBP7 inhibited the transactivation of the p21 promoter mediated by FL BRCA1, while the association of BRCA1 and RBBP7 was disrupted in cells treated with DNA damaging agents, suggesting that RBBP7 may act as a modulator of the BRCA1 transactivation activity in response to DNA damage. Yarden and Brody confirmed that BRCA1 interacts *in vivo* and *in vitro* with RBBP7, RB (§2.4.1) and also with another member of the remodeling complex, the histone-binding protein RBBP4 (RBBP4, Fig. 26, top right (R. I. Yarden & Brody, 1999)). Moreover, they proved that the BRCA1 BRCT domain associated with the histone deacetylases 1 and 2 (HDAC1 and HDAC2, Fig. 26 bottom), two enzymes that catalyzes the deacetylation of lysine residues on the N-terminal part of the core histones (S.-Y. Park & Kim, 2020).

A number of additional studies reported a connection between BRCA1 and chromatin remodeling. For example, Ye and coworkers showed that targeting BRCA1 to an amplified area on a mammalian chromosome resulted in large-scale chromatin decondensation, and that this unfolding activity was conferred by the BRCA1 BRCT repeats (Ye et al., 2001). In addition, they demonstrated that cancer-predisposing mutations of BRCA1 displayed an allele-specific effect on chromatin unfolding; specifically, 5′ mutations that result in gross truncation of the protein abolished the chromatin unfolding activity, whereas those in the 3′ region of the gene markedly enhanced this activity. The same group also discussed the role of COBRA1 (§2.6.2.3) in this mechanism, and showed that the first BRCT repeat of BRCA1 recruits COBRA1 to the chromosome site, and that COBRA1 is sufficient in itself to stimulate chromatin unfolding. Importantly, BRCA1 mutations that elicit the unfolding of chromatin also enhance both BRCA1 affinity for and its recruitment capacity of COBRA1. Overall, these evidences suggest that the rearrangement of higher levels of chromatin structure is a key controlled stage in the nuclear activities mediated by BRCA1. Histone acetyltransferases (HATs) are enzymes essential for the optimum transcriptional activity of several transcription factors, as they acetylate the amino-terminal lysine residues of core histones, decreasing their positive charge and therefore their binding affinity for DNA (S. Y. Roth, Denu, & Allis, 2001). BRCA1 has been shown to physically interact with two HATs, the histone acetyltransferase p300 (EP300, Fig. 27, left) and the CREB binding protein (CBP, Fig. 27, right) (Pao, Janknecht, Ruffner, Hunter, & Verma, 2000). Both the N- and C-terminal of BRCA1 were shown to interact with EP300/CBP, and the transcriptional activation activity of BRCA1 was found to be further enhanced by these two HATs (Bernabei et al., 2003). The interaction of BRCA1 with p300/CBP places BRCA1 in close proximity to both the core transcriptional machinery and a large complex of chromatin remodeling proteins, allowing for optimum transactivation.

While investigating the still unknown molecular basis for the gender and tissue specificity of the BRCA1 cancer syndrome, Ganesan and coworkers discovered that, in female cells, a portion of BRCA1 in female was found to be located on the inactive X chromosome (Xi) (Ganesan et al., 2004). They also reported that BRCA1 established physical contacts with the Xi-specific transcript (XIST) RNA, a non-coding RNA that, by coating Xi, promotes the initiation of its inactivation during early embryogenesis, according to their ChIP studies. Xi anomalies were seen in cells missing WT BRCA1, including a lack of appropriate XIST RNA localization, and this deficiency in XIST localization in these cells could be corrected by reintroducing WT, but not mutant, BRCA1. BRCA1 depletion in female diploid cells resulted in a deficiency in XIST localization on Xi and in the formation of normal heterochromatic superstructure on the same inactive sex chromosome. Furthermore, BRCA1 deletion enhanced the probability of a green fluorescent protein (GFP) reporter gene inserted on Xi being re-expressed. Taken together,

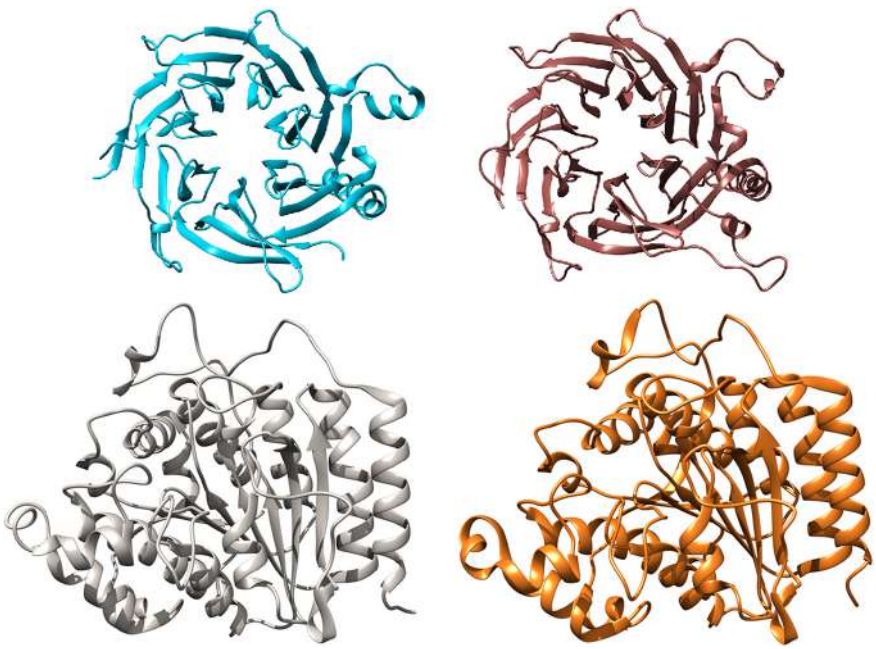

Fig. 26. Crystal structures of RBBP7 (top left, scuba blue, PDB: 7M3X (Righetto et al., 2021)), RBBP4 (top right, Marsala, PDB: 7M40 (Perveen et al., 2021)), HDAC1 (bottom left, silver cloud, PDB: 4BKX (Millard et al., 2013)), and HDAC2 (bottom right, russet orange, PDB: 5IX0 (Wagner et al., 2016)).

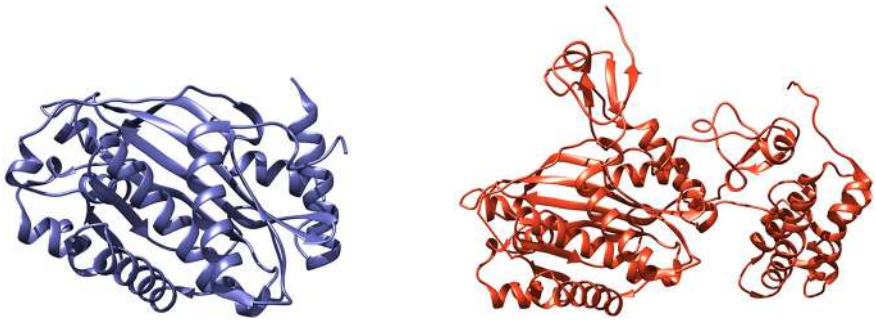

Fig. 27. Crystal structures of EP300 (left, blue iris, PDB: 6V8K (Huhn et al., 2020)) and the catalytic core of CBP (right, tangerine tango, PDB: 5U7G (S. Park et al., 2017)).

these results support a paradigm in which BRCA1 activity aids in the preservation of appropriate Xi heterochromatin superstructure. The same group confirmed that RNAi of BRCA1 decreased XIST concentration on Xi by extending the RNAi results from human to mouse cells using different target sequences (Silver et al., 2007). In addition, they re-established the relationship of BRCA1 to XIST concentration on Xi by demonstrating that depleting BRCA1 by CRE[29]-mediated excision also decreased XIST concentration on Xi, and revealed that mouse tumor lines derived from true BRCA1 null mammary tumors did not show XIST RNA concentration on Xi despite the presence of two X chromosomes.

2.6.2.6. BRCA1 mutations in cancer. The entry "*BRCA1 mutations in cancer*" on PubMed at the time of writing returned 12,659 items, testifying the enormous scientific and medical interest for and efforts focused on the role and functions of BRCA1 in oncology. At the same time, such mole of literature references makes it impracticable, if not impossible, to review the entire wealth of knowledge in the field. Therefore, what follows will offer only a succinct survey on this topic, while the interested reader is referred to a selection from the multitude of excellent recent review works on the specific subject (N. Armstrong, Ryder, Forbes, Ross, & Quek, 2019; De Talhouet et al., 2020; Gorodetska et al., 2019; Hatano, Tamada, Matsuo, & Hara, 2020; Krais & Johnson, 2020; Melchor & Benítez, 2013; Semmler, Reiter-Brennan, & Klein, 2019; Stoppa-Lyonnet, 2016). So far, almost 12,000 BRCA1 mutations are reported in the ClinVar database by searching as single gene, mainly in relation to BC and OC (https://www.ncbi.nlm.nih.gov/clinvar/), of which 711 are classified as benign, 1635 as likely benign, 3356 as pathogenic, 270 as likely pathogenic, 3050 as of uncertain significance, and 411 with conflicting interpretations; these numbers clearly support the increased risk to develop these types of malignancies along one's life time. Among the listed mutations, the major types are missense variations (4703), followed by frameshift (2015) and nonsense (748) mutations; interestingly, the database also lists 361 splice sites and, remarkably, 8968 ncRNA and 714 UTR variations. Concerning the allele origin, 9169 mutations are classified as germline variations, 84 as somatic and 5 as *de novo* mutations. The two BRCA1 domains most subjected to mutations are the RING and the BRCT domains, as already mentioned in §2.3.1 and §2.3.2. Variations in the former domain are mostly linked the disruption of the BRCA1/BARD1 heterodimer, the relative abrogation of its E3 ubiquitin ligase activity, and the consequent predisposition to BC and OC (§3.1 (Brzovic, Meza, King, & Klevit, 2001; Hashizume et al., 2001; Ruffner, Joazeiro, Hemmati, Hunter, & Verma, 2001)). In particular, the structural and biochemical investigation carried out by the Brzovic group showed that the cancer-predisposing missense mutations in the RING domain of BRCA1 primarily target Zn^{2+}-binding residues (Fig. 2). Interestingly, they reported that each of the BRCA1 RING Site II (§2.3.1) mutants still interacted and formed a stable heterodimer with BARD1, causing only a local structural perturbation primarily confined to this second Zn^{2+} binding loop of the BRCA1 subunit, in line with evidence that this region is well removed from the helices required for dimerization with BARD1. However, these mutations can predispose to cancer by altering a region of BRCA1 required for interaction with ubiquitin-conjugating enzymes. Mutations in BRCA1 exons 11–13 – which comprise most of the protein-coding region of the BRCA1 gene (§2.3.3, §2.3.4.1, and §2.3.4.2) – negatively reflect on the interaction of BRCA1 with its different substrates, and, hence, with the different functions of the protein in an array of cellular pathways, as already detailed in the previous sections. Of note, this region also includes the BRCA1 two NLSs (§2.3.3) and, although not linked to a DDR function of BRCA1 (which can obviously take place only in the cell nucleus), in this respect other associations between cancer and BRCA1 cytoplasmic mislocalization have been proposed. For instance, Santivasi et al. found an opposite relationship between the expression of BRCA1 in the cytoplasm and the metastasis-free survival affected individuals over the age of 40 (Santivasi et al., 2015). Additional analysis of BRCA1 subcellular expression within a group of patients with BC metastatic disease performed by this group showed that i) BCs that metastasized to the lung were characterized by a cytosolic BRCA1 content of 36.0%, ii) the cytosolic BRCA1 distribution in primary BCs and their corresponding lung metastases were similarly high according to both paired and unpaired analyses, and iii) genetically induced BRCA1 cytosolic sequestration (achieved using the cytosol-sequestering BRCA1 c.5266dup, p.Gln1756fs functional variant) increased cell invasion efficiency *in vitro*. The authors then proposed that these results support a model where BRCA1 cytosolic mislocalization promotes BC metastasis, making it a potential biomarker of metastatic disease. Other mutations in the BRCA1 C-terminal, and particularly in the protein BRCT domain, were linked to its cytoplasmic localization. In their study, Rodriguez et al. reported that the relocalization of BRCA1 from nucleus to cytoplasm was caused exclusively by a subgroup of clinically relevant cancer mutations that disrupted or deleted the protein BRCT domains but no other regions of BRCA1 (Rodriguez, Au, & Henderson, 2004). In particular, five different BRCA1 constructs (Pro1749Arg, Met1775Arg, Tyr1853*, Gln1756fs and Δ1751) that contained single amino acid mutations or short deletions (including the removal of 11 amino acids within the C-terminal tandem BRCT domains in the Tyr1853* mutant) were seen to promote BRCA1 cytoplasmic mislocalization. Interestingly, two of the studied mutations – Met1775Arg and Tyr1853* – have been discussed above as linked to HR activity of BRCA1 (§ 2.6.1.3) and both were shown to fail in associating with ERα (§ 2.6.2.3). Wiener and colleagues also analyzed the sub-cellular localization of BRCA1 and BARD1 in BCs, and determined the level of expression of their splice variants BRCA1-Δ11q and BARD1α and BARD1β in 103 BC samples (Wiener et al., 2015). They found BRCA1 localized in the cytoplasm with BARD1 in 51.4% of tumors, while an exclusive nuclear localization of both proteins was observed in 7/103 samples (6.8%). In relation to splice variants, they

[29] The CRE recombinase is a tyrosine member of the integrase family of site-specific recombinase and it is known to catalyze the site-specific recombination event between two DNA recognition sites (LoxP sites). This 34 base pair (bp) loxP recognition site consists of two 13 bp palindromic sequences which flank an 8 bp spacer region (Nagy, 2000).

reported a tendency to an overexpression of BARD1α mRNA (30% of cases) and a decreased expression of BARD1β (41%); on the other hand, 63% of cancers were characterized by downregulated FL BRCA1, while the BRCA1-Δ11q variant was overexpressed in the remaining 37% of tumors. Interestingly, BRCA1/BARD1 was reported to be unaffected in 58.2% of tumors in which the proteins colocalized, while BRCA1 loss in 41% of malignant samples was suggestive of a BRCA1-negative (aka *BRCA-less*) phenotype.

As mentioned above, mutations in BRCA1 and BRCA2 have been linked to hereditary instances of BCs and OCs since the 1990s, and patients with inactivating germline mutations in these genes – typically, SNPs or small indels causing frameshifts in the ORF and premature stop codons – have an increased risk of developing BCs and, to a lesser extent, OCs. Following the traditional *two-hit* model of tumor suppressor inactivation (L. H. Wang, Wu, Rajasekaran, & Shin, 2018), malignancies from these individuals tend to lose functioning of the remaining BRCA WT allele, often *via* LOH (Nielsen, van Overeem Hansen, & Sørensen, 2016b) and, given the BRCA1 critical role in DNA replication-fork protection and HR repair just discussed, this *BRCAness* phenotype promotes genomic instability in tumors (Chen, Feng, Lim, Kass, & Jasin, 2018; Lord & Ashworth, 2016). Although the loss of BRCA function may benefit tumor growth, it also renders tumor cells very susceptible to DNA ICL-inducing agents, *e.g.*, platinum-based compounds. Since ICLs depend on HR for effective repair (§2.6.2.1), this explains why platinum-based treatments have proved helpful in the clinical treatment of BRCA mutant carriers (Hollis, Churchman, & Gourley, 2017). However, likely one of the most important breakthroughs in *BRCAness* cancer therapeutics was the relatively recent discovery that inhibiting the DNA damage sensor PARP1 (§1) in BRCA-deficient patients causes synthetic lethality,[30] which has also been related to HR defects (Bryant et al., 2005; Farmer et al., 2005). The clinical development of PARP inhibitors (PARPi) in individuals with hereditary BRCA1/2 mutations was motivated on the accurate hypothesis that, in these individuals, *BRCAness* cancer cells would rely on PARP activity for survival, while normal cells (with a fully functional copy of either BRCA1 or BRCA2) would not. While these evidences were originally ascribed to the dependence of BRCA1/2 mutant cells on SSB-repair for their survival, it has since been recognized that PARP inhibition and the successive establishment of replication-dependent DSBs also significantly support the synthetic lethal connection between PARP and BRCA. Furthermore, the family of PARP enzymes is implicated in a plethora of fundamental cellular pathways other than DDR, including cellular differentiation and division (mitosis), DNA transcription, inflammatory response, apoptosis, and metabolic events, which also may contribute to the antitumor activity of PARPis (Bai, 2015; Bock & Chang, 2016; Gupte, Liu, & Kraus, 2017; Lord & Ashworth, 2016; A. N. Weaver & Yang, 2013). Reviewing the treasure of knowledge about the underlying mechanisms of action and therapeutic uses of PARPis is unavoidably beyond the scope of our present effort; however, a selection of excellent literature works on the subject is referred hereafter for the interested reader (J. S. Brown, O'Carrigan, Jackson, & Yap, 2017; Dias, Moser, Ganesan, & Jonkers, 2021; D.-S. Kim, Camacho, & Kraus, 2021; Paluch-Shimon & Cardoso, 2021; Rouleau, Patel, Hendzel, Kaufmann, & Poirier, 2010; Scott, Swisher, & Kaufmann, 2015). We must however mention here that the adoption of PARPi-based regimens to treat patients with HR-deficient cancers likely represents one of the most successful examples of targeted therapy clinical translation. At present, the success of this approach led to the approval of four different PARPis and a total of 7 different compounds are currently under clinical investigation for the treatment of several types of cancers (including BC, OC, PC, and pancreatic cancer (PANC)) (Slade, 2020). Clinical trials offer promising response rates among patients receiving PARPis although – as sadly often happens with many anticancer therapeutics – treated individuals ultimately develop resistance and relapse. Preclinical/clinical data have highlighted different resistance mechanisms, and currently enormous efforts are focused on developing strategies to address this challenge (D'Andrea, 2018; Dias et al., 2021; Janysek, Kim, Duijf, & Dray, 2021; S. M. Noordermeer & van Attikum, 2019); nonetheless, so far the resistance mechanism currently best validated in the clinics is the acquisition of secondary mutations in BRCA genes restoring the ORF detected upon treatment progression, with the first examples documented in the literature more than a decade ago (Sakai et al., 2008; Swisher et al., 2008). As a recent study in this arena, Tobalina and colleagues examined different clinical examples of secondary mutations acquired in BRCA genes described in the literature to gain insight into the mutational mechanisms driving their acquisition, as well as the importance of the different BRCA protein domains in generating drug resistance (Tobalina, Armenia, Irving, O'Connor, & Forment, 2021). Specifically, the group analyzed sequencing data of BRCA genes (from tumor or circulating tumor DNA, as available in the literature) in 327 patients with malignancies harboring mutations in BRCA1 or BRCA2 (234 patients with OC, 27 with BC, 13 with PANC, 11 with PC and 42 with a cancer of unknown origin) that progressed on platinum or PARPi treatment. As a result, these authors reported 269 cases in 86 patients in this cohort (26.3%) in which reversion secondary mutations with the capacity of restoring BRCA protein expression constitute the *bona fide* mechanism of resistance to these treatments. A detailed investigation of the reversion events led this group to suggest the following, illuminating conclusions: i) most amino acid sequences encoded by exon 11 in BRCA1 and BRCA2 are dispensable to generate resistance to platinum or PARPi, while other regions are more refractory to sizeable amino acid losses; ii) the error-prone NHEJ DDR pathway plays a key role in generating such reversions, especially in those affecting BRCA2 (as supported by the significant accumulation of DNA sequence microhomologies surrounding deletions leading to the reversion events; iii) the drug-based inhibition of NHEJ-reliant pathways could increase efficacy and resilience of anticancer therapeutics by avoiding the acquisition of BRCA gene reversion mutations, and iv) in the event that reversions result in the production of hypomorphic forms of the BRCA proteins, new therapeutic routes could be paved, particularly with medicines that target the DNA replication stress response.

2.7. Cellular functions of the BARD1 protein

2.7.1. BARD1 function in cell cycle progression

Besides the chaperone role in shuttling BRCA1 into the nucleus and sequestering it within the same cell organelle (as discussed in *e.g.*, §2.3.3), its link to S-phase progression and generic stability (§2.7.2), and its cooperation with BRCA1 in the regulation of centrosome amplification (§3.2) BARD1 has been shown to be a key, BRCA1-independent player in the later stages of mitosis for the completion of cytokinesis. In particular, Ryser and coworkers found that BARD1, but not BRCA1, localizes to the midbody at telophase and cytokinesis, where it colocalizes with the serine/threonine protein kinase aurora kinase B (AURKB (Willems et al., 2018)), a component of the chromosomal passenger complex (CPC)[31] (Ryser et al., 2009). Interestingly, the BARD1β isoform was found to coimmunoprecipitate with AURKB and BRCA2, while FL BARD1 coimmunoprecipitated with BRCA1. Accordingly, the authors

[30] Geneticists coined the phrase *synthetic lethality*, which refers to cell death induced by simultaneous perturbations of two genes (*e.g.*, loss of function mutations, RNAi, pharmacological therapy, etc.), each of which is nonlethal on its own. As a result, synthetic lethal interactions may broaden the repertory of anticancer treatment targets by allowing for the indirect targeting of non-druggable oncogenes, for example, by identifying a second-site synthetic lethal target that may be druggable.

[31] During mitotic cell division, the key regulator CPC complex has indispensable activities at the centromere in safeguarding the correct alignment and segregation of chromosomes, and is essential for the chromatin-mediated stabilization of MTs and spindle assembly (Carmena, Wheelock, Funabiki, & Earnshaw, 2012).

used selective siRNAs to differentiate the roles of FL BARD1 and BARD1β. They discovered that reduction of FL BARD1 had very modest impacts on cell proliferation and did not result in the loss of BARD1 midbody localization, although it did reflect in substantial AURKB upregulation. Contrary to this, suppression of FL BARD1 and BARD1β led to the arrest of cell proliferation and was associated with a variety of mitotic abnormalities and loss of BARD1 midbody localization. In the light of these data, Ryser and colleagues suggested a novel function of FL BARD1 in AURKB ubiquitination and degradation, opposing a pro-proliferative function of BARD1β in scaffolding AURKB and BRCA2. Moreover, loss of FL BARD1 and upregulation of AURKB, as observed in cancer cells, could be also be ascribed to an imbalance of FL BARD1 and BARD1β. Pilyugin and colleagues showed that, in non-malignant cells, the expression of BARD1δ prompts cell cycle arrest both *in vitro* and *in vivo* conditions (Maxim Pilyugin et al., 2017). Using cell culture and transgenic mice models, as well as cells derived from BC and OC patients with BARD1 mutations, these researchers investigated the mechanism that causes proliferation arrest and discovered that overexpression of the that BARD1δ isoform resulted in mitotic arrest characterized by both chromosome and telomere abnormalities. They also reported that BARD1δ is more efficient than BARD1 in associating with telomere binding proteins and in eliciting their detachment from telomeres, ultimately resulting in chromosomal and telomeric instability. Notably, while this induces the arrest of the cell cycle, malignant cells that lack G2/M checkpoint controls can still proliferate notwithstanding the chromosomal instability elicited by BARD1δ. The authors accordingly suggested that these features may render BARD1δ a *genome permutator* and a driver of indefinitely continuous and uncontrolled cancer cell growth.

2.7.2. BARD1 functions in tumor suppression, regulation of p53 and apoptosis

The role of BARD1 in tumor suppression has been originally proposed on the basis of experiments that down-regulated its expression (Irminger-Finger et al., 1998). According to the results, BARD1-repressed cells showed a prolonged S-phase – supporting the role of BARD1 in normal proliferation – and exhibited genetic instability, loss of growth inhibition by contact, and loss of morphogenetic properties, consistent with a loss of function (LOF) in tumor suppression pathways. The involvement of BARD1 in regulating apoptosis was also ascertained by the group of Irminger-Finger, who firstly identified BARD1 as an apoptosis mediator (Irminger-Finger et al., 2001) since, according to their data, *in vitro/in vivo* cell death is accompanied by increased levels of BARD1 protein and mRNA and BARD1 overexpression results in cell death characterized by all apoptotic features (Gautier et al., 2000b). Moreover, BARD1-repressed cells are defective for the apoptotic response to genotoxic stress whereas BARD1 levels increase in response to the same insult, and are necessary and sufficient for upregulation of the cellular tumor antigen p53 which, in turn, results in apoptotic response (Aubrey et al., 2018). Importantly, the authors established that the function of BARD1 as apoptosis inducer had to be attributed to BARD1 independently of its association with BRCA1. This fundamental conclusion was based on the evidences that only BARD1 but not BRCA1 levels increased after cell death *in vivo* (ischemic stroke), BRCA1/BARD1 cell co-transfection did not enhance BARD1-induced apoptosis, a BARD1 tumor-associated mutation (Glu564His) was found to be deficient in apoptosis induction, and, decidedly, BARD1 induced apoptosis in BRCA1-deficient cells. This groundbreaking work led Irminger-Finger and collaborators to propose a dual mode of action model of BARD1 function. Accordingly, when engaged in the heterodimeric complex with BRCA1, BARD1 operates in the survival mode, and is actively involved in DNA repair and cell survival. Alternatively, when in the death mode, BARD1 acts independently of BRCA1 and induces apoptosis. According to this concept, the ratio of BRCA1 and BARD1 levels of expression in a cell dictates its destiny, as high BRCA1/BARD1 ratios will foster DNA repair and survival, whereas low values of the same ratio will drive cells to death. This model also yields a rationale for the finding that BARD1 but not BRCA1 is expressed in physio/pathological conditions resulting in cell death (*e.g.*, stroke, see above); moreover, it would also be consistent with the development of an *in vitro* premalignant phenotype (Irminger-Finger et al., 1998) and carcinogenesis in the presence of low levels of BARD1 expression (Yoshikawa et al., 2000). The Ewing sarcoma family of tumors (ESFT) comprises a set of cancers distinguished by the presence of round-cells with similar morphology and chromosomal translocation. Although classified as rare diseases, ESFT is more frequent among children and young individual in their adolescence, and is the 3rd most frequent bone primary sarcoma following osteosarcoma and chondrosarcoma (Maheshwari & Cheng, 2010). In 85% of ESFT, the N-terminal of the RNA-binding protein EWS (EWSR1) – a protein that modulates cellular function and aging through genetic/epigenetic mechanisms (J. Lee et al., 2019) – is fused to the DNA-binding domain of the friend leukemia integration 1 transcription factor (FLI1), a sequence-specific transcriptional activator member of the ETS[32] transcription factors (Sharrocks, 2001), and the resultant chimeric protein is a powerful activator of transcription with transforming potential. Spahn and collaborators reported that EWSR1 and EWSR1-FLI1 interact via their common N-terminal regions with the C-terminal of BARD1 both *in vitro* and *in vivo*. Given the evidence that the BRCA1/BARD1 heterodimer constitues a hub for DDR and checkpoint control proteins, the authors concluded that their findings establish a connection between EWSR1-FLI1 and the genome surveillance complex. Irminger-Finger and her group have also highlighted the link between BARD1-related apoptosis and BARD1 binding to Ku70 and p53 (Feki et al., 2005; Irminger-Finger et al., 2001). The latter interaction involves both the ANK-BRCT linker and the BRCT domains of BARD1 (Jefford, Feki, Harb, Krause, & Irminger-Finger, 2004; V. Tembe et al., 2015), and is instrumental for the phosphorylation of p53 at Ser15 (pSer15). Overexpression of BARD1 in cell lines that are resistant to apoptosis and depleted in pSer15 (such as the NuTu-19 and HEK 293 T cell lines) may restore the phosphorylation capability of the p53 oncosuppressor. BARD1 also seems to promote the formation of DNA-PKcs and p53 assemblies through its interaction with Ku70 (§1), thus eliciting phosphorylation of p53 by ATM (§2.3.3) and the induction of apoptosis (Feki et al., 2005). Additionally, mutations linked with BC, OC, and uterine cancers lack sequences in the ANK-BRCT linker region, which is a critical component of BARD1-induced apoptosis (Jefford et al., 2004). Two works from Tembe and colleagues investigated in detail the liaison between BARD1, p53 and apoptosis (Varsha Tembe & Henderson, 2007; V. Tembe et al., 2015). This group showed that the BARD1/p53 complex localizes to the mitochondria, suggesting a cellular location for p53 regulation of BARD1 apoptotic activity. Most importantly, they confirmed that the deletion of the BRCT sequence has major detrimental effects on both BARD1 activity and localization, in that it decreases the affinity of BARD1 for p53 and prevents BARD1 shuttling from the nucleus and its subsequent localization to the cytoplasm and the mitochondria (V. Tembe et al., 2015). They also discovered that the BARD1 apoptotic function was associated with the apoptosis regulator BAX (BAX, Fig. 26, top left),[33] specifically at mitochondria where BARD1 promotes BAX oligomerization. This role is distinctive of BARD1 as its partner BRCA1 does not induce BAX oligomerization despite being endowed with pre-apoptotic functions as well (Varsha Tembe & Henderson, 2007). In the same study, the authors discovered that a cancer-related splice variant of BARD1 defective for both BRCA1 binding region and ANK domains was still found in mitochondria but it lost its capacity to trigger apoptosis or to modify the permeability of

[32] ETS domains are DNA binding motifs that recognize purine-rich core DNA sequences (F. D. Karim et al., 1990).

[33] BAX is involved in apoptotic process at mitochondria. Under physiological conditions, BAX is mainly a cytoplasmic protein due its continuous mitochondria-to-cytosol retrotranslocation, which prevents hazardous BAX levels from accumulating at the mitochondrial outer membrane. Under pathological circumstances such as stress conditions, BAX undergoes a conformational shift that induces its translocation to the mitochondrial membrane, resulting in the release of cytochrome c, which subsequently initiates apoptosis. BAX also promotes apoptosis by increasing caspase-3 (Peña-Blanco & García-Sáez, 2018).

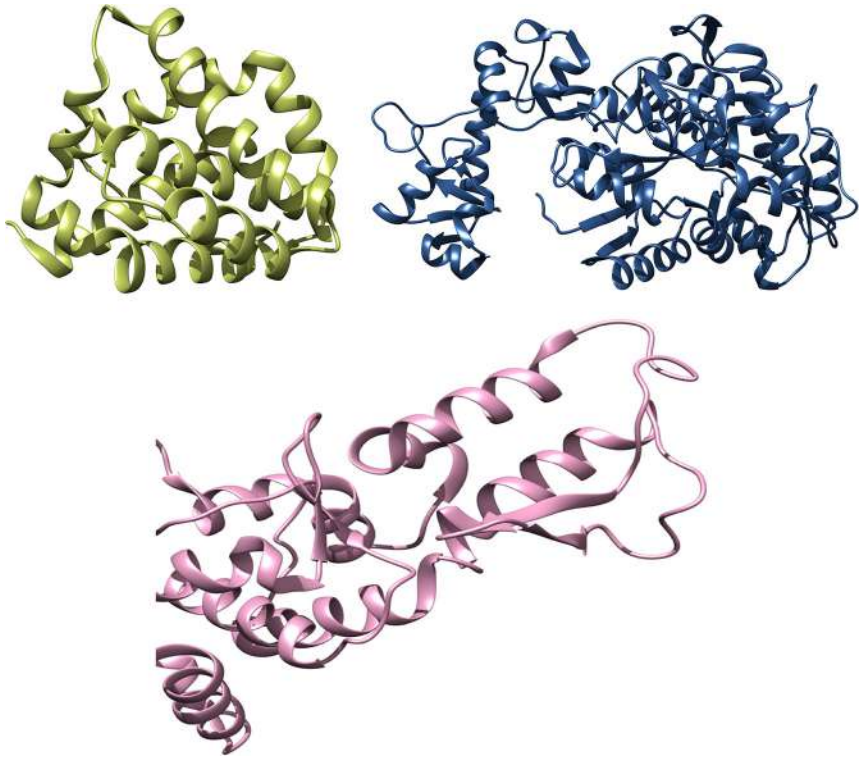

Fig. 28. Crystal structures of BAX (top left, dark citron PDB: 4S0O (Garner et al., 2016)), E6 (top right, galaxy blue PDB: 6SLM (Conrady et al., 2020)), and UBE3A (bottom, moonlite mauve, PDB: 1D5F (L. Huang et al., 1999)).

the mitochondrial membrane, reinforcing the notion that BARD1 with these intact domains is required for inducing BAX oligomerization and apoptosis. The authors also proposed that BARD1 has two major sites of action in its biological response to DNA damage: the nucleus (where it supports cell survival through DDR) and the mitochondria (where it controls apoptosis).

Interestingly, BARD1 has been demonstrated to stabilize p53 in a somewhat different clinical context. For instance, Yim and coworkers (Yim et al., 2007) identified BARD1 as a binding partner of protein E6 (E6, Fig. 28, top right (Fiamma Mantovani & Banks, 2001)), a transforming protein encoded by human papillomaviruses (HPVs) which play an established etiological role in the malignancies of the cervix, penis, vulva, vagina, anus and oropharynx (Chan, Aimagambetova, Ukybassova, Kongrtay, & Azizan, 2019). E6 primarily functions as an oncoprotein, promoting the degradation of many proteins with critical cellular roles. In particular, E6 associates with the ubiquitin-protein ligase E3A (UBE3A/E6AP, Fig. 28, bottom) of the host cells, and inactivates p53 by targeting the oncosuppressor to the 26S proteasome for degradation (S. Li et al., 2019; Sailer et al., 2018). In the same effort, using systems in which BARD1 was either knockdown or overexpressed Yim et al. were able to examine the impact of BARD1 on the transcriptional activity of p53. They found that i) not only E6 did not degrade BARD1 but the two proteins formed a complex, the formation of which was compromised for E6 isoforms with mutated E6 zinc-finger region; ii) despite the presence of E6, the p53-mediated activation of CDK1/p21 (§2.6.1.2, a main target of p53 and so associated with the link between DNA damage/cell cycle arrest (Shamloo & Usluer, 2019)) increased upon transient BARD1 transfection; and iii) the presence of BARD1 led to E6 suppression in cervical cancer cells. All these findings establish BARD1 as a regulator of the transcriptional activities of p53 as tumor suppressor and the BARD1/p53 collaboration in p53 against HPV infection (Yim et al., 2007).

Nuclear-cytoplasmic shuttling also regulates BARD1 cellular localization and activity in apoptosis. As shown by Rodriguez and coworkers, the BARD1 functional CRM1-dependent NES is part of the BRCA1 dimerization motif (§2.3.1 and 2.3.3), and co-expression of BRCA1 led to the burial of BARD1 NES and the subsequent nuclear retention of the protein (Rodriguez, Schüchner, et al., 2004). These authors reported that, in transient expression experiments, nuclear export increased the apoptotic activity of BARD1, whereas BRCA1 significantly decreased both BARD1 apoptotic function and nuclear export, and comparable results were observed for endogenous BARD1. In line with this, suppressing BRCA1 expression using siRNA or altering the endogenous BARD1/BRCA1 relationship via peptide competitive binding reduced BARD1 nuclear localization and foci formation while increasing cytoplasmic BARD1 levels, which correlated with increased apoptosis.

2.7.3. BARD1 inhibition of mRNA processing in response to DNA damage

To ensure a swift reaction to biological stimuli, gene expression is a tightly regulated process. In eukaryotes, the presence of a long chain of adenine nucleotides (poly(A) tail) at the 3'end of the mRNAs has key fucntions in post-transcriptional control (Manley & Di Giammartino, 2020). The addition/removal of poly(A) tails to modulate stability and translation efficiency of mRNAs (Ananthanarayanan Kumar, Clerici, Muckenfuss, Passmore, & Jinek, 2019) is substantially governed by the cleavage stimulation factor (CStF),[34] the carbon catabolite repression-negative on TATA-less (CCR4-NOT) (Raisch et al., 2019), and the Pan2-Pan3 (Wolf & Passmore, 2014) multiprotein complex[35] that functions in gene expression. In this context, Kleiman and Manley showed that BARD1 can elicit DDR pathways by regulating the polyadenylation

[34] CStF is a 3-subunit protein composed (CSTF1/CSTF-50 (50 kDa), CSTF2/CSTF-64 (77 kDa) and CSTF3 (77 kDa)) implicated in the cleavage of a newly synthesized pre-mRNA molecule 3'signaling region. The cleavage and polyadenylation specificity factor (CPSF) recruits CstF and forms a protein complex at the 3'end to stimulate the production of a poly (A) tail, resulting in a mature mRNA ready for nucleus-to-cytosol shuttling and subsequent translation.

[35] This protein assembly also regulates the expression of several genes involved in inflammatory processes, silencing of miRNA-targeted genes, and maternal mRNA expression during oocyte development (Y. Zhang et al., 2021). All these processes are deregulated in many diseases, including cancer (Yuan, Hankey, Wagner, Li, & Wang, 2021) and neurological disorders (Patel, Brophy, Hickling, Neve, & Furger, 2019).

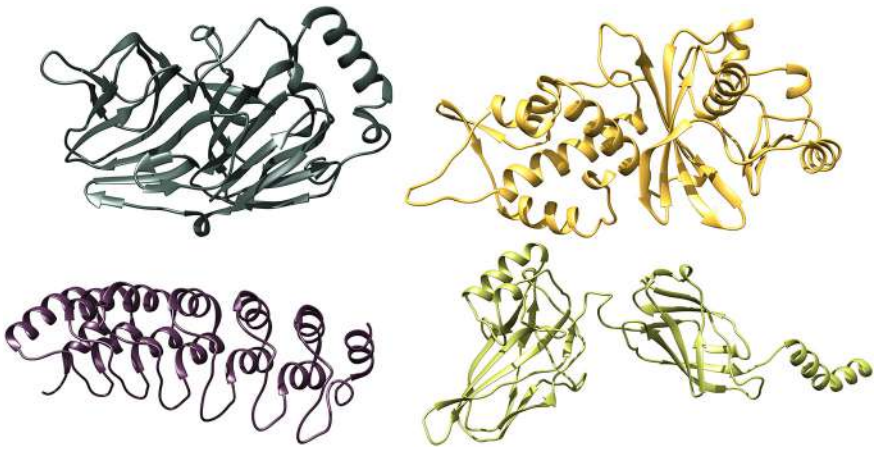

Fig. 29. (Top left) Crystal structure of PIR (jungle, PDB: 6H1H (Meyers et al., 2018)). (Top right) 3D structure of TIP60 (daffodil, AlphaFold2 PDB: Q92993) as predicted by AlphaFold2 (Jumper et al., 2021). (Bottom left) Crystal structure of the ANK repeat domain of BCL-3 (plum purple, PDB: 1K1A (Michel et al., 2001)). (Bottom right) 3D structure of p65 (bright chartreuse, AlphaFold2 PDB: Q04206) as predicted by AlphaFold2 ((Jumper et al., 2021).

machinery via binding to CSTF1 in a BRCA1-independent way (Kleiman & Manley, 1999). Upon phosphorylation of BARD1 at Thr734 by ATM (Kim et al., 2006), CSTF1 binds the ANK-BRCT linker region of BARD1 (§2.3.4.3) and engages it in mRNA processing and RNAPII stability in response to DNA damage caused by UV or IR (Cevher & Kleiman, 2010; Edwards et al., 2008). BARD1 recognizes DNA damage sites within the RNAPII holoenzyme and, by inhibiting mRNA polyadenylation, prevents the processing of immature transcripts that could otherwise be translated into potentially harmful proteins (Kleiman & Manley, 2001). The ubiquitination of RNAPII, which is mediated by BRCA1 and BARD1, further contributes to the suppression of transcription, and cells exhibit reduced levels of polyadenylation in response to DNA damage, which is accompanied by elevated levels of the CSTF1-BARD1-BRCA1 complex (Kleiman et al., 2005). To support the physiological relevance of these findings, Kleiman and Manley also studied a previously discovered tumor-associated germline mutation in BARD1 (Gln564His, which has been identified in ovarian, breast, and uterine cancers), and verified its decreased binding to CSTF1 and a consequent abolishment of polyadenylation inhibition, thereby establishing a link between BARD1 and mRNA 3'processing, DNA repair, and tumor suppression (Kleiman & Manley, 2001). Interestingly, in the same context of UV-induced DNA damage, Nazeer et al. reported that p53 interacts with the BARD1-CSTF1 complex, and tumor-associated p53 mutations decrease BARD1-CSTF1 interaction as well as mRNA cleavage (Nazeer et al., 2011).

2.7.4. BARD1 functions at the telomeres

In 2017, the group of Irminger-Finger demonstrated that overexpression of the BARD1δ splice variant (§2.2 and 2.7.1) led to chromosomal abnormalities and aneuploidy, and that this BARD1 isoform caused these adverse effects on chromosomal stability by undermining telomere integrity, eventually reflecting in telomere attrition/fusion and cell cycle arrest. They reproduced similar evidences *in vivo*, by showing that following BARD1δ induction transgenic mice presented telomeric anomalies, and mice embryonic development was fully abrogated upon BARD1δ constitutive expression. In the same effort, the authors reported that both FL BARD1 and BARD1δ bound to the members of shelterin (*e.g.*, TRF1 and TRF2 (§2.6.2.2) and the poly(ADP-ribose) polymerase tankyrase-1 (TNKS), a poly-ADP-ribosyltransferase involved in various processes including the regulation of telomere length), which are indispensable for telomere integrity maintenance. In contrast to FL BARD1, which had no appreciable effect on shelterin functions, BARD1δ expression resulted in shelterin component depletion from the telomeres. It has previously been reported that telomeric shelterin depletion causes end-to-end chromosomal fusions to occur, which are prompted by DDR pathways (Rai et al., 2010). In agreement with this, Irminger-Finger and colleagues found that BARD1δ-dependent telomeric depletion of TRF1, TRF2, and TNKS was connected to augmented chromosomal abnormalities and reduced cell growth. As a conclusion, the authors proposed that BARD1δ is an antagonist of FL BARD1 chromosome/telomere protection functions, and that BARD1δ exerts its pathological activity by promoting chromosomal instability *via* shelterin component sequestration. Notably, they also verified that TRF2 could be bound by the region (linker) connecting the BARD1 BRCT and ANK domains, which is present in BARD1δ, and that the improved binding of BARD1δ to telomeric proteins is dependent on the particular conformation assumed by this linker in BARD1δ, which differs from the structure it adopts in FL BARD1 or BARD1ω (§ 2.2). Indeed, although both FL BARD1 and BARD1ω feature the linker motifs, both of these proteins exhibited decreased affinity to telomeric proteins and did not promoted instability of the telomeres. Accordingly, the authors concluded that BARD1δ counteracts FL BARD1-BRCA1 functions on essential molecules that are critical for in preserving chromosome segregation and integrity. Whilst normal cell cycle arrest is promoted in the presence of BARD1δ- promoted genomic instability, when the cell cycle is compromised (*e.g.*, due to p53 or RB deficiencies) BARD1δ enables cells with genetic instability to grow and develop oncogenic functions, thus marking BARD1δ a driver of cancer-associated genomic instability, paving the way for tumorigenesis *via* the sustained induction of genetic aberrations.

2.7.5. BARD1 interaction with NF-κB and PAR

Another BRCA1-independent function of BARD1 is the interaction with NF-κB (§2.3.4.3 and 2.6.2.4) (Irminger-Finger & Leung, 2002) – a protein complex that controls transcription of DNA, cytokine production (and hence inflammatory processes and autoimmune diseases) and cell survival – and the modulation of its transcriptional activity via the B-cell lymphoma 3 protein (BCL-3), a predominantly nuclear proto-oncoprotein member of the inhibitor of κB (IκB) family of proteins (Hinz & Scheidereit, 2014) that controls NF-κB-dependent transcription by preventing its DNA binding and promoting its cytoplasmic localization (Dechend et al., 1999). Along with the NF-κB transcriptional co-regulators pirin (PIR, Fig. 29, top left (F. Liu et al., 2013)) and histone acetyltransferase Tip60 (TIP60, Fig. 29, top right), that plays a number of roles including cellular signaling, DDR, cell cycle and checkpoint control and apoptosis (Sapountzi, Logan, & Robson, 2006; Squatrito, Gorrini, & Amati, 2006), BARD1 forms the so-called BCL-3 interacting protein (BIP) network which, in turn, is sequestered into a super-complex with BCL-3 and the mature NF-κB p50 subunit bound to an NF-κB DNA binding site (Dechend et al., 1999). Within

this protein assembly, a BARD1 fragment composed of half of the ANK-through-BRCT domains (residues 464–777) binds the ANK repeats of BCL-3 (Fig. 29, bottom left). The super-complex subsequently binds to NF-κB gene promoter thereby activating its transcription. Because BARD1 and NF-κB both bind the N-terminal region of BRCA1 (in particular, the Rel homology domain (RHD)[36] of the p65 (aka RelA) subunit of NF-κB interacts with multiple sites within the BRCA1 N-terminal domain, Fig. 29, bottom right), Benezra and coworkers examined whether BARD1 could alter the functional relationship between the tumor suppressor and the transcription factor (M. Benezra et al., 2003). To the purpose, 293 T cells were transfected with BARD1 alone or with BRCA1, and the authors reported a 4-fold increase in reporter activity induced by BRCA1 when cells were treated with the tumor necrosis factor alpha (TNFα); on the other hand, BARD1 had no significant effect on the ability of TNFα to activate the reporter gene in the absence of BRCA1. However, they also found that BARD1 expression, although generally increasing BRCA1 protein accumulation within the cell, actually inhibited the ability of BRCA1 to activate transcription through NF-κB.

The interaction of the NF-κB p50 subunit with BARD1 was recently reinvestigated by the group of Yadav (Yadav et al., 2020). They confirmed that NF-κB p50 interacts with BARD1 BRCT directly through a C-terminal pSer motif. This association was discovered to be triggered by ATR and promotes BRCA1/BARD1-mediated NF-κB p50 mono-ubiquitination, and loss of this PTM accelerates S-phase advancement and chromosomal breakage. This study also revealed that, during S-phase there is a significant reduction in NF-κB p50 chromatin enrichment, and cyclin E has been reported as a factor mastered by NF-κB p50 during the G1/S transition. From the functional standpoint, the authors described that the BARD1/NF-κB p50 association enhances the stability of the transcription factor; in line with this, low BARD1 nuclear localization coincided with low nuclear levels of NF-κB p50 in human cancer specimens, implying that BARD1/BRCA1-mediated NF-κB p50 mono-ubiquitination during cell cycle modulates S-phase progression in genome integrity maintenance.

In 2013, Li and Yu established that, unlike the BRCA1 BRCTs, the isolated BARD1 BRCTs swiftly (20 s) relocated to DNA damage sites independently of the status of H2AX after laser micro-irradiation, although they also rapidly dissociated (5 min) from the same sites (M. Li & Yu, 2013). Since the relocation kinetics of the BARD1 BRCTs to DNA damage sites was found to be similar to that reported for PAR (Gibson and Kraus, 2012; Kim et al., 2005), these authors hypothesized that the BARD1 BRCTs may recognize PAR. Using *in vitro* assays, they found that purified recombinant BARD1 BRCTs could directly co-immunoprecipitate PAR, while reciprocal pull down further confirmed the direct interaction between the two proteins. Importantly, Li and Yu also measured BARD1 BRCTs/PAR affinity using isothermal titration calorimetry (ITC), and the relevant dissociation constant (K_d) of ~0.16 mM they reported falls in the range of values reported for similar systems, *e.g.*, for the affinity between the BRCA1 BRCTs and a pSer peptide (Wu, Jubb, & Blundell, 2015), and between PAR and its other binding partners (Karras et al., 2005; Yu et al., 2003). Most importantly, under the same experimental conditions, the BRCA1 BRCTs showed no interaction with PAR.

2.7.6. BARD1 mutations in cancer (and other diseases)

The knowledge of BARD1 mutations and their implication in cancer and other human maladies is markedly more recent and, hence, less explored with respect to its partner BRCA1. For comparison, the entry "*BARD1 mutations in cancer*" on PubMed at the time of writing returned only 287 results. At the same time, a single gene search on ClinVar listed 2453 mutations, of which 51 are classified as benign, 602 as likely benign, 225 as pathogenic, 100 as likely pathogenic, 1418 as of uncertain significant and 113 with conflicting interpretations. Among the reported mutations, the major types are missense variations (1325), followed by frameshift (119) and nonsense (98) mutations; the database also lists 54 splice sites and 2085 ncRNA and 27 UTR variations. Concerning the allele origin, 2413 mutations are classified as germline variations and 2 as somatic. Following the same approach adopted in §2.6.2.6, for the sake of brevity here we will focus only on the latest reports on BARD1 mutations, leaving the interested reader to recent literature surveys on the subject (Alenezi et al., 2020; Cimmino et al., 2017; Watters, et al., 2020).

Early this year, using integrated genomics/transcriptomics analysis of germline and tumor specimens from an early-onset TNBC patient Zheng and colleagues reported an uncommon BARD1 germline missense mutation (c.403G > A/p.Asp135Asn) and classified it as pathogenic (Y. Zheng et al., 2021). Compared to WT BARD1, the authors showed that c.403G > A BARD1 mutant cell lines were more sensitive to IR, to a DNA damage agent, and to a PARP inhibitor, and that the same mutation prevented BARD1 interaction with RAD51 but not with BRCA1. Moreover, BARD1 c.403G > A mutant mice were also found to be hypersensitive to IR. To date, a large number of mutations have been screened from breast and ovarian cancer patients. Two years ago, Adamovich and collaborators (Adamovich et al., 2019) identified 76 potentially cancer-associated BARD1 missense and truncation mutants (64 germline and 12 somatic) along the FL BARD1 sequence by analyzing a large dataset containing exome-sequencing data on matched germline and tumor samples (K. L. Huang et al., 2018; C. Lu et al., 2015). They tested all these BARD1 variants for DDR function – HR in particular – by considering those variants whose HR activity was <0.6 and whose expression was greater than or equal to endogenous BARD1 to be repair-deficient. Concerning the eight detected variants located in the RING domain (Cys62Ser, Glu67Lys, Ile69Met, Val85Leu, Ser103Asn, Met104Ile and Ser109Arg) (§2.3.1), and the 22 mutations found in the region between the RING and the ARD motifs (Asn118Ser, Lys140Asn, Ser151Asn, Asp190Asn/Tyr, Arg194Lys, Glu223Gly, Asp230Glu, Leu239Gln, Ser241Cys, Ile249Val, Ile258Thr, Arg322His, Asn326Asp/Ser, Ser339Asn/Thr, Ser342Asn, Thr343Ile, Thr351Met, Glu361Asp and Ser389Cys) (§3.2.4.3), all had HR activity similar to WT BARD1. Interestingly, in a previous paper from the same group the RING domain variants Leu44Arg, Cys53Trp, and Cys71Tyr were also found to be defective in HR due to impaired binding to BRCA1 (C. Lee et al., 2015). Rather unexpectedly, four (Ala460Thr, Leu465Phe, Leu480Ser, and Pro530Leu) out of the 17 total variants (Ile434Phe, Ala435Val, Leu447Cys, Asn450His, His483Arg, Asn488Ser, His506Arg, Val507Ala, Val510laA, Ala518Val, Val523Ila/Ala, and Tyr533Phe) in the ARD, which has no reported DNA repair function, were found to express FL BARD1 and be substantially defective in HR. On the contrary, the five variants (Lys540Asn, His556Asp, Ser6558Pro, Arg565Cys, and Arg565His) located between the BARD1 ARD and BRCT domains were found to be competent in DNA HR exception made for the Arg565Cys mutant, whose HR activity value was just below the 0.6 cutoff. Within the 19 missense variants identified in the BRCT domain (Gly574Asp, Ser575Asn, Thr598Ile, Asp612Val, Ser616Asn, Leu625Ile, Arg641Gln, Arg642Gly, Gly656Arg, Ser660Arg, Arg664Thr, Phe677Leu, Gly698Asp, Pro707Ser, Val713Met, Thr719Ala, Arg731Cys/His, Gly753Asp) (§2.3.2), involved in recruiting and retaining the BRCA1/BARD1 heterodimer to DNA damage sites (§3.3), five (Ser575Asn, Ser660Arg, Gly698Asp, Pro707Ser and Gly753Asp) were found to be defective in HR. Of these, BARD1 mutants Ser660Arg and Gly698Asp had HR function similar to that observed in cells transfected with an empty vector, while the remaining three exhibited an HR activity higher than empty vector but still significantly lower than that measure for endogenous BARD1. The authors also tested five truncated variants (Val154fs, Gly451fs, Ser551*, Gln564* and Val767fs), and their HR activity was almost equally defective as the empty vector. Finally, six of BARD1 mutants discussed above (Ser339Thr, Thr343Ile, Val523Ia, Asn450His, Gly451fs and Leu239Gln) were reported to have significantly higher

[36] The RHD is a conserved DNA-binding domain composed of ~ 300 residues (Moorthy, Huang, Wang, Vu, & Ghosh, 2007), first identified in the product of the REL oncogene (a transcription factor itself (Hunter, Leslie, & Perkins, 2016)), and subsequently found to be characteristic of eukaryotic transcription factors such as NF-κB and NFAT (nuclear factor of activated T cells, (Müller & Rao, 2010)).

LOH, and the authors suggested to take this evidence as an indication that these protein isoforms might have an increased likelihood of being pathogenic. Of note, previous work using data filtering based on high LOH led to the identification of BRCA1 variants defective in HR (C. Lu et al., 2015). However, in the case of BARD1, all variants found in the study of Adamovich et al. to have high LOH were also all functional, with the exception of truncation variant G451fs (Adamovich et al., 2019). The pathogenic nonsense BARD1 variant Gln564* was also found by Rosenthal et al. in 1 out of 500 hereditary cancer syndromes specimens using next-generation sequencing (NGS) (Rosenthal et al., 2020), while a study centered on the Brazilian population with HBOC 5 revealed BARD1 variants, including 4 missense mutations (Arg658Cys, Lys423Arg, Asn255Ser, and Leu239Gln) and 1 premature start codon (rs71579840, c.-83C > T) at the BARD1 5'UTR (da Costa et al., 2020). The last three missense variants are currently described as variants of unknown significance (VUSs) on ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), although Asn255Ser and Lys423Arg still deserve more studies characterizing their effects on protein functions before reaching an ultimate classification. Leu239Gln was already described as a germline mutation in BC patients belonging to the North American population and was also characterized as a VUS (Tung et al., 2016). The group of Da Costa then used different software to classify these BARD1 mutants, finding discordant results since all were predicted both as likely benign and pathogenic by the different *in silico* tools employed (da Costa et al., 2020). Of note, i) all missense substitutions are located either between the critical RING and ARD BARD1 domains (Leu239Gln and Asn255Ser) or within the ARD domain (Lys423Arg), and ii) while Leu239Gln was found in double heterozygosis with the pathogenic variant Trp1836* in BRCA1, Asn255Ser was identified in a non-BRCA1/BRCA2 BC patient. Further, the nonsense BARD1 mutation p.Arg641* was also associate to BRCA1/BRCA2-negative BCs (Shahi et al., 2019), as was the nonsense BARD1 variant (c.1345C > T/p.Gln449*) described by Park and coworkers in their study of variants of cancer susceptibility genes in Korean BRCA1/2 mutation-negative patients with high risk for HBC (J. S. Park et al., 2018). BARD1 p.Arg641* was also reported in another study focused on HBOCs, in a family with cancer history comprising both BC and PC (Feliubadaló et al., 2017). Germline BARD1 likely LOF and therefore oncogenic mutations R112*, S541*, G41* and Y739Lfs* were also found in BC patients belonging to the Latin-American population (Urbina-Jara et al., 2019).

Scarpitta et al. conducted a germline study on 81 male BC patients in order to further characterize the BC genetic risk factors in men. They used NGS to screen the 24 genes implicated in BC susceptibility, genome stability maintenance, and DDR mechanisms (Scarpitta et al., 2019). In this context, they found the BARD1 missense Cys639Arg, that was predicted as potentially pathogenic by *in silico* analysis. Another missense mutation – Lys670Asn – was also reported as novel SNPs connected to male BC (Kaur et al., 2019).

During the screening of BARD1 variant occurrence in almost 200 families with high-risk BOC, De Brakeleer et al. reported eleven intron SNPs and fifteen exon variants (9 missense, 4 silent, 1 in-frame deletion and 1 frameshift mutation), four of which were described for the first time (De Brakeleer et al., 2010). Among intron SNPs, six occurred with comparable frequency (>9%) both in cancer patients and in healthy controls, whilst the remaining five rare intron variants were considered to be neutral based on the consolidated results of three different splice site prediction software. Within the exon variants, this group identified six mutations that they classified as candidate BC-predisposing variants: Val85Leu, Ile509Thr, Glu652fs, Arg658Cys, Ile738Val, and Ser 761Asn. Since i) pedigree analysis showed segregation of the previously unreported Ile509Thr mutant allele within the BARD1 ARD (§2.3.4.3) with a BC phenotype, ii) ARDs in proteins are involved in a diverse set of cellular functions (A. Kumar & Balbach, 2021), and iii) defects in ankyrin repeats have been found in many tumor suppressor genes (Mosavi, Cammett, Desrosiers, & Peng, 2004), the authors considered the Ile509Thr variant as a strong candidate cancer predisposing mutation. The Arg658Cys mutation, located in the short central α-helix (residues 655–668) connecting the BARD1 BRCT1 and BRCT2 motifs (§2.3.2), was found in 2.6% of the high-risk families they analyzed, and also in 5% of the group of families with only two BC cases, while none of the controls tested featured this variant. The incomplete mutant allele segregation with the disease observed in these families, and its even more pronounced occurrence in families with only two BC cases led the authors to conclude that BARD1 Arg658Cys is a moderate BC allele. Of note, the same BARD1 mutation was already reported in the literature as linked to BC and OC malignancies (§2.2) (Karppinen et al., 2006; Thai et al., 1998; Vahteristo et al., 2006). Lastly, the authors described for the first time a BARD1 truncating mutation (p.Glu652fs), resulting in the removal of the protein BRCT2 which is instrumental in performing a correct HR in DDR and in preserving chromosomal stability (Laufer et al., 2007). The segregation of the Glu652fs allele with BC in the affected family analyzed, coupled with the importance of the BARD1 BRCT domains, allowed the authors to hypothesize this frameshift mutation to be the dominant cancer predisposing factor in that particular family. Another previously undescribed mutation involving the same region – specifically the BARD1 truncating variant (c.1935-1954dup20/p.Glu652Val*69) – was also reported as an incidental finding in a late-onset OC (Cavaillé et al., 2021). The same group searched for mutations in the BRCA1, BRCA2, and BARD1 genes in germline DNA from 61 estrogen receptor negative individuals (42 of whom were affected by TNBC) (De Brakeleer et al., 2016). BRCA1/2 mutations were discovered in 19% of TNBC patients (8/42), but not in the ER-/HER2 + group. In the TNBC cohort, they also discovered four potential pathogenic BARD1 variants, including two protein-truncating mutations (p.Gln564* and p.Arg641*). Their findings thus indicate that TNBC patients are more likely to bear pathogenic BARD1 pathogenic germline mutations than control samples and families with high BC risk.

The same group analyzed germline DNA from 61 estrogen receptor negative patients (of which 42 were TNBC) for the presence of mutations in the BRCA1, BRCA2 and BARD1 gene. BRCA1/2 mutations were found in 8 out of 42 (19%) TNBC patients, but not in the ER-/HER2+ cohort. They also found four good candidate pathogenic BARD1 mutations in the TNBC group, including two protein-truncating mutations (p.Gln564* and p.Arg641*). Their data thus suggest that TNBC patients are enriched for pathogenic BARD1 germline mutations as compared to control samples and high BC risk families.

A work from a Polish group also described 16 BARD1 mutations in BRCA1/BRCA2 negative high-risk BOC patients in the population of their own country (Ratajska et al., 2012), which included the nonsense mutation c.1690C > T/p.Gln564* resulting in the loss of both BRCT domains. Further, two interesting non-SNP variants were reported. The former is a splice variant (c.1315-2A > G) located in intron four, which translates in the skipping of the entire exon 5 and disruption of the first two ANK repeats known to be required for interactions with other proteins and in the onset of apoptosis induction. Since the BARD1 sequences required for apoptosis induction map between its ARD and BRCT domains (Jefford et al., 2004) (§2.3.2, 2.3.4.3, and 2.7.2), the authors proposed that this mutated BARD1 isoform could be endowed with a decreased ability to induce apoptosis and, as such, should be regarded as clinically significant. The second interesting variant is a silent (c.1977A > G/p.=) substitution that altered different exonic splicing enhancers (ESE)[37] motifs located within BARD1 exon 10; as a result, the corresponding transcript lacks exons 2–9.

The same group examined a sample of 255 individuals for the existence of previously known mutations in BARD1 exons 5, 8, and 10 with the goal of assessing the impact of any germline mutation possibly found in these BARD1 gene on OC susceptibility (Ratajska et al., 2015).

[37] ESEs are DNA sequence motifs consisting of 6 bases within an exon that direct, or enhance, accurate splicing of heterogeneous nuclear RNA or pre-mRNA into mRNA (Z. Wang, et al., 2004).

Within this cohort, they reported single-patients carrying a mutation in exon 8 (c.1690C > T/p.Gln564*), two different variations in exon 10 (c.1972C > T/p.Arg658Cys; c.1977A > G/p.=) and a patient with a new exon 5 missense mutation (c.1361C > T/p.Pro454Leu). Three of these mutations affected the ESE motifs, resulting in the expression of erroneous splicing skipping of exons 5, 8, and 2–9, respectively. Although based on available data the authors could not estimate their actual penetrance, the authors concluded that such BARD1 variants may predispose to OC in limited number of patients. In a study of a family with HBOC, Li et al. reported two BARD1 mutations located outside any BARD1 motif - P24S and R378S – that simultaneously exist in cis in surviving cancer patients (W. Li et al., 2021). According to the data, each single mutation does not reflect into a functional alteration of the protein; however, together they act in synergy in impairing DDR both *in vitro* and *in vivo*. Another recent study investigated the prevalence of disease-causing genes in Japanese patients with BRCA1/2-wildtype HBOC (Kaneyasu et al., 2020). These authors identified a large BARD1 deletion variant, in which exons 5 to 7 were missing, along with a nonsense mutation R150*, and they surmise that these variants were specific to Japanese/east-Asian HBOC patients, since they could not be found in non-cancer east-Asian populations and European familial BC cohorts they analyzed for comparison. Another Asian-related study focused on Chinese women showed that 2.5% of the cohort had a pathogenic variant in genes other than BRCA1/BRCA2, including BARD1 (C. Zeng et al., 2020). Concerning BARD1, these authors reported three nonsense mutations (p.Q176* (exon 4), p.R232* (exon 8), and p.R581* (exon 12)), along with a frameshift insertion mutation (c.271_272insTA/p.K91fs). Along the same line, Schoolmeester et al. reported the BARD1 nonsense variation c.448C > T/p.Arg150* in a patient with familiar cases of BC and OC in both first-degree and second-degree relatives (Schoolmeester et al., 2017). In another largely European–Caucasian multi-institutional cohort of BC gene group, with respect to controls BARD1 mutations were found with a significantly higher frequency in patients with HBC (OR = 3.18), marking this work as the first large study in which the BARD1 gene was associated to a moderate risk for HBC predisposition (Slavin et al., 2017). According to reported statistics, African American BC patients are more likely to be affected at a young age, to develop aggressive TNBC, and to die as a consequence of this malignancy with respect to BC-afflicted individuals from other populations (Menashe, Anderson, Jatoi, & Rosenberg, 2009). Churpek and coworkers examined 289 self-identified African American patients with primary invasive BC and with personal/familiar cancer history or tumor features linked with high genetic risk for all germline mutations in genes with known BC susceptibility (Churpek et al., 2015). The mutational allelic spectrum they identified was highly heterogeneous, with 57 different mutations in 65 patients, and S551* was the only BARD1 mutation they found in that cohort. DeLeonardis and coworkers reported on a family with early onset BC and primary peritoneal cancer (PPC) that was found to carry the deleterious germline mutation in BARD1 (c.947 T > G/p.Leu316*), and their LOH studies suggested a causative role of this BARD1 variant in the development of PPC (DeLeonardis et al., 2017). Epithelial ovarian cancer (EOC) is the gynecological malignancy with the worst fatal *exitus* (*e.g.*, 13,000 EOC-related deaths/year in the United States only); accordingly, risk prediction based on detecting germline mutations in genes linked to OC susceptibility has the potential to have a substantial clinical effect on diminishing the mortality from this malignancy. With this goal, Ramus et al. analyzed 3236 invasive EOC case patients, 3431 controls patients of European ancestry, and 2000 unaffected high-risk female subjects from an OC clinical screening trial (UKFOCSS) (Ramus et al., 2015). They reported 8 EOC-associated BARD1 mutations, four of which were frameshift variations (c.623dupA/p.Lys208fs, c.627_628delAA/p.Lys209fs, c.2300_2301delTG/p.Val767fs, and c.2291_2294delTAGA/p.Ile764fs) and the other four were nonsense mutations (c.1690C > T/p.Gln564*, c.1996C > T/p.Gln666*, c.1212C > G/p.Tyr404*, and c.1921C > T/p.Arg641*). Of note, all these BARD1 variants were predicted by the authors to be deleterious; however, based on their data the authors reported no statistically significant differences in BARD1 deleterious mutation frequency in affected patients *vs.* controls (4 case patients, 0.12%; 2 control patients, 0.06%, *P*-value[38] = 0.39). In the same study, the group also identified a large number of BARD1 missense mutations in the analyzed cohort and, by consolidating the results using at least three *in silico* prediction methods, the authors classified these mutations as deleterious (D)/non-deleterious (ND) as follows: Gln11His (ND), Arg13Lys (ND), Glu19Asp (ND), Arg21Gly (ND), Pro24Ser (ND), Arg31His (ND), Ala33Pro (ND), Arg38His (D), Ala40Val (D), Pro89Leu (D), Gln164Glu (ND), Pro167Leu (D), Ala189Val (ND), Asp190Asn (ND), Arg194Lys (ND), Lys205Arg/Asn (D), Gln206Lys (ND), Lys207Arg (ND), Lys209Arg (ND), Leu211Ser (D), Leu220Ser (ND), Asp230Glu (ND), Ser231Pro (ND), Ile258Thr (ND), Thr309Ala (ND), Leu316Val (ND), Lys321Arg (ND), Gly323Ser (ND), Asn326Ser (ND), Thr343Ile (ND), Ser363Tyr (D), Ser364Thr/Leu (ND), Ser376Leu (ND), Arg378Ser (ND), Pro396Ser (ND), Ser397Cys (ND), Val422Ala (ND), Leu432Phe (D), His433Pro (D), Leu447Val (D), Trp462Ser (D), Thr463Ile (D), Pro464Ser (D), Hir466Arg (D), Ans470Ser (ND), His471Tyr (D), Val477Ala (ND), His483Arg (ND), Val507Met (ND), Gly517Arg (D), Ser519Tyr (D), Ile525Met (D), Ser538Asn (ND), His556Ans (ND), Cys557Ser (ND), Ser558Thr (ND), Thr562Ile (ND), Arg565His (ND), Gly574Ser (ND), Ser586Ile (ND), Thr605Ala (D), Asp612Val (ND), Trp629Arg (D), Glu652Gly (D), Arg658Cys (D), Gly681Val (D), Lys706Glu (ND), Asp710Val (D), Thr714Ile (D), Ile738Val (ND), Gln752Lys (ND), Tyr745Asp (ND), Ser760Leu (D), Ser761Asn (ND), and Leu722Trp (D). Interestingly, of these only Pro24Ser, Arg378Ser, Val507Met and Cys577Ser had a minor allele frequency greater than 1%, and all of them were classified as non-deleterious. Clearly, a lot of work lies ahead to confirm the predicted pathogenicity and the clinical significance of this plethora of BARD1 variants in EOCs and/or other malignancies.

Pathogenic BARD1 mutations have also been linked to neuroblastoma (NB). Neuroblastoma is the most frequent extracranial solid tumor in infancy, and it is characterized by a neoplastic growth of neural crest cells in the developing sympathetic nervous system (Louis & Shohet, 2015). In 2009, Capasso and colleagues detected a new significant association of six BARD1 intronic SNPs (rs6435862, rs3768716, rs17487792, rs6712055, rs7587476, and rs6715570) with aggressive neuroblastoma in 397 high-risk cases compared to 2043 controls, which was confirmed in a second series of 189 high-risk cases and 1178 controls (Capasso et al., 2009). Of these, rs6435862 and rs3768716 were found to be the two most important SNPs in two further independent high-risk NB case series, giving a combined allelic OR of 1.68 each. The same two SNPs at the BARD1 locus showed a strongly significant association in African-American (Latorre et al., 2012), Southern Chinese (R. Zhang et al., 2016) and Spanish patients (Cimmino et al., 2018). In a follow-up work, using whole exome and deep targeted sequencing the group of Capasso confirmed that BARD1 was enriched in rare, potentially pathogenic, germline variants in clinically aggressive NB (Lasorsa et al., 2016). In particular, they found the same BARD1 LOF mutation (p.Arg641*) originally associated to NB by Pugh et al. (Pugh et al., 2013), who also reported another NB BARD1 LOF variant (p.Arg112*). Another rare, germline nonsense mutation (p.Q545*), located at the terminal part of the BARD1 ARD, was recently discovered in highly aggressive, recurrent NB patients (Fransson et al., 2020); the authors suggested that this could be a LOF variant and that it might cooperate with somatically acquired mutations in young NB patients. Interestingly, another potential functional risk allele of the

[38] The *P* value is defined as the likelihood of getting a result equal to or more extreme than what was actually observed under the premise of no effect or difference (null hypothesis). P, an abbreviation for probability, quantifies the likelihood that any observed difference between groups is attributable to chance. Because P is a probability, it may have any value between 0 and 1: a *P* value near 0 indicates that the observed difference is unlikely to be attributable to chance, while a P value close to 1 indicates that there is no difference between the groups other than that due to chance (Dahiru, 2008).

rs17489363 SNP in the 5'UTR of BARD1 (c.-48 T > C) was proposed by Bosse and colleagues (Bosse et al., 2012b) to correlate with decreased expression of the FL BARD1 (and hence with decreased protein oncosuppressor functions), and indeed its decreased expression was linked to advanced malignancies, as well as enhanced NB cell proliferation and invasion capacity. Conversely, the same group reported that, in NB cell lines, the risk SNP rs6435862 on BARD1 intron 1 is related to augmented expression of BARD1β oncogenic isoform, which can stimulate cell proliferation and stabilize the Aurora family of kinases. These evidence, in turn, provide convincing support for the ongoing development of Aurora kinase inhibitors for the clinical treatment of aggressive neuroblastoma. Shi et al. also performed a thorough search for a link between BARD1 SNPs and NB risk in Han Chinese population that included 339 NB patients and 778 disease-free controls (Shi et al., 2019). They identified 7/11 BARD1 SNPs that could be significantly related with NB risk, including one SNP in the 5'-UTR region (rs17489363), 2 SNPs in coding exons (rs2229571/p.Arg378Ser and rs3738888/p.Arg658Cys), and four intronic SNPs (rs3768716, rs6435862, rs3768707 and rs17487792). These seven SNPs were found by these authors to be significant associated with stage III/IV NB/ganglioneuroblastoma (GNB), and with an adrenal gland origin of NB. The fact that both rs17489363 and rs6435862 were already associated to NB (in particular rs6435862 was linked with increased expression of the oncogenic isoform BARD1β by the group of Bosse, as discussed few lines above) supports the idea that these two BARD1 SNPs could be related to poor clinical outcome in NB patients.

The gene NMYC belongs to the MYC family of proto-oncogenes and encodes the N-myc proto-oncogene protein (MYCN), a transcription factor that regulates key events throughout embryonic development. The MYCN protein is found downstream of many signaling pathways that promote progenitor cell development, proliferation, and metabolism in various developing organs and tissues. Deregulated MYCN signaling, on the other hand, promotes the development of several different tumors, most of which have a childhood onset, such as NB, medulloblastoma, rhabdomyosarcoma, and Wilms tumor, but it is also linked to some cancers that occur in adulthood, such as and lung cancer. MYCN amplification is also the most persistent genetic aberration linked with poor prognosis and treatment failure in NB (Ruiz-Pérez, Henley, & Arsenian-Henriksson, 2017). In a recent work, Sakka and coworkers showed that citalopram and escitalopram, two selective serotonin reuptake inhibitors, promoted apoptosis in different NB cell lines independently of the MYCN status (Sakka et al., 2017). The interesting side of this story relies on the fact that the authors determined that, among other genes, citalopram drastically reduced BARD1β expression (almost 18-fold); this promoted the destabilization of aurora kinase A (AURKA) – another mitotic serine/threonine kinase that contributes, among a plethora of other functions, to the regulation of cell cycle progression (Willems et al., 2018) – which, in turn, destabilized the major NB oncogenic driver MYCN. When FL BARD1 was knocked-out in two different human NB cell lines, Oldridge and coworkers found that cell viability and invasion increased, consistently with the tumor suppressive function of FL BARD1 in NB (Oldridge et al., 2019). Oldridge and colleagues also showed that the binding of the heat shock factor protein 1 (HSF1 (Gomez-Pastor, Burchfiel, & Thiele, 2018), a transcription factor that can be activate upon stress and has a critical role in the transcriptional activation/regulation of the heat shock response system (HSR)[39]) at the promoter of BARD1 carrying the rs17489363 SNP T-allele was less effective, thereby reducing BARD1 expression and increasing NB susceptibility. Contextually, the same group reported that another BARD1 intronic SNP (rs6720708) was strongly associate with predisposition for NB origin at adrenal gland site. On the other hand, other BARD1 SNPs were reported to be negatively associated with NB and, as such, could play a potential protective role from the disease. One such BARD1 variant is the 3'-UTR variant rs7585356 which, according to several authors, resulted in FL BARD1 overexpression (Capasso et al., 2009; Capasso et al., 2013; R. Zhang et al., 2016), while a pathway analysis revealed that the BARD1 rs10484108 variation suppressed cellular growth and controlled apoptosis; in particular, this variant exhibited no change in binding to BRCA1 when compared to its WT counterpart, suggesting that the protective effect of the BARD1 SNP was independent of BRCA1 (Y. H. Lee, Kim, & Song, 2014; Shi et al., 2019). A comprehensive review on chromosome instability and genetic predisposition in neuroblastoma – including BARD1 – has been published recently by Tonini and Capasso (Tonini & Capasso, 2020).

The data on BARD1 significance in childhood cancer is still limited. In a recent study, Jasiak et al. determined the expression level of BARD1 and its β isoform in three different histogenetic groups of pediatric cancer – neuroblastic tumors, and for the first time in chosen germ cell tumors (GCT), and rhabdomyosarcoma (RMS) – using quantitative polymerase chain reaction (Jasiak et al., 2021). Compared to healthy tissues, they reported higher BARD1β expression in tumor samples, with no such changes concerning FL BARD1. Additionally, variations in BARD1β expression were found across histological categories of neuroblastic tumors, with greater levels reported in ganglioneuroblastoma and ganglioneuroma, respectively. Additional data revealed that yolk sac tumors (GCT type) and RMSs were characterized by a greater expression of BARD1β as compared to non-neoplastic tissues, and the TERT gene (§2.6.2.2) was found to be highly expressed in these malignancies as well. Further, in two RMS cases this group found a marked decrease of BARD1β in post-chemotherapy samples. In aggregate, this work highlights the oncogenic role of BARD1β in pediatric cancers, and demonstrates that BARD1β differential expression is dependent on neoplasm histological type and the stage of development in neuroblastic tumors.

As mentioned above, NB originates from neural crest cells. Other congenital conditions including heart defects, *e.g.*, tetralogy of fallot (ToF) and coarctation of aorta,[40] are also related to tissues that originate from these cells. Interestingly, different copy number variants of BARD1 loci (nssv1608180, 2q32.3–35, copy number loss; and nssv1604018, 2q31.3–36.2, copy number gain) were also reported to be associated with these congenital conditions (Silversides et al., 2012). Since these defects were demonstrated to be frequent in NB patients (van Engelen et al., 2009), Joucov and coworkers investigated this aspect and showed that depleting BARD1 (but also BRCA1) in frog embryos resulted in widely defective developmental phenotypes (including malformed neural tube and eye structures) (Joukov, Chen, Fox, Green, & Livingston, 2001), establishing a possible role of BARD1 in early organogenesis. In addition, very recently the BARD1 mutation R749K was reported in a fatal case of lymphomatous adult T-cell lymphoma with an ambiguous myocardial involvement (Hashemi Zonouz, Abdulbaki, Bandyopadhyay, & Nava, 2021).

Neuroendocrine neoplasms (NENs) constitute a heterogeneous category of rare cancers that develop from neuroendocrine cells of the gastro-entero-pancreatic sites in two-thirds of cases and from the bronchopulmonary tree and thymus in the remaining one-third. Other

[39] Protein damage may be caused by a variety of stress factors, such as thermal shock, oxidative stress, heavy metals, or pathologic circumstances (*e.g.* ischemia and reperfusion, inflammatory conditions, tissue damage, viral infection, and mutant proteins linked with genetic disorders, among other causes. All these induce the activation of an evolutionarily conserved cell protective mechanism termed the heat shock response (HSR) to maintain protein homeostasis in virtually all eukaryotic cells. HSR activation results in the inducible expression of heat shock proteins (HSPs) that function as molecular chaperones or proteases, which contribute to stress recovery by either promoting the refolding of the damaged proteins or eliciting their degradation, thereby re-establishing protein homeostasis and supporting cell survival (Jolly & Morimoto, 2000).

[40] ToF is a congenital abnormality that manifests as pulmonary stenosis, interventricular defects, biventricular origin of the aorta, and hypertrophy of the right ventriculus (van der Ven, van den Bosch, Bogers, & Helbing, 2019). Coarctation of aorta refers to a congenital constriction of the proximal thoracic aorta that may manifest itself at any age with different clinical signs, either on its own or in conjunction with other cardiac abnormalities (Torok, Campbell, Fleming, & Hill, 2015).

frequent sources of genesis for NEN include the adrenal, thyroid, parathyroid, pituitary, sympathetic/parasympathetic ganglia, and, in rare instances, the ovary, testis, and middle ear (Shah et al., 2018). Szybovska et al. revealed a pathogenic variant in the BARD1 gene denoted as c.69_70delins25 (p.Ala25Glyfs*41) specifically linked to pancreaticoduodenal NEN (Szybowska, Mete, Weber, Silver, & Kim, 2019). While novel, this specific variant is expected by the authors to cause premature termination of protein synthesis and is predicted to be pathogenic. Pancreatic cancer is a life-threatening illness that ranks fourth on the list of cancer-related deaths in the United States of America. Among all cancers, PANC has the lowest overall survival rate in Europe (EU), and it is responsible for more than 95,000 deaths in the Old Continent each year, with a median survival time at the time of diagnosis of only 4.6 months. It has been predicted by Rahib et al. that PANC would overtake lung cancer as the second most frequent cause of cancer-related death by 2030 (Rahib et al., 2014). Within PANCs, pancreatic ductal adenocarcinomas (PDAC) account for about 95% of all pancreatic neoplasms, and they are the most difficult to cure. In this context, Chaffee et al. estimated the prevalence of mutations in PDAC patients with positive family history and, among all other genes found mutated in the analyzed cohort, they reported a novel BARD1 mutation, c.632 T > A, p.Leu211*, which corresponds to a grossly truncated BARD1 isoform missing most of its functional motifs. Other groups have also published some limited observation of pathogenic variants in BARD1 among patients with PANC. For instance, the BARD1 variation c.1921C > T, p.Arg641* already found in BC and NB (see above) was reported in a PDAC patient with familiar history by Hu et al. (Chunling Hu et al., 2016), while the BARD1 c.1935_1954dup, p.Glu652Valfs*69 – previously discussed in a late OC onset – was found by Smith and coworkers in a study aimed at identifying candidate DNA repair susceptibility genes by exome sequencing in high-risk PANC (A. L. Smith et al., 2016).

In order to clarify the clinical relevance of the low-penetrance BARD1 gene, using patient-derived lymphoblastoid cells Toh and coworkers functionally characterized two BARD1 pathogenic (c.1833dupT/p.Asp612*; c.2099delG/p.Gly700fs) and three variants of unknown significance (VUSs) (c.73G > C/p.Ala25Pro; c.1217G > A/p.Arg406Gln; and c.1918C > A/p.Leu640Ile) in 6 patients, three afflicted with BC and three with colorectal cancer (Toh et al., 2019). The carriers of the pathogenic variants developed aggressive disease forms like TNBC and high cancer grades. Variants Leu640Ile, p.Asp612*, Arg406Gln, and Gly700fs, located within or proximal to the BARD1 ARD and BRCT domains, were related to faulty apoptotic processes. Conversely, the authors found that the apoptotic function was retained in the missense Ala25Pro, which is distant from the BARD1 ARD domain. Interestingly, according to their data all mutants exhibited normal BRCA1/BARD1 heterodimer formation and colocalized with RAD51, consistent with their distant position with respect to the BRCA1- and RAD51-binding domains. In view of the detected deficient apoptosis, the authors proposed that the Arg406Gln and Leu640Ile BARD1 mutants could also be classified as pathogenic or likely pathogenic variants. Another very uncommon BARD1 variation with a significant colorectal cancer inheritance pattern (c.1811–2A > G) resulted in the loss of exon 9 (which is part of the BARD1 BRCT domain) owing to exon skipping, (Esteban-Jurado et al., 2015). Since, in general terms, colorectal cancer is related to a faulty DDR system and one of the reasons for this failure could be ascribed – among others – to the presence of BARD1 mutations or BARD1 isoforms with defective or abrogated BRCA1 or other protein (*e.g.*, p53) binding, the current view supports the notion that BARD1 pathogenic variant could have both a BRCA1-dependent and independent role in colorectal carcinogenesis (Ozden et al., 2016; Sporn et al., 2011; Zhang, Pilyugin, et al., 2012).

Esophageal squamous cell carcinoma (ESCC) accounts for ~90% of the 456,000 new cases of esophageal cancer diagnosed every year (Abnet, Arnold, & Wei, 2018), and is linked with a 5-year survival rate of approximately 5% (F. L. Huang & Yu, 2018). The melanoma-associated antigen D1 (MAGED1/NRAGE is considerably expressed in the nucleus of radioresistant esophageal tumor cells, and its remarkable upregulation promotes cell proliferation in esophageal cancers. Yang et al. showed that NRAGE is necessary for an efficient HR of DNA DSBs, and that it not just controls the stability of RNF8 (§2.6.1.1) and BARD1 *via* a ubiquitin-proteolytic route, but also promotes the BARD1/RNF8 interaction through their mutual RING domains, ultimately forming a novel ternary assembly (Q. Yang et al., 2016). Additionally, the expression of NRAGE was found by these authors to be closely correlated with RNF8 and BARD1 in esophageal tumor tissues. Cisplatin is a front-line drug employed for the treatment ESCC. However, the occurrence of cisplatin resistance and metastasis remains a clinical challenge. To investigate the mechanism involved in cisplatin resistance, Hou and coworkers established cisplatin resistant cell lines (Res) and found that i) the expression of integrin α5 is remarkably upregulated in Res cells, and ii) inhibition of α5 results in more apoptosis and resensitizes Res cells to cisplatin both *in vitro* and *in vivo* (Hou et al., 2019). In a mechanistic manner, these authors found that the expression of BARD1 was significantly increased in Res cells, and silencing of BARD1 reversed the effects of integrin α5 on cisplatin resistance. Moreover, they reported that the integrin α5/focal adhesion kinase 1 (FADK1/PTK2)/PI3K/AKT signal axis (§2.6.2.3) is activated in Res cells, which mediates the increased expression of BARD1, as well as the cisplatin resistance and cell survival. Accordingly, these results demonstrate that integrin α5 is required for cisplatin resistance through the promotion of the FADK1/PI3K/AKT/BARD1 signaling to prevent cells from apoptosis and enhance the DNA damage repair ability.

The implication of non-coding gene variants in the development of human diseases, and cancer in particular, is also attracting a lot of interest and efforts (Ahadi, 2021; Huarte, 2015; Lin & He, 2017; Piraino & Furney, 2016; Schmitt & Chang, 2016). Although the contribution of rare BARD1 non-coding variants and their relation to cancer is still a relatively unexplored field, the sequencing of 20 whole genes in HBOC individuals, which included BARD1 noncoding and flanking sequences (among a plethora of other proteins mostly involved in DDR) led to the identification of the single nucleotide variant in the 5'-UTR of BARD1 (rs143914387, c.-53G > T) as an exemplar of a mutation in the gene noncoding region that was proposed to alter the relevant mRNA structure (Caminsky et al., 2016). Given the liaison between BARD1 and NB, Fu et al. postulated that polymorphisms in the BARD1 gene might influence the predisposition to nephroblastoma (Fu et al., 2017b), another pediatric developmental malignancy of the kidney that affects approximately 1 in 10,000 children between 1 and 6 years of age (Re, Hazen-Martin, Sens, & Garvin, 1994). Their study of 145 cases and 531 controls showed that the rs7585356G > A polymorphism in the 3'-UTR of BARD1 significantly correlated with increased susceptibility to this malignancy (OR = 1.78); moreover, according to their stratified analysis rs7585356AA carriers were shown to be at higher risk of developing clinical stage I + II nephroblastoma, supporting the idea that the BARD1 rs7585356G > A is linked with a risk for nephroblastoma.

BARD1 mutations have also been discovered to play a role in human pathologies other that cancer. For example, nontuberculous mycobacterial (NTM) lung diseases (*e.g.*, those caused by the *Mycobacterium avium* complex (MAC)), constitute a rising public health concern in North America and throughout the globe (Larsson et al., 2017). Poor resistance to these infections, which may be caused by a variety of factors including other pre-existing lung pathologies, immunodeficiency, and immune-modulating therapies among others, renders the population susceptible to developing pulmonary NTM maladies. A recent study reported that a women group with persistent NTM infections progressed to BC later in their lives, supporting the concept that NTM infections could be a plausible risk factor for a condition of chronic inflammation and, ultimately, neoplastic cell transformation (J. V. Philley et al., 2017) with a mechanism similar to that characterizing *Helicobacter pylori* associated gastrointestinal transformation (Abreu & Peek Jr., 2014).

Using NGS, Philley et al. identified 4 BARD1 missense mutations - Pro24Ser, Ala40Val, Arg378Ser, and Val507Met - in patients with NTM-BC cases (Julie V. Philley et al., 2018), with BARD1 being among the genes that most frequently harbored somatic mutations together with BRCA2 and HER2, a gene whose alterations, including overexpression, amplifications and other mutations, are found in a variety of solid tumors, BC in particular (§ 2.1).

As discussed above, certain BARD1 germline mutations increase the expression of alternatively spliced BARD1 mRNAs while decreasing the expression of FL BARD1 mRNAs (Ratajska et al., 2012; Ratajska et al., 2015). The implication of these mutations in telomere integrity was investigated by Pilyugin and coworkers (Maxim Pilyugin et al., 2017). They conducted telomere fluorescence in situ hybridization (FISH) experiments using peripheral blood lymphocytes obtained from patients afflicted by BC and OC carrying the BARD1 germline mutations – *i.e.*, c.1690C > T/p.Q564*, c.1972C > T/p.Arg658Cys, and c.1977A > G/p. = — and from healthy control subjects. As already discussed a few paragraphs above, all of these BARD1 variants result in the deletion/or deficiency of the protein BRCT domains. In line with this, the authors found increased expression of BARD1δ and telomere abnormalities in the cells derived from these carriers. Accordingly, their data indicate that human BARD1 germline mutations that reduce FL BARD1 expression promote BARD1δ upregulation and, ultimately, telomeric aberrations (§2.7.4).

3. BRCA1/BARD1: the biological activities of an odd couple

3.1. The ubiquitin ligase activity of the BRCA1/BARD1 heterodimer

Ubiquitylation is a form of post-translational modification in which ubiquitin – a small, globular protein of 76 amino acids – is covalently bonded to different protein substrates (Hershko & Ciechanover, 1998). It is a highly regulated and reversible event induced by various stimuli that not only regulates protein stability but also functional interaction, localization, and signaling dynamics (Swatek & Komander, 2016). These changes in protein activity by ubiquitination are governed by the number of linked ubiquitin molecules and the nature of the bond involved (Komander & Rape, 2012). The ubiquitin system comprises three key protein types: enzymes with ubiquitin-activating functions (E1), enzymes with ubiquitin-conjugating functions (E2), and enzymes with ubiquitin ligase functions (E3). RING domains are hypothesized to contribute to the specificity of ubiquitin conjugation events as components of E3 ligases (Deshaies & Joazeiro, 2009). Indeed, E3 catalyzes the synthesis of polyubiquitin chains (occasionally monoubiquitin chains), by using activated by the E1 and E2 enzymes, and connecting them to specific substrate(s) through the formation of isopeptide bonds (Pickart & Eddins, 2004). Although ubiquitylation has long been thought to be a method for directing proteins to the proteasome for degradation, several types of ubiquitin modification have been discovered, each of which may have a particular purpose. Some proteins are modified by a single ubiquitin molecule (monoubiquitylation), while others are modified by several chains of ubiquitin molecules (polyubiquitylation). Furthermore, depending on how the ubiquitin molecules are joined together, these chains can take on a variety of shapes. It is now well understood that ubiquitin modification, in its different forms, plays a function in a variety of cellular activities, ranging from protein transport to DNA repair.

As discussed in detail in §2.6, BRCA1 is indeed involved in multiple cellular functions, *e.g.*, transcription, heterochromatin structure formation, replication fork stability, homologous recombination repair, centrosome regulation, and mitotic spindle formation (Bunting et al., 2010; Gorodetska et al., 2019; Joukov et al., 2006; Sankaran, Starita, Groen, Ko, & Parvin, 2005; Scully et al., 1997; Willis et al., 2014). These diverse roles are specified by the BRCA1/BARD1 RING/RING heterodimeric complex (§2.3.1) that greatly enhances the E3 ubiquitin ligase activity of this protein/protein assembly (Baer & Ludwig, 2002; Brzovic et al., 2003; Hashizume et al., 2001; Y. Xia, Pao, Chen, Verma, & Hunter, 2003). The enzymatic activity of BRCA1/BARD1 ubiquitin ligase complex is also unique in its own because it can generate different kinds of atypical ubiquitin linkages at specific BRCA1 lysine residues (Lys6, Lys29, Lys48, and Lys63) depending on the substrate and interacting E2 subunits (Christensen, Brzovic, & Klevit, 2007; Nishikawa et al., 2004; Swatek & Komander, 2016; W. Wu, Koike, Takeshita, & Ohta, 2008). Although identifying E3 substrates is still a main subject, new data suggests that it is the E2 enzyme that determines which ubiquitin modification will occurs on a given substrate. The human genome contains about 30 E2 proteins, many of which have yet to be characterized (Kliza & Husnjak, 2020), and the RING E3 BRCA1/BARD1 heterocomplex can interact with a total of 10 distinct E2s. The capacity of BRCA1 to interact with several E2s is a property likely shared by other RING and U-box E3s. Moreover, some E2 enzymes are more specific in linking the first ubiquitin to a lysine residue on a given substrate or ubiquitin to itself, advocating a roles for E2s in directing product formation (Christensen & Klevit, 2009). Furthermore, BARD1 interaction is a prerequisite for maintaining the correct conformation of the BRCA1 RING domain required for its for E3 ligase activity (Brzovic et al., 2003; A. Chen, Kleiman, Manley, Ouchi, & Pan, 2002; Y. Xia et al., 2003). Indeed, BRCA1/BARD1 heterodimeric assemblies elicit the transfer of ubiquitin far more efficiently than either protein alone and since the loss of one protein significantly reduces the quantity of the other, the creation of heterodimers may be indeed critical for the stability of both polypeptides (Y. Xia et al., 2003).

Besides promoting ubiquitin polymerization on a variety of proteins via a Lys6 linkage, the BRCA1/BARD1 heterodimer also elicits BARD1 and BRCA1 auto-ubiquitination (A. Chen et al., 2002; Wu-Baer, Lagrazon, Yuan, & Baer, 2003). Auto-ubiquitination does not result in BARD1 or BRCA1 degradation, but rather in increased ubiquitin ligase activity (>20-fold) (Mallery, Vandenberg, & Hiom, 2002), BARD1/BRCA1 stability (Y. Xia et al., 2003), and improved DNA damage response (Sankaran, Starita, Simons, & Parvin, 2006). While the BRCA1/BARD1 ubiquitin ligase activity is positively regulated by heterodimer formation and ubiquitination, phosphorylation of BARD1 on its -NH_2 terminus by CDK1 and CDK2 (§2.5.2) fully abolishes the ubiquitin ligase activity (Hayami et al., 2005). According to Hayami and coworkers, the suppression of the BRCA1/BARD1 E3 activity is not a consequence of the direct action of these two kinases on BARD1 because a polymutant BARD1 isoform unable to undergo phosphorylation (S148A/S251A/S288A/T299A) is still inhibited by CDK2 (Hayami et al., 2005). BRCA1/BARD1 E3 ligase activity is also abrogated by the BRCA1 associated protein 1 (BAP1, Fig. 30, top left), as reported by Nishikawa and colleagues (Nishikawa et al., 2009). BAP1 is a DUB that has been identified as a tumor suppressor: by using its DUB activity, this protein can indeed modulate a variety of processes including DDR, cell cycle progression, chromatin remodeling, apoptosis, and the immune response (Louie & Kurzrock, 2020). Specifically, residues 182–365 of BAP1 binds to BARD1 and in so doing inhibits BRCA1/BARD1 E3 ligase function, as shown by surface plasmon resonance spectroscopy investigations (Nishikawa et al., 2009). The same group of Nishikawa also found that the BRCA1/BARD1 heterodimer perturbation by BAP1 resulted in inhibition of BRCA1 autoubiquitination and the ubiquitination by BRCA1/BARD1 of nucleophosmin (NPM1/B23), a multifunctional nuclear acidic chaperone active in several stages of ribosome biogenesis, chromatin remodeling, and mitosis as well as in DNA repair, replication and transcription (Lindström, 2011). Although, still according to Nishikawa et al., *in vitro* BAP1 was able to deubiquitinate those polyubiquitin chains generated by the E3 ligase activity of the BRCA1/BARD1 heterodimers, the BAP1 Cys91Ser catalytically inactive variant was nevertheless able to suppress ubiquitination both *in vitro* and *in vivo*, suggesting a second mode of action of this DUB. Notably, suppression of BAP1 expression by short hairpin led to cell hypersensitivity to IR and a delay in cell cycle progression to the S-phase. Together, these results prompted the authors to hypothesize that BAP1 and BRCA1/BARD1 act in concert while controlling

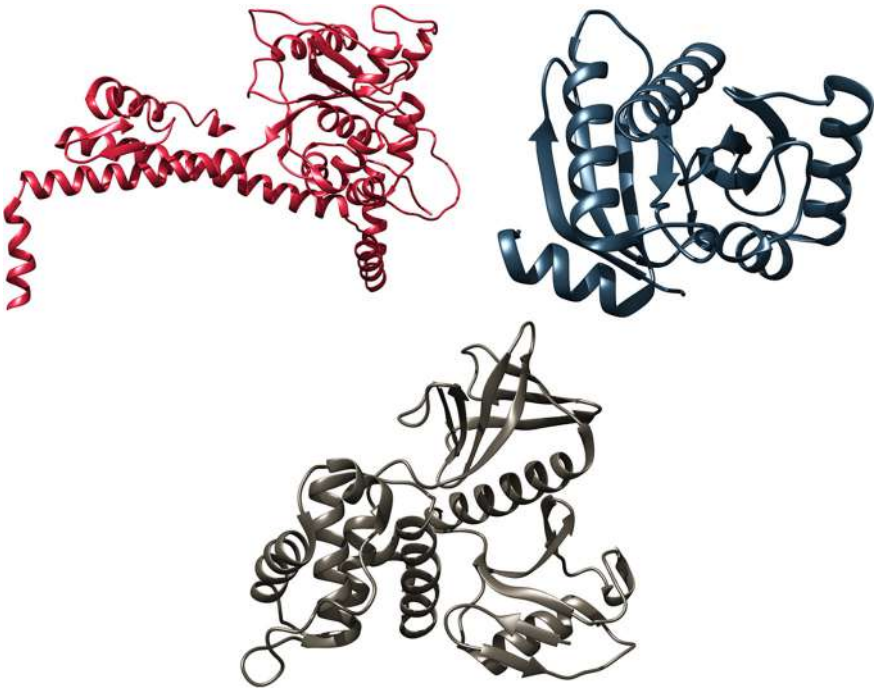

Fig. 30. (Top left) 3D structure of BAP1 (Chinese red, AlphaFold2 PDB: Q92560) as predicted by AlphaFold2 (Jumper et al., 2021). Crystal structures of MACROH2A1 (top right, majolica blue PDB: 3IID (Timinszky et al., 2009)) and merlin/NF2 (bottom, tarmac, PDB: 3WA0 (Mori, Gotoh, Shirakawa, & Hakoshima, 2014)).

ubiquitination through the cell cycle and the DDR, two aspects which will be discussed in details in §3.2 and 3.3, respectively.

Many other confirmed/putative substrates of the BRCA1/BARD1 E3 ligase activity have been identified through the years (B. J. Kim et al., 2017) which include, among several others, claspin (§2.6.1.2), RNAPII (§2.6.2.1, 2.6.2.3, and 2.7.3), the histone H2A (§2.6.1.1) and its variant core histone macro-H2A.1 (MACROH2A1, Fig. 30, top right), and merlin (NF2, Fig. 30, bottom), a tumor suppressor protein that acts through many different mechanisms connected to cell growth, survival and death, motility/adhesion, and invasion (Morrow & Shevde, 2012). Ubiquitination of claspin and RNAPII is connected with BRCA1/BARD1 E3 ligase activity in the DDR pathway, while ubiquitination of H2A/macro-H2A.1 is related to their functions in chromatin remodeling; accordingly, these topics will be discussed in more detail in the relevant sections (§3.3 and 3.4).

The Hippo signaling pathway is the main controller of mammal organ dimensions through the modulation of cell growth and death, and the mammalian transcriptional activator yes-associated protein (YAP) and the transcriptional co-activator with PDZ-binding motif (TAZ) are two key protein kinases along the Hippo route (S. Ma, Meng, Chen, & Guan, 2019). In cancer, Hippo signaling is deactivated, and this entails YAP and TAZ nuclear translocation and activation to sustain cell proliferation. Moreover, activated nuclear YAP and TAZ stimulate or repress different transcription factors that master target genes implicated in cell replication, tissue development, organ size/shape or cancer metastasis development (Zanconato, Cordenonsi, & Piccolo, 2019). Verma and coworkers established that BRCA/BARD1 facilitate stabilization of the YAP protein and switching off the Hippo pathway via merlin/NF2 ubiquitination while the Hippo pathways is active in BRCA1-deficient cells (Verma et al., 2019), thereby establishing a role of BRCA1 in regulating stability of YAP protein that correlates positively with cell proliferation.

3.2. BRCA1/BARD1 and the cell cycle

During mitosis, centrosomes and their associated microtubules govern the underlying events, and during interphase, they regulate the organization of animal cell structures and movement. The centrosome replicates once during a cell cycle, controls the formation of bipolar mitotic spindles, and is critical for cell division fidelity. Mitosis is assisted by the ubiquitin ligase activity of the BRCA1/BARD1 heterodimeric complex in different instances. For example, several groups have reported that BRCA1/BARD1 can localize to the centrosome through the cell cycle (Hsu & White, 1998;Sankaran et al., 2005; Sankaran et al., 2006) and ubiquitinates centrosome proteins – especially γ-tubulin and the multiprotein γ-tubulin ring complex (γ-TuRC, a protein ensemble that templates and catalyzes the otherwise kinetically unfavorable assembly of MT filaments (Tovey & Conduit, 2018), Fig. 31, top (Hsu et al., 2001; Sankaran, Crone, Palazzo, & Parvin, 2007)), and that this impedes microtubule nucleation at the centrosomes (Kais & Parvin, 2008; Sankaran et al., 2005; Starita et al., 2004). As mentioned in §2.5.1, two regions of BRCA1 comprising residues 504–803 and 802–1002, respectively, mediate its binding to γ-tubulin, as reported by two different groups (Hsu et al., 2001; Tarapore, Hanashiro, & Fukasawa, 2012). Brodie et al. reported that i) both BRCA1 N- and C-terminal regions are needed for the localization of this protein to the centrosome, ii) the BRCA1 translocation to the centrosome is independent of BARD1 and γ-tubulin, and iii) BRCA1 BRCT mutations abolish its centrosomal localization (Brodie & Henderson, 2012). A dynamic pool of ectopic BRCA1 at the centrosome (60%) was also discovered by these researchers along with an immobilized pool (40%), both of which were controlled by the nuclear export receptor CRM1 – as anticipated in §2.3.3 – and BARD1. In more detail, CRM1 (Fig. 31, bottom left) mediates nuclear export of BRCA1 by binding to BARD1-free BRCA1, and mutation in the BRCA1 NES block BRCA1 regulation of centrosome amplification in cells subjected to IR. Further data from this group supported the involvement of CRM1 in guiding monomeric BRCA1 towards the centrosome and showed that its subsequent heterodimerization with BARD1 results in centrosome depletion of, indicating a plausible mechanism to foster the release of BRCA1 after active heterodimer formation. Lastly, BRCA1 binding and phosphorylation by AURKA (§2.7.6) improved BRCA1 centrosome retention and its centrosome amplification control. Thus, CRM1, BARD1 and AURKA all contribute in promoting the targeting and function of BRCA1 at centrosomes. In analogy with its partner,

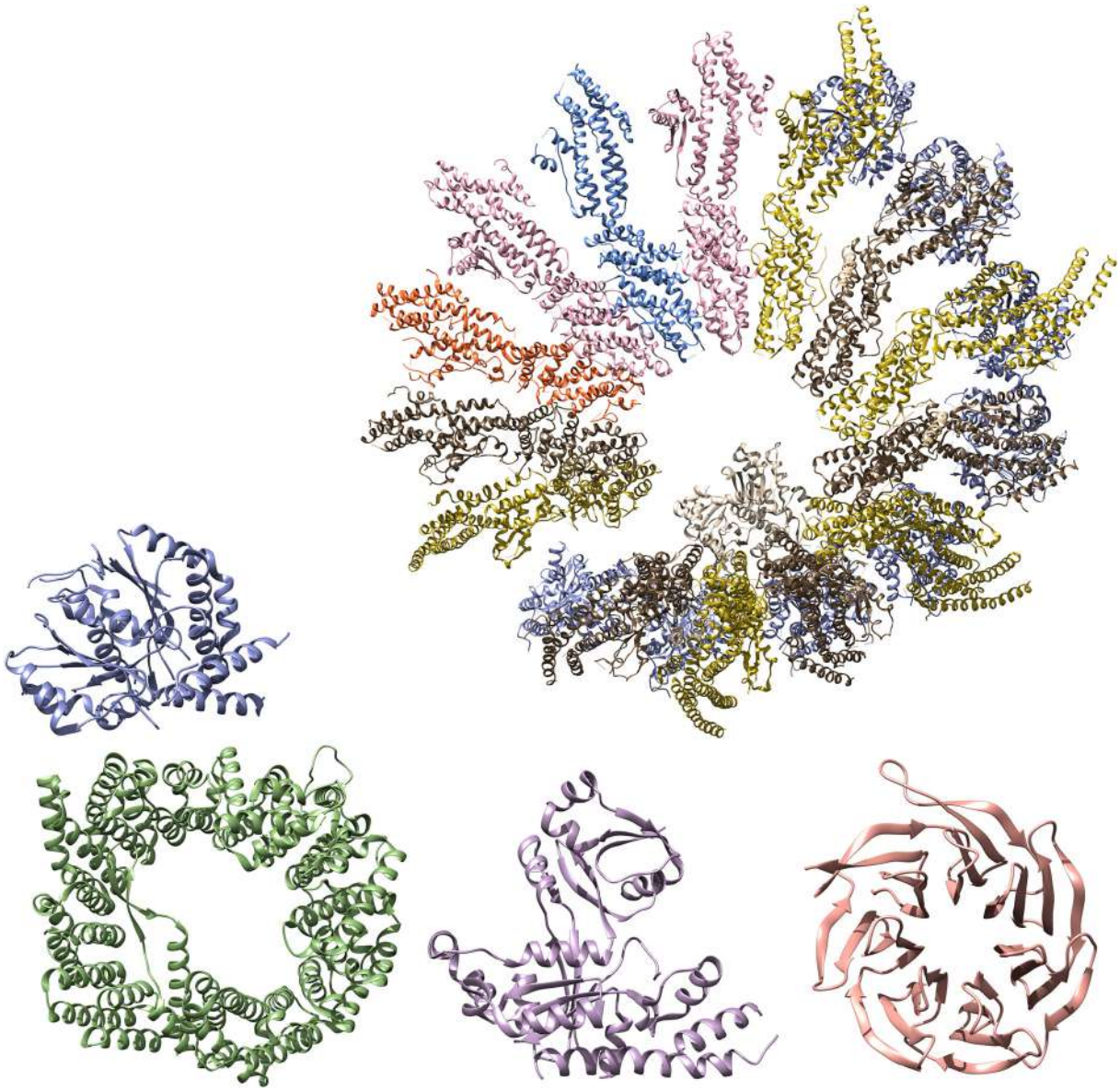

Fig. 31. (Top) EM-derived structures of γ-tubulin (left, deep periwinkle, PDB: 6V5V) in the native γ-TuRC (right, PDB: 6V6S) (Wieczorek et al., 2020). In the γ-TuRC, γ-tubulin chains are in deep periwinkle, the chains of the γ-tubulin components 2, 3, 4, 5 and 6 are in sepia, antique moss, sea pink, ultramarine and carrot, respectively, while β-actin is in frosted almond. (Bottom) Crystal structures of CRM1 (left, green tea PDB: 6TVO (Shaikhqasem, Dickmanns, Neumann, & Ficner, 2020)), OLA1 (middle, regal orchid, PDB: 2OHF (Koller-Eichhorn et al., 2007)), and RACK1 (right, lobster red, PDB: 4AOW (Ruiz Carrillo et al., 2012)).

BARD1 N- and C- terminal domains yet not the RING motif were demonstrated to be instrumental for its BRCA1-independent centrosome localization in another work by Brodie et al. (Brodie, Mok, & Henderson, 2012). They also showed that mutations in the BARD1 NES reduce its centrosome localization by 50%, indicating that CRM1 is also implicated in this mechanism, as already presented in §2.7.2. According to their further results based on fluorescence recovery after photobleaching tests BARD1 has a retained centrosomal pool that is half of that reported for BRCA1, supporting the notion that BARD1 is a very highly mobile protein the centrosome. The Parvin laboratory has also discovered that the E3 activity of BRCA1 is indispensable for the negative control of centrosome overduplication and for promoting γ-tubulin localization at the centrosome. (Parvin, 2009). Indeed, ubiquitination as a posttranslational alteration of γ-tubulin (particularly its monoubiquitination at Lys48 and Lys344 (Starita et al., 2004)) that is required for MT nucleation and duplication of the centrosome, and inhibiting the BRCA1/BARD1-mediated γ-tubulin ubiquitination results in centrosome amplification. In this respect, Zarrizi and colleagues further study the role of BAP1 (§3.1) as a DUB enzyme for γ-tubulin (Zarrizi, Menard, Belting, & Massoumi, 2014). They reported the downregulation of BAP1 in metastatic BC cell lines with respect to normal control cells, and that such low BAP1 levels were related to decreased overall survival (OS) of BC patients. The same study also revealed that BAP1 downregulation in BC cells was accompanied by mitotic aberrations; however, rescue experiments that included the expression of FL BAP1 rather than a catalytic inactive mutant isoform led to decreased γ-tubulin ubiquitination and averted mitotic flaws, underlying the important role of BAP1 in preventing abnormal mitotic spindle formation and genome instability.

At the centrosome, BRCA1/BARD1 exert their E3 ubiquitin ligase activity on different substrates that include, besides γ-tubulin, NPM1/B23 (§3.1), and receptor for hyaluronan (HA)-mediated motility (RHAMM)/HA-mediated motility receptor (HMMR). Concerning NPM1, Sato et al. found that it contacts the N-terminal regions of BRCA1 and BARD1

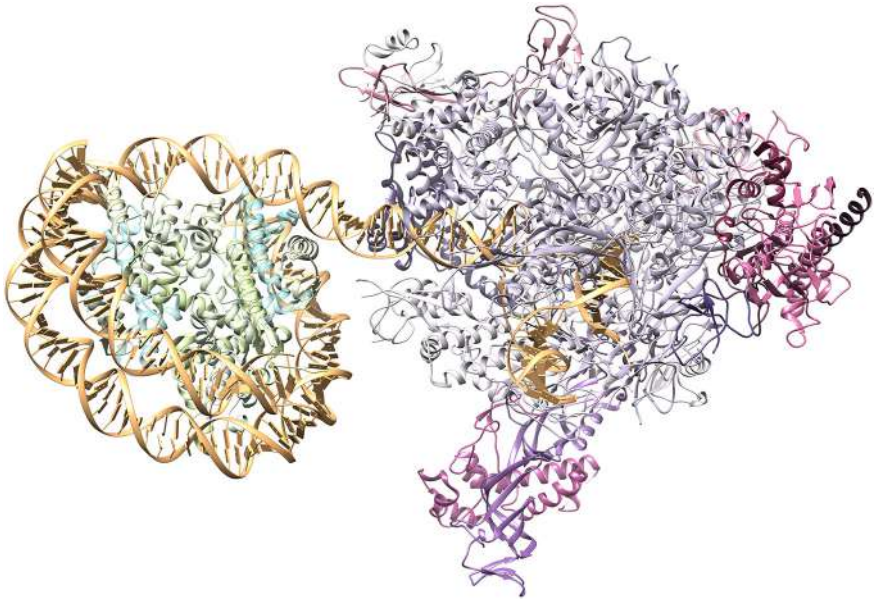

Fig. 32. X-ray solved structure of RNAPII elongation complex at the nucleosome (PDB: 6INQ (Kujirai et al., 2018)). RNAPII subunits are in purple shades; H3 chains in seafoam green; H4 chains in aqua sky; H2A chains in greenhouse glass; H2B chains in butterfly green, and nucleic acid in buff orange.

with a mechanism that is reliant on the formation of the BRCA1/BARD1 heterodimer (Sato et al., 2004). In mitotic cells, NPM1 colocalizes with BRCA1 and BARD1 supporting a possible function of BRCA1/BARD1 in the regulation of NPM1 during mitotic division. The ubiquitination of NMP1 by the BRCA1/BARD1 was also reported *in vivo* by the same authors, and the cellular co-expression of BRCA1/BARD1 resulted in an increase in NPM1 stability rather than in the degradation of this protein, in agreement with with the view thatBRCA1/BARD1 heterodimers promote the formation of non-traditional polyubiquitin chains in their E3 activity. The hyaluronan mediated motility receptor (HMMR, also known as RHAMM) is a MT-associated, spindle assembly factor that organizes protein assemblies to enhance/regulate the activities of mitotic kinases, dynein and kinesin motors. (He, Mei, Connell, & Maxwell, 2020). Because of their common localization to centrosomes and mitotic spindle poles, and their prominence during G2/M, Pujana and coworkers investigated the potential role of HMMR as a substrate of the BRCA1/BARD1 E3 activity (Pujana et al., 2007). This group found that the BRCA1/BARD1 heterodimer effectively polyubiquitinated HMMR both *in vitro* and *in vivo*, indicating that HMMR may play a role in the function of centrosome *via* BRCA1/BARD1 polyubiquitination. They further reported that BRCA1 and HMMR genetically interact to regulate the number of centrosomes both in BC and normal MECs, and that there is a connection between BRCA1 and HMMR and AURKA in the development of BCs. Notably, observations made in *Xenopus laevis* by Joukov and coworkers have related the HMMR ortholog XRHAMM to the regulation of the BRCA1/BARD1 heterodimer-mediated spindle-pole assembly (Joukov et al., 2006).

In cells derived from mammary tissues, BRCA1/BARD1 suppression or overexpression leads to centrosome amplification. In particular, Ko et al. showed that BRCA1 inhibition generated premature centriole separation and reduplication (Ko, Murata, Hwang, & Parvin, 2006). By blocking cells in early S-phase they discovered that BRCA1 inhibition induced centrosome amplification between late S-phase and G2/M, just before cell division. Based on these findings, the authors concluded regular BRCA1 activity is required in these cell lines in order to avoid centriole separation and centrosome reduplication before the onset of mitotic division. While exploring further functions of BRCA1/BARD1 at the centrosome, Matsuzawa et al. identified Obg-like ATPase 1 (OLA1, an ATP hydrolase, Fig. 31, bottom middle) as a protein that interacts with the C-terminal region of BARD1 (residues 546–777) (Matsuzawa et al., 2014). According to their work, OLA1 also directly associated with the BRCA1 N-terminal domain and with γ-tubulin. During interphase OLA1 was seen to localize to centrosomes, while during the mitotic stage it locatecd to the spindle pole; in line, OLA1 knockdown led to centrosome amplification and the formation of MT asters. Critically, the BC-related Glu168Gln OLA1 variant was unable to bind BRCA1 and to revert the centrosome amplification induced by the loss of OLA1. Contextually, the BRCA1 RING variant Ile42Val also abolished BRCA1/OLA1 binding, supporting the notion that, together with BRCA1 and BARD1, OLA1 plays an important fucntion in centrosome regulation. In a more recent work on the subject, Yoshino and coworkers investigated the effect of mutating nine OLA1 candidate phosphorylation sites and the potential role of these OLA1 mutant overexpression in centrosome amplification, and discovered five point variations that are defective in centrosome number regulation (Yoshino et al., 2018). Interestingly, working with purified proteins these authors found that three of these OLA1 mutants – Thr124Ala, Glu168Gln, and Lys242Arg – were still able to bind BARD1, whereas the three remaining mutants – Ser36Ala, Phe127Ala, and Thr325Ala – lose their BARD1 affinity. By contrast, all OLA1 mutants bound to γ-tubulin and to the BRCA1 N-terminal (residues 1–304). When the binding assays were performed in cells, they found that the affinity of the OLA1 Ser36Ala and Phe127Ala for BARD1 was still drastically reduced, while the Glu168Gln mutation slightly diminished the association to BARD1, γ-tubulin, and BRCA1, and the Thr325Ala mutation reduced the binding to BARD1 and γ-tubulin. More, knockdown and overexpression of BARD1 also triggered centrosome amplification while, upon transfection, a cancer-related BARD1 variant was no longer able to bind OLA1, to revert centrosome amplification resulting from WT BARD1 loss, and to nornally locate at the centrosome. These results allowed the authors to conclude that the interaction of OLA1 with the BARD1 C-terminal region is central to the formation of the cellular BRCA1/BARD1/OLA1/γ-tubulin complex, and that the BRCA1/BARD1/OLA1-controlled regulation of centrosome number is vital in preserving the integrity of the genome and in preventing tumorigenesis. Besides OLA1, Otsuka and coworkers identified the receptor for activated C kinase (RACK1, Fig. 31, bottom right) as another BRCA1/BARD1-interating protein that binds to BARD1 and BRCA1 and localizes to the centrosomes during the cell cycle (Otsuka,

Yoshino, Qi, & Chiba, 2020). They showed that BRCA1, BARD1, OLA1, and RACK1 cancer variants were unable to establish mutual interactions, and their abnormal expression in cells derived from mammary tissues ingenerated centrosome amplification owing to centriole overduplication. The number of centrioles in breast tissue-derived cells was greater than in cells from other tissues during the S-G2 phase, indicating that centrioles are involved in tissue-specific tumor promotion caused by BRCA1 and BARD1 germline mutations.

The BRCA1/BARD1 heterodimer also has an inhibitory effect on the centrosome-dependent MT organizing activity, and the BARD1 C-terminal region is again indispensable for this inhibition (Sankaran et al., 2005; Sankaran et al., 2006). This issue was further explored by Sankaran and colleagues by detecting aster formation by centrosomes *in vitro* (Sankaran et al., 2007). They found that AURKA inhibits BRCA1/BARD1 E3 ubiquitin ligase activity and, hence, its inhibitory impact on MT aster formation, whereas the serine/threonine-protein phosphatase PP1 (PP1) promoted this action. They further described that BRCA1/BARD1 E3 activity is necessary in both roles: the control of centriole duplication and the inhibition of MT aster formation. Centrosomal aster formation follows several distinct steps, including MT nucleation, MT anchoring and elongation/release of MTs. γ-TuRC initiates MT nucleation and then the MT anchoring machinery at the subdistal appendages, which is present only at mother centrioles, anchors the MT-nucleated γ-TuRCs. The nucleated MTs then grow into MT asters. In this context, Terapore and collaborators found that BRCA1 suppresses aster formation not by targeting MT nucleation, but by targeting either MT anchoring or elongation (Tarapore et al., 2012). Also, they found that BRCA1 has the ability to physically associate with not only γ-tubulin, but also the γ-tubulin complex component 3 (TUBGCP3), one of the components of γ-TuRC (Fig. 31, top right). Thus, the authors proposed that BRCA1 may directly block the anchoring of the MT-nucleated γ-TuRC at mother centrioles. As an alternative mechanism, they suggested that MT-nucleated γ-TuRC can be anchored at mother centrioles, but BRCA1 may block the elongation of MTs nucleated by γ-TuRC. Further studies required to better understand the aster forming process at centrosomes will clarify this issue in the future.

3.3. BRCA1/BARD1 and the DDR pathways

3.3.1. BRCA1/BARD1 E3 ubiquitin ligase activity and the DDR – still a controversial role?

As anticipated in §2.6.2.1, BRCA1 in tandem with BARD1 targets RNAPII (Fig. 32) for degradation via ubiquitination to allow the DDR machinery to access the damaged sites for repair, thereby preventing transcription of damaged genes (Tufegdžić Vidaković et al., 2020). Kleiman et al. also found that RNAPIIO, the elongating form of the RNAPII (Q. Zhou, Li, & Price, 2012), is a specific *in vitro* target of the BRCA1/BARD1 E3 activity (Kleiman et al., 2005). Silencing of BRCA1 and BARD1 *via* RNAi led to RNAPII stabilization after DNA damage. Moreover, the DNA damage-induced inhibition of 3'cleavage was reverted in the extracts from cells depleted in BRCA1-, BARD1-, or both BRCA1 and BARD1, leading to the hypothesis whereby the presence of a BRCA1/BARD1 complex promotes stalled RNAPIIO degradation, thus halting the coupled transcription-RNA processing machinery and enabling DDR. Concomitantly, Starita and colleagues showed that the BRCA1/BARD1 heterodimer ubiquitinates a hyperphosphorylated form of RPB1 (Fig. 33, left), the largest subunit of RNAPII (Starita et al., 2005). Two major phosphorylation sites have been reported by these authors within the RPB1 C-terminal domain, that is Ser2 or Ser5 of the Tyr-Ser-Pro-Tyr-Ser-Pro-Ser heptapeptide repeat, of which only the Ser5 hyperphosphorylated form is ubiquitinated by BRCA1/BARD1. Interestingly the RPB1 ubiquitination induced by DNA damage was stimulated by BRCA1 overexpression in cells; however, the authors found that BRCA1-induced RPB1 ubiquitination occurred only on those proteins characterized by hyperphosphorylation on Ser5 of the heptapeptide repeat. These authors also confirmed that the BRCA1 C-terminal is critical for its efficient ubiquitination of RPB1 *in vitro*, suggesting that the protein-protein contacts mediated by this BRCA1 motif were essential in the nucleus complex milieu.

As mentioned in §3.1, claspin is one of the targets of the E3 activity of BRCA1/BARD1. In this respect, Sato and colleagues differentiated DDR mechanisms requiring BRCA1/BARD1 E3 activity from those that did not using a BRCA1 mutated isoform devoid of catalytic activity yet still fully able to heterodimerize with BARD1 (Sato et al., 2012). They found that BRCA1 ubiquitylated claspin (an essential CHK1 coactivator) following topoisomerase inhibition, but this did not occur after DNA crosslinking which was caused by mitomycin C in their instance. Abrogation of the BRCA1/BARD1 E3 activity reduced the amounts of chromatin-bound claspin and compromised HR DDR by negatively interfering with the transduction of signal from the DDR-activated ATR to its effector CHK1 effector (§2.6.1.2). Accordingly, their study i) identified claspin as a BRCA1/BARD1 E3 ligase substrate *in vivo* ii) proposed that claspin modifications specifically elicit the activation of CHK1 for the HR repair of a subgroup of DNA lesions, and iii) established a fundamental yet selective role for the E3 ligase activity of the BRCA1/BARD1heterodimer in the cellular DDR response. Steward and coworkers discovered three BARD1 inherited missense mutations (Cys53Trp, Cys71Tyr, and Cys83Arg) in families with severe forms of BC, and verified that all these BARD1 mutant isoforms preserved the capacity to form E3-active heterodimers with BRCA1; however, the resulting BRCA1/BARD1 complexes were no longer ablet to bind nucleosomes and to ubiquitinate histone H2A (M. D. Stewart et al., 2018). These BARD1 variants also resulted in the abrogation of transcriptional suppression of two genes belonging to the ES metabolic pathways and controlled by BRCA1 (CYP1A1 and CYP3A4), and restoration of WT BARD1 into these cells repristinated their normal transcription levels.

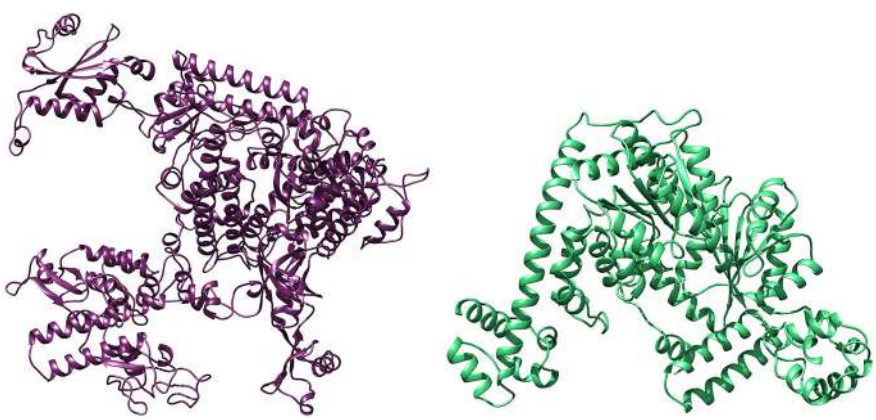

Fig. 33. 3D structures of RBP1 (left, grape juice, AlphaFold2 PDB: P24928) and SMARCAD1 (right, Irish green, AlphaFold2 PDB: Q9H4L7) as predicted by AlphaFold2 (Jumper et al., 2021).

These results therefore indicated that an integral BARD1 RING domain is instrumental for nucleosome binding and H2A ubiquitylation by BRCA1/BARD1, and to the BRCA1-mediated transcriptional repression of ES metabolism-linked genes.

Notwithstanding the intense research efforts in the field, the role of the BRCA1/BARD1 E3 ligase activity in DDR, and specifically in HR, remains an open and somewhat controversial issue. For example, in their Science paper of 2011 Shakya and collaborators generated genetically engineered mice (GEM) that expressed different BRCA1 mutants with the specific purpose of investigating the BRCA1/BARD1 E3 ligase/HR relationship (Shakya et al., 2011). In particular, one of the BRCA1 variants expressed was an enzymatically deficient protein characterized by the presence of the Ile126Ala missense mutation (Ile26Ala) in the protein RING domain that still allowed BRCA1/BARD1 heterodimer formation but prevented it from performing its E3 ligase function (Brzovic et al., 2003), and the authors verified that this mutant BRCA1 inhibited tumor development to the same extent as WT BRCA1 in different GEM cancer models. The other mutant carried the S1598F missense substitution, located on the BRCA1 BRCT domain, since susceptibility to HBC susceptibility was ascribed to single missense mutations in this region that disrupt the interaction between the BRCT domain and its cognate phospho-ligands. In this case, the related ablation phosphoprotein recognition by S1598F mutated BRCA1 BRCT domains elicited tumors in each of the three GEM animals. Accordingly, the authors came to the conclusion that, for BRCA1 tumor suppression function, the protein E3 ligase activity was dispensable while, on the contrary, recognition of the phosphorylated BRCT domains was an obligatory step. While this conclusion may indeed be correct, in contrast Stewart et al. showed that the Ile26Ala-BRCA1 was not ligase-dead with all E2s tested *in vitro*, thereby reopening the question (Mikaela D. Stewart et al., 2017). As another example, Densham et al. identified the site of BRCA1/BARD1 required for priming ubiquitin transfer from E2 ~ ubiquitin and demonstrated that BRCA1/BARD1 E3 activity was required for repositioning 53BP1 (§2.6.2.1) on damaged chromatin (Densham et al., 2016). These authors confirmed H2A ubiquitination by BRCA1/BARD1 and showed that an H2A-ubiquitin fusion protein promoted DNA resection and repair in BARD1-deficient cells. Further, they showed that BRCA1/BARD1 function in HR required the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1 (SMARCAD1, Fig. 33, right), a DNA helicase with intrinsic ATP-dependent nucleosome-remodeling function and that is both required for DDR and organization of heterochromatin. The authors showed that the ubiquitin-binding domains of SMARCAD1 were indispensable for its binding to H2A-ubiquitin, its ideal localization to DDSs and DDR activity, and 53BP1 repositioning. Accordingly, they concluded that the BRCA1/BARD1 ligase activity and the consequent chromatin remodeling mediated by SMARCAD1 are key regulators of DDR. A recent work by Densham and Morris focuses on chromatin and chromatin-bound complexes which are obstacles that prevent DNA resection with a special emphasis on how BRCA1 contributes to DDR outcome success through overcoming these blocks (Densham & Morris, 2019).

In previous years, other papers on this subject were published reporting data in apparent conflict. Again, for example, back in 2003 Dong and coworkers isolated a holoenzyme complex termed BRCC constituted by BRCA1, BRCA2, and RAD51 (Dong et al., 2003). They found that, *in vivo*, BRCC not only exhibited enhanced interaction with p53 upon DNA damage but also ubiquitinates p53. They also importantly reported that BRCC36 and BRCC45 (§2.6.1.1. and 2.6.1.3) were novel components of the complex, and their reconstitution of a recombinant four-subunit complex containing these two proteins plus BRCA1 and BARD1 exhibited enhanced E3 activity when compared with the BRCA1/BARD1 heterodimer alone. Silencing of BRCC36 and BRCC45 via RNAi *in vivo* resulted in increased sensitivity to IR and defects in G2/M checkpoint, and led to the identification of BRCC as a ubiquitin E3 ligase complex that boosts cell survival after DNA damage. Five years later, in their PNAS paper Reid et al. generated embryonic stem cells isogenic clones that expressed or were depleted of BRCA1 polypeptide endowed with enzymatic activity, and found that cells devoid of BRCA1 E3 activity could proliferate and did not accrued spontaneous cytogenetic rearrangements (Reid et al., 2008). More, they reported that, in those cells, gene targeting efficiencies were moderately reduced and, as a response to genotoxic stress, chromosomal rearrangements occurred at high rate. However, according to their data cells in which the enzymatic activity of BRCA1 was suppressed i) were not oversensitive to agents inducing DNA cross-links (*e.g.*, mitomycin C), ii) produced RAD51 foci in response to IR exposition and iii) the efficiency of chromosomal break repair by HR was comparable to that observed in WT cells. Although they concluded that their results indicated main roles of the BRCA1 role as the

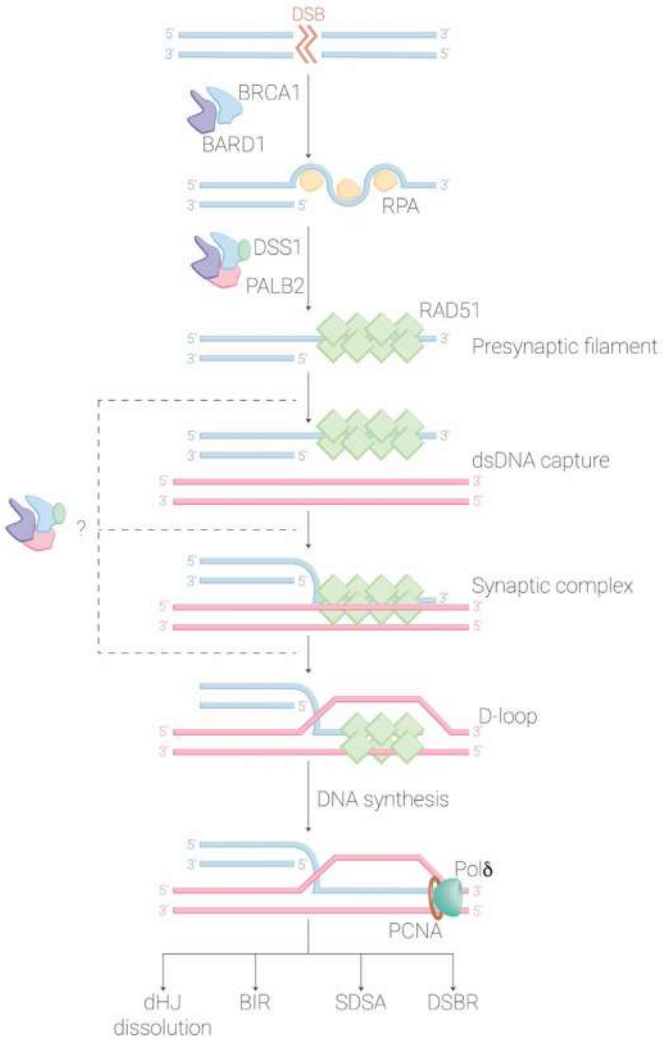

Fig. 34. Schematic representation of the role of BRCA1/BARD1 in HR of DNA DSBs. Upon DSB formation, the ssDNA overhangs resulting from BRCA1/BARD1-assisted DSB resection are rapidly coated by the RPA complex to prevent degradation or self-annealing. Subsequently, various mediator proteins, including RAD52, BRCA2-DSS1, PALB2, and BRCA1/BARD1 promote nucleation, RPA displacement, and loading of the RAD51 recombinase onto the ssDNA filament - the so-called pre-synaptic phase of HR. The physical connection between BRCA1/BARD1 and RAD51 is also expected to facilitate dsDNA engagement during the assembly of the synaptic complex, whilst the particular recognition of unwinding DNA by BRCA1/BARD1 could support the development of the nascent DNA junction in the D-loop reaction (these roles are shown as broken lines and marked by the symbol?, see text for details). The intermediate can proceed along a number of distinct sub-pathways following D-loop formation and repair DNA synthesis (performed by the DNA polymerase δ (Polδ)/PCNA ensemble). These comprise canonical DSB repair, synthesis-dependent single strand annealing (SDSA), break-induced DNA replication (BIR), and dissolution of double Holliday (dHJ) junctions,

guardian of genome integrity - including its involvement in the repair DNA DSBs *via* HR – they argued that these characteristics were independent of the BRCA1 (and BARD1) E3 ligase activity.

3.3.2. BRCA1/BARD1 in DNA HR

3.3.2.1. BRCA/BARD1 activity in DNA end resection. In BRCA1-null (BRCA1 −/−) mouse embryonic stem cells, HR deficits were identified for the first time, showing themselves as poor gene targeting and diminished repair of DNA DSBs (Moynahan et al., 1999; Moynahan et al., 2001). When it was discovered that BRCA1 interacted with the recombinase RAD51 and that the two proteins colocalized to IR-induced nuclear foci (Scully, Chen, Ochs, et al., 1997), this was considered the first evidence of BRCA1 involvement in DNA HR (Bhattacharyya, Ear, Koller, Weichselbaum, & Bishop, 2000; Huber et al., 2001). Aside from that, in both mouse and human cells knocking down BRCA1 has been shown to have an effect on the development of DNA damage-induced RAD51 foci; likewise, knocking down the BARD1 gene in mice and human cells decreased the development of damage-specific RAD51 foci and resulted in reduced HR, in a manner equivalent to that observed upon BRCA1 ablation (Laufer et al., 2007; McCarthy et al., 2003). The impairment of RAD51 foci formation upon DNA damage caused by the absence of either BRCA1 or BARD1 therefore implicates an important role of BRCA1/BARD1 in DNA end resection and RAD51 recruitment at the damage site (Bhattacharyya et al., 2000; Densham et al., 2016; W. Zhao et al., 2017). In this context, BRCA1/BARD1 is thought to be part of a higher-order HR mediator complex alongside BRCA2 and PALB2 (Chen, Feng, et al., 2018; Q. Jiang & Greenberg, 2015; Prakash, Zhang, Feng, & Jasin, 2015; W. Zhao, Wiese, Kwon, Hromas, & Sung, 2019) (Fig. 34), as discussed in detail in §2.3, 2.3.4.1, and 2.3.4.2.

After D-loop formation and repair DNA synthesis (performed by the DNA polymerase δ (Polδ)/PCNA ensemble), the intermediate can proceed along distinct sub-pathways, which include, canonical double strand break repair (DSBR),) dissolution, and break-induced DNA replication (BIR), each of these routes yielding a distinct product (Laurini et al., 2020; San Filippo et al., 2008; Scully et al., 2019). Note that, for the sake of figure readability, just one of the two DNA DSB end is shown.

As seen in §1, the initial phase of DNA 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 cooperation of the 53BP1/RIF1 pathway with the shieldin complex,[41] which direct DDR towards cNHEJ. The way in which BRCA1/BARD1 operates to remove the restriction on DNA end resection imposed by the 53BP1/RIF1/shieldin ensemble in S-phase cells and to engage DDR in HR is still a matter of investigation. A set of studies support a model according to which a direct competition between 53BP1 and BRCA1 is the key factor for channeling DSBs either through cNHEJ or HR, respectively (Bunting et al., 2010; Chapman et al., 2013; Escribano-Diaz et al., 2013). Other evidences suggest that BRCA1 may negatively interfere with DNA repair mediated by 53BP1 during the S-phase by preventing its association with chromatin proximal to the DDSs, and that the genomic instability seen in BRCA1-depleted cells could be an outcome of the inability to eliminate 53BP1 from these regions during the same cell cycle phase (Chapman, Sossick, Boulton, & Jackson, 2012), or pointed to BRCA1/BARD1 E3 ubiquitin ligase activity as one of the factors facilitating end resection, as discussed previously (§3.3.1). An alternative hypothesis invokes the interference of the shieldin complex with RAD51 loading onto DNA resected ends, establishing shieldin as the downstream effector of 53BP1/RIF1/MAD2 to promote DSB NHEJ resection and counteracting HR by antagonizing BRCA2/RAD51 loading in BRCA1-deficient cells (Dev et al., 2018). An active role for RIF was proposed by Zimmermann and collaborators, who reported that RIF1 suppresses CtIP- (§2.3.2 and 2.6.1.3), BLM- (§2.6.2.1 and 2.6.2.2) and the exonuclease 1 (EXO1)- mediated resection, limits BRCA1/BARD1 accumulation at the DDS, and specifies one of the mechanisms through which 53BP1 induces chromosomal defects in BRCA1-deficient cells (Zimmermann, Lottersberger, Buonomo, Sfeir, & de Lange, 2013). As mentioned in §2.3.2 and 2.6.1.3, BRCA1 BRCT specifically interacts with CtIP *in vivo* (Yu, Wu, Bowcock, Aronheim, & Baer, 1998) and in so doing modulates the speed of CtIP-mediated DNA-end resection (Cruz-García, López-Saavedra, & Huertas, 2014). However, the fact that cells expressing CtIP mutants defective in these interactions exhibited only moderate resection activity led to the hypothesis that they might not be essential for this function. In a very recent work, using high-throughput imaging/single cell normalization techniques Michelena and colleagues discovered that 53BP1 binding to damaged replicated chromatin was ineffective in both BRCA1-proficient and BRCA1-deficient cells (Michelena, Pellegrino, Spegg, & Altmeyer, 2021). Their findings support a *dual switch model* from a 53BP1-dominated response in unreplicated chromatin to a BRCA1/BARD1-dominated response in replicated chromatin, in which replication-coupled dilution of 53BP1 binding mark H4K20me2 (the methylated form of histone H4K20) functionally cooperates with BRCA1/BARD1-mediated suppression of 53BP1 binding.

3.3.2.2. BRCA/BARD1 activity in presynaptic and synaptic filament formation. The recent work by Zhao et al. based on pure WT and mutant BRCA1/BARD1 complexes and RAD51 directly supports the notion that this complex enhances/regulates the RAD51 recombinase activity (W. Zhao et al., 2017). Indeed, their important data not only showed that both BRCA1 and BARD1 bind DNA and directly interact with and enhance the recombinase activity of RAD51 (thereby corroborating previous evidences (Paull, Cortez, Bowers, Elledge, & Gellert, 2001; Scully et al., 1997; Simons et al., 2006), but also revealed that BRCA1/BARD1 and the BARD1 DNA binding domain have differential affinity for the different DNA structures involved in the damage/repair process, with affinity decreasing in the order: D-loop structures (*i.e.*, the heteroduplex DNA formed by RAD51 after pairing of homologous DNA strands, §1) > replication forks > dsDNA > ssDNA. From a mechanistic standpoint, these authors reported that BRCA1/BARD1 collaborates with the RAD51 presynaptic filament in promoting the synaptic complex, and that the crucial step of D-loops development requires the presence of both BRCA1 and BARD1 (Fig. 34). In particular, the physical connection between BRCA1 and RAD51 is expected to facilitate dsDNA engagement by the presynaptic filament, whilst the particular recognition of unwinding DNA by BRCA1/BARD1 enhances the development of the nascent DNA junction in the D-loop reaction. Lastly, they discovered that BRCA1/BARD1 mutants with impaired RAD51 connections had defective DNA joint formation and poor cellular HR/DDR, ultimately confirming the indispensable role of this heterodimer in genome maintenance. Nonetheless, as the same group in the same effort showed that BRCA1/BARD1 is not endowed with HR mediator activity, a likely model for the RAD51 enhanced activity of this heterodimer can be envisaged (W. Zhao et al., 2019) in terms of a global BRCA Mediator complex – made up by BRCA1, BARD1, PALB2, BRCA2 and DSS1 (§2.3) – which could 1) serve as a reservoir of RAD51 promoters and, by increasing its dwell time on ssDNA 2) could maximize the likelihood and efficiency of RPA replacement (§1) (Fig. 34).

RAD51-mediated loop creation, facilitation of DNA strand invasion and formation of the synaptic complex are also enhanced by the DNA repair and recombination protein RAD54 (RAD54, Fig. 35, top left), the RAD51-associated protein 1 (RAD51AP1), and the ubiquitin carboxyl-

[41] Shieldin is a 53BP1 effector complex that includes the shieldin complex subunits 1, 2 and 3 (SHLD1, SHLD2, and SHLD3), and the mitotic spindle assembly checkpoint protein MAD2B (MAD2B). Shieldin localization to DNA DSBs is mediated by 53BP1/RIF1, and binding to ssDNA via its SHLD2 subunit. Loss of shieldin impairs NHEJ, promotes V(D)J (§1) and causes hyper-resection (Sylvie M. Noordermeer et al., 2018).

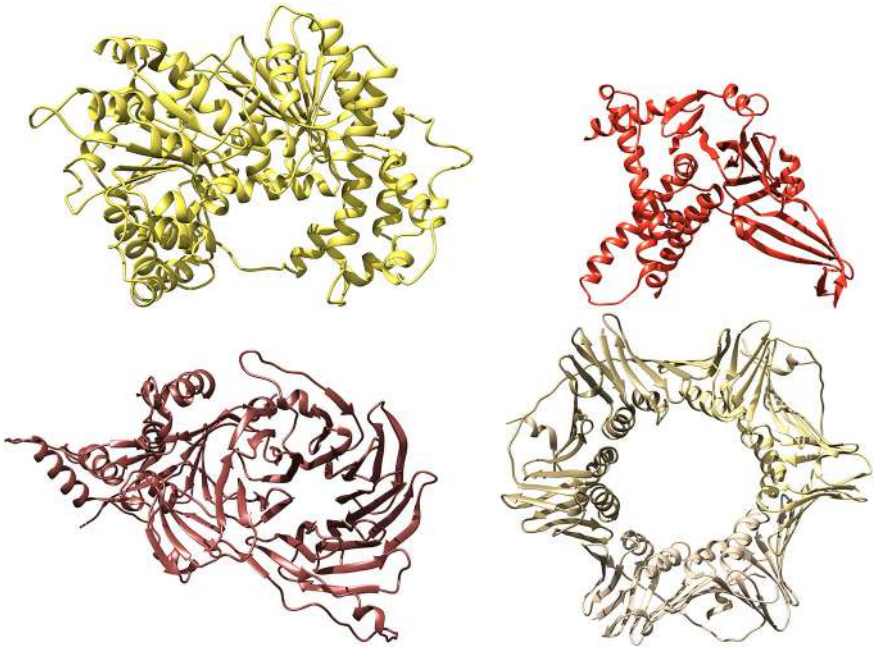

Fig. 35. 3D structures of RAD54 (top left, meadowlark, AlphaFold2 PDB: Q9Y620), USP1 (top right, cherry tomato, AlphaFold2 PDB: O94782), and UAF1 (bottom left, chili oil, AlphaFold2 PDB: A0A0G2KAW5) as predicted by AlphaFold2 (Jumper et al., 2021). (Bottom right) Homotrimeric structure of PCNA as derived from X-ray crystallography (protomers in tan shades, PDB: 5MLO (Sebesta, Cooper, Ariza, Carnie, & Ahel, 2017)).

terminal hydrolase 1 (USP1)/USP1-associated factor 1 (UAF1) (Fig. 35, top right/bottom left (Laurini et al., 2020)). As a SWI2/SNF2 enzyme,[42] RAD54 can move along the dsDNA with an ATP hydrolysis-dependent mechanism, induce superhelical torsion, and stimulate the remodeling of chromatin, thus increasing nucleosomal DNA accessibility (Amitani, Baskin, & Kowalczykowski, 2006). Kiianitsa and colleagues discovered that RAD54 physically interacts with RAD51 and elicits the DNA strand exchange activity of the recombinase (Kiianitsa, Solinger, & Heyer, 2006); furthermore, RAD54 binds dHJs (§2.6.1.3) and guides their branch migration (Goyal et al., 2018) and, by interacting with MUS81-EME1 (§2.6.2.1), rouses its DNA cleavage activity (Mazin, Mazina, Bugreev, & Rossi, 2010). RAD51P1 is a strategic HR protein that functions downstream of the RAD51 filament formation and RAD51AP1 and RAD51 foci co-localize both spontaneously and following induction of DNA damage (Wiese et al., 2007). Although RAD51AP1 can bind both ssDNA and dsDNA, yet it has the greatest affinity for DNA branched substrates and D-loops, suggesting that it plays a role in the formation of the DNA intermediates along the HR pathway. USP1 is a DDR negative regulator of DDR which, in tandem with UAF1, selectively performs the deubiquitylation of two essential DDR proteins FANCD2 (§2.6.2.1) and the proliferating cell nuclear antigen (PCNA, Fig. 35, bottom right (Cohn et al., 2007)). The USP1/UAF1 complex is a master of the cell response to DNA injury by boosting DSB repair *via* HR (Murai et al., 2011). Briefly, UAF1 binds DNA and thereby dimerizes with RAD51AP1; next, it forms a trimeric assembly with RAD51 (Liang et al., 2016) and in collaboration with PALB2 assists the recombinase in assembling the synaptic complex (Laurini et al., 2020). Given the association of BRCA1/BARD1 with PALB2 and the interaction of the last protein with BRCA2/DSS1 discussed above, unsolved questions remain as to 1) these protein assemblies have a jointed role in promoting the assembly of the synaptic complex and 2) BRCA1/BARD1/PALB2 works alone or in tandem with RAD54 or RAD51/USP1/UAF1 in mastering strand invasion (W. Zhao et al., 2019).

3.3.2.3. BRCA/BARD1 activity in replication fork repair/protection and DNA transcription. DNA DSB repair is further complicated by the presence of damaged or collapsed replication forks, which may result in one-ended DSBs or single DNA ends. In this scenario, there is no partner available for direct end joining, and the lack of a second DNA end, impossibility/inability to engage the second break end, and unsuccessful displacement of the nascent strand all exclude the potential of activating error-free repair DDR pathways. (Scully et al., 2019). During DNA replication, the replisome faces a number of hurdles that make exact copying of the genetic material difficult. *Replication stress* is defined as the slowing or stalling of a replication fork as a result of such constraints, and impaired DNA templates, difficult-to-replicate sequences (e.g., tandem repeats), RNA/DNA hybrid products, DNA/protein assemblies, and the presence of particular secondary structures in DNA all constitutes examples of endogenous replication stress (Zeman & Cimprich, 2014). As said above, at the replication fork replication stress is connected with the formation of ssDNA and recruitment ATR (§1) which, by activating and recruiting the DDR machinery, liming new origin firing, boosting replication fork stability, and driving replication restart processing, controls the replication stress response (Saldivar et al., 2017). BRCA1/BARD1 plays critical functions in the repair/restart of stalled and damaged DNA replication forks, as well as their protection from nucleolytic attack and attrition and avoidance of potentially pathogenic DNA secondary structures (*vide infra*), in addition to the DSB repair activity (Fig. 36).

A study on the response of BRCA1-mutant cells to hydroxyurea (HU, which elicits replication stress by blocking ribonucleotide reductase and hence lowering the nucleotide pools needed for DNA synthesis) provided the first evidence of BRCA1 participation in DNA replication (Scully, Chen, Ochs, et al., 1997). Using HU-treated cells, these authors found that BRCA1 colocalizes with RAD51 at S-phase-specific foci containing PCNA (§3.3.2.2), and the overt relocation of BRCA1 to PCNA-positive structures after DNA damage suggested that BRCA1 is recruited to replication forks after DNA-damaging treatment of S-phase cells. Other efforts reported BRCA1 to be colocalized with other components

[42] The SWI2/SNF2 family of proteins controls a wide range of nucleic acid transactions in eukaryotic cells by sliding, removing, and reassembling nucleosomes. Also, these proteins link the hydrolysis of ATP and DNA translocation with the remodeling of chromatin (Clapier et al., 2017; Pazin & Kadonaga, 1997).

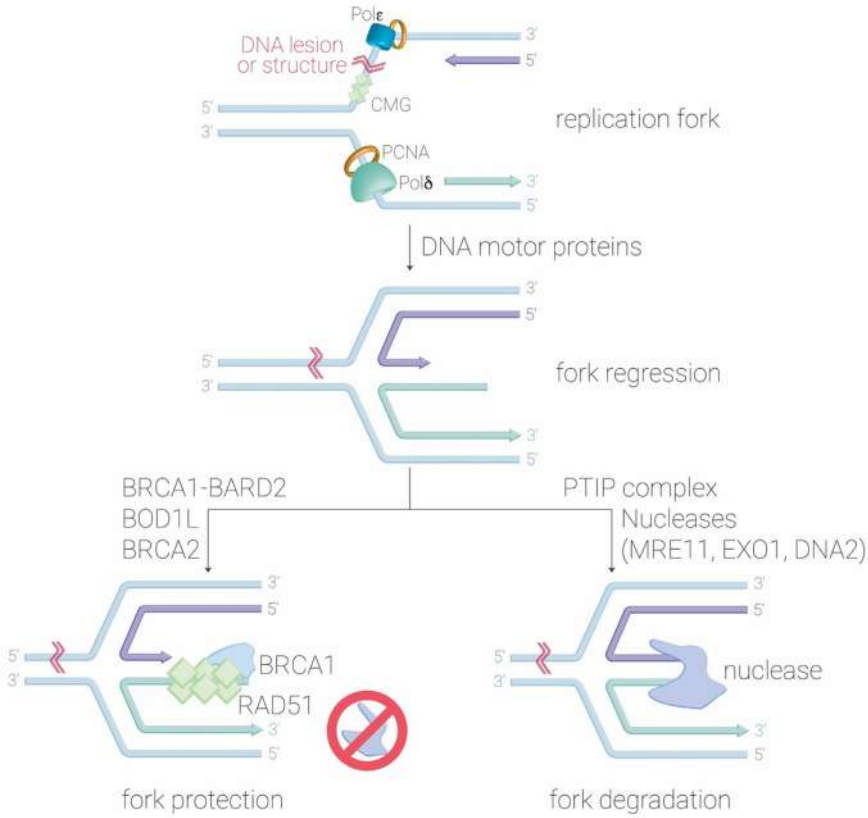

Fig. 36. Schematic representation of the role of BRCA1/BARD1 at replication forks. During the process of DNA replication, if the DNA polymerase ensemble (*i.e.*, the DNA polymerase ε (Polε), PCNA and the CGM helicase complex (The hetero-hexameric minichromosome maintenance proteins 2–7 helicase (MCM2-7), the cell division control protein 45 homolog (CDC45), and the hetero-tetrameric complex GINS (where the acronym GINS stands for the Japanese Go-Ichi-Ni-San, meaning 5–1–2-3 to indicate the four subunits of the complex SLD5 and PSF1/2/3) form the CGM helicase complex (MacNeill, 2010). During the cell cycle G1-phase, the MCM2-7 proteins in collaboration with other partners bind to and activate the origins of replication. As the DNA replication process begins, the MCM2-7 proteins move in tandem with the replication fork, consistent with their function replicative DNA helicase. The CDC45 and the GINS complex together stimulate the latent MCM2-7 helicase by promoting allosteric conformational changes upon binding, and they also act as a shield to prevent the leading strand from accidental slippage slipping away from the main channel (Petojevic et al., 2015).) on the leading strand and the DNA polymerase δ (Polδ) and PCNA on the lagging strand), is halted by the presence of an unexpected DNA structure/lesion, one of the nucleic acid motor proteins (*e.g.*, SMARCAL1, FOXN2 and ZRANB3) (In addition to other functions, the SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1) the forkhead box protein N2 (FOXN2) and the DNA annealing helicase and endonuclease ZRANB3 (ZRANB3) all promote fork regression though their annealing helicase activity to avoid replication fork degradation and DBS formation), can catalyze the reversal of the replication fork (aka *replication fork regression*). This process leads to the formation of a four-way junction (or *chicken foot*) intermediate bearing a free DNA end. In the absence of BRCA1, this intermediate can be degraded by the MRE11 complex (§1) or other nucleases (*e.g.*, EXO1 or DNA2, *vide infra*) in tandem with the PTIP, a member of the histone methyltransferase complex, a partner of 53BP1 in DSB protection (§1) and a recruiter of MRE11 at stalled forks. On the other hand, the presence of BRCA1/BARD1, BRCA2 and of the biorientation of chromosomes in cell division protein 1-like 1 (BOD1L) safeguard the loading of RAD51 on the regressed fork and presides over the stability of the RAD51/DNA interaction against spurious nucleolytic attrition (W. Zhao et al., 2019) (see text below).

that operate on stalled replication forks, including the BASC complex (§2.6.2.1) (Wang, Cortez, et al., 2000). Notably, after establishing a group of primary human BRCA1(+/+) and BRCA1(mut/+) MECs and fibroblasts, Pathania and coworkers found that all cells heterozygous for BRCA1 mutation were mostly endowed with normal BRCA1 functions - including activation the HR-directed DNA DSB repair, checkpoint tasks, control of centrosome number and spindle pole formation, and the suppression of satellite RNA (*vide infra*) (Pathania et al., 2014). In contrast, the same cells were inefficient in repairing stalled replication forks repair and/or in suppressing fork collapse, while these deficiencies were corrected by transfecting BRCA1(mut/+) cells with WT BRCA1. In addition, in BRCA1(mut/+) cells the authors witnessed *conditional* haploinsufficiency for HR-mediated DSB repair under replication stress conditions. Overall, such results support the idea that BRCA1 is a key component in ensuing correct DNA replication, a function for which a fully functional BRCA1 protein is indispensable. Alternatively, mutant BRCA1 proteins may have a dominant negative effect on WT counterparts under these circumstances.

Blocked replication forks are extremely dangerous situations as they can collapse and can abnormally ligate among themselves, and different chromosomes can fuse together causing a mitotic catastrophe and subsequently inducing cell death (Nickoloff, Jones, Lee, Williamson, & Hromas, 2017). Accordingly, upon formation stalled forks must be protected from nucleolytic degradation and stabilized until the DDR machinery can intervene for repair and restart (Fig. 36). Recently Billing et al. compared mice with mutations that ablate BRCT phospho-recognition by BARD1 (Ser563Phe and Lys607Ala) or BRCA1 (Ser1598FPhe), and observed that the BRCA1 variant abrogates both HR and stalled fork protection, indicating that both pathways are possibly compromised in the majority of cancers carrying BRCA1 mutations (Billing et al., 2018). Despite the fact that neither BARD1 mutation affected HR, both protein variations however prevented poly(ADP-ribose) from recruiting BRCA1/BARD1 to stalled replication forks, ultimately leading to resulting in fork degradation and chromosomal instability.

Although not affecting HR, both BARD1 mutations ablated the -dependent recruitment of BRCA1/BARD1 to stalled replication forks, resulting in fork degradation and chromosome instability. Yet, unlike mice homozygous for the BRCA1 Ser1598Phe mutation, animals homozygous either for BARD1 Ser563Phe or BARD1 Lys607Ala were not tumor prone, indicating that HR alone is sufficient to suppress tumor formation in the absence of stalled fork protection. Nevertheless, because stalled fork protection, unlike HR, was found to be impaired in heterozygous BRCA1/BARD1 mutant cells, the authors proposed that both stalled fork protection and HR could contribute to distinct stages of

tumorigenesis in BRCA1/BARD1 mutation carriers. Besides the already discussed role of BRCA1 in protecting stalled replication forks from degradation by MRE11 (§2.6.2.1) and EXO1 (§3.3.2.1) (Lemaçon et al., 2017), other nucleases, *e.g.*, the DNA replication ATP-dependent helicase/nuclease DNA2 (DNA2, Fig. 37, top left) and WRN (§2.6.2.1) (Iannascoli, Palermo, Murfuni, Franchitto, & Pichierri, 2015; Thangavel et al., 2015) can trigger fork attrition. With particular reference to DNA2, Higgs and colleagues identified the biorientation of chromosomes in cell division protein 1-like 1 (BOD1L) as a component of the fork protection pathway safeguarding genome stability after replication stress (Higgs et al., 2015). BOD1L loss was reported to endow cells with replication stress susceptibility and damaged replication fork uncontrolled resection, which was ascribed to the inability of stabilizing RAD51 at those forks. Critically, this was epistatic with the depletion of either BRCA1 or BRCA2, yielding the indication that that BOD1L and BRCA1/2 operate within the same replication fork protection pathways. Contextually, blocking DNA2-dependent resection, or downregulation of the helicases BLM (§2.6.2.1) and F-box DNA helicase 1 (FBH1, Fig. 37, top right) suppressed both catastrophic fork processing and the accumulation of chromosomal damage in BOD1L-deficient cells, highlighting BOD1L as a critical regulator of genome integrity that restrains nucleolytic degradation of damaged replication forks along with BRCA1 and BRCA2. Finally, a recent study by Daza-Martin and coworkers revealed that BRCA1/BARD1, and not the canonical BRCA1-PALB2 interaction, is required for fork protection (Daza-Martin et al., 2019). Most importantly, according to their data the fork protection activity of BRCA1/BARD1 is controlled through conformational changes promoted by the peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1, a protein that, by stimulating conformational modifications in a group of phosphoproteins works as a *molecular switch* in different cellular processes (Chen et al., 2018), Fig. 37, middle left). This group reported that PIN1 activity strengthens the interaction between BRCA1/BARD1 and RAD51, thus reinforcing the recombinase localization at blocked replication structures. Furthermore, in cancer patients they discovered BRCA1/BARD1 missense variants (Ser114Pro, Arg133Cys, Tyr179Cys, Ser184Cys and Ser265Tyr in BRCA1 and Lys144Asn and Phe147Cys in BARD1) that, although empowered with low efficiency in nascent strand protection, preserved their HR competency, thus highlighting the BRCA1/BARD1 domains essential in fork protection and ultimately linked to with carcinogenesis. All these evidences constitute a major milestone in establishing a route to replication force protection mediated by BRCA1.

In 2016, Masuda and coworkers identified an essential BRCA1 DNA binding region (DBR, residues 421–701) through which BRCA1 preferably fastens to splayed-arm DNA and pulls it together in a mechanism that does not depend on a specific DNA sequence (Masuda, Xu, Dimitriadis, Lahusen, & Deng, 2016). With the aim to investigate the biological role of the DBR in more detail, the group generated mouse ESCs lacking the BRCA1 DBR (ΔDBR), and reported that these cells showed

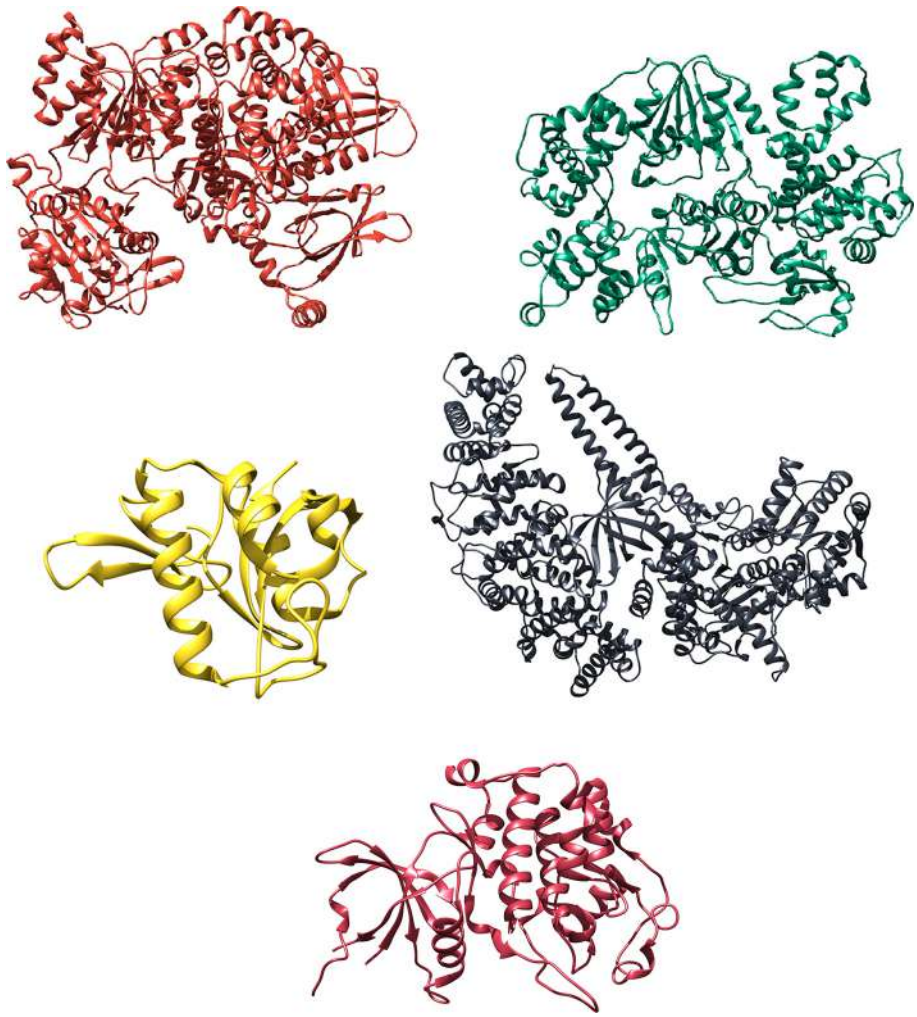

Fig. 37. (Top) 3D structures of DNA2 (left, poinciana, AlphaFold2 PDB: P51530) and FBH1 (right, golf green, AlphaFold2 PDB: Q8NFZ0) as predicted by AlphaFold2 (Jumper et al., 2021). (Middle) Crystal structure of PIN1 (left, vibrant yellow, PDB: 3TC5 (Gräber et al., 2011)) and 3D structure of SETX (right, blue nights, AlphaFold2 PDB: Q7Z333) as predicted by AlphaFold2 (Jumper et al., 2021). (Bottom) Crystal structure of P-TEFb (watermelon, PDB: 4OGR (Schulze-Gahmen, Lu, Zhou, & Alber, 2014)).

reduced survival when compared to WT controls treated with a PARPi; nonetheless, ΔDBR cells fully preserved their power to conduct HR-mediated DDR, while their ability to phosphorylate CHK1, the master regulator of S-phase checkpoint (§2.6.1.2), was slightly diminished. In addition, as compared to WT cells, ΔDBR cells had a higher number of aberrant chromosomal structures, an hallmark of increased genomic instability. These data then show that BRCA1 DBR regulates the stability of the genome *via* the intra-S-phase checkpoint triggered by replication stress.

Heterochromatic repetitive satellite RNAs (satRNAs)[43] undergo extensive translation in a plethora of human tumors, including BCs carrying BRCA1 mutations. *In vitro*, satRNA abnormal translation activates the DDR, prompts CCPs, and triggers anomalous chromosomal segregation. Nevertheless, the exact mechanism through which the expression of satRNAs contributes to genome instability is still not fully uncovered (J. Thakur et al., 2021). Zhu et al. demonstrated that higher satRNA levels in mammary glands were associated with the development of cancer in mice. These authors also showed that the genetic instability promoted by satRNAs was elicited by their interactions with the network of BRCA1-associated proteins, which are essential for DNA replication fork stability. Moreover, in cells expressing satRNAs they verified that destabilized replication forks possibly induce the production of RNA-DNA hybrids (Q. Zhu et al., 2018).

R-loops are hybrid RNA/DNA structures that include a displaced ssDNA region. These structures are mainly generated during transcription and perform a variety of essential biological functions, including gene promoter control and transcription termination, among others. On the other hand, R-loops that arise as a result of a perturbation in transcription or transcription-coupled mRNA splicing activities may negatively interfere with DNA replication and ultimately result in fatal replication fork collapse. Cells are armed to solve such conflicts by *e.g.*, preventing R-loop formation through topoisomerase 1-mediated removal of negative DNA supercoiling, digesting the RNA moiety in R-loops by ribonuclease H1 (RNASEH1, an enzyme that plays a role in RNAPII transcription termination by degrading R-loop RNA-DNA hybrid formation (Ohle et al., 2016)), and dissolving R-loops by putative RNA/DNA helicases such as a the probable helicase senataxin (SETX, Fig. 37, middle right (Cohen et al., 2018)). In 2014, Hill and coworkers discovered that BRCA1 depletion improved cell sensitivity to two transcription inhibitors and DNA damaging agents (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside and α-amanitin), leading to the idea that BRCA1 has a function a role in the cellular response to DNA damaged provoked by an anomalous arrest along the transcription process (Hill et al., 2014). To investigate the issue further, they performed a complementary systematic screen searching for new BRCA1 protein-interacting partners. In addition to BARD1, they discovered genetic connections between BRCA1 and four new interactors: the Tonsoku-like protein (TONSL, a protein that has previously been identified as being involved in the repair of stalled/collapsed replication forks (O'Donnell et al., 2010)), SETX, the transcription elongation factor A N-terminal and central domain-containing protein (TCEANC), and the transcription elongation factor A protein 2 (TCEA2, one in a group of proteins that aids RNAPII (§2.6.2.1) in crossing some transcription-pausing sites and also participates in DNA damage responses evoked during transcription (Wind & Reines, 2000)). They also found genetic interactions between BRCA1 and certain interactors of TONSL, including both members of the facilitates chromatin transactions (FACT) complex[44]: the FACT complex subunits SSRP1 (SSRP1) and SPT16 (SUPT16H), both of which are general chromatin factors that act to reorganize nucleosomes. Overall, these results and other data reported in their study highlighted a new BRCA1 role in promoting transcription restart following DNA damage and in the prevention/repair of damages due to stabilized R loops, a function that BRCA1 possibly performs in collaboration with some of its interactors listed above. The interaction of BRCA1 and SETX at stalled replication forks was confirmed one year later by Hatchi et al., who showed that BRCA1 is recruited at R-loops that naturally develop across some transcription termination areas, where it facilitates the recruitment of SETX as its specific, physiological interactor (Hatchi et al., 2015). Abrogation of BRCA1/SETX binding resulted in DNA damage induced by R-loops, as suggested by the accumulation of γ-H2AX and ssDNA breaks at the relevant R-loop untranscribed strands. Using genome-wide analysis the authors also found a substantial increase in the binding of BRCA1 at the termination regions of actively transcribed genes enriched in R-loop structures, confirming the notion that BRCA1/SETX assemblies support a DDR mechanism that targets R-loop-related DNA injury at transcriptional stalled sites. An open question however still remains whether BARCA1 partners with BARD1 in the recognition of R-loops and in synergizing with SETX or other proteins, *e.g.*, the ATP-dependent RNA helicase DDX1 (DDX1, an helicase able to unwind both RNA/RNA and hybrid RNA/DNA structures (Li et al., 2016)) or RNASEH1 in resolving this genome-threatening structures.

Very recently, Vohhodina et al. reported that BRCA1 binds TERRA RNA (a telomeric repeat-containing lncRNA),[45] directly and physically via its N-terminal NLS, as well as telomere-specific shelterin proteins (§2.6.2.2) in an R-loop-, and a cell cycle-dependent manner (Vohhodina et al., 2021). They showed that R-loop-driven BRCA1 binding to CpG-rich TERRA promoters represses TERRA transcription, prevents TERRA R-loop-associated damage, and promotes its repair, likely in association with SETX and the 5′-3′ exoribonuclease 2 (XRN2, (Eaton & West, 2018)). BRCA1 depletion upregulates TERRA expression, leading to overly abundant TERRA R-loops, telomeric replication stress, and signs of telomeric aberrancy. Moreover, BRCA1 mutations within the TERRA-binding region led to an excess of TERRA-associated R-loops and telomeric abnormalities. Thus, according to these results, normal BRCA1/TERRA binding has the fundamental role to suppress telomere-centered genome instability.

G-quadruplexes (G4) are non-Watson-Crick DNA or RNA secondary structures that form *in vitro* and *in vivo* via guanine (G) self-assembly in G-rich sequences to generate stacked tetrad structures (Spiegel, Adhikari, & Balasubramanian, 2020). In the human genome, $>7 \times 10^5$ G4s have been detected *in vitro* (Hänsel-Hertsch, Di Antonio, & Balasubramanian, 2017), and regulatory regions are particularly populated by sequences encoding, in line with their functions in replication, transcription, mRNA splicing, translation and epigenetic regulation of the genome (Varshney, Spiegel, Zyner, Tannahill, & Balasubramanian, 2020). Moreover, their over-representation in different oncogene

[43] Satellite DNA is composed of a large number of tandem repeats that are involved in a variety of biological activities, including the segregation of chromosomes, the organization of the genome, and the protection of telomeres, among others. The majority of satellite DNA repeat units are 5 to 10 bp long or are of full nucleosomal length, and locate in the centromeric/pericentromeric and telomeric areas of the genome, where they can be found embedded in highly dense heterochromatin or in other chromatin structures different from euchromatin. Nonetheless, some satellite DNAs are transcribed into non coding satellite RNAs (satRNAs), which may have important functions in satellite DNA roles. For instance, satRNAs generated by centromeric satellite DNA transcripts have been shown to regulate the organization of chromosomes and chromatin, and to influence the formation of human kinetochore. Furthermore, it was reported that, at variance with healthy tissues, the transcription products of some centromeric satRNAs are present in many tumor types, indicating that satRNAs can have a role in tumorigenesis and cancer development (J. Thakur, Packiaraj, & Henikoff, 2021).

[44] The chromatin remodeling activities of the FACT complex promote transcription elongation via chromatin regions by enabling the modification and correct replacement of the nucleosome structure. This enables transcriptional progression across chromatin areas while avoiding irreversible disruption of epigenetic markers. Furthermore, the FACT complex has been linked to various elements of transcription-associated DDR control and the resolution of replication/transcription complex collisions (Prendergast, Hong, Safina, Poe, & Gurova, 2020).

[45] TERRA is an RNAPII-transcribed telomeric DNA product that contributes in telomere length control and chromosomal end protection. G-rich TERRA molecules are transcribed from sub-telomeric regions toward the ends of chromosomes utilizing a C-rich telomeric strand as a template, and TERRA transcription is triggered by repeated CpG-rich promoter sequences found in the sub-telomeric regions of at least half of human chromosomes. CpG-rich TERRA promoter methylation is controlled by different DNA methyltransferases, the depletion of which leads to increased TERRA transcription. G-rich TERRA itself can generates R-loops with C-rich telomeric strands and, if on the one side this is an advantageous function in cancer cells that maintain telomeric length through the ALT route (§2.6.2.2) since TERRA R-loops promote HR-based telomeric replication, on the other side a large number of TERRA R-loops can result in replication stress and genomic instability.

promoters supports their important role in cellular biology and their implication in human pathogenesis, cancer *in primis* (Nakanishi & Seimiya, 2020). The formation of G4 structures is favored by processes involving transient opening of the double helix, such as DNA transcription and replication. Once formed, these structures are extremely stable and several lines of evidence indicate that the formation of G4 structures during replication impedes the progression of replication forks *in vivo*, and that specific mechanisms have developed to ensure the smooth replication of these potential *roadblocks* (Valton & Prioleau, 2016). Castillo-Bosch and colleagues showed that, after transient stalling, G4s are usually efficiently unwound and replicated but the loss of the FANCJ/BRIP1/BACH1 helicase generated prolonged stalling of the replication process at G4s, establishing a critical role for this helicase G4 resolution (Castillo Bosch et al., 2014). Interestingly, these authors verified that FANCJ/BACH1 – a BRCA1 interacting protein (§2.6.2.1) – performs its G4-resolving function independently of the classical Fanconi anemia pathway. Zimmer et al. demonstrated that i) in cells lacking HR the replication competency of G-rich telomeric repeats was significantly reduced while the presence of pyridostatin (PDS, a G4-stabilizing compound) increased telomeric fragility, and ii) cells lacking BRCA1, BRCA2, or RAD51 exhibited low survival rates upon G4 stabilization, and this was correlated to high amounts of replication stress and DNA damage. Accordingly, while the majority of genomic G4s could be resolved through alternative routes, the data from Zimmer and collaborators indicate that a G4 subgroup promotes replication fork stalling and DSB formation, both of which are highly toxic in cells with HR-impaired DDR. In such cells, DNA damage induced by G4s could be repaired by error-prone mechanisms (*i.e.*, NHEJ) which, according to their data, appeared however to be insufficient for their survival and proliferation (Zimmer et al., 2016). In line with this, Xu and coworkers showed that CX-5461, another G4 stabilizer, was endowed with high toxicity specifically against BRCA-deficient cancer cells and in polyclonal xenograft models (Xu et al., 2017). Treatments based on CX-5461 (and its related compound CX-3543) blocked replication forks, induced ssDNA gaps/breaks, and the BRCA-mediated HR or NHEJ DDR pathways were needed to repair the DNA damage provoked by these two drugs. All these evidences are consistent with a possible role of BRCA1 (and BARD1) in promoting restart of stalled replication forks at G4 sites.

In April 2021, Barrows and coworkers reported that BRCA1/BARD1 suppress transcription in a nucleoplasmic extract (NE) system (Barrows, Fullbright, & Long, 2021). This group showed that transcription suppression was independent of damage signaling, and BRCA1/BARD1 act through a histone intermediate and block transcription initiation by limiting access to chromatinized DNA. Although reduced histone H2A ubiquitination was reported in their system, the authors found that both ubiquitin and the E3 activity of the BRCA1/BARD1 heterodimer were not required for the suppression of transcription, thereby suppoting the existence of an alternative mechanism of transcriptional suppression. Using mass spectrometry, in their NE they were able to isolate different proteins whose DNA-binding properties were governed by BRCA1/BARD1. Among these, they recognized the bromodomain-containing protein 4 (BRD4, a chromatin reader protein that identifies and contacts acetylated histones and is essential for the transfer of epigenetic memory across cell divisions and in transcriptional control) as a potential candidate for regulation by BRCA1/BARD1 via interactions with i) SWI/SNF (§2.6.2.1 and 2.6.2.3), ii) the mediator of RNA polymerase II transcription complex (Mediator, a large modular complex with modular organization usually necessary in RNAPII-based transcription and a regulator of various stages of this mechanism (Soutourina, 2018)), iii) the positive transcription elongation factor b (P-TEFb, a general factor that promotes RNAPII-mediated transcription elongation, but later identified as an important cellular co-factor in the transcription of the human immunodeficiency virus (HIV) promoted by viral Tat proteins, Fig. 37, bottom (Fujinaga, 2020)), and iv) the super elongation complex (SEC, essential for the release of promoter-proximal pausing, allowing productive transcription of RNAPII-transcribed genes (Z. Luo, Lin, & Shilatifard, 2012)). Further data from this group re-established that BRCA1/BARD1 was a BRD4 negative regulator, and that the abrogation of BRD4 binding was a sufficient condition to interrupt transcription (at least in their NE). BRD4 is recruited by acetyl-histones to active genes, and there it serves as a scaffold to stabilize the transcription pre-initiation complex and stimulating elongation. Notably, when the same group examined BRD4 binding-associated histones, they found that BRCA1 specifically suppressed the level of histone H4 acetylated at Lys8 (H4K8ac), in agreement with previous evidence reporting acetylation of H4K8 to greatly increase the overall binding affinity of BRD4 for other acetyl-marks (Jung et al., 2014). Consequently, BRCA1-mediated H4K8 acetylation may function as a regulatory switch for the stable BRD4/chromatin interaction.

3.4. BRCA1/BARD1 in chromatin remodeling

Despite the link of BRCA1 and BARD1 to chromatin (§2.6.2.2, 2.6.2.5, and 2.7.4), the relevant substrates and underlying functions of the BRCA1/BARD1 E3 activity at this protein/DNA complex is still a subject of investigation. Kalb and colleagues revealed that BRCA1/BARD1 specifically ubiquitylates Lys127 and Lys129 on the C-terminal tail of histone H2A both *in vitro* and *in vivo*, and that the selectivity for these two lysine residues refers only to H2A when embedded a nucleosomal structure (Kalb, Mallery, Larkin, Huang, & Hiom, 2014). Importantly, this group found also that chromatin localization of the BRCA1/BARD1 heterodimer *via* site-specific targeting is required for foci formation by monoubiquitinated histone H2A (H2Aub) *in vivo*. Data from these authors then concurred in defining histone-H2A as a specific substrate for the E3 activity of BRCA1/BARD1, thereby offering a rationale for the localization and activities of the two RING-containing protein partners on cellular chromatin. Thakar et al. further reported that, besides H2A, also the nucleosomal H2B is ubiquitinated by BRCA1/BARD1 (although to a lesser extend with respect to H2A), supporting the prospect that, *via* its capacity of modifying histones in a nucleosomal context, the BRCA1/BARD1 heterodimer can directly regulate the structure, dynamics and function of the nucleosome (Thakar, Parvin, & Zlatanova, 2010). In a later effort, using a combination of tandem affinity purification and mass spectrometry Kim and colleagues identified 101 putative BRCA1/BARD1 E3 substrates (B. J. Kim et al., 2017). Within this ensemble, they identified the histone variant MACROH2A1 and reported the *in vitro*/*in vivo* ubiquitination of MACROH2A1 Lys123 by BRCA1/BARD1. Cells expressing a MACROH2A1 that could not undergo ubiquitination presented defects in cellular senescence compared to the WT controls; therefore, this study validated the notion that MACROH2A1 is another substrate for BRCA1BARD1 E3 ligase activity and that the selective ubiquitination of MACROH2A1 at Lys123 has a role in cellular senescence.

The group of Verma reported that BRCA1 the tumor suppression activity occurs though heterochromatin-mediated silencing (Quan Zhu et al., 2011). In their works showed that mice with BRCA1 deficiency presented tandemly repeated (TR) satellite DNA[46] transcriptional derepression. *In vivo*, BRCA1 impairment was associated with a decrease in the number of condensed DNA regions in the genome as well as a decrease in the amount of ubiquitylation of histone H2A at satellite repeats. BRCA1 deficiency was accompanied by a reduction of condensed DNA regions in the genome and loss of ubiquitylation at satellite repeats of histone H2A. Contextually, heterochromatin structure was maintained by BRCA1 *via* BRCA1/BARD1 ubiquitylation of histone H2A, which was shown to be effective when H2A linked to ubiquitin was expressed ectopically. De-repression of satellite DNA was also reported

[46] TR DNA is made up of several copies of a repeat unit (or monomer) organized head to tail. TR is found in centromeres from fission yeast to humans, and TR-rich pericentromeric areas seem to be essential for establishing heterochromatin production and correct chromosomal segregation (Hannan, 2018).

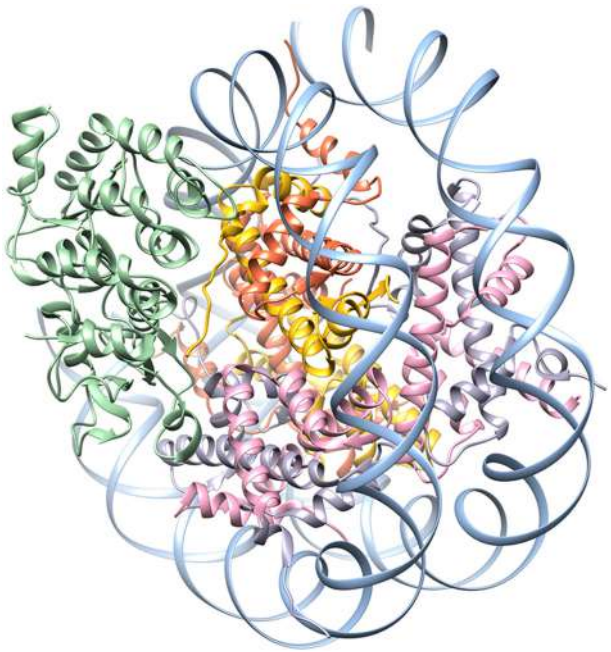

Fig. 38. Cryo-EM structure of BARD1 bound to a ubiquitinated nucleosome core particle (PDB: 7E8I (L. Dai et al., 2021)). BARD1 is in meadow; H3 chains in firecracker; H4 chains spectra yellow; H2A chains in heirloom lilac; H2B chains in prism pink, and nucleic acid in placid blue.

by the authors in murine and human BCs deficient in BRCA1, while satellite DNA ectopic expression could phenocopy the loss of BRCA1 in centrosome amplification, CCP defects, DNA damage and genetic instability. Thus, this group suggested that the involvement of BRCA1 in preserving overall heterochromatin integrity may be responsible for several of its tumor suppressor activities. Finally, as already discussed BRCA1 and BARD1 play a role in chromatin condensation control. Specifically, they contact the inactive X chromosome (Xi)-specific transcript (XIST) RNA, a ncRNA that coats Xi and known to be involved in the early stages of chromosome X inactivation during early embryogenesis (Ganesan et al., 2004).

In 2018, Fonseca and coworkers demonstrated the roles of CSTF1 (§2.3.4.3) and the ubiquitin escort factor CDC48/p97 (a chaperone that coordinates substrate recruitment, E1/E2/E3-catalyzed multi-ubiquitin chain assembly, and proteasomal targeting (Richly et al., 2005)) as BRCA1/BARD1 cofactors implicated in the remodeling of chromatin of genes differentially transcribed during DDR (Fonseca et al., 2018). These authors found that CSTF1 can directly interact not only with BRCA1/BARD1 but also with CDC48/p97 and some BRCA1/BARD1 substrates including RNAPII, H2A, and H2B. Also, along with CDC48/p97 CSTF1 elicits the monoubiquitination of histones H2A mediated by BRCA1/BARD1, the polyubiquitination of RNAPII, and last but not least BRCA1/BARD1 autoubiquitination. They also documented that the amount of monoubiquitinated H2B and H2A present in the chromatin of genes with varying degrees of expression changes during DDR, and that this is controlled by BRCA1/BARD1, CSTF1 expression, and CDC49/p97 ATPase activity. As a result, this study offers evidence that CSTF1/p97 controls the E3 activity of BRCA1/BARD1 during DDR, assisting in the formation and/or stability of the ubiquitination complex and altering chromatin structure and, therefore, gene expression.

While finalizing this review, Dai and coworkers solved the structure of BARD1 bound to a ubiquitinated nucleosome core particle (NCPUb) by cryo-EM (Fig. 38) and illustrated how, once there, BARD1 concomitantly identifies H2AK1ub and H4K20me0, two hallmarks of DNA damage and DNA replication, respectively (L. Dai et al., 2021). Further *in vitro* and *in vivo* analyses by these authors revealed that BARD1/nucleosome, BARD1/ubiquitin, and BARD1 ARD/BRTC interdomain interactions (§2.3.2 and 2.3.4.3) all stabilize the BARD1-NCPUb, and loss of these contacts impair HR-directed DDR. This group further identified numerous BARD1 disease-associated variants that prevent BARD1-NCPUb contacts and hence inhibit HR. Almost contemporarily, the group of Hu also used cryo-EM to verify that, at the nucleosome, the BARD1 ANK and BRCT motifs fold into a compact structure and bind histones, DNA and monoubiquitinated Lys13 or Lys15 on the H2A N-terminal - two specific marks of DSBs (Q. Hu et al., 2021). They further showed that the BRCA1/BARD1 RING domains predispose an E2 enzyme on the nucleosome top into a dynamic conformation that is ready for transferrin ubiquitin to the H2A and H2AX flexible C-terminals. This work then unveils a regulatory crosstalk in which the BRCA1/BARD1 identification of monoubiquitinated H2A N-termini prevents polyubiquitin chain formation and simultaneously elicits the H2A C-terminal ubiquitination. Accordingly, these results pose another milestone in elucidating the routes to chromatin recruitment and *in situ* specificity of the E3 activity of BRCA1/BARD1, highlighting key roles of BARD1 in both mechanisms and explaining the way in which BRCA1/BARD1 prompts HR by counteracting the action of the DNA repair protein 53BP1 in post-replicative chromatin.

3.5. BRCA1/BARD1 in hormone signaling

Estrogen is probably the highest risk factor for BC, and ES withdrawal constitutes the main measure of cancer prevention for individual carrying mutations in the BRCA1 or BARD1 genes. As discussed in sections §2.2 and 2.4.1, ES promotes the transcription activation of genes endowed with proliferative-supporting functions *via* ERα and ERβ, and ERα in particular has been recognized as an *in vitro* substrate for BRCA1/BARD1 E3 activity (Eakin, Maccoss, Finney, & Klevit, 2007). The results of Eakin et al. also show that i) ERα is predominantly monoubiquitinated in a reaction that involves interactions with both BRCA1 and BARD1, ii) the BRCA1/BARD1 regions required for ERα ubiquitination comprise the RING domains and at least 241 residues in BRCA1 and 170 residues in BARD1, respectively, and iii) cancer-predisposing mutations in BRCA1 abrogate ERα ubiquitination. In addition, this work established the tight link between tissue-specific malignancies in the breast and ovaries associated to even a single BRCA1 pathogenic mutation and BRCA1 suppression of ERα transcriptional activation. In a successive paper, Dizin and Irminger-Finger confirmed ERα as a BRCA1/BARD1 E3 target *in vivo* (Dizin & Irminger-Finger, 2010). They also verified that BRCA1 and BARD1 are essential for the ubiquitination and subsequent degradation of ERα, and depletion of either of the two proteins results in ERα accumulation, indicating the existence of a BRCA1/BARD1-ERα feedback loop (as both proteins are induced by this estrogen receptor). Notably, this group also ascertained that whilst the E3 ligase activity maps to the RING domains of the two proteins, the C-terminal in BARD1 plays a critical role in substrate recognition. Additionally, a *RING-less* BARD1 was also found to bind and stabilize ERα. According to these results, BRCA1 or BRAD1 deficiencies and/or upregulated BARD1 variants lead to ERα overexpression, thereby offering a functional liaison between BRCA1 deprivation, ES signaling, and carcinogenesis. The ovary is the main source of circulating ES in pre-menopausal women while ES production in post-menopausal individuals moves to peripheral tissues including fat and skin (Simpson & Davis, 2001). A single gene – CYP19A1 – encodes aromatase (Fig. 39, top left), a cytochrome P450 monooxygenase that assists in the conversion of C19 androgens, androstenedione and testosterone to the C18 estrogens, estrone and estradiol, respectively (Bulun et al., 2005). Thus, Lu and coworkers investigated whether BRCA1 could act as a protective factor by suppressing aromatase expression – and therefore ES production – by contacting the cancer-related promoter I.3/II region of the aromatase gene (M. Lu et al., 2006). By treating breast adipose fibroblasts (BAFs) with prostaglandin E2 (a major factor produced by breast tumors that mediates aromatase promoter switching (Richards & Brueggemeier, 2003)) or with a surrogate hormonal cocktail (SHC)

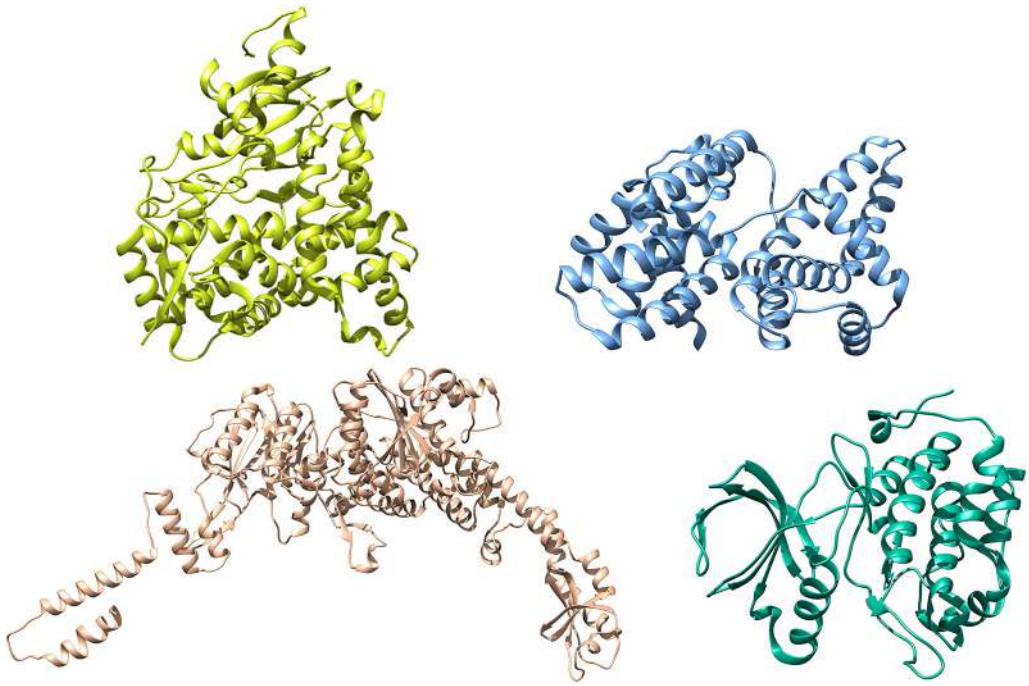

Fig. 39. (Top left) Crystal structure of aromatase (lime punch, PDB: 4GL7 (D. Ghosh et al., 2012)). 3D structures of COSA-1 (top right, little boy blue, AlphaFold2 PDB: Q9BL45) and MSH5 (bottom left, peach nougat, AlphaFold2 PDB: O43196), as predicted by AlphaFold2 (Jumper et al., 2021). (Bottom right) Crystal structure of plk-2 (arcadia, PDB: 4I5P (D. Ghosh et al., 2012)).

they observed significantly lower amounts of BRCA1 and aromatase mRNA levels. At the same time, lowering BRCA1 levels in BAFs and in different MECs of malignant origins using RNAi resulted in a considerable elevation of both aromatase mRNA levels and enzyme activity. The authors verified that the effect of BRCA1 was modulated by the selective repression of the aromatase promoters I.3 and II, which are upregulated by the prostaglandin E2 activity or upon SHC treatment. Using ChIP assays the same group discovered that BRCA1 directly contact the aromatase promoter I.3/II region and that cell treatment with SHC abrogated this interaction. In summary, these findings prompted these scientists to conclude that BRCA1 binding to the I.3/II tumorigenic promoter regions, which selectively inhibits aromatase production, may actually be an essential protective mechanism against the development of BC. In a later work the same group demonstrated that, in the KGN ovarian granulosa cell line, the steroidogenic factor 1 (SF-1, a key regulator of adrenal and reproductive development and function (Ferraz-de-Souza, Lin, & Achermann, 2011)) is obligatory for the basal activity of the aromatase PII promoter and the BRCA1 knockdown-mediated aromatase elevated expression. Furthermore, they reported that in KGN cells BRCA1 can be found mainly in its heterodimeric form with BARD1, and that the BRCA1/BARD1 assembly contacts SF-1 both *in vivo* and *in vitro*. Notably, however, SF-1 does not appear to be a substrate for the BRCA1/BARD1 E3 ubiquitin ligase activity. Thus, the authors proposed a mechanism according to which the interaction between BRCA1/BARD1 and SF-1 promotes the localization of the BRCA1/BARD1 heterodimer to the aromatase PII promoter for its transcriptional repression.

3.6. BRCA1/BARD1 in gametogenesis

To accomplish faithful segregation in the gametes during meiosis, the maternal and paternal homologous chromosomes must align along their full lengths and recombine to realize faithful segregation in the gametes. Meiotic recombination is achieved *via* the generation of DNA DSBs, a fraction of which may develop into crossovers to connect the parental homologous chromosomes and facilitate their segregation process. DDR in mitotic cells is efficiently assisted by BRCA1 and its heterodimeric companion BARD1 (§2.5.2, 2.7.1, and 3.2); however, their roles during gametogenesis are still somewhat obscure. In this respect, Janisiw and colleagues (Janisiw, Dello Stritto, Jantsch, & Silva, 2018) reported a dynamic localization of BRC-1 and BRD-1 (*i.e.*, the *Caenorhabditis elegans* orthologues of BRCA1 and BARD1) during the prophase I of meiosis. These proteins eventually accumulate in the areas around the putative crossover sites, where they colocalize with the pro-crossover factors crossover site associated (COSA-1, Fig. 39, top right), mutS protein homolog 5 (MSH5, Fig. 39, bottom left) and zip homologous protein 3 (ZHP-3). The activity of the synaptonemal complex (SyC)[47] and plk-2 (a serine/threonine-protein kinase which, by assisting the recruitment of meiotic chromosomes and their adhesion to the nuclear envelope, has a function in chromosome pairing and synapsis during oogenesis, Fig. 39, bottom right) is indispensable for recruiting BRC-1 to chromosomes and its successive redistribution near the bivalent short arms. This group also determined that BRC-1 and BRD-1 form *in vivo* complexes with the synaptonemal complex component SYP-3 (synapsis in meiosis abnormal, a protein involved in chiasma assembly and embryo development located in both the central and lateral elements of the SC) and MSH5. Furthermore, BRC-1 was also reported by these authors to be crucial for effective stage-specific localization/stabilization of RAD51 to DDSs when under impaired synapsis conditions or following exogenous DNA damage induction. These aspects were further investigated by Li and coworkers (Li et al., 2018). Using functional GFP fusions they found that i) BRC-1 and BRD-1 were nucleoplasmic in mitotically-dividing germ cells, ii) they accumulated at foci partially overlapping with those generated by RAD51, and iii) co-localization of BRC1/BRD1 with RAD51 increased under replication

[47] The synaptonemal complex (SyC) is a protein lattice that mimics train tracks and links paired homologous chromosomes in the majority of meiotic systems. Transverse filaments are proteins that connect the two side rails of the SC, also called lateral elements (LEs). It is thought that the LEs derive from the chromosome axial elements and play important functions in the condensation and pairing of chromosomes, assembly of transverse filaments, and preventing DNA DSBs from entering into recombination pathways that implicate sister (Page & Hawley, 2004).

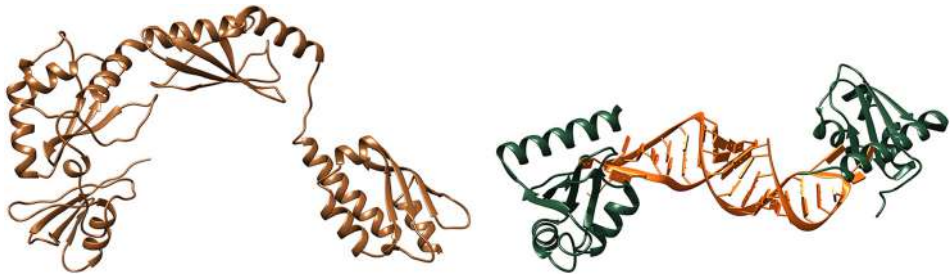

Fig. 40. (Right) 3D structure of FANCL (sugar almond, AlphaFold2 PDB: Q9NW38) as predicted by AlphaFold2 (Jumper et al., 2021). (Left) X-ray solved structure of the C-terminal RRM RNA recognition motif of LARP7 (Eden) bound to the 7SK RNA stem-loop (tiger orange, PDB: 6D12 (Eichhorn, Yang, Repeta, & Feigon, 2018)).

stress. They observed that BRC-1/BRD-1 remained nucleoplasmic and organized in foci while cells entered meiosis and, starting by mid-pachytene,[48] BRC-1/BRD-1 is seen to colocalize with the SC. Using localization dependencies the authors also discovered that BRC-1 and BRD-1 are interdependent and that the BRC-1/BRD-1 complex does not correctly localize in the presence of meiotic recombination and chromosome synapsis mutants. Consistent with the BRC-1/BRD1 function in the SC context during meiotic recombination, the authors showed that either BRC-1 or BRD-1 inactivation promotes the death of embryonies carrying chromosome synapsis-defective mutants. In agreement with the findings of Janisiw et al. just discussed above, the data from Li et al. also indicate that BRC-1/BRD-1 promotes RAD51 filament stabilization and modifies the recombination scenario, and these two BRC-1/BBRD-1 roles are genetically distinct from those played by the same heterodimer in the DDR context. These authors thus proposed that the BRC-1/BRD-1 ensemble carries out a checkpoint role at the SC, where it presides over and regulates meiotic recombination (Li, Saito, et al., 2018).

3.7. Further aspects of BRCA1/BARD1 activity in tumor suppression and DDR

To further investigate the tumor suppressor role of BRCA1/BARD1, Shakya and coworkers created mouse strains characterized by the presence of conditional alleles of either BRCA1 or BARD1 and employed CRE recombination (note 31) to suppress these genes expression in MECs (Shakya et al., 2008). Notably, both groups of engineered animals developed BCs with identical phenotypes, which were also indistinguishable from those established in double conditional BARD1/BRCA1-mutant mice with respect to all main features (*e.g.*, frequency, latency, histopathology, and cytogenetic characteristics). Moreover, these malignancies were "triple negative" for the expression of ES and progesterone receptors and amplification of HER2, in a way similar of the basal-like BCs observed in human individuals carrying BRCA1 mutations. Additionally, these cancers also expressed CK5 and CK14 (two basal cytokeratins), showed high p53 mutation frequency, and displayed elevated genetic instability. The striking resemblances between the BCs developed all these three types of murine models led the authors to conclude that the BRCA1/BARD1 heterodimer modulates both gene tumor suppressor functions.

UBE2T (§2.5.1) was reported to be one of the genes whose expression was upregulated in human fibroblasts with serum stimulation (Iyer et al., 1999). Moreover, UBE2T protein was shown to bind the E3 ubiquitin-protein ligase FANCL (FANCL, Fig. 40, top left), a member of the Fanconi anemia proteins (§2.6.2.1), and to be necessary for the efficient DNA damage–induced monoubiquitination of FANCD2 (§ 2.6.2.2), another component of the FA repair pathway (Alpi et al., 2007; Machida et al., 2006). Ueki et al. originally reported that UBE2T was highly overexpressed in the great majority of BC cells, while their immunocytochemical staining and *in vitro* binding assay revealed that UBE2T interacts and colocalizes with the BRCA1/BARD1 complex. UBE2T knockdown by RNAi substantially suppressed the growth of BC cells. Remarkably, *in vivo* ubiquitination assays revealed BRCA1 to be polyubiquitinated by incubation with WT UBE2T but not with the E2 activity-dead mutant Cys86Ala-UBE2T isoform. Also, UBE2T protein knockdown elicited upregulation of BRCA1 in BC cells, whereas its overexpression resulted in lowered BRCA1 protein amount. In all, these findings emphasize the important role of UBE2T in development and/or progression of BC via the interaction with and the regulation of the BRCA1/BARD1 heterocomplex.

Stable retention of BRCA1/BARD1 complexes at DDSs is a mandatory requirement for proper HR response to DSBs. Remarkably, Wu and colleagues demonstrated that BARD1 BRCT domain (§2.3.2) is crucial for its retention through interaction with the chromobox protein homolog 5 (CBX5/HP1) (Wu et al., 2015). CBX5 is a heterochromatin component that identifies and binds to methylated Lys9 residues on the tails of histone H3 (H3K9me), resulting in epigenetic repression; in contrast, CBX5 is removed from chromatin upon phosphorylation of Tyr41 on the same histone (H3Y41γ) (Maison & Almouzni, 2004). In response to DNA damage, BARD1 was seen to interact with Lys9-dimethylated histone H3 (H3K9me2) in an ATM-dependent mechanism but independently of RNF168 (note 19 and §2.6.1.1), and this interaction is mediated primarily by the phosphorylated form of HP1 (HP1γ). In particular, a conserved HP1-binding domain located on the BRCT motif of BARD1 directly interacts with the chromo shadow domain[49] of HP1 *in vitro*, and the authors further demonstrated that mutations in this motif (or HP1) abolishes DSB localization of BRCA1, BARD1, and CtIP (§2.3.2) and promoted the ectopic accumulation of RIF1, the NHEJ effector at damaged loci in S-phase (§1).

Back in 2004, Stark et al. investigated the interrelationship between two DSB repair pathways, *i.e.*, SSA and HR (§1), and found that SSA and HR were reduced by BC-predisposing mutation of BRCA1, whereas both pathways were increased by Ku70 (§1) mutations, which affect NHEJ (§1) (Stark, Pierce, Oh, Pastink, & Jasin, 2004). They also found that disruption of BARD1 had effects similar to those of BRCA1 mutations, yet Ku70 mutation partially suppressed the HR defects of BARD1 disruption. They thus concluded that BRCA1/BARD1 has a role in HR before the branch point of HR and SSA.

La-related protein 7 (LARP7) is a La family RNA-binding protein (Krueger et al., 2008; Markert et al., 2008) that contains two types of

[48] Pachytene, also known as pachynema, is the third stage of the prophase of meiosis, during which the homologous chromosomes are synapsed, or have been fully zipped up and are connected from end to end by the synaptonemal complex.

[49] The chromo shadow is a protein domain which is distantly related to the chromodomain (*i.e.*, another protein structural motif of about 40–50 amino acids commonly found in proteins associated with the remodeling and manipulation of chromatin), and these two domains are always found in association (Aasland & Stewart, 1995). Aside HP1, other two proteins contain a chromo shadow domain - the histone-lysine N-methyltransferases SUV39H1 and SUV39H2 - that specifically trimethylate Lys9 of histone H3 using monomethylated H3 Lys9 as substrate. Chromo shadow domain containing proteins are bound to nucleosomes and, upon their self-assembly, they promote the condensation of those chromatin areas they are associated with. Since condensed chromatin prevent transcription factors and other effectors from accessing DNA, chromo shadow domain containing proteins are usually regarded as repressors of gene transcription.

RNA binding domains: the RNA recognition motif (RRM, (Nowacka et al., 2019)) and the HTH La-type RNA-binding domains (other RNA-binding, winged helix-turn-helix (wHTH) domains of about 90 residues (Aravind, Anantharaman, Balaji, Babu, & Iyer, 2005)). Binding of LARP7 stabilizes the 3'hairpin of the most abundant mammalian ncRNA, i.e., 7SK RNA (Fig. 40, right), and in so doing it originates the core of the 7SK small nuclear ribonucleoprotein (7SK snRNP). To date, the 7SK snRNP has been recognize to act as a negative regulator in RNAPII pausing release, a fundamental checkpoint along the RNA transcription process found in many organisms, by sequestering the P-TEFb complex (§3.3.2.3) in the nucleoplasm (Egloff, Studniarek, & Kiss, 2018). Decreased LARP7 levels leads to 7SK snRNP disruption, chromatin relocalization of P-TEFb, and removal of RNAPII pausing. However, the functions of LARP7 and 7SK snRNP beyond RNAPII pausing (*e.g.*, in the DDR) remain unknown. In a recent effort, Zhang and coworkers discovered that when BRCA1 and BARD1 are activated by genotoxic stress, they catalyze the Lys48 polyubiquitination of LARP7, thereby flagging it for destruction via the 26S ubiquitin-proteasome pathway (F. Zhang et al., 2020). Moreover, the 7SK snRNP disassembly induced by LARP7 loss promotes the suppression of the CDK1 complex expression and halts the cell cycle at the G2/M checkpoint. CDK1 complex depletion also decreases BRCA2 phosphorylation which, in turn, fosters the recruitment of RAD51 to DDSs and boosts HR-directed DDR. Importantly, these authors also reported that the reduced levels of LARP7 seen in patients with BC reflect into resistance to chemoradiotherapy both *in vitro* and *in vivo*. Altogether, this study unveiled another route by which BRCA1/BARD1 master DNA HR and the cell cycle.

Different studies indicate that, when DSBs occur in mammals, bidirectional transcription activities close to the breaks produce small RNAs that activate the DDR through local RNA/RNA interactions (Bader, Hawley, Wilczynska, & Bushell, 2020; Hawley, Lu, Wilczynska, & Bushell, 2017). At the same time, lncRNAs (§2.4.2) are also emerging as genome stability regulators (Durut & Mittelsten Scheid, 2019; Khanduja, Calvo, Joh, Hill, & Motamedi, 2016). Nevertheless, the exact mechanism of action for individual lncRNAs in the DDR has still to be revealed. In this respect, Hu et al. showed that the lncRNA BGL3 – a critical regulator of the BCR-ABL oncogene in chronic myeloid leukemia (Guo et al., 2015) – binds to PARP1 (§1) and BARD1, exhibiting an unexpected role in HR (Z. Hu et al., 2020). From a mechanistic standpoint, the authors found that PARP1 recruits BGL3 to DNA DSBs at an early time point, and this interaction involves the DNA-binding domain of PARP1. Importantly, BGL3 also interacts with both the BARD1 BRCT domain and an protein internal region (residues 127–424), which modulate the contacts of the BRCA1/BARD1 heterodimer with its binding partners such as HP1γ and RAD51, resulting in the retention of BRCA1/BARD1 at the DSB sites. Cells in which BGL3 was exhibited genetic instability and sensitivity to DNA-damaging agents, and in aggregate these findings underline the biological adaptability of RNA in mediating DDR response, which impact BRCA1/BARD1 localization at DSBs. Along a similar line, Sharma et al. identified the lncRNA DDSR1 (DNA damage-sensitive RNA1), which is stimulated – following DNA damage provoked by different DSB-inducing agents – in a manner that is reliant on the ATM-NF-κB pathway (Sharma et al., 2015). DDSR1 loss impaired cell growth, DDR signaling, and the ability to repair DNA in a HR-directed mechanisms, with HR defects characterized by abnormal BRCA1 and RAP80 accumulation at the sites of DSBs. In line with a role in HR regulation, DDSR1 was found to directly interact with BRCA1 and with the heterogeneous nuclear ribonucleoprotein U-like protein 1 (HNRNPUL1, an RNA-binding protein implicated in the resection of DNA ends), advocating a function for the lncRNA DDSR1 in modulating DNA repair by HR *via* BRCA1.

4. Conclusions

In this assay we have reviewed the main roles played by BRCA1 and BARD1 in preserving human genome maintenance, the different mechanisms underlying the biological activities of these two proteins, and the impact of a range of BRCA1/BARD1 variations on cancer inception and progression. Yet, since BRCA1/BARD1 continue to offer new, exciting and challenging perspectives, and each single contact of BRCA1 and BARD1 with the myriads of other proteins and interactors described in this work could be a potential source of future therapeutic intervention, we sincerely hope that this work could serve as a motivational boost in focusing the efforts of scientists active in different fields on this incredible subject in the near future.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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